



Moss-cyanobacteria associations as a novel source of biological N₂-fixation in temperate grasslands

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Abstract

Aims We investigated N₂-fixation by moss-cyanobacterial associations in a North American prairie ecosystem, identifying cyanobacteria associated with moss species, and evaluating spatio-temporal dynamics in N₂-fixation rates.

Methods We confirmed the presence and abundance of N₂-fixing cyanobacteria on three moss species (*Pleurozium schreberi* (Brid.) Mitt., *Racomitrium elongatum* Frisvoll, *Rhytidiadelphus triquetrus* (Hedw) Warnst.) using epi-fluorescence light microscopy. To estimate monthly N₂-fixation rates on mosses from three sites, we conducted laboratory-based acetylene reduction assays with constant incubation temperatures and natural daylight. We evaluated the relationship between

daylength and N₂-fixation, and daylength and weather variables.

Results N₂-fixation rates varied by species, site, and month. *R. elongatum* exhibited the highest rates and *P. schreberi* had the lowest. Rates for *R. elongatum* and *R. triquetrus* were positively correlated with daylength, with peaks occurring at 13–14 h daylight, suggesting spring and fall conditions support N₂-fixation in this system. Annual median N₂-fixation for *R. elongatum* and *R. triquetrus* ranged from 0.008–0.124 kg N ha⁻¹ yr⁻¹ based on cover of 11–100%.

Conclusions Our results highlight a previously undescribed source of biological N₂-fixation in temperate grasslands. Changes in the distribution or activity of N₂-fixing moss-cyanobacterial associations due to management practices and climate change could impact future stand-level nitrogen dynamics.

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Introduction

Ground-dwelling bryophytes (mosses, liverworts, and hornworts) significantly influence global carbon (C) and nitrogen (N) cycles through contributions to above-ground biomass and associations with N₂-fixing cyanobacteria (Cornelissen et al. 2007; Elbert et al. 2012). In boreal forest and arctic ecosystems, N₂-fixation by moss-cyanobacteria associations is an important source of bioavailable N, where they contribute greater

than 2 kg N ha⁻¹ yr⁻¹ (DeLuca et al. 2002; Rousk and Michelsen 2017). The association is thought to be driven by N limitation in the host bryophyte which secretes chemical attractants to draw cyanobacteria (Bay et al. 2013). This relationship has been most commonly observed in the mosses *Pleurozium schreberi* and *Hylocomium splendens* (DeLuca et al. 2002; Markham 2009; Zackrisson et al. 2009), and typically involves the cyanobacterial genera *Nostoc*, *Stigonema*, and *Calothrix* (Gentili et al. 2005; Houle et al. 2006). After colonization, cyanobacteria live epiphytically providing N, which accrues within the moss tissue and is either released rapidly through leaching from live tissue or released slowly through decomposition (Berg et al. 2013; Liu et al. 2020).

Temporal and spatial variability in N₂-fixation rates of bryophyte-cyanobacteria associations have been well-documented in boreal and arctic ecosystems where major drivers include temperature, moisture and nutrient availability as well as forest stand age and type (deciduous vs. coniferous) (DeLuca et al. 2008; Jean et al. 2018; Rousk and Michelsen 2017). In boreal forests, N₂-fixation by moss-cyanobacteria associations show a seasonally driven pattern, where fixation rates begin to climb just after snowmelt in May, followed by a brief mid-summer drop, before spiking to the highest fixation activity in late summer (Aug-September) (DeLuca et al. 2002). Experimental warming has been reported to both increase and decrease N₂-fixation rates at an ecosystem level, depending on other seasonal factors (Gundale et al. 2012; Lett and Michelsen 2014; Sorensen and Michelsen 2011). However, because warming has also been reported to shift vegetation cover, the subsequent changes in nutrient input through leaf litter and shading of ground-dwelling bryophytes may reduce their long-term N₂-fixation potential (Jean et al. 2013; Rousk and Michelsen 2017). In a study examining the sensitivity of nitrogen fixation to litter and moisture variability in boreal forest feather moss cyanobacteria associations, prolonged drought (45 days) decreased nitrogen fixation rates, whereas daily watering resulted in increased nitrogen fixation. (Gundale et al. 2009). A growth chamber experiment examining the interactive effects of temperature and moisture on nitrogen fixation in temperate-arctic moss cyanobacteria associations found varying effects of moisture on nitrogenase activity, depending on the moss species and ecosystem (Rousk et al. 2017). These results suggest that more regional and species-specific studies will be needed for determining future

temporal and spatial shifts in N₂-fixation by bryophyte-cyanobacteria associations worldwide.

Bryophyte-cyanobacteria associations extend into temperate forests of North America and New Zealand occurring in a broad range of moss and liverwort genera, with reported N₂-fixation rates ranging from 0.009 to 10 kg N ha⁻¹ yr⁻¹ (Bidwell 2017; Deane-Coe and Sparks 2016; Jean et al. 2012; Lindo and Whiteley 2011; Menge and Hedin 2009); these rates are highly variable compared to those reported for boreal forests moss-cyanobacteria associations (0.5–2 kg N ha⁻¹ yr⁻¹) (DeLuca et al. 2002; Zackrisson et al. 2004). This variation could be partially explained by the fact that temperate forest studies include not only forest floor mosses but also canopy-dwelling mosses whose cyanobacterial diversity may vary as well as differing light, moisture, and nutrient availability. N₂-fixation rates in the canopy moss *Isoetes stoloniferum* (Brid.) were found to vary spatially and temporally across an urban to wildland gradient from Seattle, Washington (WA), USA to the temperate Hoh Rainforest on the Olympia Peninsula, WA, USA with the highest N₂-fixation observed in pristine forests in spring (4786 μmol m⁻² d⁻¹) and summer (344 μmol m⁻² d⁻¹) and the lowest N₂-fixation observed in the fall in urban areas (3 μmol m⁻² d⁻¹). Elevated heavy metal concentrations in moss samples were inversely correlated with N₂-fixation rates (Bidwell 2017).

Interestingly, relatively few studies have reported biological N₂-fixation by cyanobacteria-moss associations in temperate grasslands, despite the fact that grasslands worldwide are N-limited and host diverse bryophyte communities (Calabria et al. 2015; During and Willems 1986; Merrill 1991; van der Linden and Farrar 1983; Zales 1971). Early in situ studies of turf mosses (*Ceratodon purpureus* (Hedw) Brid., *Bryum argenteum* Hedw and *Weissia controversa* Hedw.) from temperate prairies in Saskatchewan, Canada (Vlassak et al. 1973) and Georgia, USA (Reddy and Giddens 1981) demonstrated that individual prairie mosses were capable of fixing up to 146 ng N g⁻¹ of dry weight of moss hr⁻¹ under laboratory conditions. Assuming peak activity throughout one-third of the year and moderate coverage, just one moss species could contribute up to 0.05% of the total N budget, which has been estimated at <1 to 5 kg N ha⁻¹ year⁻¹ for North American grasslands (Vlassak et al. 1973). Global mean symbiotic N₂-fixation rates for mixed grass prairies have been reported with a range of 0.2–0.9 kg N ha⁻¹ year⁻¹ (Cleveland

et al. 1999), however these estimates do not account for moss-cyanobacteria associations.

In Washington State, USA, Puget Sound prairies are considered critically threatened and of great conservation interest because of their high biodiversity, cultural importance, and preponderance of state- and federally-listed plant, mammal, avian, and invertebrate species. As such, prairies have been the focus of intensive restoration and management practices such as prescribed burning, herbicide application, and seeding with native grasses and forbs (Stanley et al. 2011). Puget Sound prairies are characterized by a diverse cryptogamic layer of bryophytes and lichens, often dominated by the moss *Racomitrium elongatum* (Chappell and Crawford 1997; Del Moral and Deardorff 1976). *Pleurozium schreberi*, a common associate with N₂-fixing cyanobacteria in boreal regions, also occurs on Puget Sound prairies, although the abundance is much lower (Calabria et al. 2015). Nitrogen budgets for Puget Sound prairies have not been quantified and the potential contribution of moss-cyanobacteria symbiosis is unknown.

The primary objectives of our study were to: 1) Identify the N₂-fixing moss-cyanobacteria associations that occur on Pacific Northwest prairies and quantify species-specific N₂-fixation rates, 2) evaluate spatial variation in N₂-fixation rates and estimate the annual N contribution of prairie N₂-fixing moss-cyanobacteria associations, and 3) evaluate the seasonal fluctuations for all moss-cyanobacteria associations.

Materials and methods

Site description

The study was carried out at three prairie preserves in western Washington, USA: Glacial Heritage Preserve (46.865861°N, 123.041178 °W), Mima Mounds Natural Area Preserve (46.903076°N, 123.05028°W), and Scatter Creek Natural Wildlife Area (46.831527 °N, 123.0226644 °W). These sites, located approximately 32 km south of Olympia, WA, are situated roughly 8–16 km apart and range in size from 257 to 459 ha (Fig. 1).

The climate of this region is typified by mild, wet winters and warm, dry summers, with a characteristic summer drought lasting up to 3 months. The closest weather station in Tenino, Washington recorded a mean annual temperature of 10.02 °C and mean annual

precipitation of 129 cm for 2017 (Western Regional Climate Center 2017). Fall rains typically begin about mid-October and continue with few interruptions through spring. Daytime temperatures in winter months (December–January–February) range from 4 to 12 °C, with nighttime temperatures between –1 to 4 °C. In summer months (July–August–September) daily high temperatures range from 21 to 27 °C with nighttime lows around 10 °C; temperatures equal or exceed 32 °C about 6 days each summer.

Characteristic native vascular prairie plant species in the area include *Festuca roemerii* (Pavlick) E.B. Alexeev, *Camassia quamash* (Pursh) Greene, *Lomatium utriculatum* (Nutt. ex Torr. & A. Gray) J.M. Coult. & Rose, *Eriophyllum lanatum* (Pursh) J. Forbes, *Quercus garryana* Douglas ex Hook., *Racomitrium elongatum* Frisvoll (*Racomitrium* s.l. is recognized here, following Larraín et al. (2013), and lichens, including several state-listed rare *Cladonia* P. Browne species (Calabria et al. 2015; Chappell and Crawford 1997; Del Moral and Deardorff 1976; Washington Natural Heritage Program 2011). Restoration and management strategies over the past two decades have varied among sites but have typically included a combination of mowing, herbicide application, prescribed burning, and native plant seeding and planting to reduce exotic species and enhance the native plant community. All sites are bordered by encroaching conifer forests, consisting mainly of *Pseudotsuga menziesii* (Mirb.) Franco. Although there is no available N deposition data for the study sites, total wet inorganic N deposition for the Puget Sound region ranges from 1 to 3 kg ha⁻¹ yr⁻¹ (Geiser and Neitlich 2007). Puget Sound prairie soils are categorized as either Nisqually or Spanaway types, consisting of extremely well-drained gravelly, sandy loam and glacial outwash soils.

Cyanobacteria identification and abundance

We initially collected samples of six common and abundant moss species from Scatter Creek Wildlife Recreation Area and Glacial Heritage Preserve during fall 2015 to confirm the presence and abundance of cyanobacteria. These species included *Dicranum scoparium* (Hedw.), *Kindbergia oregana* (Sull.) Ochyra, *Racomitrium elongatum* Frisvoll, *Polytrichum juniperinum* Hedw., *Rhytidiadelphus triquetrus* Hedw. (Warnst.), and *Pleurozium schreberi* (Brid.) Mitt. Samples were strategically collected from areas with no

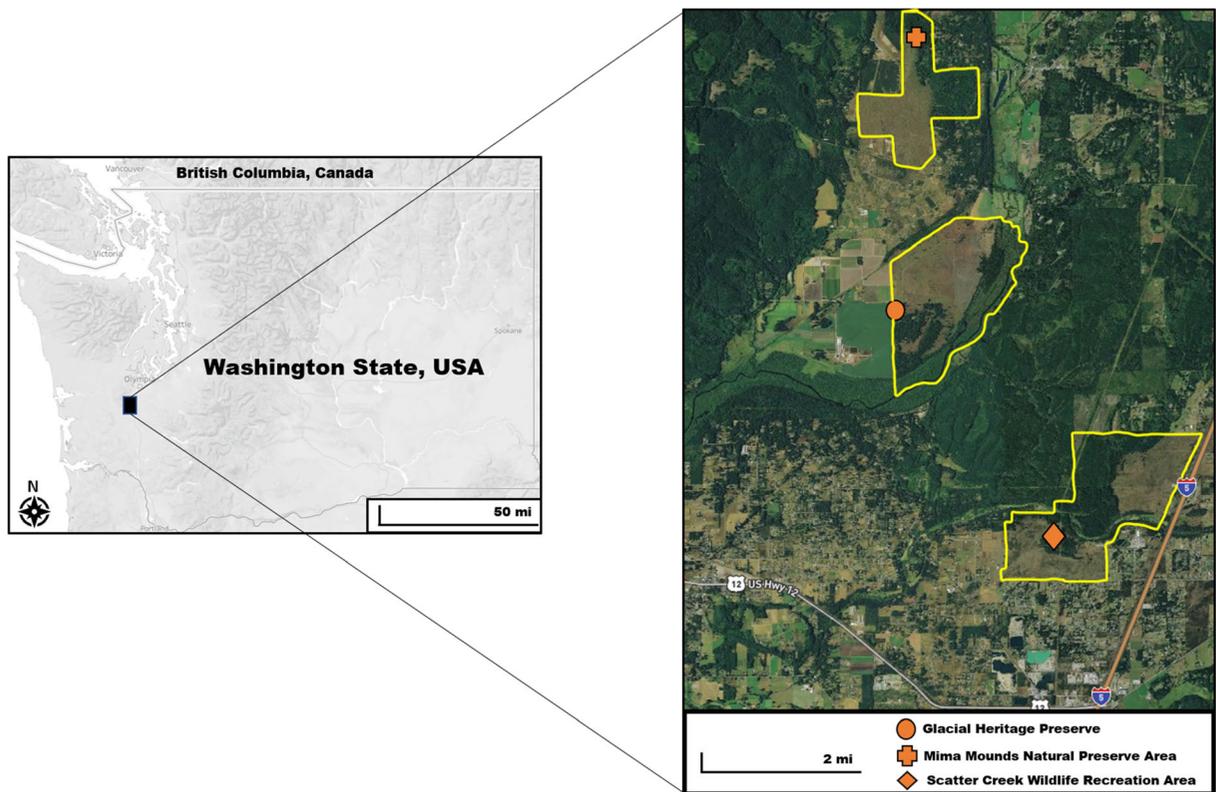


Fig. 1 Map showing the location of prairie study sites, Glacial Heritage Preserve, Mima Mounds Natural Area Preserve, and Scatter Creek Natural Wildlife Area located in the Puget Sound region of western Washington, U.S.A.

recent burn activity (more than 10 years since fire) and where cryptogamic layers had not been disturbed by recent restoration activities. We collected samples by removing a 5–10 cm diameter plug from an intact, monospecific moss mat, including the top 2–8 cm of the moss shoots, depending on the species. We then placed samples into clean paper bags for transport back to the laboratory.

To estimate the degree of cyanobacteria colonization for each moss species, we randomly selected nine branches from each plug, prepared a slide with the branches evenly spread out and then recorded the number of branches hosting cyanobacteria using a UV-fluorescent light microscope fitted with a green excitation filter (Olympus BH-2). Leaves were examined at 100–400 \times magnification and this process was repeated with 3–8 replicate slides ($n = 27$ –72 branches scanned depending on the size and growth form of the moss species). This initial screening for cyanobacteria in six prairie moss species indicated varying degrees of colonization, expressed as a percentage of stems colonized (± 1 SE): *Dicranum scoparium* and *Polytrichum*

juniperinum (0%), *Kindbergia oregana* ($7\% \pm 0.01$) *Racomitrium elongatum* ($40\% \pm 0.04$) *Rhytidiadelphus triquetrus* ($87\% \pm 0.04$) and *Pleurozium schreberi* ($49\% \pm 0.02$) (Table 1; Online Resource 1). The latter three moss species were selected for further investigation based on their widespread distribution across our sites and consistent presence of cyanobacteria. For the three chosen moss species, we identified cyanobacterial genera by examining moss samples at 100–400 \times magnification using the same microscope conditions and model described above and through comparison with the literature (Rippka et al. 1979).

To further quantify cyanobacteria abundance we determined the number of moss leaves colonized with cyanobacteria for *Racomitrium elongatum*, *Rhytidiadelphus triquetrus*, and *Pleurozium schreberi*.

For each moss species, we randomly selected five separate moss shoots and from each shoot leaves were sampled from three different branches. The number of leaves observed varied by the size of the moss leaves, but generally, we filled the area underneath a standard square cover slip with leaves and counted five slides for

Table 1 Results from initial screening of cyanobacterial colonization for common prairie moss species *Kindbergia oregana*, *Racomitrium elongatum*, *Rhytidiadelphus triquetrus*, and *Pleurozium schreberi*. Two species not included in this table (*Dicranum scoparium* and *Polytrichum juniperinum*) were screened and showed no colonization

Moss species	% of branches colonized	% of leaves colonized
<i>Kindbergia oregana</i>	7 (\pm 0.01)	n/a
<i>Pleurozium schreberi</i>	49 (\pm 0.02)	33 (\pm 0.02)
<i>Racomitrium elongatum</i>	40 (\pm 0.04)	87 (\pm 0.01)
<i>Rhytidiadelphus triquetrus</i>	87 (\pm 0.04)	79 (\pm 0.02)

Degree of cyanobacteria colonization was measured in two ways: 1) Percentage of branches colonized by cyanobacteria colonies ($n = 27$ – 72 branches counted, depending on the size and growth form of the moss species) and 2) Percentage of leaves colonized calculated as the number of leaves with cyanobacteria cells ($n = 69$ – 117 leaves/species). Values in parentheses represent (\pm 1 SE)

each species ($n = 69$ – 117 leaves/species). The number of leaves with cyanobacteria was recorded for each slide and the percentage of leaves colonized was calculated for each species (Table 1; Online Resource 2).

Monthly ARA sample collection

In June 2016, we established three 0.378-ha ocular plots (68 m diameter) at each of the three prairie sites based on a modified Ground Layer Indicator method (Smith et al. 2015). We randomly selected plot center points in prairie areas that met our criteria (areas greater than 50m² that were colonized by at least two of our three target moss species and had not been burned in at least 10 years). Within each of the three ocular plots at the three study sites we collected triplicate samples of each moss species present, barring a few occasions where samples proved to be a mixture of more than one moss species, of insufficient size, or where moss tissue was mostly dead (brown) and were therefore excluded from our analysis. We collected samples at one-month intervals (June 2016–May 2017), except in January 2017 when inclement weather prohibited our team from accessing sites, for a total of 11 sampling collections. Samples were collected as described above for initial identification and abundance estimates.

In total we collected 252 moss samples over the sampling period: 93 samples of *Racomitrium elongatum*, 99 samples of *Rhytidiadelphus triquetrus* and 60 samples of *Pleurozium schreberi*. As *Pleurozium*

schreberi was only growing in abundance at the transition from prairie to forest at two of our study sites (Glacial Heritage Preserve and Scatter Creek Wildlife Natural Area), only two of the nine plots contained this species. Therefore, this species had insufficient replication and was excluded from the analysis of N₂-fixation rates by site.

Acetylene reduction assay (ARA)

Samples were stored at room temperature for 24 h prior to conducting acetylene reduction assays to estimate N₂-fixation rates. After the short-term storage, all moss samples in this study were rehydrated immediately prior to incubation by spritzing each sample 3 \times with distilled water. We placed moss samples in 20 mL vials to fill roughly 10% of the headspace, sealed them with crimp caps and recorded the number of shoots for each tube. Ten percent of the headspace was removed using a syringe and replaced with reagent grade acetylene (Praxair, 99.6% purity). All samples were incubated for 24 h at 16 °C on the lab bench immediately adjacent to a window, reflecting natural light and day length conditions. After incubation, we measured ethylene concentration by removing a 1 mL sample of headspace from each tube and analyzing it in a Thermo Scientific Trace Gas Chromatograph equipped with a flame ionization detector (FID) and a Poropak Q column, which uses N₂ as carrier gas at 10 mL/min. We used ethylene standards (0, 5.7, 28.5, 57 ppm) to estimate the ethylene generated by bacterial fixation in each sample. Instrument performance tests were carried out daily to ensure proper calibration. Controls consisted of blank samples containing acetylene gas and moss samples without acetylene gas to ensure that ethylene in sample tubes was derived from biological process and did not represent ethylene contamination from our acetylene source.

To be able to convert the amount of N₂ fixed per moss shoot to a unit area basis, we first counted the number of shoots of each species within three 28.27 cm⁻² monospecific cores collected from each site. Cores were collected from the center of homogenous mats that were typically greater than 40 cm in diameter within our study plots at each site. Three replicate core samples were collected from each study site for *Racomitrium elongatum* and *Rhytidiadelphus triquetrus*. Only one core sample was collected for *Pleurozium schreberi* at two of our three study sites because its abundance at all three sites was low and

did not occur in consistent monospecific mats in our study plots. The average number of moss stems per cm² was calculated for each species at each site and these values were used to convert the amount N₂ fixed per moss shoot to units of μmol m⁻² d⁻¹ of ethylene produced based on a molar ratio of 3:1.

acetylene reduced to N₂ (DeLuca et al. 2002) (Online Resource 3). Thus, site and species-specific N₂-fixation values reported in our results assume a 100% cover of that moss species in a given m² area.

To determine whether the measured rates of N₂-fixation corresponded to the degree of colonization by cyanobacteria on moss leaves we first selected six samples with varying acetylene reduction rates (ranging from 0 to 321 μmol m⁻² d⁻¹) for each of our target moss species. We counted the number of individual cyanobacterial cells on ten leaves for the six moss samples ($n = 52\text{--}60$ leaves per species, depending on the leaf size) (Online Resource 4).

Statistical analysis

We investigated the relationship between cells per leaf and acetylene reduction using linear regression analysis in JMP© 14.0 (2018). One outlier with exceedingly high N₂ fixation rates and cyanobacterial counts was determined using a Grubb's test and removed for the regression of *Racomitrium elongatum* (Online Resource 4). To determine if acetylene reduction varied by species and site, we ran a mixed effects ANOVA with species and prairie as fixed effects and month as a random effect. For species-specific responses for *Racomitrium elongatum* and *Rhytidiadelphus triquetrus* we ran mixed effects ANOVAs with prairie and month as fixed effects and plot as a random effect. *Pleurozium schreberi* was only sampled from one plot per site so we ran a one-way ANOVA to evaluate effects of month on activity, using sample ($n = 3$ per plot) as the level of replication. All mixed effects models were run with the *lmer* and *lmerTest* packages (Bates et al. 2015) in Rstudio version 1.2.5033 (R Core Team 2017). We conducted post-hoc analyses using pairwise comparisons and t-tests with pooled standard deviations. We log-transformed the N₂-fixation rate data prior to all analyses in order to meet assumptions of normality and variance.

To evaluate the potential relationships between N₂-fixation rates and environmental variables, we first conducted non-parametric Spearman Rank correlation tests for all active (non-zero) samples from each species

against daylength, which we did not manipulate in this study. We used sample-level data for this analysis, giving a sample size of 187 for *R. elongatum*, 121 for *R. triquetrus* and 29 for *P. schreberi*. We then ran Pearson's correlation tests between daylength and a suite of weather variables to determine the relationship between daylength and environmental variables that could influence nitrogenase activity. All correlations were run using the *devtools* package in Rstudio. We obtained daily temperature and precipitation values for the study period (June 2016 to May 2017) from the Olympia Airport field station (46.9733°, -122.9033°; NOAA 2019). We calculated temperature averages as well as precipitation sums and frequencies for the week and month prior to each sampling date, resulting in the following eight variables: day-of, weekly and monthly average temperatures, day-of, weekly and monthly precipitation sums, and frequency of precipitation over the previous week and month.

Annual N₂-fixation rates

We calculated annual N₂-fixation rates to assess contributions of *Racomitrium elongatum* and *Rhytidiadelphus triquetrus* to the N budget for Puget Sound prairies using median monthly N₂-fixation (μmol m⁻² d⁻¹), mean moss cover (%), and moss shoot density (stems cm⁻²). Measurements were taken monthly, as described above, from samples collected at each site. Because ecosystem N pools and fluxes have not been quantified for Puget Sound prairies, we compare our annual estimates to the total annual N budget for North American temperate grasslands which ranges from <1 to 5 kg N ha⁻¹ year⁻¹ (Vlassak et al. 1973) and global mean symbiotic N₂-fixation rates for mixed grass prairies which are reported as 0.2–0.9 kg N ha⁻¹ year⁻¹ (Cleveland et al. 1999) (Online Resource 5).

Considering that previous studies have shown temperature-mediated effects on N₂-fixation rates under experimental conditions (Gentili et al. 2005; Rousk and Michelsen 2017), it's possible that lab incubation at room temperature could result in an overestimation of N₂-fixation for winter months and underestimation for warmer months. Therefore, when calculating annual stand-level N₂-fixation to assess contributions of moss-associated N₂-fixation to N cycling in Puget Sound prairies we exercise caution by using median fixation rates (instead of mean) to provide a conservative estimate that could account for any potential over- or

underestimation. Using median N_2 -fixation also helps to address extreme outliers that could skew the distribution when using the mean. Because bryophyte cover varies widely between prairie preserves and depending on management strategies, we provide a range of potential fixation rates based on cover estimates for target moss species reported in the literature for Puget Sound prairies (Del Moral and Deardorff 1976) and from a survey conducted in spring 2016 at two of our study sites. Moss cover for *Racomitrium elongatum*, *Rhytidiadelphus triquetrus* and *Pleurozium schreberi* averaged 7.4% (SE = 0.91), 3.6% (SE = 0.63) and 2.9% (SE = 0.60), respectively (Online Resource 6).

Results

Based on morphological characteristics, we identified two cyanobacteria genera, *Nostoc* and *Stigonema*, growing epiphytically on leaves of all three moss species (Fig. 2). We found a weak positive correlation between the number of cyanobacteria cells per leaf and N_2 -fixation rates in *Racomitrium elongatum* ($R^2 = 0.35$; $P < 0.0001$) and *Rhytidiadelphus triquetrus* ($R^2 = 0.27$; $P < 0.0001$). Leaf colonization rates for *Pleurozium schreberi* showed no significant correlation with N_2 -fixation ($R^2 = 0.005$; $P = 0.6307$) (Online Resource 4).

When averaged across the sampling period, N_2 -fixation rates varied significantly by species ($F_{2,542} = 45.42$, $P < 0.001$; Fig. 3). The average daily N_2 -fixation rate for *Racomitrium elongatum* was $32.0 \pm 4.4 \mu\text{mol m}^{-2} \text{d}^{-1}$, while the average daily N_2 -fixation rates were significantly lower for *Rhytidiadelphus triquetrus* ($3.8 \pm 0.65 \mu\text{mol m}^{-2} \text{d}^{-1}$) and *Pleurozium schreberi* ($3.5 \pm 1.3 \mu\text{mol m}^{-2} \text{d}^{-1}$).

Overall N_2 -fixation rates (averaged across species and months) varied by site ($F_{2,544} = 6.19$, $P = 0.002$), with Glacial Heritage and Mima Mounds both having higher rates than Scatter Creek (Fig. 3). Glacial Heritage had an average N_2 -fixation rate of $28.8 \pm 5.7 \mu\text{mol m}^{-2} \text{d}^{-1}$, followed by Mima Mounds Natural Area Preserve ($16.9 \pm 3.2 \mu\text{mol m}^{-2} \text{d}^{-1}$), and Scatter Creek Wildlife Area ($8.8 \pm 2.3 \mu\text{mol m}^{-2} \text{d}^{-1}$). When separated by species, site was not statistically significant for either *Racomitrium elongatum* ($F_{2,7} = 4.24$, $P = 0.063$) or *Rhytidiadelphus triquetrus* ($F_{2,6} = 0.95$, $P = 0.440$).

N_2 -fixation rates for *Racomitrium elongatum* varied significantly by sampling month ($F_{10,249} = 3.69$, $P < 0.001$), with the highest average N_2 -fixation rates

occurring in July of 2016 ($73.50 \pm 31.74 \mu\text{mol m}^{-2} \text{d}^{-1}$) and April of 2017 ($75.50 \pm 19.9 \mu\text{mol m}^{-2} \text{d}^{-1}$) (Fig. 4a). Sampling month was also a significant factor for N_2 -fixation rates in *Rhytidiadelphus triquetrus* ($F_{10,203} = 5.49$, $P < 0.001$), but not for *Pleurozium schreberi* ($F_{10,49} = 1.17$, $P = 0.331$). The highest average N_2 -fixation rates for *Rhytidiadelphus triquetrus* and *Pleurozium schreberi* were $9.88 \pm 5.88 \mu\text{mol m}^{-2} \text{d}^{-1}$ in April 2017 and $14.41 \pm 14.41 \mu\text{mol m}^{-2} \text{d}^{-1}$ in August 2016, respectively. The highest N_2 -fixation activity measured in an individual sample over the 11-months period was observed in *Racomitrium elongatum* in August ($567.67 \mu\text{mol m}^{-2} \text{d}^{-1}$). The highest N_2 -fixation rate measured for a sample of *Rhytidiadelphus triquetrus* was $80.00 \mu\text{mol m}^{-2} \text{d}^{-1}$ in April and for *Pleurozium schreberi* was $72.01 \mu\text{mol m}^{-2} \text{d}^{-1}$ in August.

Daylength ranged from 8.5 h to 15.8 h throughout our study period (Fig. 4b). As described above, the Mediterranean climate of western Washington involves cool, wet winters and warm, dry summers. This means that daylength is positively correlated with temperature variables and negatively correlated with precipitation variables (Table 2). Because we could not directly evaluate the relationship between field weather conditions on N_2 -fixation rates, due to our decision to incubate samples under controlled laboratory conditions, we instead evaluated how daylength was related to N_2 -fixation for each species. We found that N_2 -fixation rates were significantly, positively correlated with daylength for both *R. elongatum* and *R. triquetrus*, but not for *P. schreberi* (Table 2). The highest recorded daily activity rates occurred for all three species when daylength was between 13.5 and 14.5 h long. This corresponds to the months of April and August.

The annual median nitrogen fixation rates (given a molar ratio of 3:1 acetylene reduced to N_2) for *Racomitrium elongatum* and for *Rhytidiadelphus triquetrus* range between $0.008 - 0.106 \text{ kg N ha}^{-1} \text{ year}^{-1}$ and $0.001 - 0.018 \text{ kg N ha}^{-1} \text{ year}^{-1}$, respectively based on percent cover estimates ranging from 11 to 100% (Online Resource 5).

Discussion

This study is the first to examine patterns in N_2 -fixation rates of moss-cyanobacteria associations in a Pacific

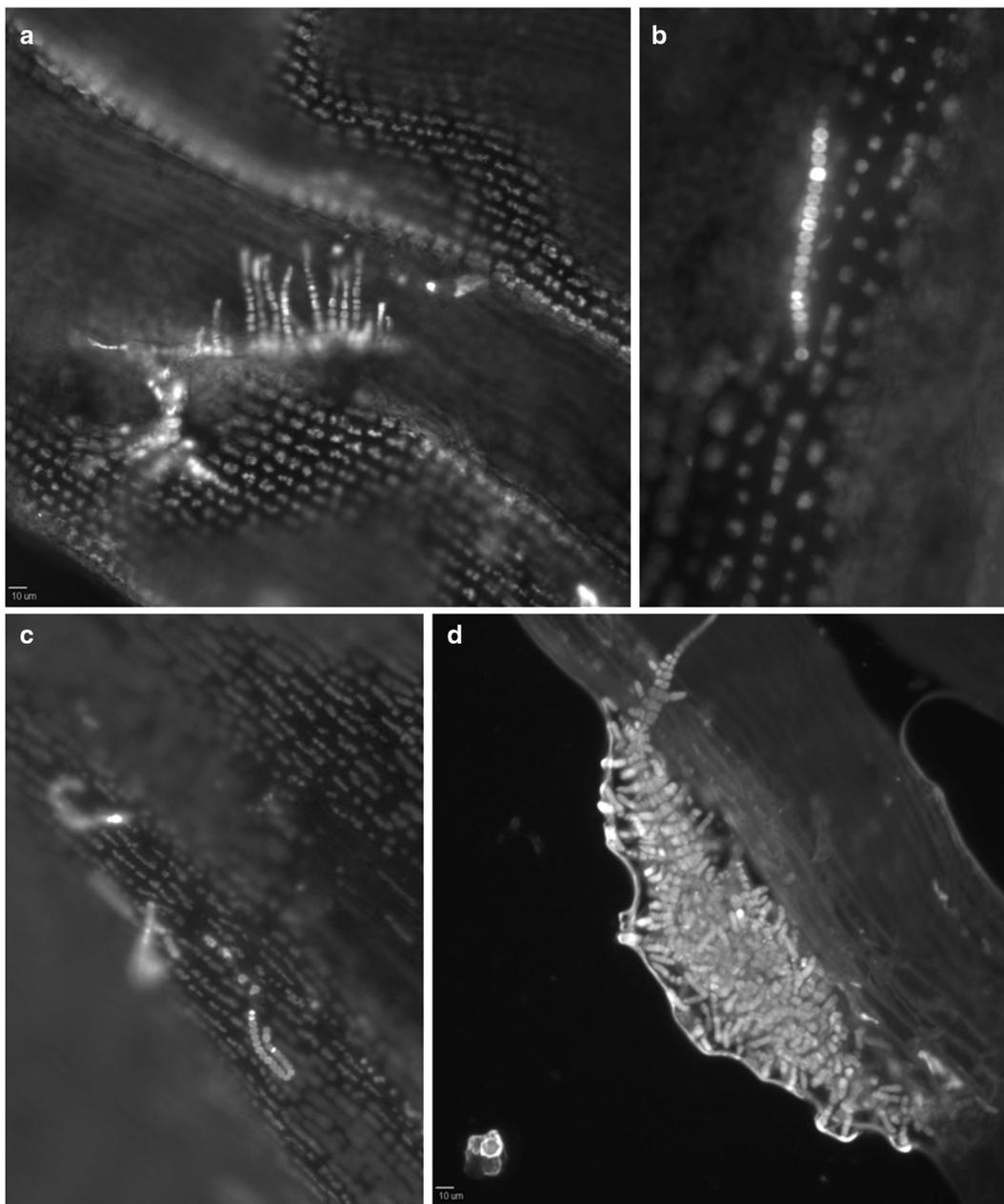


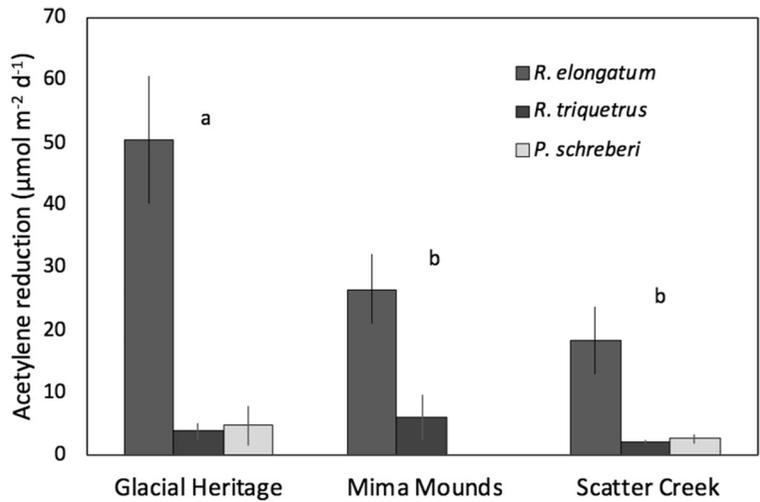
Fig. 2 Epi-fluorescence microscopic images of cyanobacteria cells on moss leaves at 100 \times magnification. *Stigonema* branching cells on leaves of *Racomitrium elongatum* (a) and *Pleurozium*

schreberi (d) Chains of *Nostoc* on *R. elongatum* (b) and *Rhytidiadelphus triquetrus* (c). (Photo credit: C. Allison and S. Pierce)

Northwest U.S. prairie ecosystem. Our results highlight a previously undescribed source of biological N₂-fixation in Puget Sound prairies and extend the geographic ranges of reported N₂-fixing cyanobacterial associations with the mosses we studied. We found both *Nostoc* and *Stigonema* cyanobacteria associating with three prairie mosses: *Racomitrium elongatum*, *Rhytidiadelphus*

triquetrus, and *Pleurozium schreberi* in Puget Sound prairies. *Racomitrium elongatum* is one of the most common and abundant mosses in cold regions of the world and is also widely distributed in temperate regions (Noguchi et al. 1988). Our findings suggest that associations between N₂-fixing cyanobacteria and *Racomitrium elongatum* (and potentially other

Fig. 3 Acetylene reduction ($\mu\text{mol C}_2\text{H}_2 \text{ m}^{-2} \text{ day}^{-1}$) for each of the prairie moss species at each of the prairie sites. No *P. schreberi* samples were collected from Mima Mounds. Site averages (across months) \pm 1SE are presented and different letters represent significant site differences, using $\alpha = 0.05$



Grimmiaceae) may be more widespread than previously reported, which could have broad implications for understanding global C and N cycling.

N_2 -fixation rates varied significantly by moss species in our study. Most studies of bryophyte-associated cyanobacterial N_2 -fixation have focused on

feathermosses belonging to the Hylocomiaceae (i.e., *Pleurozium* and *Hylocomium*) (Lindo et al. 2013), which share morphological features such as a relatively large, pleurocarpous growth habit, often forming a monospecific carpet across vast areas. In a study reporting N_2 -fixation by cyanobacteria associated with

Fig. 4 Variation between June 2016 and May 2017 in a) acetylene reduction ($\mu\text{mol C}_2\text{H}_2 \text{ m}^{-2} \text{ day}^{-1}$) averaged across sites for each of the prairie moss species and b) average daylength, average monthly temperature and total monthly precipitation for south Puget Sound prairies, Washington State, USA. Species-specific averages are stacked for each month and one standard error for each species is displayed

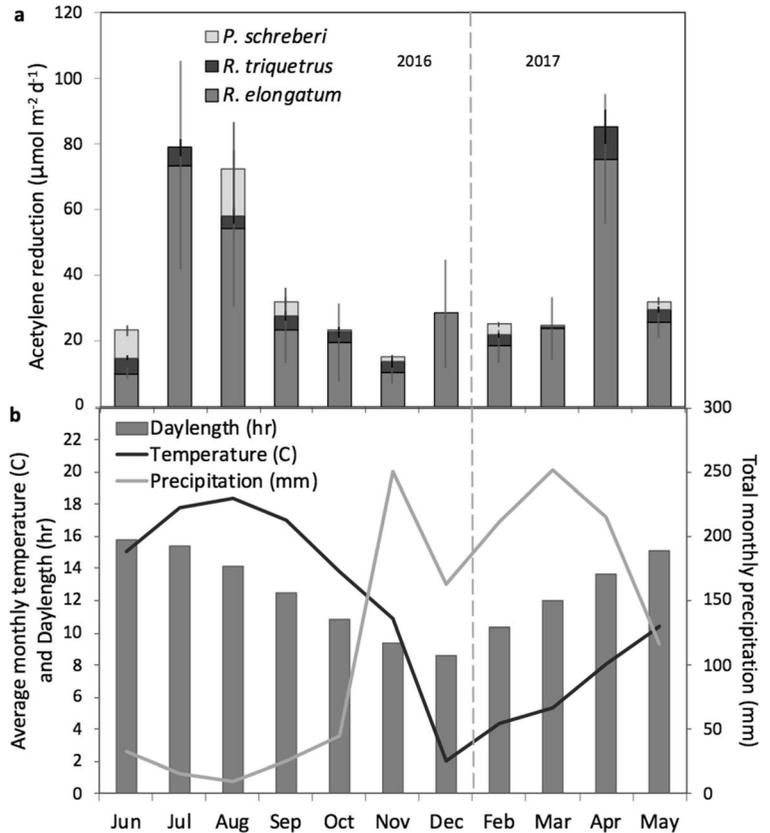


Table 2 Correlation coefficients and associated *P* values for the relationships between daylength and activity rates for each species (using Spearman rank-order correlations) and weather variables for the south Puget Sound prairies (using Pearson's product moment correlations)

		Daylength	
		Corr. coeff.	<i>P</i> value
Activity rates	<i>Racomitrium elongatum</i>	0.456	0.013
	<i>Rhytidiadelphus triquetrus</i>	0.442	0.013
	<i>Pleurozium schreberi</i>	0.135	0.550
Weather variables	Day-of sampling temperature	0.659	<0.001
	Average weekly temperature	0.688	<0.001
	Average monthly temperature	0.595	<0.001
	Day-of sampling precipitation	−0.407	<0.001
	Sum of weekly precipitation	−0.389	<0.001
	Sum of monthly precipitation	−0.453	<0.001
	Precipitation frequency week-prior to sampling	−0.426	<0.001
	Precipitation frequency month-prior to sampling	−0.531	<0.001

All of the daily, weekly and monthly temperature and precipitation measurements reflected conditions immediately leading up to the sampling day

epiphytic and forest floor bryophytes in the coastal temperate rainforest of North America, *Rhytidiadelphus* spp. (also a member of the Hylocomiaceae) were found as hosts for N₂-fixing cyanobacteria (Lindo and Whiteley 2011). The current study corroborates these findings, further expanding our knowledge of this association in the Hylocomiaceae.

In contrast to the Hylocomiaceae, mosses belonging to the Grimmiaceae (e.g., *Racomitrium*) are typically xerophytic adapted species that form dense tufts or cushions on exposed rocks, with leaves adapted to retain moisture and reflect high light levels (Flora of North America Editorial Committee 1993+). Cyanobacteria symbiosis in members of the Grimmiaceae has not been extensively documented and therefore the physiological parameters that control N₂-fixation rates are less understood. In the current study, average daily N₂-fixation rates for *Racomitrium elongatum* were an order of magnitude higher than *Rhytidiadelphus triquetrus* and *Pleurozium schreberi*. This may be an important predictor of tolerance to climate warming, whereby the denser, cushion-like growth form of *Racomitrium elongatum* provides better moisture retention for cyanobacteria than the looser, quicker-drying growth form of the feathermosses under drought conditions typical of summers in the Pacific Northwest prairies.

The observation that N₂-fixation rates for two species were variable throughout the sampling period and peaked in April and July/August is similar to a pattern

reported in a Pacific Northwest forest canopy moss, *Isoetecium stoloniferum*, collected in the same sampling year (Bidwell 2017). In Scandinavian boreal forests a bimodal-type distribution pattern was also observed, however peak fixation occurred in June and September, tracking the moist spring and fall seasons (DeLuca et al. 2002; Zackrisson et al. 2009). Interestingly, seasonal peaks in N₂-fixation in the Pacific Northwest United States and those in boreal forests of Sweden do not align with temperature and precipitation averages during peak fixation months and therefore, cannot fully explain the bimodal patterns observed N₂-fixation (Swedish Meteorological Institute, SMHI).

It is pertinent to point out again that we conducted our acetylene reduction assay (ARA) in a laboratory under room-temperature conditions, while in most studies of N₂-fixation by moss-cyanobacterial associations, the ARA incubations are made under natural conditions in the field (e.g., DeLuca et al. 2002). While spatial and temporal variation in N₂-fixation have been measured using laboratory-based ARA to isolate the effects of individual factors on fixation rates (Chapin et al. 1991; Markham 2009), and studies comparing fixation rates between lab- and field-incubated samples have found little difference between the two methods for growing season measurements (Bidwell unpublished (see Online Resource 3); Markham 2009), it is possible that laboratory conditions (stable temperature and moisture) could confound our results. As an example, warmer

conditions in the lab could result in artificially elevated N_2 -fixation rates in the winter months. However, while laboratory conditions were static throughout the year, the patterns in N_2 -fixation that we observed showed marked seasonal patterns and significant positive correlation with daylength, which itself is significantly positively correlated with field temperatures and negatively correlated with precipitation.

We hypothesize that changes in day length may be an important environmental cue for initiating N_2 -fixation in moss-cyanobacteria associations. Photoperiodism has not been well-documented in N_2 -fixing moss-cyanobacterial associations (Billington and Alexander 1978), however many biological processes, such as flowering in angiosperms, are known to be controlled by day length and the phenomenon of photoperiodism has been shown to positively regulate N_2 -fixation activity in marine symbiotic cyanobacteria associated with diatoms (Karl et al. 2012). Our analyses indicate that daylength may partially explain the temporal variation in N_2 -fixation rates observed across sampling date, despite incubation of our moss samples at a consistent temperature in the laboratory. Our weather correlations with daylength also point to the longer-term influence of moisture availability and temperature on seasonal N_2 -fixation rates.

Numerous studies have found a correlation between temperature and N_2 -fixation by moss-associated cyanobacteria, both positive and negative (Gundale et al. 2012; Jean et al. 2018; Markham 2009; Rousk et al. 2018). The patterns observed in this study indicate that the highest monthly fixation rates for *Racomitrium elongatum* and *Rhytidiadelphus triquetrus* occurred in April, when average monthly temperatures begin to reach the established temperature optima reported for cyanobacterial N_2 -fixation (between 10 and 20 °C, depending on the species and environmental conditions) (Gentili et al. 2005). The second highest average monthly N_2 -fixation rates occurred in samples collected during the two warmest and driest months of the year (July and August), suggesting that temperature may be one of the primary drivers of N_2 -fixation by cyanobacteria-associated mosses in this system.

The positive relationship between moisture availability and N_2 -fixation activity in moss-cyanobacterial association is well-established in the literature (e.g., Gundale et al. 2009). Moreover, several studies have observed a positive correlation between rainfall frequency and N_2 -fixation rates in arctic and boreal regions

(Jackson et al. 2011; Gundale et al. 2012; Rousk et al. 2014). These findings stand in contrast with those from our study system, where the drought-like conditions during summer months correspond to some of the highest N_2 -fixation rates, reinforcing that temperature and daylength are probably more important drivers of N_2 -fixation rates in our study system than water availability. Although very low precipitation is characteristic of summer months in the Pacific Northwest, the high water-retention capacity of *Racomitrium*'s cushion-like growth form may provide sufficient moisture levels to maintain N_2 -fixation during the driest months of the year. Frequent morning condensation might also partially explain why prairie moss-cyanobacteria associations are able to remain active during long-periods of drought.

It is also possible that the uniform re-wetting of moss samples prior to ARA incubations may have temporarily increased fixation rates in summer months. In contrast to studies of boreal regions where relatively high monthly precipitation corresponds with warmer months, the wettest months in our study system are also the coldest months of the year. This is when we recorded the lowest N_2 -fixation rates ($<30 \mu\text{mol m}^{-2}\text{d}^{-1}$), likely because the average monthly temperatures during the wettest months (November through March) fall below the temperature optima for nitrogenase activity.

The reproductive phenology of bryophytes may also contribute to the temporal variation observed in our study. Bay et al. (2013) found that in boreal forests, colonization of N_2 -fixing cyanobacteria on *Pleurozium schreberi* is enhanced in spring during the moss reproductive phase. Studies of *Racomitrium elongatum* reproductive phenology showed that moss sporophytes tend to emerge in spring, maturing into the fall (Maruo and Imura 2018). Peak fixation rates for *Racomitrium elongatum* and *Rhytidiadelphus triquetrus* in April were strongly correlated with high leaf cyanobacteria colonization which supports the hypothesis that recruitment of cyanobacteria colonization occurs during periods of moss reproduction (Bay et al. 2013). Interestingly, we found no month-to-month variation in N_2 -fixation rates and no correlation between *Pleurozium schreberi* N_2 -fixation rates with cyanobacteria colonization. This may be due to the greater sensitivity of *Pleurozium schreberi* to N deposition (DeLuca et al. 2007; Zackrisson et al. 2009; Zackrisson et al. 2004), potential differences in optimum temperature and moisture levels for colonizing cyanobacteria (Gentili et al. 2005; Rousk and Michelsen 2017), or simply due to the possibility that our sample

size was too low to detect existing relationships for this species. Additional focus on drivers of *P. schreberi* N₂-fixation rates with higher sampling rates may reveal stronger seasonal patterns.

Land use history and current land management practices are likely to influence both the presence and the productivity of the moss-cyanobacterial associations across our study sites. While our sampling plots had not been burned recently, they had received fire within the past 30 years, annual spot herbicide treatments and hand-pulling activities to remove invasive weeds, and they exist within a framework of very active management and research. Frequent prescribed burns reduce bryophyte cover and shift bryophyte community composition from feather mosses and *Racomitrium*-dominated to more fire-adapted, turf growth form species (e.g., *Ceratodon*, *Polytrichum*) (Calabria et al. 2016). Variation in the frequency, intensity and severity of prescribed burns and other physical disturbances can also lead to patchy bryophyte distribution and uneven release of soil inorganic N across our study sites (Hamman et al. 2011). In boreal forests, N₂-fixation rates by moss-cyanobacteria associations increased linearly with time since last fire (DeLuca et al. 2008), suggesting that increased N availability shortly after fire temporarily inhibits N₂-fixation by moss-cyanobacteria associations. In contrast, N₂-fixation by free-living cyanobacteria in tallgrass prairies of the Midwestern USA were stimulated by burning as a result of increased light levels with removal of litter (Dubois and Kapustka 1983; Knapp and Seastedt 1986). Burning also increases the availability of aboveground phosphorus (P) (Ojima et al. 1994), which can stimulate (Chapin et al. 1991; Eisele et al. 1989; Rousk et al. 2016), repress (Smith 1984) or have no effect on N₂-fixation in mosses (Zackrisson et al. 2009), depending on the species and habitat. From these data, we can deduce that N₂-fixation rates will likely be higher in prairies with lower N deposition and with longer prescribed fire intervals. Given the prevalence of prescribed burning as a restoration tool in Puget Sound prairies, N₂-fixing moss-cyanobacteria associations are likely to be impacted by these practices and land managers should develop conservation plans that include monitoring the long-term effects of burning on prairie moss-cyanobacterial associations.

In addition to prescribed burning practices, varying degrees of anthropogenic N emissions from road traffic, energy production and agriculture likely contributed to

the spatial variation in N₂-fixation rates observed in this study. Rates of N₂-fixation by moss-cyanobacteria decrease under high N availability in boreal forests (DeLuca et al. 2008; Gundale et al. 2012) and have been shown to decrease along a latitudinal N deposition gradient (Zackrisson et al. 2009). It's also possible that moss-associated cyanobacterial species of temperate prairies vary in their response to atmospheric N deposition; this has been observed for more common boreal taxa, such as *Pleurozium schreberi* (Zackrisson et al. 2009; Rousk et al. 2014; Salemaa et al. 2019). More studies are needed on moss-cyanobacterial associations in temperate latitude ecosystems to elucidate the factors that affect spatial variability in N₂-fixation.

The total amount of N₂ fixed by moss-cyanobacterial associations during our study period was at the lower range of annual fixation rates reported from northern boreal forested ecosystems (Lindo et al. 2013) and tropical montane forests of Hawaii (Matzek and Vitousek 2003), and similar to those observed in the canopy moss *Isoetes stoloniferum* collected from Seattle, WA (Bidwell 2017).

Applying the documented total grassland N budget of 1–5 kg N ha⁻¹ yr⁻¹ (Vlassak et al. 1973) to the Puget Sound prairies suggests that, depending on moss cover, the moss cyanobacterial associations in this system have the potential to contribute an additional 0.1–2.5% to the annual N budget, based on a conversion factor of 3:1 (acetylene reduced to N₂). These figures must be interpreted with caution, as many factors (eg. nutrient availability, nitrogenase type) have been shown to influence the conversion ratio (Darnajoux et al. 2017; Menge and Hedin 2009; Rousk et al. 2017).

Future studies of this system should include site and species-specific conversion ratios to convert from acetylene reduced to N₂ fixed and field-based ARA incubations to further explore temperature-mediated effects on N₂-fixation by moss-cyanobacterial associations. Researchers could also consider in situ data loggers that track temperature, moisture, and light regimes in individual moss mats. Finally, more research is necessary to quantify N inputs from this association into the south Puget Sound ecosystem. Establishing a more definitive relationship between fixation rates and environmental variables, as well as placing this particular N source in the context of the total ecosystem N flux, would facilitate modeling and management efforts for this endangered ecosystem.

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Data accessibility The datasets generated during and analyzed during the current study are available at DRYAD entry DOI <https://doi.org/10.5061/dryad.cvdncjt05>

Author contributions L.M.C. conceived the study. L.M.C., K.S.P. and S.T.H. further refined the research questions and developed methodologies; K.S.P., A.B. collected field and instrumentation data with the help of undergraduate researchers (see Acknowledgments section). S.T.H. conducted the statistical analyses. L.M.C. and S.T.H. led the writing of the manuscript and all authors contributed critically to the writing and editing of drafts and gave final approval for publication.

References

- Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. *J Stat Softw* 67:1–48
- Bay G, Nahar N, Oubre M, Whitehouse MJ, Wardle DA, Zackrisson O, Nilsson M-C, Rasmussen U (2013) Boreal feather mosses secrete chemical signals to gain nitrogen. *New Phytol* 200:54–60
- Berg A, Danielsson Å, Svensson BH (2013) Transfer of fixed-N from N₂-fixing cyanobacteria associated with the moss *Sphagnum riparium* results in enhanced growth of the moss. *Plant Soil* 362:271–278
- Bidwell AL (2017) Urbanization impacts on epiphytic nitrogen and metal cycling in *Acer macrophyllum* stands in Western Washington, USA. Thesis, University of Washington, Seattle WA
- Billington M, Alexander V (1978) Nitrogen fixation in a black spruce (*Picea mariana* [mill]) forest in Alaska. *Ecol Bull*: 209–215
- Calabria LM, Arnold A, Charatz E, Eide G, Hynson LM, Jackmond G, Nannes J, Stone D, Vilella J (2015) A checklist of soil-dwelling bryophytes and lichens of the South Puget Sound prairies of Western Washington. *Evansia* 32:30–41
- Calabria LM, Petersen K, Hamman ST, Smith RJ (2016) Prescribed fire decreases lichen and bryophyte biomass and alters functional group composition in Pacific northwest prairies. *Northwest Sci* 90:470–483
- Chapin DM, Bliss LC, Bledsoe LJ (1991) Environmental regulation of nitrogen fixation in a high arctic lowland ecosystem. *Can J Bot* 69:2744–2755
- Chappell CB, Crawford RC (1997) Native vegetation of the South Puget Sound prairie landscape. In: Dunn P, Ewing K (eds) Native vegetation of the South Puget Sound prairie landscape. The Nature Conservancy of Washington, Seattle
- Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO, Perakis SS, Latty EF, Von Fischer JC, Elseroad A, Wasson MF (1999) Global patterns of terrestrial biological nitrogen (N₂) fixation in natural ecosystems. *Glob Biogeochem Cycles* 13:623–645
- Cornelissen JHC, Lang SI, Soudzilovskaia NA, During HJ (2007) Comparative cryptogam ecology: a review of bryophyte and lichen traits that drive biogeochemistry. *Ann Bot* 99:987–1001
- Darnajoux R, Zhang X, McRose DL, Miadlikowska J, Lutzoni F, Kraepiel AM, Bellenger JP (2017) Biological nitrogen fixation by alternative nitrogenases in boreal cyanolichens: importance of molybdenum availability and implications for current biological nitrogen fixation estimates. *New Phytol* 213:680–689
- Deane-Coe KK, Sparks JP (2016) Cyanobacteria associations in temperate forest bryophytes revealed by $\delta^{15}\text{N}$ analysis. *J Torrey Bot Soc* 143:50–57
- Del Moral R, Deardorff DC (1976) Vegetation of the Mima mounds, Washington state. *Ecology* 57:520–530
- DeLuca TH, Zackrisson O, Nilsson M-C, Sellstedt A (2002) Quantifying nitrogen-fixation in feather moss carpets of boreal forests. *Nature* 419:917–920
- DeLuca TH, Zackrisson O, Gentili F, Sellstedt A, Nilsson MC (2007) Ecosystem controls on nitrogen fixation in boreal feather moss communities. *Oecologia* 152:121–130
- DeLuca TH, Zackrisson O, Gundale MJ, Nilsson M-C (2008) Ecosystem feedbacks and nitrogen fixation in boreal forests. *Science* 320:1181–1181
- DuBois JD, Kapustka LA (1983) Biological nitrogen influx in an Ohio relict prairie. *Am J Bot* 70:8–16
- During HJ, Willems JH (1986) The impoverishment of the bryophyte and lichen flora of the Dutch chalk grasslands in the thirty years 1953–1983. *Biol Conserv* 36:143–158
- Eisele KA, Schimel DS, Kapustka LA, Parton WJ (1989) Effects of available P and N:P ratios on non-symbiotic dinitrogen fixation in tallgrass prairie soils. *Oecologia* 79:471–474
- Elbert W, Weber B, Burrows S, Steinkamp J, Büdel B, Andreae MO, Pöschl U (2012) Contribution of cryptogamic covers to the global cycles of carbon and nitrogen. *Nat Geosci* 5:459–462
- Flora of North America Editorial Committee, eds. (1993) Flora of North America north of Mexico. 16+ vols. New York and Oxford. Volume 27: Bryophytes. 2007
- Geiser LH, Neitlich PN (2007) Air pollution and climate gradients in western Oregon and Washington indicated by epiphytic macrolichens. *Environ Pollut* 145:203–218
- Gentili F, Nilsson M-C, Zackrisson O, DeLuca TH, Sellstedt A (2005) Physiological and molecular diversity of feather moss associative N₂-fixing cyanobacteria. *J Exp Bot* 56:3121–3127
- Gundale MJ, Gustafsson H, Nilsson M-C (2009) The sensitivity of nitrogen fixation by a feathermoss–cyanobacteria association to litter and moisture variability in young and old boreal forests. *Can J For Res* 39:2542–2549

- Gundale MJ, Wardle DA, Nilsson M-C (2012) The effect of altered macroclimate on N-fixation by boreal feather mosses. *Biol Lett* 8:805–808
- Hamman ST, Dunwiddie PW, Nuckols JL, McKinley M (2011) Fire as a restoration tool in Pacific northwest prairies and oak woodlands: challenges, successes, and future directions. *Northwest Sci* 85:317–328
- Haubensak KA, Parker IM (2004) Soil changes accompanying invasion of the exotic shrub *Cytisus scoparius* in glacial outwash prairies of western Washington [USA]. *Plant Ecol* 175:71–79
- Houle D, Bilodeau Gauthier S, Paquet S, Planas D, Warren A (2006) Identification of two genera of N₂-fixing cyanobacteria growing on three feather moss species in boreal forests of Quebec, Canada. *Can J Bot* 84:1025–1029
- Jackson BG, Martin P, Nilsson M-C, Wardle DA (2011) Response of feather moss associated N₂ fixation and litter decomposition to variations in simulated rainfall intensity and frequency. *Oikos* 120:570–581
- Jean ME, Cassar N, Setzer C, Bellenger JP (2012) Short-term N₂ fixation kinetics in a moss-associated cyanobacteria. *Environ Sci Technol* 46:8667–8671
- Jean ME, Phalyvong K, Forest-Drolet J, Bellenger JP (2013) Molybdenum and phosphorus limitation of asymbiotic nitrogen fixation in forests of eastern Canada: influence of vegetative cover and seasonal variability. *Soil Biol Biochem* 67:140–146
- Jean M, Mack MC, Johnstone JF (2018) Spatial and temporal variation in moss-associated dinitrogen fixation in coniferous- and deciduous-dominated Alaskan boreal forests. *Plant Ecol* 219:837–851
- Karl DM, Church MJ, Dore JE, Letelier RM, Mahaffey C (2012) Predictable and efficient carbon sequestration in the North Pacific Ocean supported by symbiotic nitrogen fixation. *Proc Natl Acad Sci* 109(6):1842–1849
- Knapp AK, Seastedt TR (1986) Detritus accumulation limits productivity of Tallgrass prairie. *BioScience* 36:662–668
- Larrain J, Quandt D, Stech M, Muñoz J (2013) Lumping or splitting? The case of *Racomitrium* (Bryophytina: Grimmiaceae). *Taxon* 62:1117–1132
- Lett S, Michelsen A (2014) Seasonal variation in nitrogen fixation and effects of climate change in a subarctic heath. *Plant Soil* 379:193–204
- Lindo Z, Whiteley JA (2011) Old trees contribute bio-available nitrogen through canopy bryophytes. *Plant Soil* 342:141–148
- Lindo Z, Nilsson M-C, Gundale MJ (2013) Bryophyte-cyanobacteria associations as regulators of the northern latitude carbon balance in response to global change. *Glob Chang Biol* 19:2022–2035
- Liu X, Wang Z, Li X, Rousk K, Bao W (2020) High nitrogen resorption efficiency of forest mosses. *Ann Bot* 125:557–563
- Markham JH (2009) Variation in moss-associated nitrogen fixation in boreal forest stands. *Oecologia* 161:353–359
- Maruo F, Imura S (2018) The effect of snow cover on the phenology of the moss *Racomitrium lanuginosum*. *Bryologist* 121:148–157
- Matzek V, Vitousek P (2003) Nitrogen fixation in bryophytes, lichens, and decaying wood along a soil-age gradient in Hawaiian montane rain forest. *Biotropica* 35:12–19
- Menge DN, Hedin LO (2009) Nitrogen fixation in different biogeochemical niches along a 120 000-year chronosequence in New Zealand. *Ecology* 90:2190–2201
- Merrill GLS (1991) Bryophytes of Konza prairie research natural area, Kansas. *Bryologist* 94:383–391
- NOAA (2019) <https://www.ncdc.noaa.gov/cdo-web/datatools/normals>. Accessed 9 August 2019
- Noguchi A, Iwatsuki Z, Yamaguchi T (1988) Illustrated moss flora of Japan. Part 2. Hattori Botanical Laboratory, Nichinan
- Ojima DS, Schimel DS, Parton WJ, Owensby CE (1994) Long- and short-term effects of fire on nitrogen cycling in tallgrass prairie. *Biogeochemistry* 24:67–84
- R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
- Reddy GB, Giddens J (1981) Nitrogen fixation by moss-algal association in grassland. *Soil Biol Biochem* 13:537–538
- Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Microbiology* 111:1–61
- Rousk K, Michelsen A (2017) Ecosystem nitrogen fixation throughout the snow-free period in subarctic tundra: effects of willow and birch litter addition and warming. *Glob Chang Biol* 23:1552–1563
- Rousk K, Jones DL, DeLuca TH (2014) Exposure to nitrogen does not eliminate N₂ fixation in the feather moss *Pleurozium schreberi* (Brid.) Mitt. *Plant Soil* 374:513–521
- Rousk K, Sorensen PL, Michelsen A (2016) Nitrogen transfer from four nitrogen-fixer associations to plants and soils. *Ecosystems* 19:1491–1504
- Rousk K, Degboe J, Michelsen A, Bradley R, Bellenger JP (2017) Molybdenum and phosphorus limitation of moss-associated nitrogen fixation in boreal ecosystems. *New Phytol* 214:97–107
- Rousk K, Sorensen PL, Michelsen A (2018) What drives biological nitrogen fixation in high arctic tundra: moisture or temperature? *Ecosphere* 9:e02117
- Salemaa M, Lindroos A-J, Merilä P, Mäkipää R, Smolander A (2019) N₂ fixation associated with the bryophyte layer is suppressed by low levels of nitrogen deposition in boreal forests. *Sci Total Environ* 653:995–1004
- Smith VR (1984) Effects of abiotic factors on acetylene reduction by cyanobacteria epiphytic on Moss at a Subantarctic Island. *Appl Environ Microbiol* 48:594–600
- Smith RJ, Benavides JC, Jovan S, Amacher M, McCune B (2015) A rapid method for landscape assessment of carbon storage and ecosystem function in moss and lichen ground layers. *Bryo* 118:32–45
- Sorensen PL, Michelsen A (2011) Long-term warming and litter addition affects nitrogen fixation in a subarctic heath. *Glob Chang Biol* 17:528–537
- Stanley AG, Dunwiddie PW, Kaye TN (2011) Restoring invaded Pacific northwest prairies: management recommendations from a region-wide experiment. *Northwest Sci* 85:233–246
- van der Linden JO, Farrar DR (1983) An ecological study of the bryophytes of a natural prairie in northwestern Iowa. *Bryologist* 86:1–13
- Vlassak K, Paul EA, Harris RE (1973) Assessment of biological nitrogen fixation in grassland and associated sites. *Plant Soil* 38:637–649

- Washington Natural Heritage Program (2011) Proposed list of rare lichens. <https://www.dnr.wa.gov/NHPlists>. Accessed 17 December 2015
- Western Regional Climate Center (2017) Climate Summaries. <https://wrcc.dri.edu/Climate/summaries.php>. Accessed July 20 2017
- Zackrisson O, DeLuca TH, Nilsson M-C, Sellstedt A, Berglund LM (2004) Nitrogen fixation increases with successional age in boreal forests. *Ecology* 85:3327–3334
- Zackrisson O, DeLuca TH, Gentili F, Sellstedt A, Jäderlund A (2009) Nitrogen fixation in mixed *Hylocomium splendens* Moss communities. *Oecologia* 160:309–319
- Zales WM (1971) Bryophytes of goose Lake prairie, Illinois. *Trans Illinois State Acad Sci* 64:222–224

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