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RESEARCH PAPER

Short-term fate of nitrogen fixed by moss-cyanobacteria associations under different rainfall regimes

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ABSTRACT

Nitrogen (N) fixation by moss-cyanobacteria associations has been recognized as an important N input pathway in many ecosystems from arctic tundra to tropical forests. However, the transfer of fixed N_2 from mosses to the soil as well as the effects of rainfall frequency and volume on this N transfer has hardly been studied – even though mosses can leach nutrients upon rewetting. In this study, we investigated the transfer of fixed N_2 by moss-cyanobacteria associations in one month under four watering regimes with a combination of high and low volume and frequency. For this, we used two morphologically similar moss species collected from ecosystems with different climate and N availability (subarctic - *Hylocomium splendens*; and tropical - *Thuidium delicatulum*). Acetylene reduction assays were conducted as a measure of N_2 fixation rates in mosses, and ^{15}N-N_2 tracing was used to follow the fixed N_2 from moss to the underlying substrate. Nitrogen fixation rates were higher in *T. delicatulum* than in *H. splendens*, but rainfall volume and frequency did not show strong effects on N_2 fixation rates. Nonetheless, the extent of N leached from mosses was more sensitive to an increase in rainfall volume than to an increase in frequency, and more N was lost from *T. delicatulum* under high volume precipitation than from *H. splendens*. Both total nitrogen and ^{15}N enrichment results demonstrate that the fixed N_2 was mostly stored in moss tissues with less than 1 % leached to the substrate. Our results show that both moss species retain almost all fixed N_2 within their tissues under small rainfall disturbances within one month, while increased N availability under higher precipitation volume renders some moss species an important N source for the soil.

Introduction

Nitrogen is a key element in biogeochemical cycles and essential for net primary production in terrestrial ecosystems (Du et al., 2020; Li et al., 2018; Xu et al., 2020). Massive N loss associated with soil erosion can cause a reduction in soil fertility, therefore affecting the ecological and economic value of lands (An et al., 2022; Teixeira & Misra, 2005). Apart from protecting surface soil from wind and runoff erosion, mosses may also compensate for soil N loss by hosting N_2-fixing bacteria (diazotrophs) which fix atmospheric N (Alvarenga & Rousk, 2022; Rousk & Michelsen, 2017). Although diverse microorganisms are found on mosses (Chen & Nelson, 2022), cyanobacteria colonizing mosses are among the most important diazotrophs contributing to the ecosystems' N pools in many biomes, especially in forests where atmospheric N deposition is relatively low (Renaudin et al., 2022). Nitrogen fixation rates via moss-cyanobacteria associations can reach 2.2 ± 0.2 kg N ha^{-1} yr^{-1} in subarctic tundra (Rousk & Michelsen, 2017), which can account for up to 50 % of new N (Gundale et al., 2011). In tropical forests, moss-cyanobacteria associations can also be important N sources, as N_2 fixation rates were recorded as reaching two orders of magnitudes higher than those found in soil, as well as four times higher than the rates measured in the forest floor covered by woody tissues and decomposing leaves (Zheng et al., 2020) and contribute up to 2 kg N ha^{-1} yr^{-1} to ecosystem N input (Permin et al., 2022). Nonetheless, if this fixed N_2 is available to the soil N pool remains unknown. Precipitation plays an important role in preventing mosses from desiccation given that mosses lack a vascular system and roots that prevent the absorption of water directly from soil (Carleton & Read, 1991). In addition, moisture is key for sustaining N_2 fixation in mosses (Permin et al., 2022; Rousk et al., 2018). A recent study found that 130–190 % of moss dry weight is the optimal water content for N_2 fixation (Fan et al., 2022). On the other hand, while a positive correlation between precipitation volume and cyanobacterial biomass on mosses in a boreal forest in Quebec was found, no effects on N_2 fixation rates could be seen (Renaudin et al.,

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2022). In short, with the help of colonizing, N_2-fixing cyanobacteria, mosses could have the potential to increase soil N pools via N_2 fixation, in which precipitation is a crucial factor determining this process.

Boreal forests and arctic tundra are predicted by climate models to experience less frequent but heavier rainfall events in the coming decades (Gundale et al., 2012; IPCC, 2021), with arctic regions, in general, expected to receive 50 % more precipitation by the end of this century (Bintanja & Selten, 2014). Therefore, changes in precipitation pattern worldwide may alter the N_2 fixation ability by mosses affecting the N pool in different ecosystems.

Besides precipitation, atmospheric N deposition is another key driver of moss- associated N_2 fixation. Nitrogen fixation rates in the widespread mosses *H. splendens* and *P. schreberi* commonly decreases with increasing N deposition (Rousk et al., 2013), and can be suppressed at N deposition rates as low as 3 kg ha^{-1} $year^{-1}$ (Salemaa et al., 2019). Nitrogen loss via leaching from mosses as a result of different precipitation regimes in habitats with different N availability, however, is unknown to date.

While N_2 fixation has been widely studied under different environmental conditions, the fate of the fixed N_2, has rarely been investigated. To explore the capacity of mosses to contribute to the soil nutrient pool, it is necessary to understand to what extent the fixed N_2 is transferred to the surrounding soil, as once fixed by cyanobacteria, the N likely becomes available to the mosses and the epiphytic microbial community. A minimal share of fixed N_2 might be released back into the atmosphere through denitrification in the form of N_2 and N_2O (Rousk et al., 2016). Other possible pathways are the use and storage in moss tissues, leaching to soil, reabsorption by the moss or other plants, or release to the soil after moss decomposition (DeLuca et al., 2022).

Since moss tissue decomposes rather slowly, leaching of N from mosses might be important in soil nutrient cycling in the short term. However, previous studies report mixed results. In a temperate alpine forest, N loss via leaching reached an annual rate of 13.2 % of N from live tissues of *H. splendens* (Liu et al., 2020). In arctic tundra, tracing of ^{15}N-N_2 showed that the fixed N was mostly retained in moss tissues, and very limited N was found in the soil after five weeks (Rousk et al., 2016). But drying-rewetting and freeze-thaw cycles can temporally impair the moss' cell membrane, thereby promoting leaching of intracellular nutrients to the environment (Coxson et al., 1992; Wilson & Coxson, 1999). Hence, mosses could be an N source upon disturbances such as large rainfall events.

Herein, we investigate the short-term fate of fixed N by cyanobacteria on two morphologically similar moss species from ecosystems with different climates and nutrient status, subarctic tundra and tropical cloud forest, to explore how different watering regimes influence N transfer from the moss to the substrate. Based on that, we hypothesized that N_2 fixation rates would be higher under high volume treatment due to better moisture conditions (H1). Fixed N would be mostly retained in moss tissues, but N leaching would increase with higher watering frequency when compared to higher watering volume since the number of wet/dry cycles is likely responsible for N leaching from moss (H2). The moss *H. splendens* from an N-limited subarctic tundra site will have higher N_2 fixation rates than *Thuidium delicatulum* collected from an N-rich tropical cloud forest site but more N will be leached from the tropical moss (H3). To address our hypotheses, we conducted watering simulation experiments with the two moss species (*H. splendens* - subarctic and *T. delicatulum* - tropical) for one month. We assessed the effects of different watering regimes on N_2 fixation by acetylene reduction assays, and ^{15}N incubation was initiated before watering treatments to explore the vertical distribution of fixed ^{15}N-N_2 along mosses, substrate, and leachates in the short-term.

Materials and methods

Sampling

Hylocomium splendens and *Thuidium delicatulum* were chosen in this study to investigate N transfer patterns of moss species from different climate regions with different N status (arctic low vs. tropical high). *Hylocomium splendens* and *T. delicatulum* are common species in subarctic tundra and tropical rainforest ecosystem, respectively. Both species belong to the Hypnales order, which are commonly named as feather mosses, sharing similar morphologies and showing highly branched and pinnate leaves. Those morphological traits were previously linked to high cyanobacteria colonization (Liu & Rousk, 2021), as such, *H. splendens* and *T. delicatulum* are known to associate with N_2-fixing cyanobacteria, rendering both species good candidates in increasing the ecosystems' N pool via N_2 fixation.

Four replicates (consisting of >10 shoots from a 1 × 1 m plot, the plots were several meters apart from each other; $n = 4$) of *H. splendens* were collected from a birch forest near Abisko Scientific Research Station (68°33′N, 18° 85′ E) in August 2021. The climate is subarctic, with annual mean temperature of 0 °C and annual precipitation of 361 mm (10-year mean 2012–2021, see below). Similarly, four field replicates of *T. delicatulum* were collected, each a few meters apart, in the Chirripó National Park, a tropical montane cloud forest in Costa Rica (9°26′35″N, 83°31′53″W, 3000 m above sea level) in November 2021 (end of the wet season). The average temperature for the site is approximately 10.9 °C, with annual precipitation reaching 2812 mm (Kappelle et al., 1995). All samples collected were transported to the University of Copenhagen, Denmark, within a week and stored at 4 °C in dark condition until the experiment started.

Experimental setup

Prior to the start of the experiment, all samples were soaked in double distilled water (ddH_2O) for 30 min to ensure saturated conditions. Single intact moss shoots of *H. splendens* (H) and *T. delicatulum* (T) were thoroughly cleaned to remove substrate materials and surface water. To investigate the effect of rainfall frequency and volume on N vertical transfer, four treatments were setup: (1) standard volume ($V_{standard}$) & standard frequency ($F_{standard}$) (referred to as H-$V_{standard}$-$F_{standard}$ and T-$V_{standard}$-$F_{standard}$ hereinafter, where the first letter "H" stands for *H. splendens* and "T" for *T. delicatulum*); (2) standard volume & low frequency (F_{low}) (0.3 × standard frequency; referred as H-$V_{standard}$-F_{low} and T-$V_{standard}$-F_{low} hereinafter); (3) high volume (V_{high}) & standard frequency (5 × standard volume; referred to as H-V_{high}-$F_{standard}$ and T-V_{high}-$F_{standard}$ hereinafter) and (4) high volume & low frequency (5 × standard volume and 0.3 × standard frequency; referred to as H-V_{high}-F_{low} and T-V_{high}-F_{low} hereinafter). The volume and frequency factors were chosen for comparison with previous studies (Gundale et al., 2012). The average rainfall amount and frequency from the Abisko meteorological station was adopted as a standard precipitation pattern in this experiment for both species. The high-volume treatment is comparable to the precipitation volume of Chirripó National Park where *T. delicatulum* was sampled (see below).

Each treatment was applied to the two moss species, each with four replicates consisting of several moss shoots. For this, the water saturated moss shoots were randomly distributed into 20 ml glass vials so that each contained 2 g of *H. splendens*. For *T. delicatulum*, we had less material available from the field and therefore only 1.3 g of fresh weight per vial were used. All samples were placed in a greenhouse for 72 h to acclimatize to the new environment prior to any measurements or treatments. Samples were kept in the same greenhouse throughout the whole experiment. Results were calculated on dry weight basis for inter-species comparison.

Acetylene reduction assay

To estimate the effects of watering regimes on N_2 fixation of moss-cyanobacteria associations, acetylene reduction assays (ARA) were conducted twice, one day before the start and right after the end of the treatments. The first ARA was run to confirm nitrogenase activity

associated with the mosses, while the second one to assess the effects of the watering treatments on N_2 fixation rates. The ARA method is widely used to quantify the nitrogenase enzyme activity, the enzyme reduces N_2, as well as acetylene gas (C_2H_2). The reduction of acetylene is exclusive to N_2-fixing organisms, and the produced ethylene is stable in the system (Leppänen et al., 2013). For the ARA measurements, the 20 ml glass vials with the mosses were sealed with rubber lids. Ten percent of the air in each vial was replaced by acetylene gas and incubated in the greenhouse (24 h, 22.7 °C, 234 μmol photons · m^{-2}· s^{-1}). Additional three vials with 10 % acetylene gas-only were placed in the same greenhouse for 24 h to account for any ethylene background present in the acetylene gas. Three extra moss-only vials, for each species, were also incubated to ensure no ethylene was being naturally produced by the mosses, which was not the case. Total ethylene production was measured using an Agilent 8890 GC System gas chromatographer with a J&W CarboBOND column (Agilent, Santa Clara, USA). Finally, we calculated the amount of ethylene produced by comparison with a 300 ppm ethylene standard curve.

^{15}N-N_2 labeling and watering treatments

Samples were sprayed with ddH_2O ensuring the mosses to be moist before ^{15}N-N_2 incubations, which were conducted one day after the first ARA. Specifically, all vials were sealed with rubber lids again, and 4 ml of air in each vial were replaced by 4 ml of 99 % ^{15}N-N_2 (corresponding to 20 % of the air in the vial; obtained from Cambridge Isotope Lab). The incubations were conducted in the greenhouse for 24 h.

After ^{15}N-N_2 labelling, each moss sample was carefully transferred from the glass vials to a "dual-tube system" (Fig. 1), where each upper 50 ml tube was filled with 1 g substrate (vermiculate) onto which the mosses were placed. The lower 50 ml tube was designed as a sink to collect leachate from the upper tube. The upper tube was opened at both ends allowing water to enter and leave the tube. Also, a net was attached at the bottom of the upper tube to prevent any material to be lost. Finally, the two tubes were secured with parafilm to ensure no loss of evaporated leachate from the lower tube. The dual-tube system was then placed in the greenhouse (23.4 ± 3 °C, ca. 16 h/light and 8 h/dark) during the precipitation treatments.

To simulate natural precipitation, watering regimes were initiated one day after the ^{15}N-N_2 labeling when all moss samples were in semi-dry condition. As previously stated, the rainfall amount and frequency from Abisko were adopted as a baseline in this experiment for both species, with the watering regime for the four treatments calculated based on the precipitation data from the recent 10 years (1.1.2012 – 31.12.2021) of Abisko meteorological station (Abisko Scientific Research Station climate records). Specifically, a day is counted as a rainy day when precipitation is higher than 0.3 mm during the calendar day, resulting in a total of 119 days and an annual precipitation of 360.77 mm. The corresponding volume of ddH_2O were added for each treatment and the leachates were collected 24 h after watering to ensure that the percolated leachates were collected as much as possible. Thereafter, the leachates were stored in a freezer (−20 °C) until further analyses. The watering regimes lasted 30 days allowing the treatment groups that differed only in rainfall frequency to receive the same volume of water by the end of the treatments (Fig. 2).

After the precipitation treatments, all moss samples were immediately transferred to 20 ml glass vials once more to conduct the second ARA, using the same methodology as described previously. Following ARA, ^{15}N enrichment analyses were conducted for mosses, vermiculates and leachates. All leachate samples from the same treatment were combined across time points, and freeze-dried, weighing approximately 3.5 mg. Vermiculate and moss samples were dried in an oven (70 °C, 24 h). Vermiculate samples were further ground and homogenized with a blender, while the desiccated moss samples were cut into a fine powder with scissors and weighed also to approximately 3.5 mg in tin capsules for ^{15}N analyses.

The samples were analyzed for total N as well as 15 N/14 N ratios on an Isoprime isotope ratio mass spectrometer (Isoprime Ltd, Cheadle Hulme, Stockport, UK) connected to a CN elemental analyzer (Eurovector, Milan, Italy) with continuous flow. The natural abundances of the 15 N isotope (see Appendix A: Table S1) are presented in the δ notation relative to international standards (atmospheric N2: δXsample (‰) = 1000 × [(Rsample/Rstandard) − 1], where R represents the molar ratio of heavy X to light X). The analysis was drift-corrected using peach leaves (NIST) as an internal standard. The standard deviation of isotope measurements for the standards was ± 0.2 ‰ for δ15N (Schmidt., 2018).

Data analysis

The calculations of total nitrogen (TN) and excess ^{15}N were conducted for moss and leachate samples only since there was no N and excess ^{15}N detected in the vermiculite samples. The TN concentration in mosses was calculated as TN/dry weight, while TN in leachates was expressed as TN corresponding to 1 g of moss to facilitate the comparison between species. ^{15}N contents were calculated by atom% excess

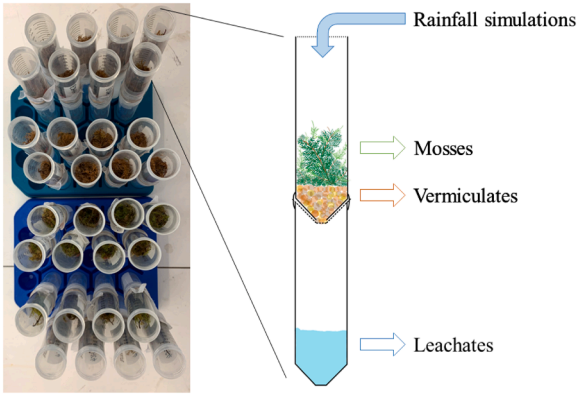


Fig. 1. The "dual-tube system" used for the precipitation treatments to assess N transfer from moss to the substrate below, including loss of N in leachate. The moss samples (green) and the underlying substrate material (vermiculite, brown) were placed in the upper tube exposed to watering treatments, whereas the lower tubes were used to collect the leachate (blue).

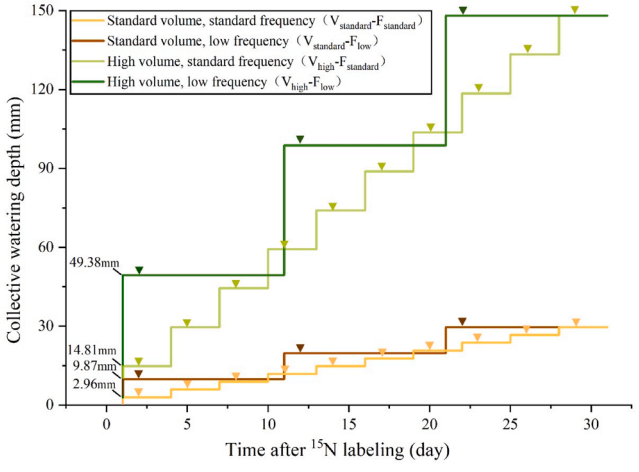


Fig. 2. Scheme of the four watering regimes applied after the ^{15}N-N_2 labeling of mosses. The inversed triangles indicate the leachate collection after each watering event. Different colors indicate the four different watering regimes: Standard volume, standard frequency: yellow; Standard volume, low frequency: brown; High volume, standard frequency: light green; High volume, low frequency: dark green.

(APE%), subtracting atom% ^{15}N natural abundance value from the atom % ^{15}N detected. Values of APE% below 0 are considered 0 since ^{15}N was not enriched in this case. To compare the distribution of ^{15}N enrichment in moss vs. leachates, the sum of excess ^{15}N in moss and leachate (total excess ^{15}N; APE% multiplied with TN in the sample) was calculated and the respective fraction of excess ^{15}N in moss and leachates was determined. In addition, correlations between moss and leachate excess ^{15}N were conducted. Statistical differences in nitrogenase activity and TN distribution in mosses and leachates were tested by three-way ANOVAs with watering volume, frequency, and species as the factors. ANOVAs were followed by Tukey HSD tests to identify significant differences between treatments (watering regimes combined with different species). Tests of normal distribution of the data were conducted in advance. Correlations between nitrogenase activity, TN, excess ^{15}N were conducted in OriginPro 2020. To further analyze the correlation between the N distribution results and environmental variables, a redundancy analysis (RDA) was performed in Canoco 5 based on Euclidean distance between a subset of sample properties (TN of leachates and mosses, excess ^{15}N of mosses, N_2 fixation ability before and after the treatments) and the independent environmental variables.

Results

Nitrogenase activity in moss-cyanobacteria associations

Nitrogen fixation rates, expressed as ethylene produced (nitrogenase activity assessed with ARA), were not significantly different between the samples before the treatments started, but increased significantly under all watering regimes in *T. delicatulum* ($T\text{-}V_{standard}\text{-}F_{standard}$: $F = 55.96$, $p = 0.0003$; $T\text{-}V_{standard}\text{-}F_{low}$: $F = 14.98$, $p = 0.008$; $T\text{-}V_{high}\text{-}F_{standard}$: $F = 20.55$, $p = 0.003$; $T\text{-}V_{high}\text{-}F_{low}$: $F = 13.27$, $p = 0.01$) but not in *H. splendens* (Supplementary material, **Fig. S1**).

Even though nitrogenase activity increased in all samples after the watering regimes had been applied for 30 days (e.g. in *T. delicatulum*, activity increased 437x in the treatments receiving high volume watering compared to the standard precipitation; **Fig. S1**), the precipitation treatments did not lead to any significant differences in nitrogenase activity (Fig. 3**; Table S2**). However, ethylene production rates associated with *T. delicatulum* were up to 4 times higher compared to rates associated with *H. splendens* ($p < 0.0001$; $F = 18.06$), except in the high volume, low frequency watering regime (Fig. 3).

Total nitrogen distribution

Total nitrogen (TN) was quantified to assess the transfer of N in the different parts of the experimental set-up (moss vs. leachate). For each treatment, TN of *T. delicatulum* was on average 2.11 times higher than that of *H. splendens* ($p < 0.0001$; Fig. 4**A; Table S3**). After standardization to the corresponding moss dry weight, leachate TN was two orders of magnitudes lower than TN detected in mosses (Fig. 4**B**). Increased watering volume lead to higher TN in leachate for both mosses ($p < 0.0001$; $F = 57.9$), except in the combination with low frequency watering. Here, average leachate TN (0.03 ± 0.02 mg/g moss DW) was only slightly higher than the leachate TN in the standard volume, low frequency for *H. splendens* (0.003 ± 0.002; Fig. 4**B**). Low frequency watering regimes generally decreased TN in leachates ($p = 0.05$; $F = 4.4$; Fig. 4**B; Table S4**). Unlike the moss TN pattern, leachate TN only showed significant differences between species when the volume and species factor were considered together, with higher leachate TN recorded for *T. delicatulum* samples under high watering volume. The highest average TN in leachate was recorded in the high volume, standard frequency regime for *T. delicatulum* (0.09±0.02 mg/g moss DW).

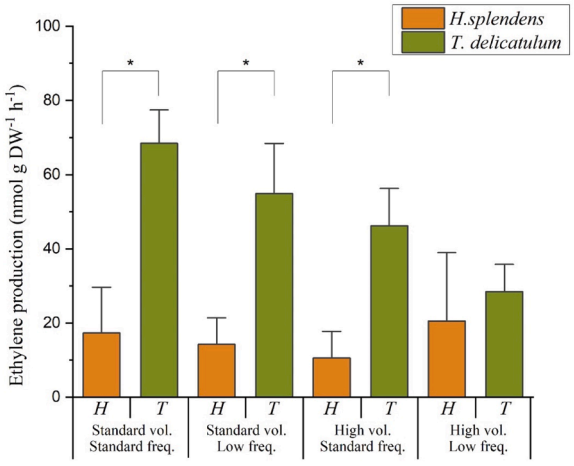


Fig. 3. The effects of watering regimes on nitrogen fixation rates (measured as ethylene production; nmol g dw^{-1} h^{-1}) in the two investigated mosses, *Hylocomium splendens* ("H", orange bars) and *Thuidium delicatulum* ("T", green bars) after the watering regimes were applied. Vol. and freq. are the abbreviations for volume and frequency, respectively. The asterisks above the bars indicate statistically significant differences between the species ($p < 0.05$). Shown are means ($n = 4$) ± error bars. The error bars represent standard errors.

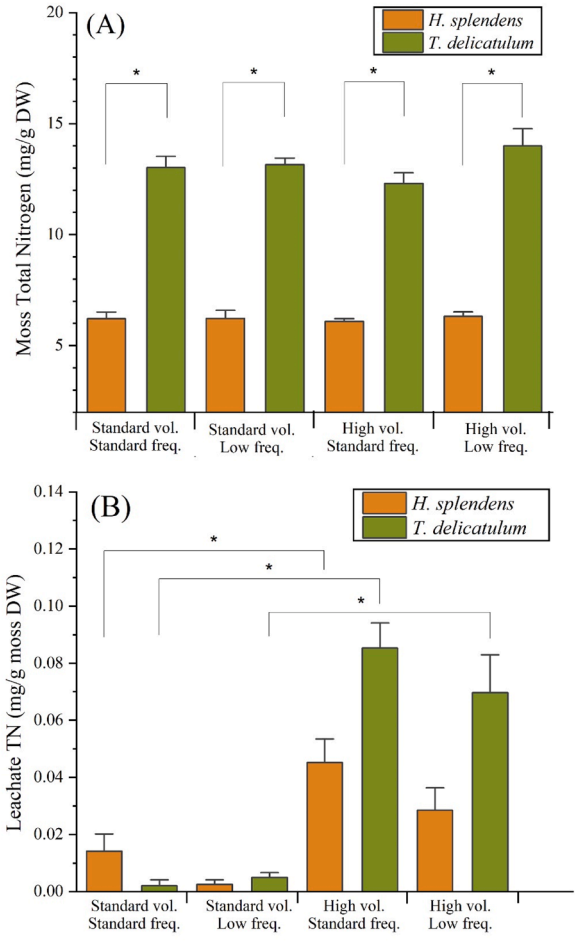


Fig. 4. Effects of watering regimes on Total Nitrogen (TN) in the two investigated mosses, *Hylocomium splendens* (orange bars) and *Thuidium delicatulum* (green bars). Moss TN (A); Leachate TN (B) Vol. and freq. are the abbreviations for volume and frequency, respectively. The asterisks above the bars indicate statistically significant differences between the treatments and species ($p < 0.05$). Shown are means ($n = 4$) ± error bars. The error bars represent standard errors.

Distribution of excess ^{15}N

Excess ^{15}N accumulation was tested to assess the effects of different watering regimes on the transfer of newly fixed N (Fig. 5). There was not enough ^{15}N content detected in the vermiculite samples. Neither moss ^{15}N nor leachate ^{15}N showed any significant differences between the watering treatments. No excess ^{15}N was found in the T-V_{high}-$F_{standard}$ and T-V_{high}-F_{low} treatments from both moss and leachate samples. Leachate ^{15}N constituted less than 1 % (ranging from 0.01 % to 0.71 %) of the total excess ^{15}N pool (Fig. 5). The highest share of excess ^{15}N was found under the H-$V_{standard}$-F_{low} treatment (0.71 %).

Correlation between ^{15}N fixation and ^{15}N in leachates

Linear regression analysis showed a positive and significant relation between excess ^{15}N in one of the investigated mosses, *H. splendens* and the excess ^{15}N in their corresponding leachates ($p = 0.0037$; $F = 12.1$; r^2=0.46), indicating that 46 % of the variation of the excess leachate ^{15}N can be explained by the excess moss ^{15}N (Fig. 6). On the other hand, *T. delicatulum* showed no significant relations between excess moss ^{15}N and excess leachate ^{15}N.

Redundancy analysis

To summarize the differences in moss species, watering frequency and volume on N_2 fixation (nitrogenase activity) and leachate, a redundancy analysis (RDA) was performed based on Euclidean distance between a subset of properties (TN of leachates and mosses, excess ^{15}N of mosses, N_2 fixation rates before and after the treatments), and the independent environmental variables, including watering frequency and volume, moss wet weight, and moss species (Fig. 7). The independent variables collectively explained 39 % of the total variation. Axes 1 and 2 in the graph explained 19 % and 18 % of the total variation, respectively. Leachate TN was positively correlated with watering volume. On the other hand, watering frequency explained less compared to volume but positively affected moss TN and N_2 fixation rates after the treatments. Moss species also affected the excess ^{15}N in mosses, moss TN and N_2 fixation, though it is difficult to disentangle the effects of species from the wet weight of moss samples before the treatments were applied.

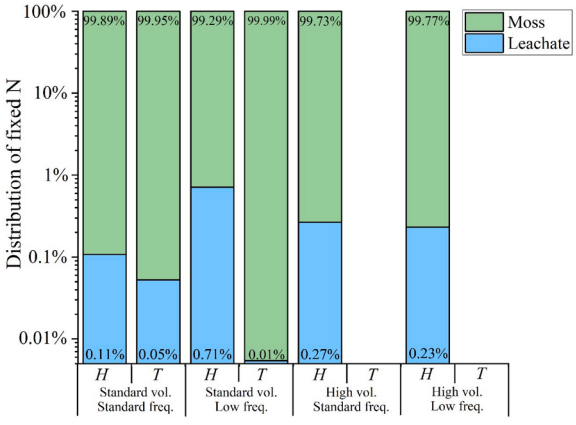


Fig. 5. The effects of watering regimes on the distribution of excess ^{15}N in moss (green) and leachates (blue). The percentages represent the share of ^{15}N in leachates and moss. Vol. and freq. are the abbreviations for volume and frequency, respectively. *H* and *T* represent *Hylocomium splendens* and *Thuidium delicatulum*, respectively. There was no excess 15 N found under the T-Vhigh-Fstandard and T-Vhigh-Flow treatments from both moss and leachate samples.

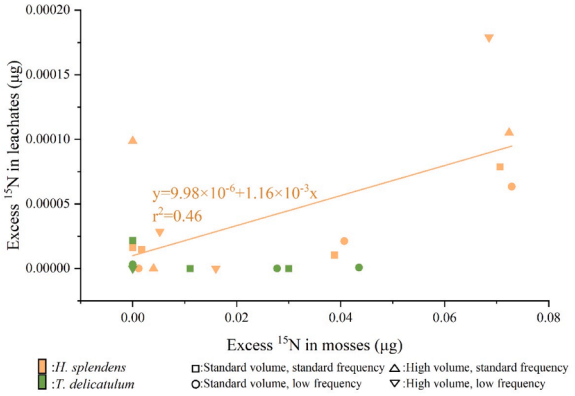


Fig. 6. Linear regression analysis between excess ^{15}N in the mosses (*Hylocomium splendens*), (yellow), *Thuidium delicatulum* (green) and the corresponding leachates. Trendline is given when a significant relationship was found.

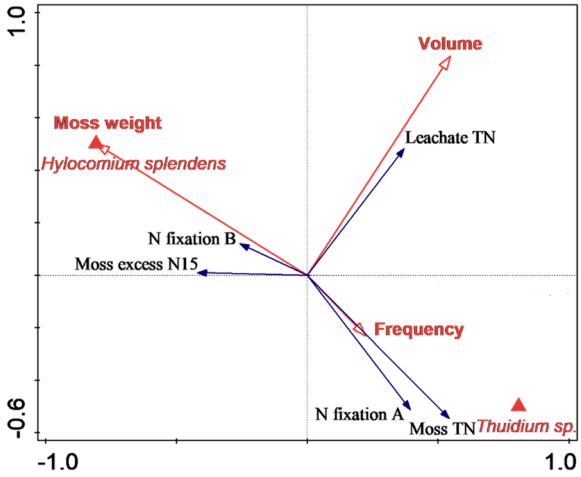


Fig. 7. Redundancy analysis based on Euclidean distance between sample properties including N_2 fixation (nitrogenase activity), TN, ^{15}N (blue arrows) and the environmental variables including watering volume, frequency, moss weight before experiments and moss species (red arrows and triangles). N_2 fixation B stands for N_2 fixation before the treatments and N_2 fixation A stands for N_2 fixation after the treatments.

Discussion

The effects of watering regimes on N_2 fixation

Nitrogen fixation in moss-cyanobacteria associations increased in all watering regimes with averages ranging from 5 to 438 times during the 30-day experiment. Even though N_2 fixation rates increased dramatically in *T. delicatulum* in the high-volume, standard frequency precipitation over the course of the experiment, there were no significant differences in N_2 fixation rates between the experimental watering regimes. Hence, we cannot confirm H1 (N_2 fixation rates are higher under high volume precipitation).

Nitrogen fixation performed by cyanobacteria and moss metabolisms require a moist environment that is closely related to rainfall volume and frequency. However, our results showed little effects of different precipitation events on N_2 fixation rates in mosses. Rather, standard precipitation led to slightly higher N_2 fixation rates (in *T. delicatulum*) or had no effects at all (in *H. splendens*). This is in contrast with previous results that identified rainfall volume as an important factor in controlling N_2 fixation in moss-cyanobacteria associations (Gundale et al., 2012; Li et al., 2018; Peter et al., 2002). The lack of a treatment effect in

our experiment may be due to the fact that the standard precipitation (volume and frequency) maintained moss moisture at least as well as the high volume precipitation, or that the high volume watering regime may have been above the suggested threshold of moisture content of mosses (below).

Our experimental set-up kept the mosses moist for several days which may have limited the (negative) effects of lower watering frequency and volume on moss water content and thereby, associated N_2 fixation. On the other hand, higher precipitation frequency with less volume may not have been enough to hydrate the entire moss tissue and the vermiculites for a long period, leading to a faster water loss. By maintaining high water content within the moss in all precipitation regimes, moss photosynthesis was likely kept high and could support cyanobacterial N_2 fixation by providing energy and carbohydrates (Li et al., 2018). Previous studies also showed that, by increasing water content from 50 % to 200 % of dry weight, nitrogenase activity of moss-associated cyanobacteria was enhanced in four investigated moss species (Fan et al., 2022). However, there is likely a threshold of moss-water content above which N_2 fixation, and photosynthesis, may be reduced or inhibited. Fan et al. (2022) suggested that there is an optimal water content for mosses (around 130 % - 190 % dry weight) for maximizing nitrogenase activity. The substrate used in our study (vermiculites) has a relatively high saturated hydraulic conductivity (6×10^{-4} m/s) compared to silt (10^{-9}–10^{-5} m/s) or clay soil ($<10^{-9}$ m/s) (Vijayaraghavan & Badavane, 2017), enabling leachates to rapidly pass through the substance in seconds, which should have created ideal near-saturated condition for N_2 fixation in moss-cyanobacteria associations.

We found significant differences between moss species in N_2 fixation rates after the watering regimes were applied. Even though N_2 fixation of both species increased during the one-month experiment, a much larger increase was observed in *T. delicatulum* compared to *H. splendens*. The most likely explanation for the observed differences in N_2 fixation between the moss species could be that N accumulated in *T. delicatulum* - collected from a high N deposition area - was removed through the watering during the experiment. It has been shown that N_2 fixation rates in *H. splendens* and *P. schreberi* commonly decreases with increasing N deposition (Rousk et al. 2023), and can be suppressed at N deposition rates as low as 3 kg ha^{-1} $year^{-1}$ (Salemaa et al., 2019). However, N_2 fixation on mosses can recover from N stress after removal of the stressor (Rousk et al., 2014a; Wang et al., 2022).

The effects of watering regimes on N transfer

A few ^{15}N tracing studies have been conducted to elucidate how fixed N of moss-cyanobacteria associations is leached into the soil as well as the factors influencing this process. In a ^{15}N-NH_4^+ labeling study in a boreal forest, the common feather moss *P. schreberi* retained most of added ^{15}N at least for one year, along an N_2 fixation gradient, while no excess ^{15}N was detected in the soil beneath the moss (Rousk et al., 2014b). *Sphagnum* sp. mosses also retained most fixed N five weeks after ^{15}N-N_2 labeling (Rousk et al., 2016). DeLuca et al., (2022) also could not find traces of fixed N in the O horizon of boreal soil until 5 years after ^{15}N-N_2 labeling on *P. schreberi* dominated moss layers. In contrast, another study found that although the moss layers acts as the primary sink for the first year, it developed into an N source losing 23 % fixed N in the second year in a heathland system in Germany (Friedrich et al., 2011). After N_2 fixation, the N stored in the senescent moss tissues could eventually be accessed by mycorrhizal fungi as organic N or leach to the soil after disturbances like drying-wetting cycles. Our results support the majority of these studies and the first part of our second hypothesis (H2), demonstrating that the fixed N is mainly stored in the moss in the short term, with less than 1 % leached within a month, regardless of the differences in rainfall treatments and moss species (Fig. 5).

The ability to conserve fixed N_2 in moss tissues might result from the non-vascular morphology of mosses. Since mosses mainly access N from atmospheric deposition and potentially from associated N_2 fixation, instead of uptake from soil, many species developed nutrient retention mechanisms to prevent N loss. For instance, *Dicranum majus, H. splendens, Actinothuidium hookeri* and *Sphagnum* sp. mosses can redistribute N from senescing moss tissues to young tissues (Bakken, 1995; Liu et al., 2020). A 2-year study found that 78 % of the moss N pool was recycled from senescing brown segments of *H. splendens* (Eckstein, 2000). Also, *H. splendens* has an estimated ramet life span of three years, which is close to the leaf life span of evergreen shrubs, the long segment life span and the high N retention ratio seems to allow the moss to accommodate chronically nutrient-poor habitats, while at the same time, prevent N from leaching (Aerts & Peijl, 1993; Eckstein, 2000).

The second part of our second hypothesis (N leaching is increased by higher watering frequency) could not be confirmed since the effects of watering frequency on N transfer were not clear (Fig. 4**B**). Perhaps the moss samples did not experience complete desiccation in this study due to the experimental set-up stated above. This way, the effect of rehydration of moss-cyanobacteria associations on nutrient release was mediated. Nonetheless, there might be a watering frequency threshold controlling rehydration from desiccation state for specific moss species and, only when the watering frequency is below the threshold, a drying-wetting process is initiated. The frequency threshold may also be linked to moss morphology, density, and the substrate properties like soil hydraulic conductivity, which determines the infiltration process and soil water holding capacity, and eventually the evaporation rate. In other words, the drying-wetting disturbance introduced by watering frequency was not severe enough to stimulate substantial N leaching.

Unlike moss excess ^{15}N, moss TN was positively related to N_2 fixation rates in *T. delicatulum* only after the treatments (Fig. 7). This suggests that the excess ^{15}N indicates the distribution of newly fixed N, while TN in mosses and leachates also includes fixed N_2 before the experiment started, in addition to increased N_2 fixation during the experiment. The increased N leaching by high volume precipitation could be the result from over-saturation of moss tissue, therefore any previously fixed N is lost with the excess water. The results indicate that the newly fixed N is probably less prone to leaching compared to the previously fixed N, at least in *T. delicatulum* (see below). Still, leaching of N from mosses can be potentially enhanced in the long term via increased cyanobacterial N_2 fixation, which is further controlled by precipitation volume.

Differences in N transfer between moss species as a result of different precipitation regimes

To date there have been limited efforts to investigate the short-term fate of fixed N in different mosses under different precipitation regimes. We found that higher TN leaching was associated with higher precipitation volume for both species (Fig. 7), indicating that precipitation volume may be an important factor for mobilizing the moss' N pool. Only under high volume watering treatments was N leaching from *T. delicatulum* considerably higher than the N leaching from *H. splendens*. Thus, our third hypothesis could not be confirmed – *H. splendens* from the N-limited site did not have higher N_2 fixation rates, and did not leach less N compared to the tropical moss. However, excess ^{15}N in leachate and in moss were linked in *H. splendens*, indicating a link between N_2 fixation rates (higher ^{15}N values) and the potential to lose the newly fixed N. This suggests the moss does not use the newly fixed N_2, even though one would have expected a more efficient N resorption and recycling strategy to prevent N loss compared to the tropical counterpart *T. delicatulum*, originating from a more N saturated habitat. In contrast, the lack of a link between excess ^{15}N in moss and leachates for *T. delicatulum*, and the higher loss of N - but less excess ^{15}N - suggests that *T. delicatulum* might have a more efficient strategy retaining the newly-fixed N but not the historically accumulated N in moss tissues. Considering that no significant differences of excess ^{15}N were found between the watering treatments, our results suggests that the fixed N_2 could be leached from the surface of the moss during longer

precipitation events before it can be used by the mosses. As it is gradually taken up by the mosses, ^{15}N is incorporated into moss tissue and therefore was not susceptible to leaching after precipitation. In addition, the higher moss TN of *T. delicatulum* likely results from the originally higher moss N pool from tropical cloud forests. Total N of *T. delicatulum* (10.3 mg/g dry weight) from the same sampling site is double that of *H. splendens* (5.72±0.28 mg/g dry weight). Hence, while there is potential to leach more N from *T. delicatulum*, this did not happen in our study possibly because the wetting-drying events in our experiment were minor disturbances for the moss-cyanobacteria association and did not trigger cell membrane damage, which is believed to be one of the mechanisms causing N leaching. However, investigating additional moss species from contrasting ecosystems in terms of climate and N availability would help shed light on these questions.

Climate change will lead to increased precipitation in northern ecosystems, and higher temperatures across the globe, which may facilitate N leaching by promoting both, N_2 fixation and decomposition of senescent tissue (Eckstein, 2000). Hence, high volume watering could promote moss growth and increase N_2 fixation rates, which eventually will contribute to increase the soil N pool since disturbance and higher precipitation could lead to N release from the moss carpet. However, current climate models project high spatial variations of precipitation change across the globe, with a 50 % increase of precipitation in the Arctic, and a decrease of precipitation in Central America by 10–40 % under the SSP5–8.5 scenario (fossil-fueled development) (Almazroui et al., 2021). As our results suggest that N leaching from *T. delicatulum* is more sensitive to the change of precipitation volume, areas colonized by *T. delicatulum* in tropical montane forests could experience less N input to the soils. On the other hand, though less prone to N leaching, *H. splendens* colonized areas in subarctic regions may eventually receive a greater N input to the soils as consequence of increased precipitation volume and temperature.

Conclusion

Understanding the transfer of fixed N by moss-cyanobacteria associations is of great interest since it can reflect on how much N is potentially readily available for soil nutrient cycling. While watering regimes had little impact on moss-associated N_2 fixation, N_2 fixation rates increased dramatically over time with high volume precipitation (5 times of standard volume) for *T. delicatulum*, which is likely due to the removal of N, and an increase in moisture levels. This translated into a larger loss of N from the tropical species. Nitrogen fixation associated with *H. splendens*, on the other hand, was relatively low and stable across time and watering regimes, suggesting that the background ecosystems' N deposition and the moss species may be crucial factors determining the moss-associated N pool. Independently of the moss species, newly fixed N as well as N stored in mosses is not easily leached but remains within the moss tissue. However, larger volumes, but not higher frequency, of precipitation have the potential to release some of the stored N into the environment. In the context of rapid climate change, N_2 fixation and N loss from moss-cyanobacteria association can be expected to change with the current projections indicating a large shift in precipitation by the end of this century, especially in northern regions, which will affect the moss-N_2 fixation pathway and thereby, soil fertility.

CRediT authorship contribution statement

Song Guo: Conceptualization, Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft. **Lina Avila Clasen:** Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Kathrin Rousk:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, 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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.baae.2024.04.009.

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