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Nitrogen fixation in Arctic lichens and mosses: A survey across circumpolar subzones

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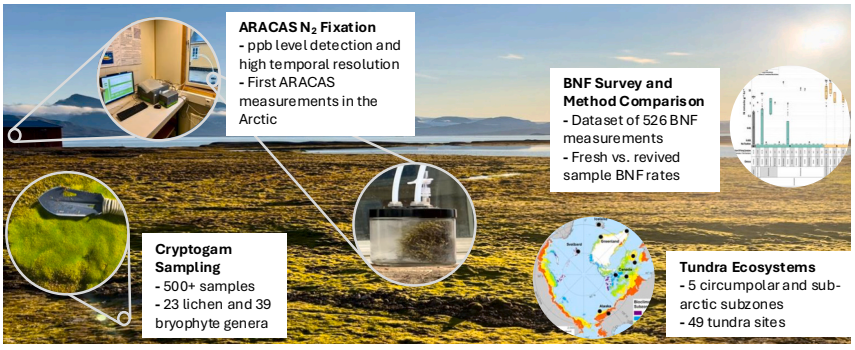
HIGHLIGHTS

- Most extensive dataset of Arctic-wide BNF rates emphasizing the variability between in situ and laboratory BNF conditions
- Current models may underrepresent lichen BNF rates and over-rely on stress-tolerant bryophyte genera
- High-subzone cryptogams may have the capacity to increase their in situ fixation rates as Arctic conditions change

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrogen bioavailability frequently constrains primary production in the Arctic with tundra communities vulnerable to ecological and metabolic disruption from climate variability. Diazotrophs associated with lichens and mosses are the primary source of new nitrogen (N) in the Arctic. We made 526 laboratory measurements of biological nitrogen fixation (BNF) in 272 lichens and 254 bryophytes representing 23 and 39 genera, respectively. These samples were collected from 49 tundra sites across the Arctic. We found 65 % of lichen and 44 % of bryophyte genera analyzed fixed N. We also identified potentially new cryptogam-diazotroph relationships in the lichen genera *Asahinea*, *Nephromopsis* and *Thamnolia* and the bryophyte genera *Dicranoweisia* and *Amphidium*. We found that while over 95 % of individual bryophyte samples fixed N within three months of storage, this dropped to less than 15 % after one year at room temperature. Individual lichen samples maintained a stable ~45 %

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fixation rate over nearly two years of frozen storage. Our experiments highlight the complexity in establishing robust BNF measurements required for model simulations.

1. Introduction

Through associations with diazotrophic archaea and bacteria, cryptogamic covers, including bryophytes and lichens, support Arctic tundra communities by providing a crucial source of bioavailable nitrogen (N), a macronutrient that frequently limits primary production in the Arctic (Rousk et al., 2021). Extreme swings in Arctic conditions (Jacobs et al., 2021; Rousk et al., 2018) can alter nutrient availability, as respiration and photosynthesis respond in tandem to maintain equilibrium (Crous et al., 2022). The Arctic's role in global biogeochemical cycling depends in part on the biological nitrogen fixation (BNF) capacity of diazotrophs in association with Arctic cryptogams and their ability to respond to changes in their environment (De Sisto and MacDougall, 2024).

Already, Arctic greening is driving new species of vascular plants to establish at higher latitudes (Hupperts et al., 2021). Some plants, such as shrubs, shade cryptogams and alter established nutrient cycling processes. With 50 % of the world's underground organic carbon (C) stored in Arctic permafrost, cryptogamic covers are important insulators that reduce permafrost degradation and C release while promoting native insulating vegetation through N cycling (Rousk et al., 2016; Schuuring et al., 2024). Climate variability increases the importance of understanding the effect of biotic and abiotic factors on BNF (Rousk et al., 2018). For example, Rousk and Michelsen (2017) found that birch litter, which has a low N content, increased BNF, while willow litter, which has a comparably higher N content, decreased BNF. Combined with changes in temperature and precipitation patterns, these effects will shift the circumpolar bioclimate subzones northward and change each subzone's geographic location and surface area, affecting the native species that reside in each subzone and their impact on local nutrient cycles (Walker et al., 2017).

Diazotrophs have evolved a variety of life strategies to occupy diverse environmental niches. In terrestrial Arctic environments, they are found free-living in soil, in biological crusts, in the root nodules of legumes and in associations with various lichens and bryophytes (mosses, liverworts and hornworts) (Rousk et al., 2016). Lichens are symbiotic organisms formed from at least one fungus and one photoautotroph (algae or cyanobacteria). For most lichens, the fungus forms the main structural body, called a thallus. In symbiosis with the fungus, there can be three photoautotrophic associations: (1) chlorolichens consisting only of green algae to fix C, (2) cyanolichens containing solely cyanobacteria to fix C and N, or (3) tri-membered lichens having both green algae to fix C (as the main or co-primary photoautotroph) and cyanobacteria to fix N (usually restricted to small structures called cephalodia) and, occasionally, to fix C (as the co-primary photoautotroph) (Almendras et al., 2018; Henskens et al., 2012). Bryophytes, on the other hand, are a group of land plants (embryophytes) that often form associations with diazotrophs that live endophytically (*e.g.*, within most thallose hornworts) or epiphytically (*e.g.*, on surfaces of liverworts or mosses) (Chen and Nelson, 2022; Meeks, 1998; Sprent and Meeks, 2013).

While various studies have measured BNF rates of Arctic cryptogams, none have reported Arctic-wide BNF rates, especially from both lichen and bryophyte genera. Furthermore, current regional and global models that incorporate BNF rates do not distinguish field (fresh) and laboratory-simulated measurements (using rehydrated samples collected within a few days to weeks), even though it is unclear whether fresh and rehydrated rates are comparable. It is important to determine whether we should distinguish between BNF rates obtained from fresh *versus* rehydrated specimens to more accurately predict changes to Arctic biogeochemical cycling.

In this study, we present a pan-Arctic survey of cryptogam BNF capacity based on 526 field- and laboratory-measured BNF rates from Arctic and sub-Arctic cryptogam samples collected from 49 tundra sites across Norway, Iceland, Greenland, Canada and the United States (Fig. 1). To our knowledge, this represents the largest single survey of Arctic cryptogams for BNF analysis. We developed our sampling and experimental design to test the hypotheses that:

(**H1**) The BNF capacity from multiple measurements of a cryptogam genus matches the BNF capacity of that genus from other circumpolar bioclimate subzones when measured at the same temperature.

(**H2**) Fresh cryptogam BNF rates are equal to rehydrated cryptogam rates under simulated in situ conditions.

To address these hypotheses we used a lightweight, mobile method (Cassar et al., 2012) to measure BNF in Arctic cryptogams at a field station with samples collected within 8 h, followed by BNF measurements of the same samples after our return to our home laboratory at Duke University (Durham, NC, USA). In conjunction with other samples, this allowed us to assess Arctic BNF capacity in dozens of cryptogam genera and identify potentially new cryptogam-diazotroph relationships that have not been reported to fix N.

2. Methods

2.1. Sample subzones and sample types

To facilitate analysis of BNF rates from similar ecological areas of the Arctic, we subdivided our observations into circumpolar bioclimate subzones as defined by Walker et al. (2017), who group samples by their vegetation profile and Mean July Temperature (MJT, Table 1). Specifically, we surveyed subzones B, C, D, and E, as well the sub-Arctic (S) (Table 2).

We used these subzones to conduct a broad survey of cryptogam BNF

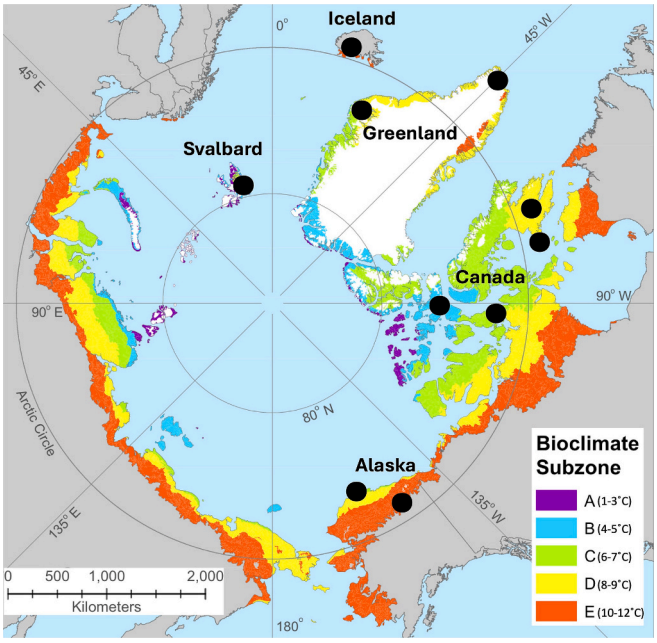

Fig. 1. Arctic and sub-Arctic collection sites used in this study (adapted from Walker et al., 2017). Sample sites are compressed for clarity: 17 sites in Alaska, 5 sites in Canada, 7 sites in Greenland, 11 sites in Iceland and 9 sites in Svalbard. Temperatures represent Mean July Temperature (MJT) for each respective subzone.

Table 1
Circumpolar bioclimate subzones defined by Walker et al. (2017) with an additional sub-Arctic (S) subzone. MJT stands for Mean July Temperature, a common measure of average peak summer temperatures.

Subzone	MJT	Vegetation profile
A	1–3 °C	Mostly barren. In favorable microsites, 1 lichen or moss layer <2 cm tall, very scattered vascular plants hardly exceeding the moss layer.
B	4–5 °C	2 vegetative layers, moss layer 1–3 cm thick and herbaceous layer, 5–10 cm tall, prostrate dwarf shrubs <5 cm tall.
C	6–7 °C	2 layers, moss layer 3–5 cm thick and herbaceous layer 5–10 cm tall, prostrate and hemiprostrate dwarf shrubs <15 cm tall.
D	8–9 °C	2 layers, moss layer 5–10 cm thick and herbaceous and erect dwarf-shrub layer 10–40 cm tall.
E	10–12 °C	2–3 layers, moss layer 5–10 cm thick, herbaceous/erect dwarf-shrub layer 20–50 cm tall, sometimes with low-shrub layer to 80 cm.
S	13–15 °C	Moss layer often >10 cm thick, dense herbaceous and shrub layers, tall shrubs and occasional trees.

Table 2
Collection sites with corresponding circumpolar bioclimate subzones used in this study.

Collection site	Subzone range
Alaska	C – E
Canada	B – D
Greenland	C – D
Iceland	S
Svalbard	B – C

rates across the Arctic (H1). Using a subset of these samples, we also analyzed the effect of rehydration on BNF (H2). Addressing these questions required different sample types defined by various collection and storage methods (Table 3).

The differences between these three groups is outlined in more detail in subsections 2.2–2.3. Briefly, fresh samples refer to 2023 Svalbard samples measured in Ny-Ålesund, Svalbard within 8 h of collection. Rehydrated samples refer to 2023 Svalbard samples that were first measured in Svalbard then air dried, shipped to Duke University and upon arrival immediately rehydrated and measured. Finally, stored samples refer to all other samples that were collected in the field, air dried, shipped to Duke University and upon arrival stored for at least three months before being reactivated and measured.

2.2. Sample collection

Using a serrated trowel, we collected 10 cm-by-10 cm patches of cryptogamic cover from multiple locations within each field site to minimize disturbance to native tundra flora and fauna following polar fieldwork guidelines (Lønstrup Frendrup et al., 2021). While most genera were sampled with replicates from nearby tundra sites, we were only able to collect one sample of a few rare genera (Figs. 2–3).

We selected samples that (1) reflected the diversity of genera at the collection site, (2) represented the prevalence (total ground cover) of each genus, and (3) were homogenous (contained almost exclusively one genus). We took care to remove as much of the underlying material as possible while keeping the thallus or gametophytes intact. However, crustose lichen samples were usually growing on bryophytes or soil. While most of these samples consisted of a crustose lichen, it was impossible to totally separate the crustose thallus from the underlying material (bryophytes or soil). Finally, while crustose lichens were not identified to the family or genus rank, they were all chlorolichens. Because of this, we have clustered crustose lichens adjacent to the more taxonomically-defined chlorolichens life form group (Fig. 2).

We then transported samples to a field station in a paper bag (stored samples) or plastic bag (fresh and rehydrated) to preserve in situ moisture and more accurately capture in situ BNF capacity (Hupperts et al., 2021). At the field station, we measured the BNF rates of fresh samples (see Sections 2.3–2.4) before they were air dried and individually wrapped in paper bags for shipment. Stored samples were air dried then wrapped immediately after collection.

Once the samples arrived at Duke University, they were identified to the genus level based on morphology, where possible (a few samples were only identified to the family or life form level). Stored bryophyte samples were kept at room temperature (Akther and Rousk, 2019) whereas stored lichen samples were wrapped in a plastic moisture barrier and stored at −20 °C (Bidussi et al., 2013; Colesie et al., 2014, 2018). This difference reflects storage procedures of previous BNF studies of lichens and bryophytes. Finally, we measured the dry weight (Mettler PM200, ± 0.001 g) of all cryptogam samples prior to rehydration, since subsequent experiments prohibited oven-drying the samples after BNF measurements. Vouchers of each cryptogam genus are stored in the Duke University Herbarium.

2.3. Reactivation and rehydration for BNF measurements

Following previously-published protocols (Akther and Rousk, 2019; Colesie et al., 2014, 2018), we reactivated stored lichen samples by transferring them to a Percival climate chamber (Model AL36L4C9) set to 0 °C (± 0.5 °C) and spraying them with Milli-Q water (17.5 MΩ-cm) until saturated. Gradually, we increased the temperature to 10 °C while introducing light up to 45 μmol s^{-1} m^{-2} (LI-250 A with Q 48417 photometer, ± 0.6 % and ± 0.03 μmol s^{-1} m^{-2}) and gently spraying them with Milli-Q water (Bidussi et al., 2013; Permin et al., 2022). We then moved stored or rehydrated lichen samples to an acclimation climate chamber (EGC, M Series Walk-in) in the Duke Phytotron set to a 12-h day/night cycle with peak light intensity of 145 μmol s^{-1} m^{-2} (full spectrum), 80 % (± 5 %) relative humidity (RH) and 10 °C (± 0.5 °C). We moved stored or rehydrated bryophyte samples directly to the acclimation climate chamber on paper towels after they were soaked until saturated (in order to reach the thick inner layers of gametophytes) in Type II filtered water (≥ 1 MΩ-cm). All cryptogam samples in the acclimation climate chamber were sprayed three times a day with Type II filtered water. Previous laboratory experiments, especially with lichens, have found that matching daily humidity and temperature cycles (diel cycles) is important for the longevity and health of lichen and bryophyte samples in the laboratory (Baldauf et al., 2021; Inoue et al., 2014; Pearson and Benson, 1977).

2.4. Nitrogen fixation measurements

To measure BNF in our samples, we used ARACAS (Acetylene Reduction Assays by Cavity ring down laser Absorption Spectroscopy, Cassar et al., 2012). ARACAS uses an ethylene-sensitive cavity ring-down spectrometer with a high temporal resolution that allows for continuous BNF measurements with time intervals on the order of

Table 3
The two analyses conducted in this study with reference to the sample types: fresh, rehydrated and stored.

Hypothesis	Analysis	Sample types	Measurement location	Temperature	Water content
H1	BNF survey	Rehydrated and stored	Duke University	11 °C	Sprayed
H2	Effect of rehydration	Fresh	Svalbard	In situ (11 °C)	In situ
		Rehydrated	Duke University	11 °C	Sprayed

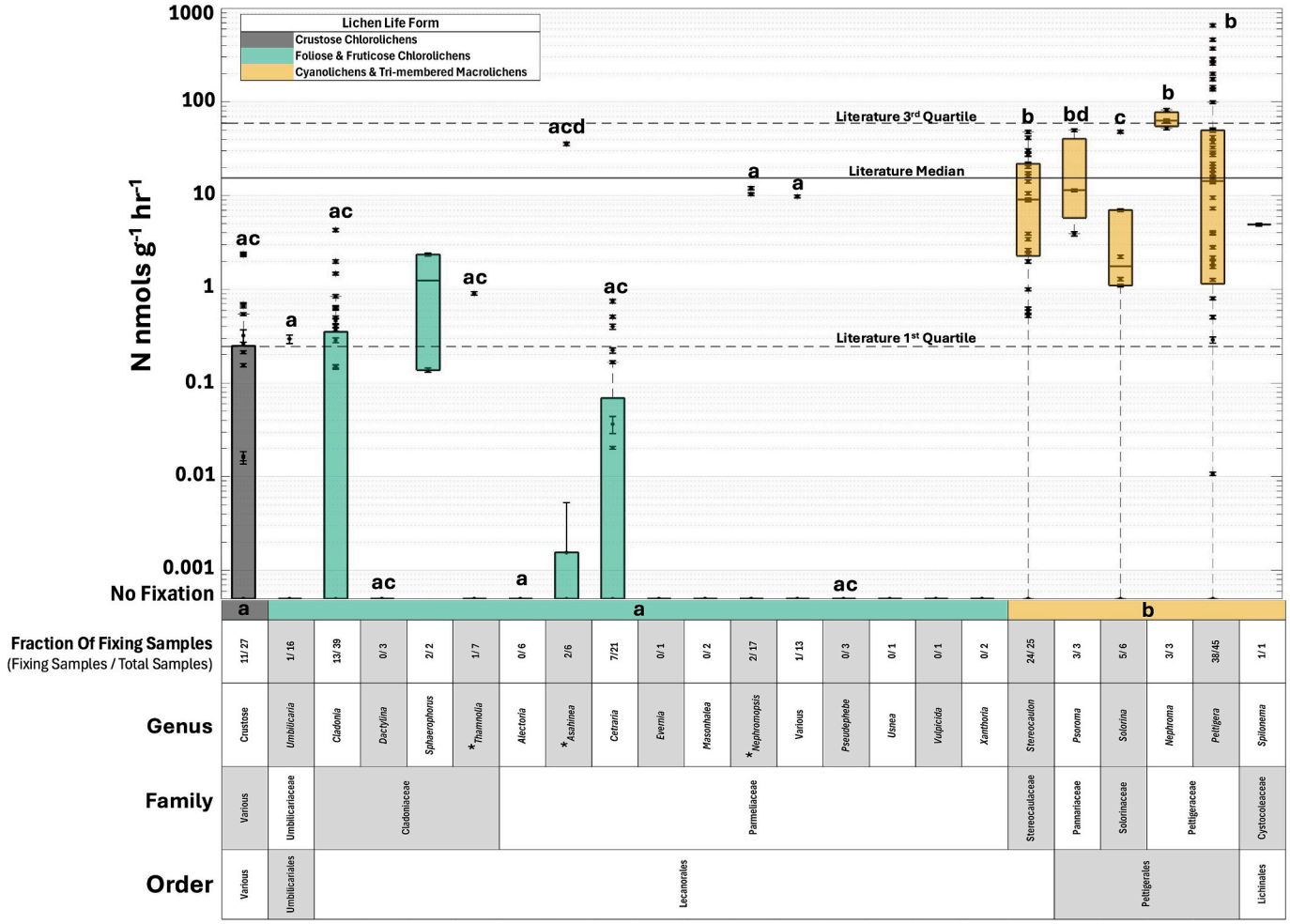

Fig. 2. Comparison of BNF rates among lichens. Letters (a, b, c, d) signify a significant difference (p-value <0.05) in BNF rates among genera or lichen life forms as the result of a Kruskal-Wallis test followed by a Dunn's post-hoc test. Genera without letters had fewer than three samples and were excluded. Asterisks indicate potentially new lichen-diazotroph interactions, where at least one sample was detected to fix N. Samples aligned with the "No Fixation" label did not fix N and were assigned an arbitrarily small positive rate (smaller than any measured rate) in order to plot on the logarithmic y-axis. Literature median and quartile reference lines were generated from previously published comparable (per dry weight) BNF measurements (Antoine, 2004; Crittenden et al., 2023).

seconds (~1.8 s) as opposed to hours or days for traditional acetylene reduction assays (ARA) using Flame Ionization Detector Gas Chromatography (GC-FID) (Vitousek, 1994). In addition, ARACAS has 1,000 times the sensitivity of traditional ARAs and is easily portable. This technique has been used to successfully measure BNF in cryptogams (Jean et al., 2012) and vascular plants (Bytnerowicz et al., 2019).

To prepare samples for ARACAS, we first measured their wet weight then sealed the samples in a glass jar with an air-tight lid containing a septum system with three ports: one for acetylene injection, one for airflow to the spectrometer (Picarro model G2106) and one for return airflow *via* a pump (Picarro model A0702-V). We then replaced 15 % (90 mL) of the system's gas volume (~0.6 L) by injecting acetylene produced from the reaction between calcium carbide pellets and Milli-Q water (17.5 MΩ-cm) into the head space (Cassar et al., 2012). To measure the fresh samples, we placed the glass jar outside the field station to replicate the in situ light field and temperature. To measure the rehydrated and stored samples at Duke University, we placed the glass jar within a separate test climate chamber (EGC, M Series Walk-in) simulating the in situ Svalbard conditions (~150 μmol s^{-1} m^{-2}, 10 °C and 80 % RH) (Gebauer et al., 1998). Finally, we accounted for a slight greenhouse effect that warmed the incubation jar 1.22 °C above the climate chamber (measured with a Govee thermohygrometer, ± 0.3 °C). We chose to make all BNF measurements at Duke University at 11 °C (after accounting for the offset) in order to use the rehydrated samples and the stored samples in our survey of BNF capacity and assess the effect of storage on BNF. While 11 °C is warm for the Arctic, it falls within the upper bound (10°-12 °C) of normally defined Arctic MJT, allowing us to measure BNF capacity on the edge of the Arctic while aligning with previous BNF studies (Akther and Rousk, 2019).

We used a minimum of 15 % acetylene to fully saturate the Mo-Nase and V-Nase enzymes; otherwise the amount of acetylene could become limiting and artificially lower BNF rates (Darnajoux et al., 2022). We confirmed nitrogenase saturation at 15 % acetylene by partially replicating experiments performed by Darnajoux et al. (2022). Using the same sample, we found no change in the rates of ethylene production for 10 %, 15 % and 20 % acetylene. In addition, to control for the natural production of ethylene by cryptogam samples, we measured ethylene production prior to acetylene injection under the same conditions. We then subtracted background ethylene production rates from ethylene production rates during sample incubation under 15 % acetylene. We converted this net rate of ethylene production to an equivalent BNF rate using the R Ratio, which describes the conversion factor between the reduction of acetylene to ethylene and the reduction of N_2 to ammonia. Mo-Nase has a theoretical R Ratio of 3:1–4:1 while alternative nitrogenases like V-Nase have lower theoretical R Ratios (2.4:1) (Darnajoux et al., 2022). To account for this range, we used an R Ratio of 3:1 in our calculations as recommended by Darnajoux et al. (2022).

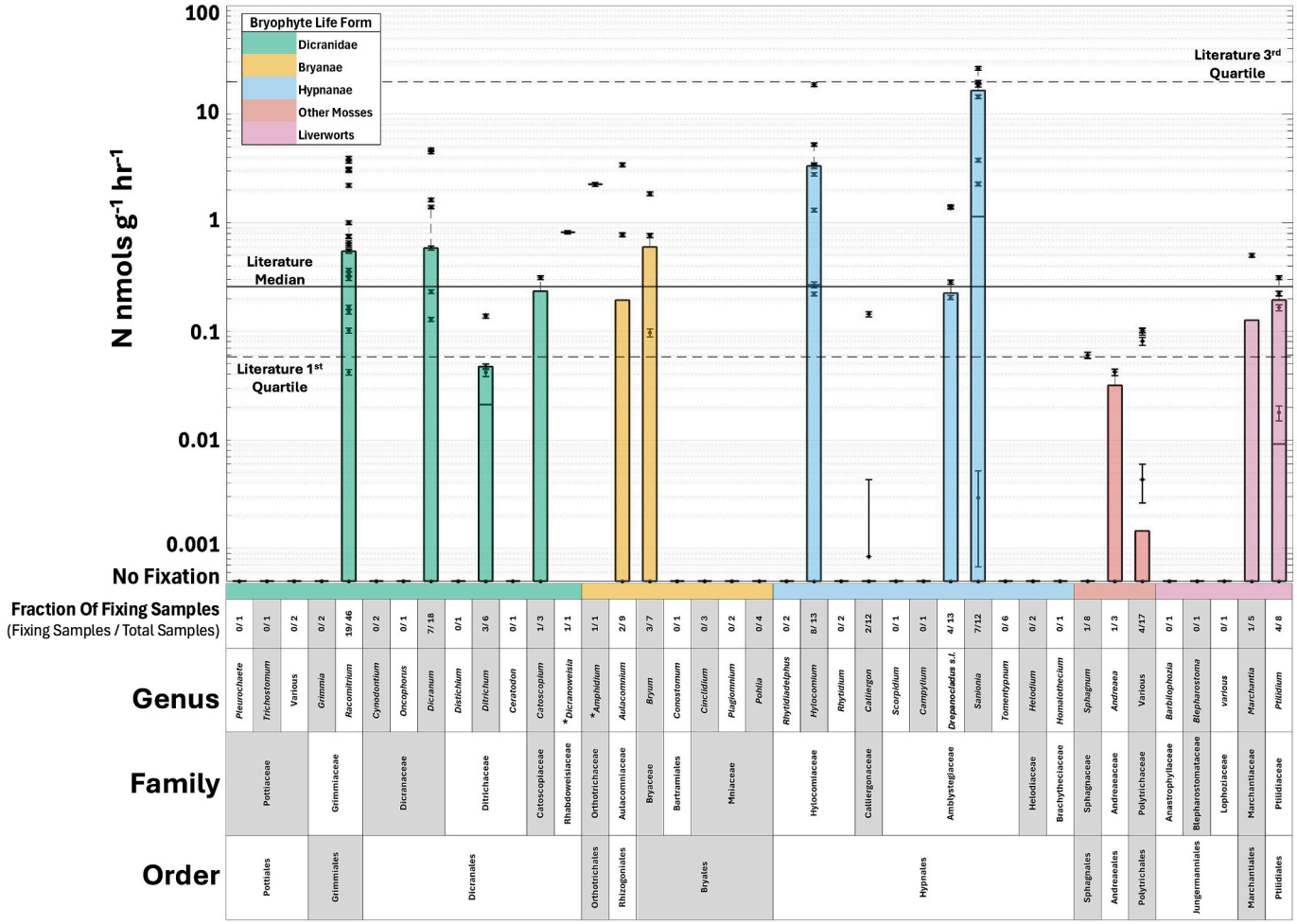

Fig. 3. Comparison of BNF rates among bryophytes. There were no significant differences (p-values >0.05) in BNF rates among genera or bryophyte life forms. Asterisks indicate potentially new bryophyte-diazotroph interactions, where at least one sample was detected to fix N. Samples aligned with the "No Fixation" label did not fix N and were assigned an arbitrarily small positive rate (smaller than any measured rate) in order to plot on the logarithmic y-axis. Literature median and quartile reference lines were generated from previously published comparable (per dry weight) BNF measurements (Akther and Rousk, 2019; Alvarenga and Rousk, 2021; Liu and Rousk, 2022).

2.5. Analysis and uncertainty

We used MATLAB (version R2024b) for all data processing, statistical analyses and figure preparation. ARACAS returns ethylene concentrations which we converted to equivalent N production using Eq. 1 adapted from Cassar et al. (2012):

$$N = 2^{*}\frac{(mC_2H_4 - bC_2H_4)^{*}V^{*}P}{R^{*}T^{*}R\ Ratio^{*}W_{Dry}}$$

where 2 refers to the 2:1 conversion of N:N_2, mC_2H_4 is the *measured* ethylene buildup rate in ppb(v) hour^{-1} (± 3 %) during the acetylene injection, bC_2H_4 is the *background* ethylene buildup rate in ppb(v) hour^{-1} (± 3 %) before the acetylene injection, V is the total volume of the chamber in liters (± 2 %), P is the pressure of the system in atmospheres (± 0.7 %) at the beginning of the experiment, R is the gas constant in L atm mol^{-1} K^{-1}, T is the temperature in °K (± 0.05 K) at the beginning of the experiment, R *Ratio* is the ethylene to N_2 conversion factor and W_{Dry} is the dry weight of the sample in grams (± 0.001 g). This yields a BNF rate in nmols g^{-1} h^{-1} of N.

Typical of closed-loop systems (Bytnerowicz et al., 2019; Jean et al., 2012), experiments with ARACAS suggested there was a small leak of ~5 ppb(v) hr^{-1} during the BNF measurements. A leak of this magnitude would normally go undetected with a ppm-level ARA method. Additional experiments at multiple ethylene concentrations suggested the leak was first order (*i.e.*, concentration dependent) governed by Eq. 2:

$$C(t) = C_{ext} + (C_0 - C_{ext})e^{-kt}$$

where $C(t)$ is the ethylene concentration inside the chamber in ppb(v), C_0 is the ethylene concentration inside the chamber at the beginning of the experiment in ppb(v), C_{ext} is the ethylene concentration outside the chamber in ppb(v), k is the leak constant in hr^{-1} and t is the time in hours. Using Eq. 2, which assumes no active ethylene production, we calculated the average expected loss of ethylene due to the leak over the course of the *background* and *measured* components and added these to bC_2H_4 and mC_2H_4, respectively. Uncertainty in the leak is considered in the ±3 % associated with bC_2H_4 and mC_2H_4.

Propagating the uncertainties of each variable through Eq. 1, the total uncertainty δN (nmols g^{-1} h^{-1}) is expressed by Eq. 3:

$$\delta N = N^{*}\sqrt{\left(\frac{\delta(mC_2H_4 - bC_2H_4)}{mC_2H_4 - bC_2H_4}\right)^2 + \left(\frac{\delta V}{V}\right)^2 + \left(\frac{\delta P}{P}\right)^2 + \left(\frac{\delta T}{T}\right)^2 + \left(\frac{\delta W_{Dry}}{W_{Dry}}\right)^2}$$

where the uncertainty (*e.g.*, δV) is expressed as a fraction of the measured value (*e.g.*, V). The variables R *Ratio* and R in Eq. 1 do not have uncertainty terms in Eq. 3, because these factors are assumed to be

idealized (*R*) or the uncertainty is poorly constrained (*R Ratio*, Soper et al., 2021).

We used non-parametric analyses for all group comparisons based on extremely low *p*-values from Shapiro-Wilk tests and visual inspection from Q-Q plots, indicating our dataset was not normally distributed (zero-inflated and right skewed). Non-parametric tests make few assumptions about the underlying data distribution and often work with the null hypothesis that both groups are equal. Importantly, non-parametric tests are still valid for normally distributed data. For analyses of two groups ($n = 2$), we used a Mann Whitney *U* test (significance level of 0.05, similar to a Student's *t*-test). For analyses of more than two groups ($n > 2$), we used a Kruskal-Wallis test (significance level of 0.05, similar to a one-way ANOVA) followed by a Dunn's post-hoc test to uncover which groups were significantly different. The Kruskal-Wallis test included a Benjamini-Hochberg correction to the p-values to account for the tendency of tests with many groups to find spurious significant differences. We then assigned a letter (or asterisk) to each group (with a minimum of three replicates) to note significant differences. Groups that do not share any similar letters were significantly different. Finally, the different requirements for storing lichens (frozen, Colesie et al., 2014, 2018) and bryophytes (room temperature, Akther and Rousk, 2019) meant that we could not directly compare these two groups. We therefore conducted separate analyses of lichens and bryophytes.

3. Results and discussion

3.1. Survey and new cryptogam-diazotroph relationships

We made 526 laboratory BNF measurements from 39 bryophyte and 23 lichen genera, families or life form groups. Around 44 % (17) of the bryophyte and 65 % (15) of the lichen groups fixed N. Our measurements show high variability in BNF rates (Renaudin et al., 2022) with median lichen BNF of 3.68 nmol g^{-1} h^{-1} and median bryophyte BNF of 0.58 nmol g^{-1} h^{-1}. Rousk et al. (2016) and Gavazov et al. (2010) reported lichens had significantly greater BNF rates than bryophytes growing in the same environment. While lichen BNF rates may surpass those of bryophytes in many tundra environments, bryophyte biomass may far exceed lichen biomass and contribute more to the bioavailable N flux (Oechel et al., 1997).

We also identified potentially new cryptogam-diazotroph relationships with the lichen genera *Asahinea*, *Nephromopsis* and *Thamnolia*, and the bryophyte genera *Dicranoweisia* and *Amphidium*. These genera had at least one sample fixing N and, to our knowledge, have not previously been reported to have BNF or nitrogenase activity (Figs. 2 and 3). Follow-up experiments and molecular sequencing are necessary to confirm these associations, as we were unable to fully remove every sample from the underlying material (see Section 2.2).

Under simulated Arctic conditions, the great majority of cyanolichen and tri-membered lichen samples fixed N. This was not the case for foliose and fruticose chlorolichens, except for *Sphaerophorus* (Fig. 2). In contrast, there were no groups of bryophytes where most samples fixed N (with $n > 1$). Hypnanae had the highest average bryophyte fixation rates of 1.9 nmol g^{-1} h^{-1}. Average rates of BNF in Dicranidae and Bryanae bryophytes were similar to rates in crustose chlorolichens and foliose and fruticose chlorolichens (0.28–0.61 nmol g^{-1} h^{-1}) but were below the cyanolichens and tri-membered lichens (average rate of 46.8 nmol g^{-1} h^{-1}) and well below the highest *Peltigera* rate (~660 nmol g^{-1} h^{-1}). Liverworts and a grouping of three other bryophytes (*Sphagnum*, *Andreaea* and Polytrichaceae) had the lowest fixation rates (0.01–0.08 nmol g^{-1} h^{-1} on average).

3.2. BNF capacity across circumpolar subzones

We sampled four of five circumpolar bioclimate subzones defined by Walker et al. (2017) as well as sub-Arctic field sites in Iceland. Analysis of all samples (fixing and non-fixing) found no significant differences in BNF rates among subzones for either bryophytes or lichens after controlling for elevated BNF rates from rehydrated 2023 Svalbard samples from the B subzone due to storage effects (see Section 3.4 below).

At the genus level, while there were differences in the average BNF rates, variation in rates within a single subzone overshadowed differences between subzones (Fig. 4). This trend continued after subdividing these groups by their collection location, suggesting that intra-subzone variation outweighed inter-subzone variation (Fig. S1). For example, a few of the intra-subzone coefficients of variation (CVs) were greater than the inter-subzone CVs for *Racomitrium* (1.16 intra-subzone D *vs.* 1.06 inter-subzone), *Peltigera* (1.98 intra-subzone B and 1.79 intra-subzone E *vs.* 1.73 inter-subzone) and *Stereocaulon* (1.19 intra-subzone B and 1.15 intra-subzone D *vs.* 1.04 inter-subzone). While there were significant differences in BNF rates among groups defined by a specific subzone-genus (Fig. 4) or specific subzone-genus-location (Fig. S1), there were no differences within the same genus or within the same subzone-genus, respectively.

The measured cryptogam BNF rates suggest that the BNF capacity of a cryptogam genus is the same as the BNF capacity of that genus from other circumpolar bioclimate subzones, consistent with our hypothesis (**H1**). For example, our findings show that *Peltigera* from subzone E has the same capacity for BNF at 11 °C as *Peltigera* from subzone B. While we found that multiple samples from a cryptogam genus from different subzones do not have significantly different BNF capacity at 11 °C, this does not mean their in situ rates are the same. Low-subzone cryptogams from a genus likely have greater in situ BNF rates due to warmer average temperatures than high-subzone cryptogams from the same genus (Hupperts et al., 2021). Therefore, high-subzone cryptogams may have the capacity to increase their in situ fixation rates as Arctic conditions change.

Climate variability and new ecological competition from range-expanding shrubs may inhibit high-subzone cryptogams from fully realizing their BNF capacity. Such colonizing plants may not affect cryptogams equally. Cannone et al. (2022) found that two types of vascular plants on sub-Antarctic Signy Island colonized moss and lichen habitats at different rates. This suggests that some native Arctic cryptogam species may be more vulnerable to competition with non-native plants, perhaps from direct effects on cryptogams through changes in nutrient cycling or indirect effects on the diazotrophs through changes in BNF factors like light intensity due to shading.

The quantities and intensities of abiotic factors that influence BNF (*i.e.*, metal and nutrient availability (Darnajoux et al., 2019; Rousk et al., 2016), light intensity (Jean et al., 2012), temperature (Calabria et al., 2020) and water content (Hupperts et al., 2021)) are expected to change in the coming decades. Soil drying and a reduction in relative humidity may spur wildfires that deposit metals like Mo on downwind tundra communities (Kim et al., 2024). Changing intensity and frequency of precipitation may alter the growing season and demand for N (Hupperts et al., 2021). Rousk and Michelsen (2017) suggested a shift in the relative composition of diazotroph colonizers during their experiments between the treatment and control groups could represent acclimation of existing diazotrophs to different environmental regimes. These findings highlight that specific diazotroph-cryptogram associations are important for determining the BNF capacity and adaptability to change.

3.3. Rehydration effects on BNF rates

We made over 50 field (fresh) measurements of BNF in Svalbard, representing the first in situ measurements of BNF with ARACAS. Measurements of the same samples (rehydrated 2023 Svalbard samples) were then repeated in the laboratory at Duke University. Among samples that fixed N, we found that BNF rates in fresh lichens were higher than rehydrated rates ($p = 0.035$, $Z = 2.109$), while BNF rates in fresh and rehydrated bryophytes were largely the same ($p = 0.728$, $Z = 0.35$) (Fig. 5 and Fig. S2). Lichen BNF rates were slightly more varied than

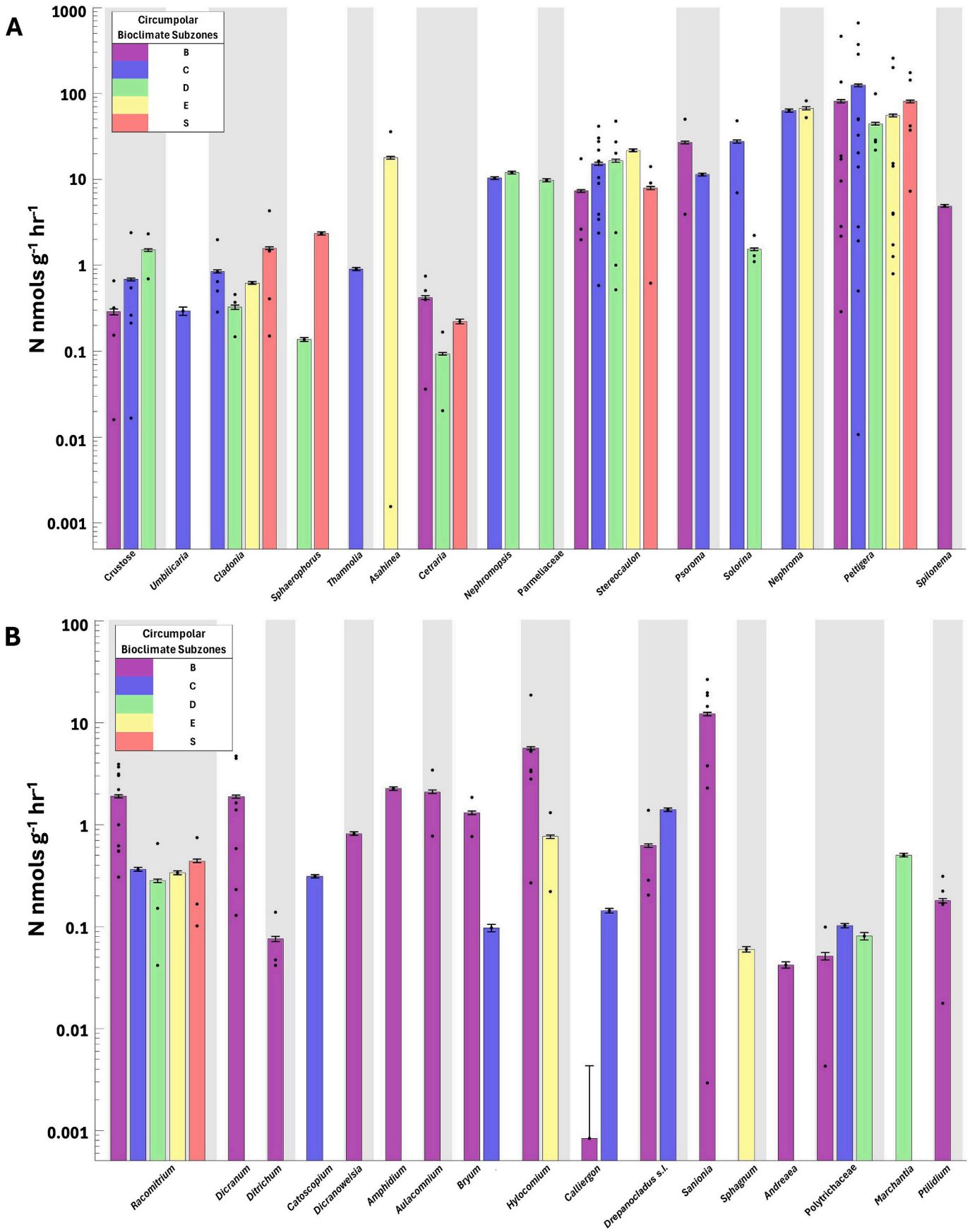

Fig. 4. BNF rates of fixing lichen (A) and bryophyte (B) genera grouped by bioclimate subzones. There were no significant differences (p-value >0.05) in BNF rates among groups defined by a specific subzone-genus as the result of a Kruskal-Wallis test followed by a Dunn's post-hoc test.

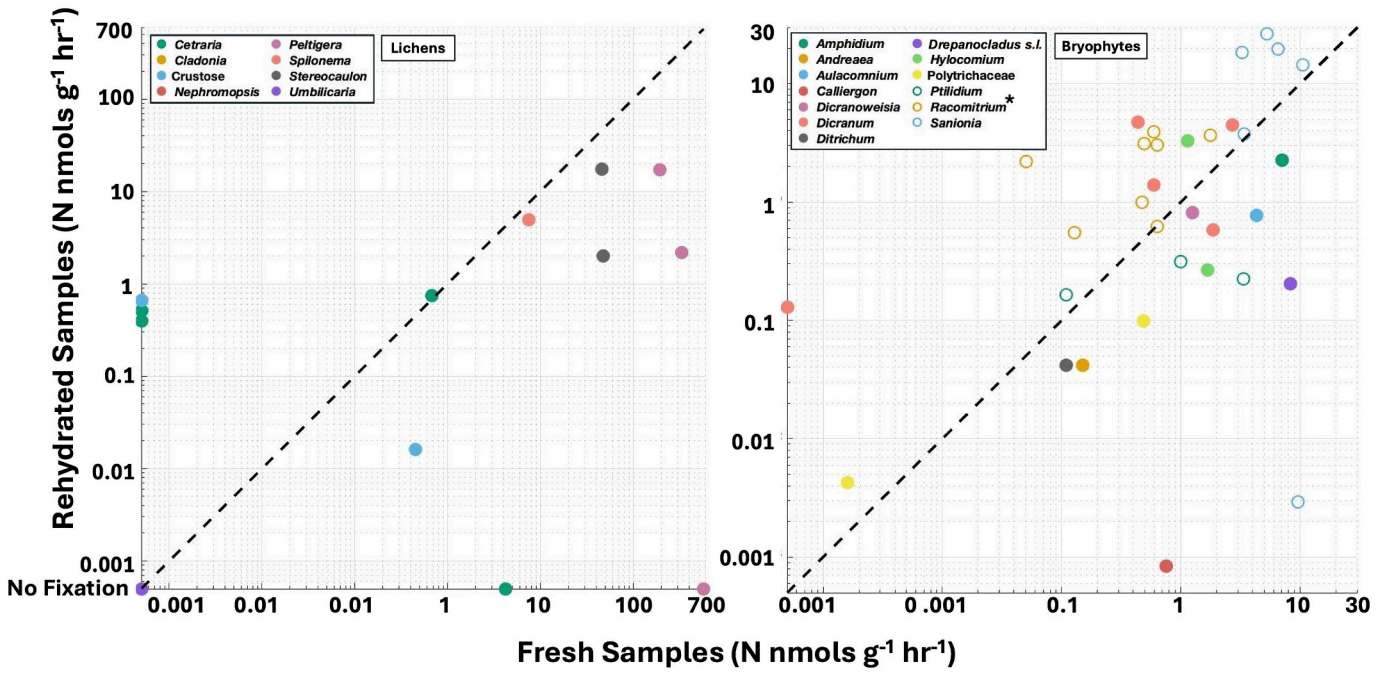

Fig. 5. Comparison of fresh and rehydrated BNF rates for lichens (left) and bryophytes (right). Dashed black identity line represents equal (1:1) BNF fresh and rehydrated. Samples above the line fixed more rehydrated than fresh, and samples below the line fixed more fresh than rehydrated. Asterisk (*) signifies a significant difference (p-value <0.05) in a genus's BNF rates fresh *versus* rehydrated as the result of a Mann Whitney *U* test. Samples without fixation were assigned an arbitrarily small positive value (smaller than any fixing value) in order to plot on the logarithmic y-axis.

bryophyte rates relative to their means (8.72 *vs*. 8.63, relative range) with fresh *Peltigera* and rehydrated *Sanionia* having the highest BNF rates, respecitvely (Fig. 5).

Peltigera BNF rates were more than an order of magnitude lower across all samples after rehydration, while some genera like *Umbilicaria* never fixed N under fresh or rehydrated conditions (Fig. 5). A non-fixing result is more expected for chlorolichens, like *Umbilicaria*, that form looser relationships with diazotrophs and do not have the symbiotic relationships that promote BNF as in cyanolichens or tri-membered lichens.

The lack of a discernible pattern among all measurements (fixing and non-fixing) supports our hypothesis (**H2**) that fresh cryptogam BNF rates are statistically equal to rehydrated cryptogam rates (*p*-values >0.05) compared over many sample measurements. However, individual sample variability highlights the challenge to understand BNF rates using laboratory measurements, even when simulating in situ conditions. Some of the complexity could be due to genus-specific responses to changes in water content or stress from desiccation and rehydration. Fresh samples were measured using in situ water content whereas rehydrated samples were measured after being well sprayed with Type II water. On average, fresh water content (which relates the percent of the water weight to the dry weight) was 165 % for bryophytes and 101 % for lichens, whereas rehydrated water content was 230 % and 146 %, respectively. Jean et al. (2012) and Rousk et al. (2021) found that mosses and their diazotrophs are resilient to desiccation and recover quickly once rehydrated. This might explain why rehydrated *Racomitrium* rates exceeded fresh *Racomitrium* rates (p-value = 0.010, Z = −2.562). It is possible that samples that did not fix N when fresh but did fix N when rehydrated were water-limited in the field (Rousk et al., 2018). This aligns with Hupperts et al. (2021) who suggested moss moisture content positively correlates with BNF rates. Care should be taken to match in situ water content (through wet weight) when making laboratory BNF measurements that attempt to describe in situ BNF rates. Since global land surface models (Zha and Zhuang, 2021) currently do not distinguish fresh and rehydrated cryptogam BNF responses to abiotic factors, our results suggest that model projections may underrepresent lichen BNF in genera like *Peltigera* which fixed N at lower rates rehydrated than fresh and may be biased by BNF rates of bryophyte genera like *Racomitrium* that are more stress tolerant and fixed N more rehydrated than fresh.

3.4. Storage effects on BNF rates

In addition to rehydration, we briefly considered the effect of storage on BNF rates using rehydrated (< 3 months since collection) and stored samples (> 3 months since collection). A full investigation of the effect of storage on lichen *versus* bryophyte BNF rates using an experimental design with samples from each group kept frozen and at room temperature was outside the scope of this study. We therefore limit our discussion to separate observations of lichens and bryophytes. We found that, with the storage conditions we employed, more than 95 % of individual bryophyte samples fixed N within three months of storage, while fewer than 15 % fixed after one year at room temperature. For lichens, a little more than 45 % of individual samples fixed N within three months of storage and retained roughly the same 45 % rate after nearly two years of frozen storage. This also is seen in the BNF fixation rates with bryophyte samples, which showed significantly higher BNF after two to three months than after one year (p = 1.9E-30, Z = 11.47). There was no significant difference for lichen samples (p = 0.742, Z = 0.33). Our work highlights the need to further investigate whether bryophyte samples stored longer than two to three months better preserve BNF ability and rates if stored frozen.

4. Conclusion

Our survey tentatively identified two new bryophyte and three new lichen cryptogam-diazotroph associations. Our findings highlight the complexity in interpreting and applying laboratory-derived BNF rates and suggest models may underrepresent lichen BNF rates and overrepresent stress-tolerant bryophyte genera. We recommend laboratory BNF measurements should match in situ water content when describing in situ BNF rates. Finally, our pan-Arctic survey results suggest the BNF

capacity of a cryptogam genus matches the BNF capacity of that genus from other circumpolar bioclimate subzones when measured under the same environmental conditions. This suggests high-subzone cryptogams may have the capacity to increase their BNF rates as seasons and conditions shift across the Arctic, but changes in nutrient cycles and competition may limit their ability to realize their full BNF capacity.

CRediT authorship contribution statement

Perrin Hagge: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Elizabeth King-Doonan:** Writing – review & editing, Visualization, Supervision, Conceptualization. **François Lutzoni:** Writing – review & editing, Investigation, Conceptualization. **Jolanta Miadlikowska:** Writing – review & editing, Resources, Investigation. **Blanka Aguero:** Writing – review & editing, Resources, Investigation. **Hannah Whitby:** Writing – review & editing, Investigation. **Charles Umbanhowar:** Writing – review & editing, Investigation. **Claudia Colesie:** Writing – review & editing, Methodology. **A. Elizabeth Arnold:** Writing – review & editing, Resources, Funding acquisition. **Eric Yitong:** Validation, Investigation. **Nicolas Cassar:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.180264.

Data availability

All raw data used in this study is available on Zenodo (https://zenodo.org/records/14976433) and data transformation scripts on GitHub (https://github.com/PerrinHagge/Nitrogen-fixation-in-Arctic-lichens-and-mosses-a-survey-across-circumpolar-subzones). Additionally, physical vouchers of select cryptogam samples are available in the Duke Herbarium.

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