Studies in bryophyte-cyanobacterial symbioses: host-symbiont diversity and N fixation controls in a boreal forest and a beach-ridge complex in Baileys Harbor, Wisconsin

by

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I would like to dedicate this thesis to Lake Michigan
for lessons on the nature of impermanence.

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Dissertation abstract. This work focuses on (1) exploring how frequently cyanobacterial symbiosis occurs with bryophytes, (2) determining how often cyanobacterial symbionts belong to the N-fixing order Nostocales, and (3) characterizing potential controls on ecosystem-scale contributions of fixed N, given the possibility of bryophyte-microbial N fixation.

To explore the morphotype diversity and frequency of cyanobacterial symbiosis, 478 single-species bryophyte samples were collected over four seasons from small circular plots in a second-growth boreal forest in Baileys Harbor, Wisconsin, near the shore of Lake Michigan. Logistic regression was used to evaluate the predictive power of season and microhabitat structure for (1) the presence or absence of cyanobacteria on a given bryophyte sample, and (2) the presence or absence of Nostocalean cyanobacteria, given presence of cyanobacteria. Most predictors were not influential, but cyanobacterial symbionts were more likely to be present on bryophytes associated with rock substrates, suggesting that exposed rocks might function as “hotspots” for N fixation. 40% of all bryophyte taxa screened represented potentially novel symbioses, although cyanobacterial abundances were low for many samples.

To characterize potential controls on bryophyte-associated N fixation in a Lake Michigan beach-ridge ecosystem, paired single-species samples from 10 sites across the substrate-age gradient were subjected to the acetylene reduction assay on multiple dates. One bryophyte sample was tested at ambient moisture levels, and one at saturated moisture levels, to determine whether short-term moisture limitations might

suppress N fixation. At the landscape level, upland and wetland sites were paired along the substrate-age gradient. General linear model results indicate that wetland status is associated with a higher probability of detecting symbiotic N fixation; water saturation also increased the probability of detecting N fixation by a small but statistically significant amount. The estimated probability of detecting N fixation in wetland samples decreased with swale age. Intense N fixation in young beach wetlands was associated with mosses in Amblystegiaceae, Bartramiceae, Bryaceae, and Hypnaceae.

N fixation was rarely detected in the uplands, where mosses in Dicranaceae and Hylocomiaceae were dominant. This raises the possibility of differential response to controls such as atmospheric N deposition for the upland and wetland moss families.

Part I: A family-level overview of bryophyte-cyanobacterial associations indicates symbioses are little-studied but potentially common.

Key results from Part I. Bryophyte-cyanobacterial associations are important in nitrogen metabolism for individual plants, and in nitrogen cycling for some ecosystems. This study is an attempt to systematically assess the prevalence of such symbioses among diverse bryophyte families. A literature review was used to create a dataset with over 1000 reports of bryophyte-cyanobacterial associations from the scientific literature; this was used to identify families of mosses, liverworts, and hornworts that host or do not host cyanobacteria. Among the 170 moss, liverwort, and hornwort taxa studied, 147 species or genera (86% of all unique taxa) hosted cyanobacteria; there are additional hornwort species capable of symbiosis in literature not reviewed here. Bryophyte-cyanobacterial associations occur in at least 31 moss families, 20 liverwort families, and all 5 hornwort families. Bryophyte taxa that did not host cyanobacteria were relatively rare, encompassing 14% of the 170 bryophyte taxa examined. 77% of all species or genera screened for diazotrophy were capable of N fixation (103 of 134 unique taxa).

Bryophyte-cyanobacterial interactions are more widely distributed among the mosses, liverworts, and hornworts than previously recognized. The scarcity of negative reports neither supports nor refutes the idea that particular bryophyte taxa are consistently incapable of cyanobacterial symbioses, due to both limited taxon sampling (< 1% of all extant species) and the potential for a positive bias in the publication of results. It remains to be seen whether bryophyte-cyanobacterial associations are extremely rare,

moderately common, or perhaps nearly ubiquitous; there is a need to embrace a broader array of bryophyte taxa in future work.

INTRODUCTION

The bryophytes, a group of non-vascular plants consisting of the liverworts, the mosses, and the hornworts, are known to associate with various cyanobacteria, some of which are capable of N fixation. These organisms may live in biofilm communities on the surface of bryophyte tissues, in specialized morphological structures such as slime pits, or even inside bryophyte cells (particularly in peat moss). In some ecosystems, most notably boreal forest and mire habitats of the circumpolar north, bryophyte-cyanobacterial associations are an important source of fixed N; but we still know relatively little about the overall prevalence of bryophyte-cyanobacterial symbioses. Which of the thousands of moss, liverwort, and hornwort taxa are capable of hosting cyanobacteria?

Work by Bay et al. (2013) has highlighted the potential for species-specific differences in chemical signaling between moss plants and potential cyanobacterial symbionts. In the culture-based experiments of Bay and colleagues, some moss species were able to communicate with cyanobacteria and establish symbioses, and others were not. Are such differences truly and broadly characteristic of specific bryophyte taxa—that is, are some bryophytes generally incapable of hosting cyanobacteria under all possible environmental conditions? Are some bryophytes characteristically or consistently

involved with cyanobacterial symbionts? How common is the symbiotic condition, and what can its distribution among the hornworts, liverworts, and mosses tell us about the evolution of the bryophytes--and perhaps, of the land plants in general?

This review will contribute to an active debate regarding these questions; recent work related to bryophyte-cyanobacterial diversity includes analyses and reviews by Alvarenga & Rousk 2022 and Chen & Nelson 2022. Alvarenga & Rousk (2022) focus on the bacterial microbiome of mosses, providing insight into the genera of mosses most intensively studied based on a systematic search of the Google Scholar database. Chen & Nelson (2022) focus on the microbiome of mosses, liverworts, and hornworts, incorporating information on a wide array of microbes (diazotrophs, methylotropes, fungi, and protists) based on a systematic seach of the Web of Science. This review focuses on bryophytes in general, but with different methods and a specific focus on cyanobacteria. I utilized database search tools, but also identified papers of interest via citations in relevant papers. Potential benefits of a better understanding of the extent and diversity of bryophyte-cyanobacterial symbioses are numerous, ranging from better estimates of controls on N and C storage and transfer rates in ecosystems and landscapes to a better appreciation of the evolution of plant-microbe chemical communication among the plants.

METHODS

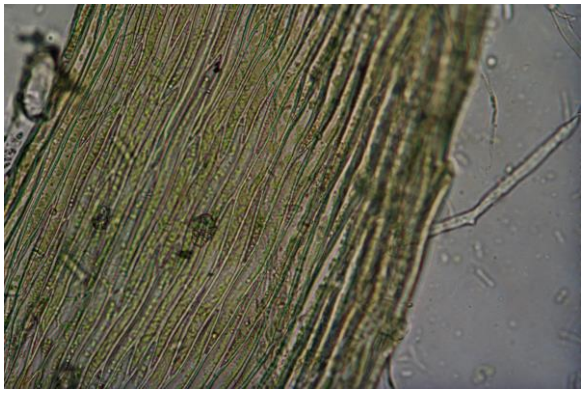
To identify bryophytes capable of hosting cyanobacteria, 190 publications, most from 1955 to 2016, were reviewed for evidence relevant to the following questions. (1) Which bryophytes are able to host cyanobacteria, either epiphytically, endophytically, or both? (2) Which bryophyte taxa have yielded evidence of N fixation activity? (3) Given that heterotrophic N-fixing bacteria have also been documented on bryophytes (e.g., Basilier et al., 1979, Opelt et al., 2007), is there evidence of bryophyte-related N fixation in the apparent absence of cyanobacteria? (4) How broad is the basis of evidence for our conclusions about bryophyte-cyanobacterial symbioses, i.e., how much of the diversity at a given taxonomic level is encompassed by published reports of symbiosis for a given family of bryophytes?

To minimize the chances of a false positive report (i.e., characterizing free-living cyanobacteria that are merely living in proximity to bryophytes as symbionts), the following criteria were applied. First, the cyanobacteria must be living directly on bryophyte tissues, or within bryophyte cells. Reports of cyanobacteria living "among" mosses, or "near" mosses, were excluded. For example, algal biofilms and cyanobacteria are sometimes mentioned in studies of slime molds (myxomycetes) and bryophytes, but the descriptions did not indicate whether an epiphytic relationship existed; these reports were excluded (e.g., Smith and Stephenson, 2007). Second, reports of cyanobacteria in loose association with bryophytes in aquatic habitats (e.g., inventories of cyanobacteria from *Sphagnum* bogwater, Malatoni, 1999) were excluded, although aquatic bryophytes with epiphytic cyanobacteria were included. Third,

bryophyte-cyanobacterial associations from soil crusts[1] were not a focus of the review, as the crust bryophytes are more or less embedded in a diverse bacterial matrix; the structure and function of these systems may be quite different from that of an epiphytic or endophytic cyanobacterial symbiosis. Fourth, light microscopy studies reporting the absence of cyanobacteria were taken at face value, even though work with epifluorescence microscopy has demonstrated that sometimes abundant and diverse cyanobacterial communities may be difficult to detect using normal light (Figure 1). A false negative was deemed preferable to a false positive.

Figure 1(a) shows that cyanobacteria on a moss leaf *(Brachythecium* sp.) may be essentially invisible under natural light (magnification 200X). In Figure 1(b), cyanobacterial photosynthetic pigments on the same moss leaf emit red light under fluoresecent illumination (magnification 200X), highlighting diverse cyanobacterial morphotypes (unicells and various filaments).

A. Moss leaf with light microscopy



B. Moss leaf with epifluorescent microscopy

Figure 1. Cyanobacterial pigments are easier to detect with epifluorescent microscopy.

[1] The literature on soil crusts is quite robust; interested readers are referred to the work of Dr. Jayne Belnap as a starting point.

Heterocysts (specializing N-fixing cells) are visible as darkened cells in some filaments, as these cells typically lack photosynthetic pigments.

In selecting papers, a few laboratory studies involving cultured strains of cyanobacteria—occasionally isolated from organisms that are not bryophytes—were deemed to provide evidence of an association, given the possibility of the broad distribution of microbes in nature. Examples of such associations occur in Stewart & Rodgers 1978, a study in which cyanobacteria isolated from a liverwort, a hornwort, and the vascular plant *Gunnera* sp. were used to infect *Blasia pusilla* L. By contrast, studies involving genetically-modified strains of cyanobacteria, often used in molecular-level work (e.g., Wong and Meeks, 2002; Chapman et al., 2008), were excluded because the mutant cyanobacteria differ from those in the natural symbiotic systems that are the focus of this review. Tests of N fixation potential (e.g., acetylene reduction assays, stable isotope studies with ^{15}N) were included only when the cyanobacteria and bryophyte were in the symbiotic state. Results from studies involving only cyanobacterial strains isolated from bryophytes, or free-living cyanobacteria living in bryophyte-dominated habitats, were excluded.

With respect to taxonomy: All microbial autotrophs currently recognized by the online database CyanoDB.cz (Komárek and Hauer, 2013) were classified as cyanobacteria, as well as observations of unnamed cyanobacteria. Names reported for bryophytes (often decades old, sometimes illegitimate or invalid) were assigned to an appropriate modern

species or genus using Tropicos (2017). Individual species or genera were assigned to an appropriate bryophyte family using specialized classifications for hornworts, liverworts, or mosses: the online Classification of the Phylum Anthocerotophyta Stotl. & Crand.-Stotl. (2023) for the hornworts, the online classification of the Phylum Marchantiophyta Stotler & Crand.-Stotl. (2017) for the liverworts, and the Online Classification of the Bryophyta by Goffinet and Buck (2017) for the mosses. It was not possible to use a single taxonomic resource, as some phylogenetic resources for bryophytes focus on taxa at the family level or higher, without incorporating species-level information.

With respect to taxonomy, I am an ecologist utilizing resources developed by bryophyte systematists with far greater expertise; I defer to that expertise and also must, in certain instances, cope with taxonomic challenges related to a lack of concensus in a rapidly-evolving field. The moss family Calliergoniaceae Vanderp., Hedenäs, C.J. Cox & A.J. Shaw, for example, is recognized by both Tropicos and Goffinet & Buck at the time of writing (2023), but Tropicos does not yet recognize any subordinate taxa. By contrast, several genera have been assigned to Calliergoniaceae in the Goffinet & Buck classification since 2017. In such situations I have deferred to the classification by Goffinet & Buck (2017).

A species count for bryophtye families with cyanobacterial associates was calculated to provide an estimate, within an order of magnitude or so (e.g., less than 0.1%, less than 1%, less than 10%), of how well-studied the species-level biodiversity of a given family

is, with respect to bryophyte-cyanobacterial symbioses. Estimates were obtained by taking the total number of valid species recognized for a family in Tropicos and (1) subtracting valid species counts from any genera excluded by Goffinet et al. (2017), then (2) adding valid species counts from any genera added to a given family by Goffinet et al. (2017). The species counts were rounded to the nearest 5 for larger families. These estimates are not definitive, as bryophyte phylogenies continue to evolve rapidly; they do, however, provide the reader with some perspective on how much work remains to be done in relation to bryophyte-cyanobacterial symbioses.

RESULTS

Organization of the review

The results will be presented as an overview of bryophyte cyanobacterial hosts at the family level for the mosses, the liverworts, and the hornworts, with occasional discussion of work on particular species. In addition, the implications of the results will be considered in light of the evolution of the bryophytes as early land plants.

Citations for some families involve a large number of publications. To avoid lengthy paragraphs consisting primarily of citations, the reader is referred to reference material in the Supplemental Information. It is possible to search by bryophyte taxon within the spreadsheet found in this section, which includes 1070 individual reports from 190 publications. These reports indicate whether I was able to locate relevant publications

for a given bryophyte species, genus, or family; taxonomic notes; site descriptions; and information on the strength of evidence (positive or negative) related to cyanobacterial presence/absence and N fixation.

Family overview: Cyanobacterial associations among the mosses

Early-divergent mosses. With the family Takakiaceae Stech & W. Frey at the base of the moss phylogeny, the other early-divergent mosses based on the classification of Goffinet et al. (2017) constitute a grade composed of the families Sphagnaceae Dumort, Flatbergiaceae A.J. Shaw, Ambuchananiaceae Seppelt & H. A. Crum, Andreaeceae Dumort, Andreaeobryaceae Steere, Oedipodiaceae Schimp., Polytrichicaeae Schwägr., Tetraphidaceae Schimp., Buxbaumiaceae Schimp., Diphysciaceae M. Fleisch., Gigispermaceae Lindb., Timmiaceae Schimp., Disceliaceae Schimp., Bryobartramiaceae Sainsb., Encalyptaceae Schimp., and Funariaceae Schwägr., ordered from the most basal to the most recently-diverged. These families are generally characterized as acrocarpous.

Overall there is evidence of bryophyte-cyanobacterial associations in four (Sphagnaceae, Andreaeceae, Polytrichaceae, Funariaceae) of these 17 early-divergent moss families (Table 1). References and species relevant to each family can be found in the Supplemental Information. Sphagnaceae is one of the most-basal early-divergent moss family; Funariaceae, the most-derived early-divergent moss family. However, we lack both convincing negative and positive reports of bryophyte-cyanobacterial

associations for most of the families within the early-divergent moss grade as a whole, although Satjarak et al. (2022) found no evidence of cyanobacteria in a metagenomic study of *Takakia lepidozioides*. Further investigation may reveal that all 17 of these early-divergent moss families are capable of forming associations with cyanobacteria; but it is also possible that such associations represent relatively unusual innovations in Sphagnaceae, Andreaeceae, Polytrichaceae, and Funariaceae. We lack clarity with respect to (1) the likely ancestral condition of the mosses, and (2) the distribution of moss-cyanobacterial associations across these 17 early-divergent families.

Evidence of N fixation associated with cyanobacteria exists for all four early-divergent host families (Sphagnaceae, Andreaeceae, Polytrichaceae, and Funariaceae). Relevant references are available in the Supplemental Information. There are also reports of N fixation in the apparent absence of cyanobacteria for three species in Andreaeaceae (Arróniz-Crespo et al., 2014), two species in Polytrichaceae (Lambert and Reiners, 1979; Bowden, 1991), and at least three species in Sphagnaceae (Basilier, 1979; Granhall and Selander, 1973; Lambert and Reiners, 1979; Schwintzer, 1983). These reports may be indicative of heterotrophic rather than cyanobacterial N fixation; it is also possible that cyanobacteria were present but not visible in light microscopy studies.

CYANOBACTERIAL SYMBIOSIS IN EARLY-DIVERGENT MOSS FAMILIES		
FAMILY	**SYMBIOTIC STATUS**	**N FIXATION STATUS**
Takakiaceae Stech & W. Frey	No evidence of cyanobactera per Satjarak et al. (2022) metagenomic study	Unknown
Sphagnaceae Dumort	Robust evidence of cyanobacterial symbiosis involving diverse taxa; cyanobacteria endophytic and epiphytic.	Abundant evidence of N fixation, sometimes in the absence of cyanobacteria (heterotrophic fixation).
Flatbergiaceae A.J. Shaw	Unknown	Unknown
Ambuchananiaceae Seppelt & H. A. Crum	Unknown	Unknown
Andreaeaceae Dumort.	Evidence of cyanobacterial symbiosis involving a few taxa; cyanobacteria epiphytic.	Evidence of N fixation involving a few taxa, occasionally in the absence of cyanobacteria (heterotrophic fixation).
Andreaeobryaceae Steere	Unknown	Unknown
Oedipodiaceae Schimp.	Unknown	Unknown
Polytrichaceae Schwägr.	Evidence of cyanobacterial symbiosis involving some taxa; cyanobacteria epiphytic.	Evidence of N fixation involving a few taxa, occasionally in the absence of cyanobacteria (heterotrophic fixation).
Tetraphidaceae Schimp.	Unknown	Unknown
Buxbaumiaceae Schimp.	Unknown	Unknown
Diphysciaceae M. Fleisch.	Unknown	Unknown
Gigaspermaceae Lindb.	Unknown	Unknown
Timmiaceae Schimp.	Unknown	Unknown
Disceliaceae Schimp.	Unknown	Unknown
Bryobartramiaceae Sainsb.	Unknown	Unknown
Encalyptaceae Schimp.	Unknown	Unknown
Funariaceae Schwägr.	Evidence of cyanobacterial symbiosis involving the pioneer moss *Funaria hygrometrica* Hedw.; cyanobacteria epiphytic.	N fixation common in *Funaria hygrometrica.*

Table 1. Cyanobacterial symbioses are evident in several early-divergent moss families.

Organizational themes for moss symbioses. Two subclasses structure this discussion of cyanobacterial associates among the more-derived moss families. The first subclass, consisting primarily of acrocarps, is Dicranidae Doweld (Goffinet et al., 2017). The second subclass, Bryidae Engl., encompasses both acrocarps and pleurocarps that arose within Bryidae (Goffinet et al., 2017). In this next section we will focus on the mostly acrocarpous families within Dicranidae.

Dicranidae. Thirty-two acrocarpous moss families comprise the order Dicranidae. About half of these families are monogeneric as currently defined; others range from modestly diverse (several genera) to highly diverse (Pottiaceae, with over 90 different genera) (Goffinet et al. 2017).

At least seven families within the acrocarpous subclass Dicranidae are capable of hosting cyanobacteria (see Supplemental Information for references). These families include Distichiaceae Schimp., Grimmiaceae Arn., Fissidentaceae Schimp., Ditrichaceae Limpr., Dicranaceae Schimp., Leucobryaceae Schimp., and Pottiaceae Schimp. (about 22% of all families within Dicranidae). Nitrogenase activity has been documented for six of these families (see Supplemental Information); the seventh, Fissidentaceae, had not been tested for N fixation at the time of writing. Strong evidence of heterotrophic N fixation appears to be rare within Dicranidae based on the information available: reports were found for just two mosses, *Ditrichum strictum* (Line, 1992) in Ditrichaceae and *Dicranum* sp. (Lambert and Reiners, 1979) in Dicranaceae. As with the early-divergent mosses, little or no information about potential cyanobacterial symbioses is available for most families in Dicranidae (~ 78%).

Bryidae: Acrocarps. Subclass Bryidae Engl. encompasses both acrocarps and the pleurocarpous mosses that arose from acrocarps. We will focus on the acrocarps in this section of our review. These families include Splachnaceae Grev. & Arn., Meesiaceae Schimp., Pulchrinodaceae D. Quandt, N.E. Bell & Stech, Bryaceae Schwägr., Phyllodrepaniaceae Crosby, Mniaceae Schwägr., Leptostomataceae Schwägr.,

Bartramiaceae Schwägr., Orthotrichaceae Arn., Hedwigiaceae Schimp., Helicophyllaceae Broth., Rhacocarpaceae Kindb., Rhizogoniaceae Broth., Aulacomniaceae Schimp., and Orthodontiaceae Goffinet. The last three families are of particular interest in an evolutionary context, as some molecular evidence supports their close relationship to the pleurocarpous mosses (Cox et al., 2010).

Overall seven of 15 families in the mostly acrocarpous grade of subclass Bryidae are capable of hosting cyanobacteria: Meesiaceae, Bryaceae, Mniaceae, Bartramiaceae, Orthotrichaceae, Aulacomniaceae, and Orthodontiaceae (see Supplemental Information for references). Dinitrogenase activity has been documented for most of these families; the exceptions include Orthotrichaceae, which was not studied, and Orthodontiaceae, which yielded a single negative report (*Leptotheca gaudichaudii* Schwägr in Brasell et al. 1986.). Collectively, the acrocarps in Bryidae yielded very few reports of nitrogenase activity in the absence of cyanobacteria; evidence suggestive of heterotrophic N fixation is sparse for these mosses. The presence of cyanobacterial associates in two (Aulacomniaceae and Orthodontiaceae) of the three families that may be closely related to pleurocarpous mosses is notable; does this evidence suggest that the ancestral condition of the pleurocarps included an ability to communicate with and host cyanobacteria? At this time we can only speculate; few studies related to potential moss-cyanobacterial associations have been conducted with these transitional taxa.

Bryidae: Pleurocarps. The pleurocarpous mosses are an extremely diverse group of bryophytes with over 6000 species; many of the traditionally-defined pleurocarpous moss families appear to be polyphyletic (Cox et al., 2010).

At a minimum, mosses in 13 of the 55 pleurocarpous families are capable of hosting cyanobacteria (~ 24%). This group of cyanobacterial hosts includes the families Hypnodendraceae Broth., Daltoniaceae Schimp., Amblystegiaceae G. Roth., Calliergoniaceae Vanderp., Hedenäs, C.J. Cox & A.J. Shaw, Thuidiaceae Schimp., Brachytheciaceae G. Roth., Hypnaceae Schimp., Hylocomiaceae M. Fleisch., Plagiotheciaceae M. Fleisch., Pylaisiadelphaceae Goffinet & W.R. Buck, Leucodontaceae Schimp., Lembophyllaceae Broth., and Anomodontaceae Kindb. (see Supplemental Information for references). In addition, reports with various spellings of the genus *Ptychomnion* (Hook. f. & Wilson) Mitt. suggest that Ptychomniaceae M. Fleisch. may include moss cyanobacterial hosts (*Ptycomnion aciculare* (Brid.) Mitt. Brasell et al. 1986) or mosses associated with N fixation (*Ptycomnion aciculare* in Brasell et al. 1986, *Ptychomnium aciculare* in Menge & Hedin 2009). However, a single report from Antartica on *Ptychomnion aciculare* (Brid.) Mitt. documents the absence of cyanobacteria (Line, 1992). For most of the pleurocarpous families with documented epiphytic cyanobacteria and N fixation, the evidence of symbiosis is sparse, involving 1-5 species in any given family and < 1% of the species-level biodiversity. The only pleurocarpous moss families with cyanobacterial symbioses documented across a broader range of species are Amblystegiaceae (8 species) and Calliergonaceae (6

species). References and species names can be found by searching for Amblystegiaceae or Calliergonaceae in the Supplemental Information.

Almost all of the pleurocarpous host families are capable of N fixation (Supplemental Information); the exception is Daltoniaceae, in which a single acetylene reduction study of *Distichophyllum* yielded negative results (Brasell et al., 1986).

There is no consistent, geographically widespread evidence of an inability to host epiphytic cyanobacteria at the family level for pleurocarps. Moss samples from six different pleurocarpous families (Pylaisiadelphaceae, Hypnaceae, Brachytheciaceae, Thuidiaceae, Amblystegiaceae, Ptychomniaceae) were found to be free of cyanobacteria in some ecosystems, but at least five of these of these families are known to host cyanobacteria under other circumstances. (Please see references in the Supplemental Information, searching by family.) If the alternative spellings for the genus *Ptychomnion* (Brasell et al. 1986, Menge & Hedin, 2009) are confirmed as synonyms, which seems likely, then there are no pleurocarpous families consistently characterized as non-hosts in the reviewed literature.

Summary of moss-cyanobacterial associations. The moss families represented in this review include 33 of the 119 extant moss families. At a minimum, 31 of these 119 moss families (~ 26%) are capable of hosting cyanobacterial symbionts. Given the existence of one or more published reports (positive or negative), the probability of a given moss family hosting cyanobacteria is 0.94. Only two of the studied moss families are

classified as non-hosts: Takakiaceae, based on evidence from a single genomic study (Satjarak et al. 2022), and Ptychomniaceae, which is characterized as such due to taxonomic uncertainty regarding the spelling of a genus name, rather than a lack of cyanobacterial symbionts in the literature. I was unable to locate published information on the potential for moss-cyanobacterial associations in over 70% of the families. Within the published information available to me, no substantial evidence supports the idea that a particular moss family is intrinsically or even typically incapable of hosting cyanobacteria.

At the level of species, information on the potential for cyanobacterial symbiosis is lacking on roughly 99% of all moss species. Among the 122 unique moss taxa that were screened for cyanobacteria, 86% were capable of hosting cyanobacteria. Only 14% of the moss taxa studied (e.g., *Dicranum* Hedw.) failed to host cyanobacteria in any study, although genera such as *Grimmia* Hedw., *Polytrichum* Hedw., and *Racomitrium* Brid. failed to host cyanobacteria at some but not all sites. Overall there is insufficient evidence to support the idea that a given moss species or genus is generally incapable of hosting cyanobacteria across the breadth of its range. Among the 102 unique moss taxa tested for N fixation, 79 moss taxa (78%) were capable of such activity. The presence of N fixation does not necessarily indicate that cyanobacteria are responsible for fixation; heterotrophic N fixation is well-documented in many bryophyte taxa (e.g., Leppänen et al., 2015).

Family overview: Cyanobacterial associations among the liverworts

Introduction to liverwort-cyanobacterial associations. Among the liverworts, literature for 22 different families was reviewed with respect to potential for cyanobacterial symbiosis; 20 of these families harbored cyanobacteria in at least one site.

Neither positive nor negative reports of cyanobacteria were found for liverworts in the most basal class, Haplomitriopsida Stotler & Crand.-Stotl., due to a lack of published information accessible to me. Cyanobacterial symbioses have been documented in at least a few thallose liverworts in Marchantiopsida Cronquist, Takht. & W. Zimm.**,** and some leafy liverworts in Jungermanniopsida Stotler & Crand.-Stotl.; more detailed information, including citations, is presented below. The published reports are largely positive; about 76% of the unique liverwort species or genera studied proved capable of cyanobacterial symbiosis. Only a small percentage (< 1%) of the total species-level liverwort diversity is reflected in the literature reviewed here; the literature on cyanobacterial symbiosis I was able to access encompasses 38 distinct liverwort taxa, a mere handful of the estimated 7500[2] liverwort species (von Konrat et al., 2014).

Marchantiopsida: Thallose liverworts. Three of the 20 thallose liverwort families in Marchantiopsida, including Blasiaceae H. Klinggr., Marchantiaceae Lindl., and Ricciaceae Rchb., are capable of cyanobacterial symbiosis. (Please see Supplemental Information for references on individual liverwort families.) These host families

[2] There is still uncertainty with respect to the number of liverwort species; the estimation methods employed by von Konrat et al. (2014) yield a 95% confidence interval of 5500-7500 species.

constitute about 15% of all thallose liverwort families. A single report from Conocephalaceae Müll. Frib. ex Grolle indicates the thallose liverwort *Conocephalum conicum* (L.) Underw. did not host cyanobacteria (Knack et al., 2015). N fixation is well-documented in Blasiaceae (Bond & Scott, 1955) and evident in Marchantiaceae (Brasell et al., 1986; Line, 1992; Egorov, 2007), but not studied or not evident in the other thallose liverwort families characterized. No reports of N fixation in the absence of cyanobacteria were found for Blasiaceae, suggesting that heterotrophic N fixation is (a) typically absent, or (b) occurs in conjunction with cyanobacterial N fixation. For other thallose liverworts, there was no information on potential heterotrophic N fixation in the publications reviewed. Although the exact number of thallose liverwort species is unknown, based on current estimates (von Konrat et al. 2014) less than 1% of the species in this group has been the subject of published research within papers accessible to me.

Jungermanniopsida: Leafy liverworts. The leafy liverworts in class Jungermanniopsida constitute the largest group within Marchantiophyta, with over three-quarters of all liverwort species. Reports of cyanobacteria involve multiple families, ranging from the most basal to the most derived.

Overall 17 of the 60 leafy liverwort families (28%) are known to host cyanobacteria; ordered from least-derived to most-derived, these include Pelliaceae H. Klinggr., Aneuraceae H. Klinggr., Mizutaniaceae Furuki & Z. Iwats., Porellaceae Cavers nom. cons., Frullaniaceae Lorch, Lejeuneaceae Cavers, Ptilidiaceae H. Klinggr.,

Lophocoleaceae Müll. Frib. ex Vanden Berghen, Plagiochilaceae Müll. Frib. & Herzog, Jamesoniellaceae He-Nygrén, Juslén, Ahonen, Glenny & Piippo, Cephaloziaceae Mig., Cephaloziellaceae Douin, Scapaniaceae Mig., Blepharidophyllaceae R.M. Schust, Jungermanniaceae Rchb., Antheliaceae R.M. Schust., and Gymnomitriaceae H. Klinggr. All of these leafy liverwort families hosted cyanobacteria in at least one site; please see Supplemental Information for references related to each family. Two liverwort taxa that are not affiliated with a legitimate name in Tropicos (2017), Crandall-Stotler (2017), or both, are also cyanobacterial hosts: *Diplolaena* sp. (Janczewski, 1879; Molisch, 1926; and Takesige, 1937 in: Duckett et al., 1977) and *Cyclolejeunia peruviana* (Lehm. & Lindemb.) A. Evans (Hentschke and Komárek, 2014). Only one of the 18 leafy liverwort families studied, Lepidoziaceae Limpr., failed to host cyanobacteria. For Lepidoziaceae, two screenings of *Bazzania trilobata* (L.) Gray failed to yield evidence of cyanobacteria (Lambert and Reiner, 1979; Deane-Coe and Sparks, 2016), but N fixation was detected in other liverworts within the family, including *Bazzania adnexa* (Lehm. & Lindenb.) Trevis., various species of *Lepidozia*, and *Telaranea gottscheana* (Lindenb.) E.A. Hodgs. (Brasell et al., 1986).

Thirteeen of the 60 leafy liverwort families have been tested for N fixation; these include include Acrobolbaceae E.A. Hodgs., Antheliaceae, Blepharidophyllaceae R.M. Schust., Cephaloziellaceae, Jamesoniellaceae, Lepidoziaceae, Lepicoleaceae R.M. Schust, Lophocoleaceae, Porellaceae, Ptilidiaceae, Scapaniaceae, and Schistochilaceae H. Buch. There is evidence of N fixation in all of these families except for Cephaloziellaceae, which was studied in Antarctica: Fogg & Stewart (1968) did not find

evidence of N fixation in *Cephaloziella varians* (Gottsche) Stephani with 15^N methods. References and species names for N fixation in the other leafy liverwort families are found in the Supplemental Information.

Overall we have evidence of symbiotic N fixation in at least 20% of the leafy liverwort families in Jungermanniopsida, although species-level representation and replication are limited. In most families, just 1-2 species have been studied, and the total number of reports per family does not exceed 5. Only *Bazzania trilobata* (Lambert and Reiners, 1979) and *Anastrophyllum involutifolium* (Mont. ex Gottsche, Lindenb. & Nees) Stephani (Arróniz-Crespo et al., 2014) were capable of N fixation in the apparent absence of cyanobacteria; the available evidence indicates that heterotrophic N fixation may not be particularly common in leafy liverworts, or may co-occur with cyanobacterial N fixation. But this assessment must be tempered by the general scarcity of symbiotic N fixation studies involving leafy liverworts.

Summary of liverwort-cyanobacterial associations. Thirty-nine liverwort species or genera were screened for cyanobacterial symbionts; approximately 77% of these thallose or leafy liverwort taxa (30 of 39) were capable of hosting. About 86% of the liverwort families screened (20 of 22) hosted cyanobacteria. Among the 82 liverwort families defined by the Crandall-Stotler classification (2017), at least 20 of 82 liverwort families (~ 24%) were cyanobacterial hosts in at least one taxon. Among the liverworts screened for N fixation (both thallose and leafy), 23 of 31 unique taxa (~ 74%) were capable of diazotrophy. Reports of N fixation in the absence of cyanobacteria were limited to *Bazzania trilobata* (Lambert and Reiners, 1979; Deane-Coe and Sparks,

2016) and *Anastrophyllum involutifolium* (Mont. ex Gottsche, Lindenb. & Nees) Stephani (Arróniz-Crespo et al., 2014). The reviewed literature encompasses symbiotic potential (evidence on presence or absence of cyanobacteria) for just 39 of the approximately 7500 liverwort species, or < 1% of the species-level diversity for Marchantiophyta.

Family overview: Cyanobacterial associations among the hornworts

Hornworts host cyanobacteria consistently (Renzaglia et al. 2007); this group of bryophytes is not particularly speciose, with an estimated 215 species worldwide (Söderström et al. 2016). In the literature I was able to access and review, reports of cyanobacterial symbiosis among the hornworts involve all five hornwort families: Anthocerotaceae Dumort. corr. Trevis. emend Hässel, Dendrocerotaceae (Milde) Hässel emend Duff et al., Leiosporocerotaceae Hässel ex Ochyra, Phymatocerotaceae (Milde) Hässel emend Duff et al., and Notothyladaceae (Milde) Müll. Frib. ex Prosk. emend Hässel. Please see the Supplemental Information for specific hornwort taxa and references, which may serve as an entryway into a larger collection of relevant literature. Model cyanobacteria-hosting hornworts within the genera *Anthoceros* (Anthocerotaceae) and *Phaeoceros* (Notothyladaceae) have been a primary focus of research (e.g., Meeks, 2009; Nelson et al. 2019; Nelson et al. 2021). In Leiosporocerotaceae, cyanobacteria have been documented on *Leiosporoceros dussii* (Stephani) Hässel de Menéndez (Villarreal & Renzaglia, 2006). In Notothyladaceae, cyanobacteria have been documented on *Hattorioceros striatisporus* (J. Haseg.) J. Haseg. (Zhang et al., 2011) and on *Notothylas* Sull. ex A. Gray (Rai et al. 2000). In

Dendrocerotaceae, *Nothoceros superbus* J. C. Villarreal, Hässel de Menéndez & N. Salazar (Villarreal et al., 2007) is a cyanobacterial host. N fixation has been documented in Anthocerotaceae in *Anthoceros punctatus* L. (e.g., Rodgers and Stewart, 1977; Enderlin and Meeks, 1983; Meeks et al., 1985; Campbell and Meeks, 1989). Accounts of cyanobacterial symbiosis in various species of *Phymatoceros* are developed in Renzaglia et al. 2009. There are no accounts of N fixation in the absence of cyanobacteria (i.e., no evidence of potential heterotrophic N fixation); but this may reflect the emphasis on model systems such as *Anthoceros,* in which *Nostoc* cyanobacteria are characteristically present.

Overview of information on mosses, liverworts, and hornworts

Our current knowledge of bryophyte-cyanobacterial symbiosis is modest due to limited taxon sampling (Figure 2). Although a substantial number of bryophyte families (5 hornwort, 20 liverwort, and 31 moss) host cyanobacteria, the published work available to this author does not encompass over 140 bryophyte families (Figure 2). About two-thirds of the extant moss and liverwort families have not been studied with respect to cyanobacterial symbiosis (Figure 2).

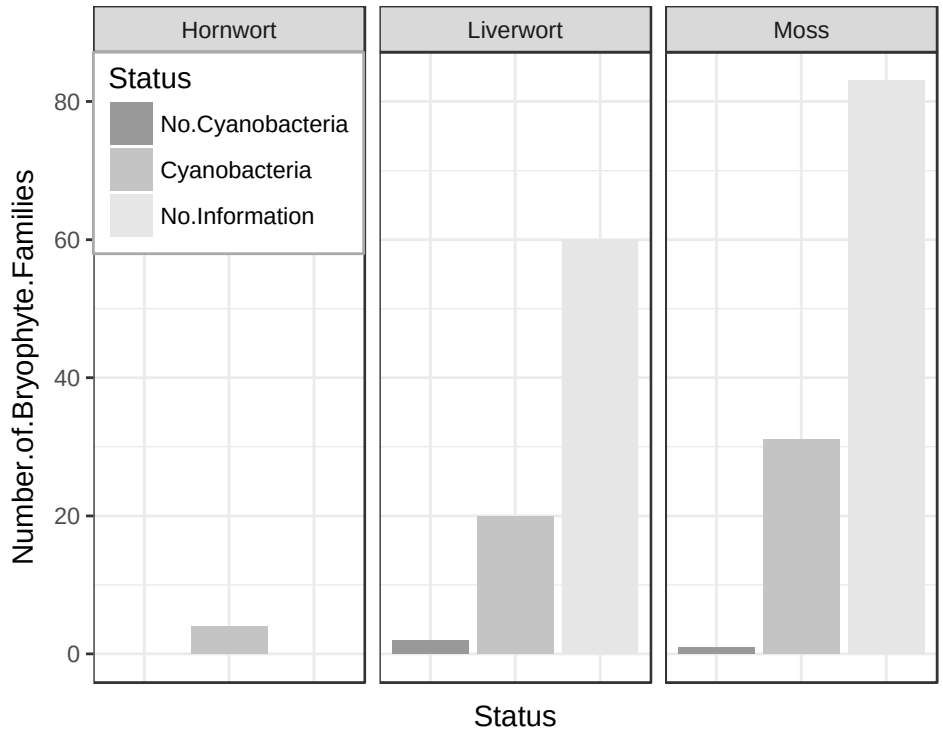


Figure 2. Magnitude of information gap on bryophyte-cyanobacterial associations by bryophyte group. Hornworts are generally capable of cyanobacterial symbiosis, but there is relatively little information on moss and liverwort bryophyte groups in the literature reviewed from 1955-2016.

The limited information available strongly supports the idea that cyanobacterial associations may be common among bryophytes. Among the hornworts, 100% of the families studied hosted cyanobacteria; among the liverworts, 90%; and among the mosses, 97%.

DISCUSSION

The large number of families capable of cyanobacterial symbioses, and their broad dispersal among the bryophytes, challenges the idea that these symbioses are anecdotal evolutionary phenomena, unique elaborations that occur in only a few

unusual taxa. How can we account for their presence in both of the studied liverwort classes (leafy and thallose), all of the major moss groups (early-divergent acrocarps, acrocarps, and pleurocarps), and both classes of the hornworts?

Consider the moss group in isolation to simplify the problem. Most of the 31 moss families known to host cyanobacteria are unlikely to be sister taxa, with the possible exception of potential sister taxa Bartramiaceae/Orthotrichaceae, Aulacomniaceae/ Orthodontiaceae, and Amblystegiaceae/Calliergonaceae. One possible explanation involves the independent evolution of moss-cyanobacterial communications in each of these families or groups of sister taxa; up to 28 distinct evolutionary events might be required under such an assumption. A second potential explanation might involve a commensialistic relationship between the cyanobacteria and moss plants for at least some of these families. If the cyanobacteria are simply living on moss tissues, without being involved in nutrient exchange or a relationship driven by chemical signaling, then there may be no genetic basis for the observed symbiosis. The moss in this case would simply be another cyanobacterial substrate, not substantially different from rock, bark, or other non-living substrata. A third potential explanation involves the possibility that genetically-mediated moss-cyanobacterial associations are feasible in many, most, or all of these families, reflecting an origin in early-divergent taxa and (perhaps) some reversals to the non-hosting condition. Future work will need to explore these three hypotheses in relation to the mosses, as well as the liverworts and hornworts. Further advances may also require studies of important evolutionary transitions within the mosses, liverworts, and hornworts.

In the mosses, broader taxon sampling for cyanobacterial symbioses among the early-divergent mosses would be helpful, as information has been published on only a few of these families. This might help resolve an important question: Is the ability to form associations with cyanobacteria the ancestral condition of the moss clade, or did bryophyte-cyanobacterial associations within the moss clade originate independently within other early-divergent moss groups, such as the peat mosses? We have one negative report (Satjarak et al. 2022) with respect to the basal genus *Takakia,* the sole genus in Takakiaceae. But environmental controls may constrain the formation of moss-cyanobacterial associations; it is not uncommon for a moss species to host cyanobacteria in one habitat but not in another. More studies exploring the potential for diazotrophic cyanobacterial symbioses among basal bryophytes would be useful.

Another area of focus might involve the moss families associated with the transition from acrocarpous to pleurocarpous forms. With further investigation, will we discover that all of the more-basal pleurocarp families (Braithwaitiaceae, Racopilaceae, and Pterobryellaceae, as well as Hypnodendraceae) are capable of these symbioses? Or is it possible that moss-cyanobacterial symbioses evolved independently one or more times in the pleurocarps? Overall, the evolutionary transition between the acrocarps and the pleurocarps remains opaque with respect to moss-cyanobacterial symbioses.

In the liverworts, we have no information about the potential for cyanobacterial symbiosis among the basal class Haplomitriopsida; none of the reviewed literature contained accounts for these taxa. Other forms of symbiosis are present in extant

representatives of Haplomitriopsida; at least two ancient fungal lineages (Mucoromycotina, Glomeromycota) are mutualistically associated with liverworts in Haplomitriopsida and capable of carbon exchange (Field et al., 2015). Did bryophyte-cyanobacterial associations also evolve early among the liverworts? If so, are these ancestral associations similar to those in *Anthoceros* and *Blasia*? And if cyanobacteria are not capable of symbiosis with these basal Haplomitriopsida liverworts, is *Blasiaceae* the liverwort taxon in which these associations first evolved?

In the more-derived liverwort classes Marchantiopsida (thallose liverworts) and Jungermanniopsida (leafy liverworts), evidence of cyanobacterial associations is sparsely but broadly distributed. Notable features include a cluster of closely-related late-divergent families with cyanobacterial associates (Jamesoniellaceae, Cephaloziaceae, Cephaloziellaceae, Scapaniaceae), and the evidence of cyanobacteria in the most-derived liverwort families, Antheliaceae and Gymnomitriaceae. Overall more intense taxon sampling in the study of potential symbioses would be beneficial in both the thallose and leafy liverwort classes.

Among the hornworts, family-level taxon sampling is relatively well-distributed and more complete than in the mosses or liverworts.

Clearly increasing our knowledge of bryophyte-cyanobacterial associations at the family level is one goal for future research, but increasing the density of taxon sampling within families—at the species level—will also be critical. Typically we have studied less than

1% of the overall species-level diversity within a given bryophyte family; building a broader base of knowledge would expand our knowledge of the evolution of plant-microbial associations, with implications for both non-vascular and vascular plants. Continuing to collect information on cyanobacterial symbioses (or the lack thereof) from researchers who routinely work with particular families may also substantially expand our basis of knowledge, simply by more formally organizing what is already known.

The nature of the plant-microbe relationships remains unclear for many bryophyte-cyanobacterial associations. Is photosynthate transferred from the moss to the cyanobacteria, and fixed N, from the cyanobacterium to the moss, in all of the 147 species documented as cyanobacterial hosts? Expanding our knowledge of potential nutrient exchange in diverse moss-cyanobacterial systems will help us begin to understand whether these relationships are typically mutualistic, commensialistic, or parasitic (or all three, in association with different symbiotic partners or environments).

The overall prevalence of cyanobacterial symbioses among the bryophytes is unclear, but their broad distribution within diverse bryophyte groups raises questions about plant-cyanobacterial associations in vascular plants. Cyanobacterial symbioses among extant vascular plants are considered rare, limited to ferns in the genus *Azolla* de Lamarck, cycads, and the angiosperm *Gunnera* L. This seems puzzling given the broad distribution of symbioses in the ancestral bryophytes, at least among bryophyte families. How and when did the bryophyte's ability to foster associations with cyanobacteria become so rare among their vascular-plant descendants? Although the fossil record of

cyanobacterial symbioses is sparse, there is evidence of a vascular plant symbiosis with cyanobacteria dating to approximately 400 Ma (Krings et al., 2009), a time period contemporaneous with the evolution of the early-divergent bryophytes. Krings et al. (2009) describe fossil evidence of filamentous cyanobacteria colonizing *Aglaeophyton major,* a small mycorrhizal plant.

The number of vascular plants known to associate with cyanobacteria has been increasing slowly in recent years, with the discovery of cyanobacteria associated with certain orchid roots (Tsavkelova et al., 2001; Ram and Shamina, 2015), a few crop plants (e.g., Venkatachalam et al., 2016), and the leaves of some tropical plants (e.g., Fürnkranz et al., 2008). How do these symbioses relate to one another, and to those among the bryophytes, with respect to potential nutrient exchange and chemical communication? What can we learn through comparative study of these diverse plant-cyanobacterial symbioses?

In the realm of agriculture, work by Venkatachalam et al. (2016) indicates that the rice phyllosphere harbors epiphytic cyanobacteria, including forms similar to *Gloeotrichia, Lyngbya, Plectonema,* and *Nostoc.* Low rates of nitrogen fixation (2-3 kg ha^{-1}) were detected, potentially attributable to cyanobacteria and other bacterial components of the phyllosphere. If such epiphytic associations have a genetic basis, exploration of potential similarities or dissimilarities between vascular plant-cyanobacterial symbioses and bryophyte-cyanobacterial symbioses could contribute to our understanding of plant evolution and plant-microbe communication. Such knowledge might prove useful in

efforts to create economically-valuable crop plants with novel epiphytic or root-associated N-fixing symbionts (e.g., in wheat: Karthikeyan et al., 2007).

Studies also highlight the role of cyanobacteria in the phyllosphere of some tropical plants, including those in Brazilian mangrove ecosystems (Rigonato et al., 2012; Alvarenga et al., 2016) and Costa Rican lowland tropical rainforests (Freiberg, 1998; Fürnkranz et al., 2008). Simple sugars from leachate are believed to be the primary source of fixed C for phyllosphere bacteria (Lindon and Brandl, 2003; Vacher, 2016). Further investigation of leaf phyllosphere microbial communities may be useful, especially given the limited study of cyanobacterial microbes in the phyllosphere. The use of epifluorescence microscopy for screening leaf tissue for cyanobacteria might complement the use of genomic methods. The latter often utilize primers that exclude cyanobacterial sequences, to avoid confounding leaf-plasmid DNA (e.g. Delmotte et al., 2014). Also, bryophytes are often abundant and little-studied components of tropical forests, sometimes present as epiphytes on trees with phyllosphere cyanobacteria (e.g., Freiberg, 1998; Fürnkranz et al., 2008). The epiphytic bryophytes and the vascular-plant leaf phyllosphere sometimes harbor similar cyanobacterial communities, suggesting that investigation of their respective symbiotic mechanisms might prove fruitful.

Overall the evidence available to us indicates that bryophyte-cyanobacterial associations may be more common than anticipated, with a broad distribution across families of liverworts, hornworts, and mosses. Cyanobacterial symbioses are found in taxa ranging from basal to late-divergent in all of these groups except for the liverworts,

where the basal taxon (Haplomitriopsida) has not been screened for cyanobacteria. Further study of potential cyanobacterial symbioses in a more diverse range of bryophyte families, and more dense sampling within bryophyte families, could yield a more complete understanding of plant-microbe communication mechanisms and evolution in both bryophytes and vascular plants.

SUMMARY

This literature review expands the number of bryophytes understood to be potential cyanobacterial hosts, by collecting information presented (often anecdotally) over the past several decades: approximately 93% of the 60 bryophyte families screened for cyanobacteria proved capable of hosting these microbial photoautotrophs. This conclusion is based on individual studies of at least 170 unique moss, liverwort, and hornwort taxa; 147 of the bryophyte species or genera examined (~ 86%) harbored cyanobacterial symbionts in at least one ecosystem. The published evidence suggests that bryophyte-cyanobacteria symbioses are potentially common, if not ubiquitous; but the published information also involves < 1% of all bryophyte species. This leaves us with substantial uncertainty about the overall prevalence of bryophyte-cyanobacterial associations. If the absence of information reflects a strong bias against publishing accounts of non-symbiotic bryophytes, it may be that bryophyte-cyanobacterial symbioses are quite rare, with a prevalence as low as 1%. But if the prevalence of such symbioses among the unstudied bryophytes is as high as that among the studied bryophyte families, the prevalence might be even greater than 90%. This suggests we

cannot rule out the possibility of a universal capability for cyanobacterial symbioses among the bryophytes with the data available for this study (1955 – 2016).

Future opportunities could involve (1) studies of symbiosis in a wider array of bryophyte taxa; (2) work in plant-microbe communications and nutrient exchange, and their evolution in both vascular and non-vascular land plants; and (3) research on the biogeochemical impacts of bryophytes in ecosystems with substantial nonvascular biomass, including both terrestial and aquatic systems.

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DATA ACCESSIBILITY

Data compiled from the literature (including citations and descriptions for all reports of symbiosis) are available in a spreadsheet (Supplemental Information).

Part II: Non-Nostocalean symbionts are relatively common in bryophyte-cyanobacterial symbioses.

Key results from Part II. Cyanobacterial symbionts for bryophytes (and for vascular plants) are typically considered to be members of the order Nostocales. In this study the diversity of cyanobacterial symbionts found in 60 families of bryophytes was assessed, and the probability of a given bryophyte family hosting cyanobacteria was calculated. A case-study approach illuminates the frequency of cyanobacterial symbiosis for individual bryophyte families. A dataset with 784 observations of bryophyte-cyanobacterial symbiosis was compiled from the scientific literature to characterize the diversity of cyanobacterial symbionts. The symbiotic cyanobacteria belong to the orders Nostocales, Oscillatoriales, Chroococcales, Pseudanabaenales, and Synechochococcales. Multicellular cyanobacteria in Nostocales, distinguished by nitrogen-fixing heterocystous cells, were abundant (56.5% of all reports). Contrary to expectation, a substantial percentage of the symbionts were unicellular and/or non-heterocystous (43.5%). 46 of the 60 bryophyte families (77%) studied hosted cyanobacteria almost always or always ($0.85 < \text{host probability} \leq 1.00$), although about 40% of the families studied were represented by just one or two reports of symbiosis. Families with strong affinities for cyanobacteria that are relatively well-studied (≥ 10 published reports) include Amblystegiaceae, Anthocerotaceae, Blasiaceae, Brachytheciaceae, Bryaceae, Calliergoniaceae, Ditrichaceae, Grimmiaceae, Hylocomiaceae, Mniaceae, Pottiaceae, and Sphagnaceae.

Existing theory does not adequately account for the robust presence of non-Nostocalean cyanobacteria; the nature of these symbioses with respect to molecular-level communication, potential nutrient exchange, and ecosystem impacts is unclear. Studies of model bryophyte taxa such as *Physcomitrella, Syntrichia,* and *Ceratodon purpureus* could be enriched by deeper consideration of their symbiotic potential.

INTRODUCTION

A review of published literature established that cyanobacterial symbioses occur in at least 147 unique bryophyte species or genera (Ederer thesis, Part I, 2023), including liverworts, hornworts, and mosses. But the presence or absence of cyanobacterial symbionts is merely the first level of inquiry. To better understand the potential ecological and biogeochemical impacts of bryophyte-cyanobacterial associations, we need to know how commonly such symbionts occur within ecologically important bryophyte taxa. A symbiosis that occurs rarely is likely to have little or no impact at a larger scale of inquiry; a symbiosis that occurs frequently may have impacts worthy of further investigation. In addition, these associations may spur investigation at the molecular and genomic level of bryophyte taxa that typically host cyanobacteria. This analysis of the published literature is organized by bryophyte family, with families grouped based the quality of evidence for cyanobacterial symbiosis.

The diversity of cyanobacterial symbionts in the published literature is also explored. Much work on bryophyte-cyanobacterial symbioses and plant-cyanobacterial symbioses

has focused on cyanobionts in the genus *Nostoc* Vaucher ex Bornet & Flahault, but the degree to which *Nostoc* associations are characteristic of bryophytes is unclear. Well-supported models posit that cyanobacterial symbioses in bryophytes are relatively rare (e.g., Santi et al. 2013), limited to just a few genera, and that cyanobionts are typically members of Nostocales (Adams et al. 2013). The ability to form hormogonia and heterocysts is likewise thought to be requisite. This review examines the prevalence of cyanobacterial symbioses that do not involve heterocystous cyanobacteria such as *Nostoc,* or involve *Nostoc* in conjunction with other cyanobacterial taxa.

METHODS

The data on bryophyte-cyanobacterial symbioses were collected from over five decades (~ 1955 to 2016) of published literature in ecology, bryology, phycology, molecular cell biology and molecular genomics, and geology. English was the primary language for the literature search. Bryophyte host taxa were differentiated from non-host taxa based on whether the plants had direct contact with cyanobacteria, either epiphytically or endophytically. More details on the host criteria are presented in Part I (Ederer thesis, 2023). In this analysis, the likelihood of finding bryophytes that host cyanobacteria within a given moss, liverwort, or hornwort family was estimated based on the prevalence of positive reports in the literature. In this context, a report is defined as a single unique combination of a bryophyte taxon and a cyanobacterial taxon at a given geographical site, as defined by the original researchers. For example, A. Pentecost (1998) detected two species of cyanobacteria on the moss *Cratoneuron commutatum* (Hedw.) G. Roth from a thermal travertine-deposition spring in England. For the

purposes of this review, these were classified as two reports: (1) *Cratoneuron commutatum* (Hedw.) G. Roth with *Aphanocapsa muscicola* (Menegh.) Wille, and (2) *Cratoneuron commutatum* with *Chroococcus turgidus* (Kütz.) Näg. In this manner, each incidence of a unique combination of bryophyte and cyanobacterium at a given physical site is counted once. Using these count data, bryophyte families were grouped into categories based on the value of the ratio of positive to total reports (positive and negative) within a given family, referred to as the *host probability*. These categories include (1) *Never*: bryophyte families that did not host cyanobacteria at any site in the published literature reviewed (host probability = 0), (2) *Sometimes*: bryophyte families in which the incidence of positive reports was less than or roughly equal to the incidence of negative reports (host probability $\leq$ 0 .5); (3) *Often*: bryophyte families in which positive reports were dominant (host probablity > 0.50 but ≤ 0.85); and (4) *Almost Always or Always*: bryophyte families in which all or almost all taxa studied hosted cyanobacteria (host probability > 0.85).

This approach to the estimation of frequency of bryophyte-cyanobacterial symbioses in individual bryophyte families has limitations. The size of a given site varies according to the definitions applied by different researchers, exploring different ecosystems or regions with various purposes. Most studies focused on one or several sites at a typical ecosystems-ecology scale, e.g., a forested area of several hectares or a particular freshwater aquatic habitat. But some studies involved much larger or smaller areas; for example, Bowden (1991) defined an ecosystem as a single patch of moss, several square meters in area; Zackrisson et al. (2009) conducted a regional-level study, pooling information on 40 diverse sites along a north-south gradient encompassing

multiple degrees of latitude. In addition, the number of published reports for a given family varies from none to over 100 (Sphagnaceae). These limitations with respect to site and sample size equivalence suggest that the results of this study should be interpreted descriptively rather than quantitatively.

For these reasons, case studies representing each of the host-frequency categories (non-host, cyanobacterial incidence ≤ 50%, cyanobacterial indicidence > 50% and ≤ 85%, and cyanobacterial incidence > 85%) are used to explore the results.

Cyanobacterial taxonomy is currently in transition from older systems of taxonomy based on morphology to newer systems based on genomic data (Komárek et al. 2014). Given the rapid pace of revision and the limitations of the published data on cyanobacterial symbiosis (e.g., sometimes identifications are limited to genus level), it is often impossible to assign an older morphotype name to an approved name. In assessing cyanobacterial diversity at the genus level, the original morphotype names were used as reported by authors. To assess broader trends in symbiotic diversity, the original reported taxa were assigned to cyanobacterial orders per current standards specified in CyanoDB.cz (2017). The goals of this analysis were to determine (1) whether the genus *Nostoc* was dominant among bryophyte symbionts, and (2) whether the order Nostocales Borzi was dominant among bryophyte symbionts.

RESULTS

Case studies of bryophyte families in symbiosis. Most of the bryophyte families studied offered positive proof of cyanobacterial symbiosis, but sometimes the evidence involved only one or two taxa within a particular family (e.g., Orthotrichaceae, Ress 2012). The case studies focus on families with more than one or two published studies, and involve a more diverse array of taxa. The symbiotic status of the 60 bryophyte families studied varied greatly, with some hosting cyanobacteria occasionally, some frequently, and others nearly always (Figure 1). Most of the studied moss and liverwort families hosted cyanobacteria almost always or always (Figure 1). Hornwort families always hosted cyanobacteria (Figure 1). Only four bryophyte families failed to provide evidence of cyanobacteria. We will consider specific families of mosses, liverworts, and hornworts as examples of each host probability category.

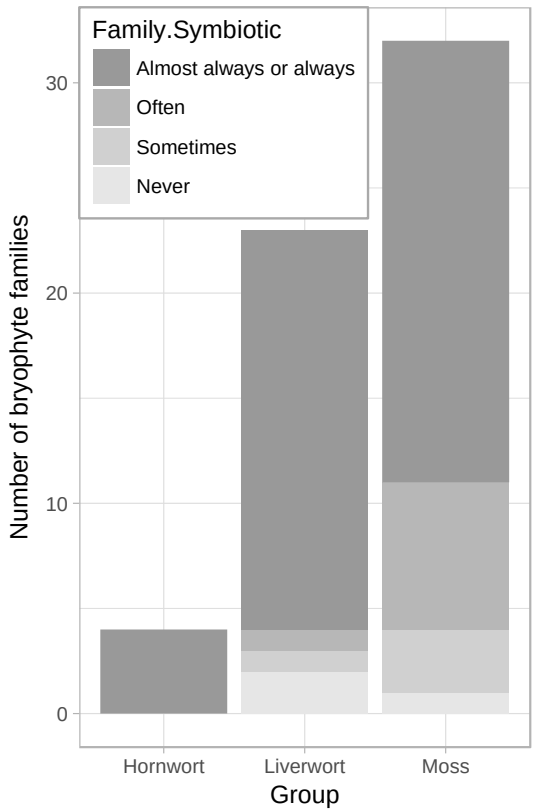

Figure 1. Among families with published research on cyanobacterial associates, most families were highly likely to host cyanobacteria.

In Figure 1, the category definitions are as follows. (1) *Never*: bryophyte families that did not host cyanobacteria at any site in the published literature reviewed (host probability = 0), (2) *Sometimes*: bryophyte families in which the incidence of positive reports was less than or roughly equal to the incidence of negative reports (host probability ≤ 0.5); (3) *Often*: bryophyte families in which positive reports were dominant (host probablity > 0.50 but ≤ 0.85); and (4) *Almost Always or Always*: bryophyte families in which all or almost all taxa studied hosted cyanobacteria (host probability > 0.85). The fact that most bryophyte families belong to the latter category may reflect a positive bias in the publication process, as well as the small number of published reports for many families (e.g., a family with just one report that is positive will be in the “Almost Always or

Always” category). An alternative explanation is that cyanobacterial associations are rather common.

Bryophyte families that did not host cyanobacteria. Among the 60 bryophyte families studied, only two liverwort families failed to host cyanobacteria (Figure 2). These include the thallose liverwort family Conocephalaceae Müll. Frib. ex Grolle (Knack et al. 2015) and the leafy liverwort family Lepidoziaceae Limpr. (Deane-Coe & Sparks, 2016). A recent microbiome study of the basal moss family, Takakiaceae, indicated that cyanobacterial sequences were not present in plants that had been carefully washed to remove exterior detritus (Satjarak et al., 2022). In addition, the moss family Ptychomniaceae M. Fleisch did not host cyanobacteria in a light microscopy study (Line 1992), although a potential synonym (*Ptycomnion aciculare* (Brid.) Mitt. in Brasell et al. 1986) harbored cyanobacteria. For these four non-hosting families, the relevant evidence involves a single species, site, and study for each taxon (Figure 2). Overall there is published evidence of bryophyte families that do not host cyanobacteria, but this evidence is often sparse or lacks replication.

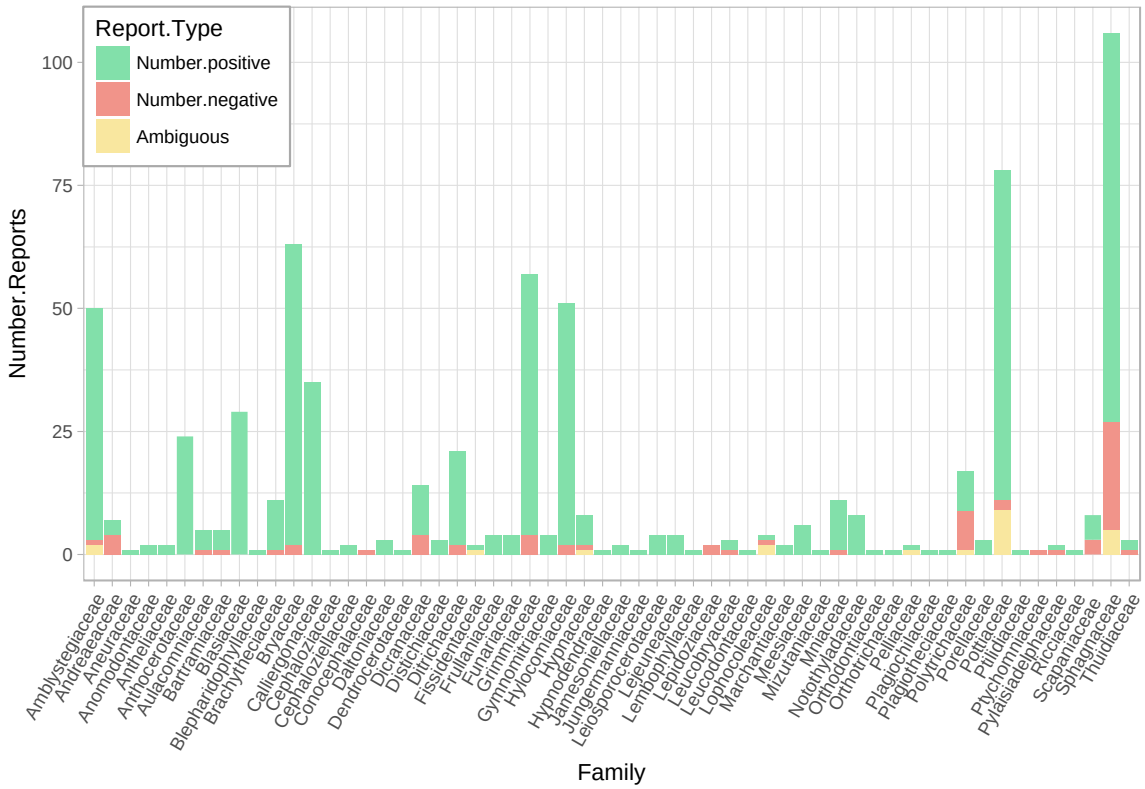

Figure 2. Families with published evidence of cyanobacterial associates. The height of the bar reflects the prevalence of reports per family. Each unit of measure along the y-axis represents a single report: one mention of cyanobacteria in association with the bryophyte taxon, not necessarily one publication. A given publication may have multiple reports (e.g., multiple cyanobacterial taxa found on a *Sphagnum* sample). Takakiaceae, studied recently (Satjarak 2022), is not visualized in these results.

Only a few bryophyte families have been studied extensively (i.e., reported more than 10 times in the literature). For about 40% of the studied bryophyte families, the information available is limited to one or two published reports.

Bryophyte families that sometimes hosted cyanobacteria. Only a few families were characterized by rare cyanobacterial presence, or by roughly equal numbers of positive and negative reports for cyanobacteria (host probability ≤ 0.5). The number of published reports per family ranges from 3 to 16, with a mean of 7.5 reports per family (Figure 2). These taxa include the moss families Andreaeaceae Dumort., Polytrichaceae Schwägr.,

and Pylaisiadelphaceae Goffinet & W.R. Buck, and the liverworts in Lophocoleaceae Vanden Berghen. We will consider the moss family Polytrichaceae as an example of this group.

Roughly half of the published reports confirm that species in the acrocarpous moss family Polytrichaceae can host cyanobacteria and are associated with N fixation. The mosses known to host cyanobacteria are *Polytrichastrum alpinum* (Hedw.) G.L. Sm. (Solheim et al. 1996), *Polytrichum alpestre* Hoppe (Broady 1977, Christie 1987), *Polytrichum commune* Hedw. (Alexander & Schell 1973, Gavazov et al. 2010), *Polytrichum juniperinum* Hedw. (Brasell et al. 1986), and *Polytrichum strictum* Menzies ex Brid. (Gavazov et al. 2010). All of these studies except for one (Broady 1977) generated evidence of N-fixation capability utilizing the acetylene reduction assay or stable isotopes methods with ^{15}N; Broady (1977) did not test for N fixation.

Reports on *Polytrichum* sp. and *Atrichum* sp. provided evidence of dinitrogenase activity via the acetylene reduction assay, but the authors did not report on the presence or absence of cyanobacteria (Lambert & Reiniers 1979). Other researchers failed to detect N fixation or cyanobacteria in Polytrichaceae. This work included negative culture studies from *Polytrichum commune* (Basilier et al. 1979), a lack of cyanobacteria in SEM/TEM studies of *Polytrichum commune* (Scheirer & Dolan 1983), a lack of cyanobacteria in light microscopy studies of *Polytrichum* sp. and *Psilopilum australe* (Hook. f. & Wilson) Mitt. (Line 1992), and light-independent dinitrogenase activity in *Polytrichum juniperinum,* suggesting heterotrophic N fixers were present (Bowden

1991). Likewise, *Polytrichum commune* proved unable to host epiphytic cyanobacteria cultured from another moss, although other potential moss hosts were successful (Bay et al., 2013); and in a study of denaturing gradient gel electrophoresis (DGGE) sequence data and cyanobacteria pigments (echinone), no evidence of cyanobacteria was found for *Dendroligotrichum squamosum* (Hook. f. & Wilson) Cardot (Arróniz-Crespo et al. 2014). Overall there is substantial evidence that the ability to host cyanobacteria varies in space and time for Polytrichaceae, even within a given species (e.g., *Polytrichum juniperinum*).

The published reports for Polytrichaceae pertain to only eight distinctive taxa, but over 505 valid moss species comprise this moss family. Our knowledge of bryophyte-cyanobacterial associations and N-oriented biogeochemistry in Polytrichaceae rests on roughly 1% of the extant biodiversity for this common moss family.

Polytrichaceae illustrates that even bryophyte families with a substantial number of negative reports for cyanobacteria tend to be capable of hosting in some taxa and/or habitats. In addition, our knowledge of bryophyte-cyanobacterial symbioses is generally based on a small percentage of the biodiversity within a given family; the picture may change, as the evidence grows to include more taxa and more geographic regions for a given moss, liverwort, or hornwort family. Finally, the percentage of host families with a substantial percentage of negative reports (50% or more) for cyanobacteria is small; only 4 of the 60 families studied (7%) fall into this category.

Bryophyte families that often hosted cyanobacteria. The families in this category had more positive reports of cyanobacteria than negative reports (host probabilities 0.51 - 0.85), with the number of reports per family ranging from 3 to 101 (Figure 2). Bryophyte taxa that meet these criteria include the moss families Aulacomniaceae Schimp., Bartramiaceae Schwägr., Dicranaceae Schimp., Hypnaceae Schimp., Leucobryaceae Schimp., Thuidiaceae Schimp., and Sphagnaceae Dumort, and the liverwort family Scapaniaceae Mig. We will consider the moss family Dicranaceae as an example of this group.

Taxa with evidence for epiphytic cyanobacteria in the acrocarpous family Dicranaceae include *Aongstroemia julaceae* (Hook.) Mitt. (Nakatsubo & Ohtani 1991), *Chorisodontium aciphyllum* (Hook. f. & Wilson) Broth. (Broady 1977, Christie 1987), *Dicranella cardotii* (R. Br. bis) Dixon (Line 1992), *Dicranoloma billardierei* (Brid.) Paris (Brasell et al. 1986), and *Dicranoloma chilense* (De Not.) Ochrya & Matteri (Arróniz-Crespo et al. 2014). Most of these studies tested for nitrogenase activity, with positive results for most of the moss taxa tested: *Aongstroemia julaceae* (Nakatsubo & Ohtani 1991), *Chorisodontium aciphyllum* (Christie 1987), *Dicranella cardotii* (Line 1992), and *Dicranoloma billardierei* (Brasell et al. 1986). *Dicranum* sp. (Lambert & Reiners 1979) was also associated with a positive ARA result, although cyanobacteria were not evident using light microscopy. By contrast, the study of *Dicranoloma chilense* failed to detect nitrogenase activity, although other bryophytes in the same habitat (post-glacial terrain in Tierra del Fuego) were associated with active N-fixing cyanobacteria (Arróniz-Crespo et al. 2014).

Other evidence indicates that some mosses in Dicranaceae may not host cyanobacteria. In 1968, Fogg & Stewart did not detect any N fixation in a stable isotope study of *Platyneuron laticostatum* (Cardot) Broth., suggesting that cyanobacteria were not present, or were not actively involved in reduction of atmospheric N_2(g) if present. For N-fixing *Dicranum* samples, cyanobacteria were deemed "unlikely" based on light microscopy screenings (Lambert & Reiners 1979). More generally, in numerous studies of the well-characterized Fennoscandian boreal-forest moss flora (e.g., Gundale et al. 2011), mosses in the genus *Dicranum* have been characteristically free of cyanobacteria. More recently Bay et al. (2013) found that *Dicranum polysetum* Sw. was not capable of developing an epiphytic relationship with cyanobacteria cultured from another moss (*Hylocomium splendens*), although other mosses studied did develop epiphytic colonies. The lack of any positive reports of cyanobacteria for the genus *Dicranum* in the literature (1955-2016) is noteworthy given the prominence of this genus in many of the intensively-studied bryophyte floras of the circumpolar north; many *Dicranum* mosses are common, robust, and relatively easy to identify to genus, even in the field. Are mosses in *Dicranum* truly incapable of the interactions necesssary to foster cyanobacterial symbioses, as evidence from Bay et al. (2013) suggests? If so, what makes *Dicranum* differ from other taxa in the family Dicranaceae, which are clearly sometimes capable of hosting cyanobacteria? Are these two distinctive habits (occasionally symbiotic versus non-symbiotic) present within other bryophyte families, too, but obscure due to limited taxon sampling?

As tantalizing as these questions are, we still have a very small basis of knowledge for the genus *Dicranum* and for the family Dicranaceae. The genus *Dicranum* has over 330 valid species; the studies of moss-cyanobacterial associations to date encompass only two species, plus samples identified as *Dicranum* sp. It is possible that we will be able to learn something fundamental about the mechanisms that enable some plants to communicate with and host N-fixing cyanobacteria, when others cannot, by studying *Dicranum.* But it is also possible that the apparent inability of *Dicranum* mosses to host cyanobacteria is a sampling artefact--one that will disappear with a broader consideration of the species-level diversity in the genus, or perhaps of *Dicranum* mosses from different ecosystems and geographic areas. The family Dicranaceae has approximately 1730 species; the reports cited here encompass less than 1% of that biodiversity.

Bryophyte families that almost always or always host cyanobacteria. In these families 85%-100% of all samples studied within a given bryophyte family hosted cyanobacteria. More than 75% of the 60 bryophyte families studied fall into this category (Figure 2). But evidence is sometimes too sparse to make good estimates of host probability: About 40% of the families studied have just one or two published reports of cyanobacterial symbiosis. Liverwort families in general tend to be little-studied. But a substantial subset (14 of 60 bryophyte families, or 23%) has been relatively intensively studied, with at least 10 published reports for each family. The best-studied family is Sphagnaceae Dumort (101 published reports).

Among the mosses, the well-studied families that almost inevitably hosted cyanobacteria include Amblystegiaceae Kindb., Bryaceae Schwägr., Brachytheciaceae G. Roth., Calliergonaceae Vanderpoorten, Hedenäs, C. J. Cox & A. J. Shaw, Ditrichaceae Limpr., Grimmiaceae Arn., Hylocomiaceae M. Fleisch., Mniaceae Schwägr., Pottiaceae Schimp., and Sphagnaceae. (Figure 2). Numerous other families (e.g., Aulacomniaceae Schimp.) also typically host cyanobacteria, but the evidence available is modest or sparse (1-9 reports per family). Among the liverworts and hornworts, Blasiaceae H. Klinggr and Anthocerotaceae Dumort have been the subject of more intensive scrutiny (Figure 2). Some of these families are intensively studied due to the utility of a single species as a model system for cyanobacterial symbiosis, such as *Anthoceros punctatus* L. in Anthocerotaceae (e.g., Meeks et al. 1998, Li et al. 2020) and *Blasia pusilla* L. in Blasiaceae (e.g., Stewart & Rodgers 1978, Liaimer et al., 2016). Blasiaceae consists of just two liverwort species, *Blasia pusilla* and *Cavicularia densa* Steph., both of which host cyanobacteria (e.g., Liaimer et al., 2016; Rikkinen & Virtanen 2008). Other bryophyte families are more speciose, and demonstrate an affinity for symbiosis in an array of species across different sites and regions; we will consider the family Pottiaceae as an example.

Mosses in the acrocarpous family Pottiaceae known to harbor cyanobacteria include *Anoectangium aestivum* (Hedw.) Mitt. (Ress 2012), *Barbula calycina* Schwägr. (Brasell et al. 1986), *Didymodon tophaceus* (Brid.) Lisa (Golubic 1957 in Golubic 2010, Pentecost 1998), *Gymnostomiella vernicosa* (Hook. ex Harv.) M. Fleisch. (Sant'Anna 1984), *Hennediella heimii* (Hedw.) R.H. Zander (Broady 1981 in Schwarz et al. 1992,

Davey 1982, Kanda & Ohtani 1991, Alfinito et al. 1998, [3]*Hymenostylium recurvirostrum* (Hedw.) Dixon (Sant'Anna, C.L. 1984, Ress 2012), *Hyophila involuta* (Hook.) A. Jaeger (Ress 2012), *Pottia austrogeorgica* Cardot (Kanda & Inoue, 1994); *Sarconeurum glaciale* (Müll. Hal.) Cardot & Bryhn (Alfinito et al. 1998), *Tortella* (Müll. Hal.) Limpr (Wojciechowski & Heimbrook 1984), *Tortula* Hedw. (Sant'Anna, C.L. 1984); *Syntrichia princeps* (De Not.) Mitt. (Alfinito et al. 1998), *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr (Snyder & Wullstein 1973), *Weissia controversa* Hedw. (Reddy & Giddens 1981), and *Weissia ovalis* (R.S. Williams) E.B. Bartram (Ress 2012). Negative reports are rare for the family Pottiaceae: mosses that failed to host cyanobacteria included *Hennediella heimii* (Hedw.) R.H. Zander (Kanda et al. 2004) and *Syntrichia ruralis* (Opelt & Berg 2004). Both of these species were capable of symbiotic relationships with cyanobacteria at other sites.

In spite of the diversity of mosses in Pottiaceae with cyanobacterial associates, only four studies of potential N fixation have been conducted. Three species in Pottiaceae are associated with nitrogenase activity: *Barbula calycina* (Brasell et al. 1986), *Hennediella heimii* (Davey 1982), and *Weissia controversa* (Reddy & Giddens 1981). By contrast, a lone attempt to assay *Syntrichia ruralis* for acetylene reduction yielded negative results (Snyder & Wullstein 1973).

The family Pottiaceae is prominent in reports from freshwater calcite deposition systems, including reports that suggest the potential for moss-cyanobacterial

[3] Additional reports of symbiotic cyanobacteria are associated with *Hymenostylium recurvirostre* (Hedw.) Dix. (Sant'Anna 1984), which is not recognized or affiliated with any bryophyte family in Tropicos.

associations in mosses such as *Eucladium verticillatum* (Hedw.) Bruch & Schimp. and *Gymnostomum calcareum* Nees & Hornsch. (Freytet & Verrecchia 1998), and *Hymenostylium recurvirostre* (Freytet & Verrecchia 1998). Most of these studies involve the analysis of moss tufa or travertine samples, which develop from extensive calcite deposition on living moss plants. It is not clear whether these epiphytic associations, in which some cyanobacteria may produce calcite, are similar to better-studied symbioses with respect to nutrient exchange. These issues are relevant to other moss families; similar reports from calcite deposition habitats involve Amblystegiaceae (*Cratoneuron filicinum* (Hedw.) Spruce in Turner & Jones 2005) and Fissidentaceae (*Fissidens crassipes* Wilson ex Bruch & Schimp. in Freytet & Verrecchia 1998). For Pottiaceae, a relatively detailed report from a freshwater calcite system involves the moss *Gymnostomiella vernicosa* (Sant'Anna 1984), which harbored over a dozen different cyanobacterial species.

Relative to the overall biodiversity of Pottiaceae, sampling is sparse; with approximately 3200 valid species in Pottiaceae, the 18 moss species studied to date (including reports from calcite-deposition systems) constitute less than 1% of the overall biodiversity for the family.

In Pottiaceae, we see that bryophyte-cyanobacterial symbioses are not only present in ecosystems where they have been studied for decades, as in Antarctica, but also in diverse ecosystems with a broad global distribution. In the United States, bryophyte-cyanobacterial symbioses for Pottiaceae have been documented in fescue-dominated

grasslands in Georgia (*Weissia controversa* in Reddy & Giddens 1981), moist shrub meadows in the Rocky Mountains (*Tortella* in Wojciechowski & Heimbrook 1984), high-elevation deserts in Utah (*Syntrichia ruralis* in Snyder & Wullstein 1973), and on a montane cliff with various moisture levels in Hawaii (*Anoectangium aestivum*, *Hymenostylium recurvirostrum,* and *Hyophila involuta* in Ress 2012). In other areas of the world, bryophyte-cyanobacterial symbioses for Pottiaceae have been found in calcite-deposition systems such as the Krka River of Croatia (Golubic 1957, in Golubic 2010), on cliffs with calcite deposition in Brazil (Sant'Anna 1984), and in clear-cut, recently burned *Eucalyptus*-dominated rainforest of Southern Tasmania, Australia (*Barbula calycina* in Brasell et al. 1986). But large areas of the world remain little-studied and poorly understood with respect to bryophyte-cyanobacterial symbioses in Pottiaceae; and among the ecosystems that have been studied, work is sparse with little replication. In addition, the potential biogeochemical impacts of Pottiaceae are little-studied; even though the first study of N fixation in symbiotic Pottiaceae occurred in the 1970s (Snyder & Wullstein 1973), only three additional studies have been conducted in the years since.

Pottiaceae illustrates issues that are broadly relevant to bryophyte families capable of hosting cyanobacteria: there is a need for more geographically and taxonomically diverse research across an array of ecosystems, to complement the more-intensive work on single species such as *Pleurozium schreberi* and *Hylocomium splendens* that is being conducted in bryophyte-dominated ecosystems of the boreal forest. The biodiversity of bryophyte-cyanobacterial symbioses, their biomass in particular

ecosystems, and their potential contributions to biotic N fixation have not been well-characterized in many ecosystems. For example, although studies have been conducted in tropical rainforests in Costa Rica (Bentley & Carpenter 1980, 1984, Fürnkranz et al. 2008), Puerto Rico (Cusack et al. 2009), Hawaii (Matzek & Vitousek 2003, Ress 2012), Guadeloupe (Fritz-Sheridan & Portécop 1987, Sheridan 1991), and Australia (Brasell et al. 1986), some tropical areas of the world were not represented in the publications reviewed. [4]Within the dataset curated for this study, there were no publications from Asia, just one from South America (Arróniz-Crespo et al. 2014), and only two studies of potential symbiosis were found for India (Sengupta et al., 1981, Rao et al., 2016). Australia, New Zealand, and the Pacific Islands were also somewhat under-represented in this literature. All of these areas may offer significant opportunities for improving our understanding of symbiosis formation and nutrient cycling. Geographic limitations of current research on bryophyte-cyanobacterial studies are more thoroughly reviewed in Chen & Nelson (2022).

For most of the frequently-hosting families, the species studied constitute ≤1% of all species within that family. It is likely that some of the cyanobacterial hosts play important roles in community structure and nutrient cycling of ecosystems. For example, *Campylopus introflexus* (Hedw.) Brid in Lembophyllaceae is a well-known invasive moss in some parts of the world; does its ability to host epiphytic N-fixing cyanobacteria (Brasell et al. 1986) have any connection to its success as an invader in lichen-rich grey dunes in Denmark (Klinck 2009) or disturbed peatlands in Lithuania (Repečkienė et al.

[4] Additional research on bryophyte-cyanobacterial N fixation from these areas has been published in scientific literature, but was unavailable to the author at the time of analysis due to limited digital access.

2012)? In the moss family Brachytheciaceae, some taxa are robust and considered fairly "weedy" for bryophytes, contributing significant biomass to some ecosystems (e.g., *Brachythecium rutabulum* in tall herbaceous communities, Al-Mufti et al., 1977; *Tomenthypnum nitens* in rich fen communities, Chee & Vitt 1989). What role do these bryophytes, which almost always host cyanobacteria, play in the biogeochemistry of such ecosystems?

Cyanobiont diversity and the potential ecophysiology of the symbiosis. From this boreal forest site, 784 reports of symbiosis (one cyanobacterial taxonomic unit, one bryophyte species or genus per report) were analyzed after the exclusion of 11 reports with uncertain cyanobacterial taxonomic status. 55 different genera of cyanobacteria were present as epiphytic or endophytic symbionts. *Nostoc* was the most common cyanobacterial symbiont, cited in 32% of the reports (Figure 3). Collectively, non-*Nostoc* taxa constitute a larger portion of the symbiotic cyanobacterial diversity, outnumbering *Nostoc* by roughly 2:1 (Figure 3). This conclusion is robust to taxonomic uncertainties regarding the distinctions between *Anabaena* and *Nostoc;* even lumping the two taxa together results in a *Nostoc* prevalence of only 36%. This is surprising, as *Nostoc* is generally characterized as the most common plant cyanobiont for both bryophytes and vascular plants (e.g., Adams et al. 2013; Syiem & Rai, 2013). Furthermore, some of the more common symbionts lack heterocysts (e.g. *Phormidium* Kützing ex Gomont; *Plectonema* Thuret ex Gomont). Others lack both heterocysts and a hormogonial life-stage (e.g., *Gloeocapsa* Kützing, which may be unicellular or colonial). *Gloeocapsa* is

the second most abundant genus among the cyanobacterial symbionts studied, constituting about 8% of all reports (Figure 3).

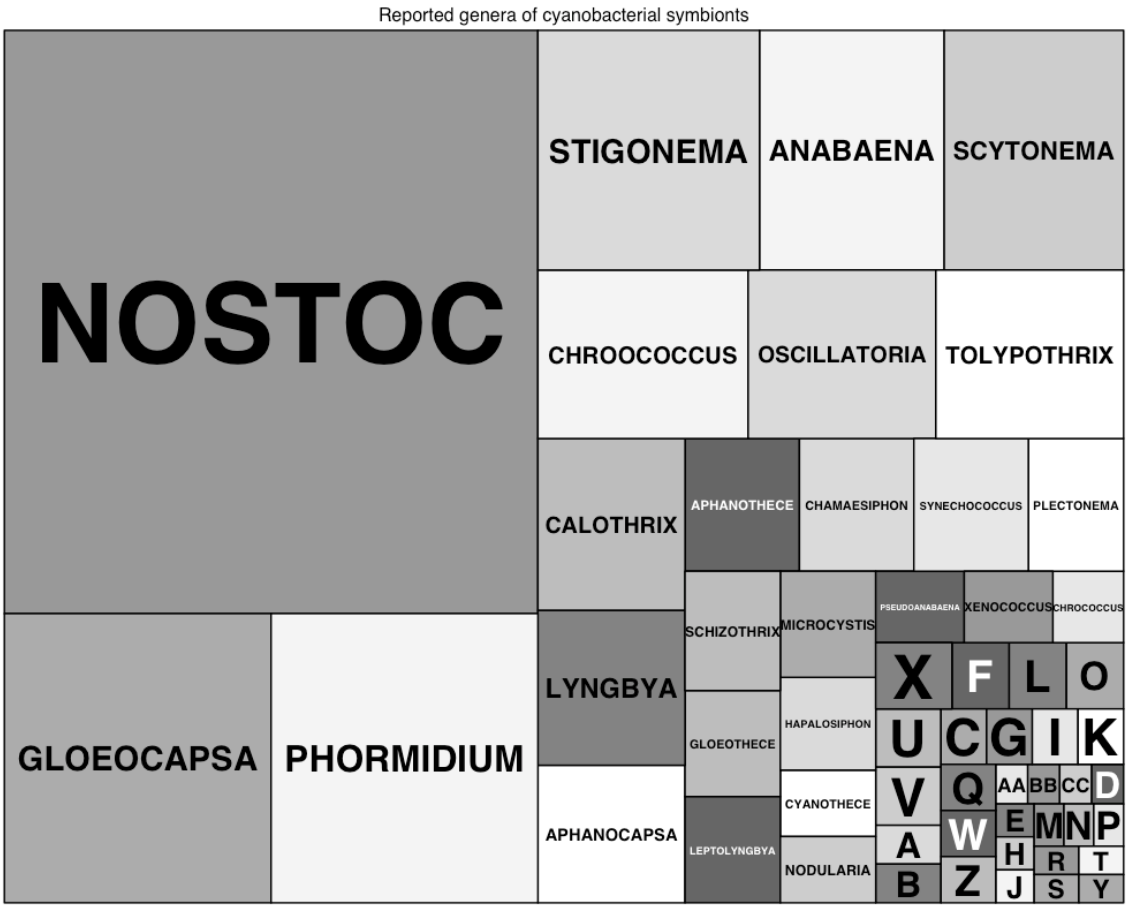

Figure 3. The area allocated to each cyanobacterial genus is proportional to the abundance of reports for that taxon in the published literature. Color-coding is for aesthetic purposes only.

The morphotype genus *Nostoc* in Nostocales is the most abundant cyanobacterial symbiont for the studied bryophytes. Some of the other abundant genera (*Stigomena, Anabaena, Scytonema, Tolypothrix, Calothrix)* are also heterocystous and belong to Nostocales. Other abundant genera include *Gloeocapsa* and *Chroococcus* in Chroococcales, and *Phormidium, Oscillatoria,* and *Lyngbya* in Oscillatoriales. The

relatively rare genera (< 5 occurences, or < 0.65% abundance) are labeled with letters (Table 1).

Symbol	Cyanobacterial Genus
A	*Anabaenopsis*
B	*Aphanizomenon*
C	*Asterocapsa*
D	*Borzia*
E	*Chlorogeopsis*
F	*Chlorogloea*
G	*Clastidium*
H	*Crinalium*
I	*Cyanobacterium*
J	*Cyanobium*
K	*Cyanosarcina*
L	*Cylindrospermum*
M	*Fortiea*
N	*Gloeotrichia*
O	*Gomphosphaeria*
P	*Isocystis*
Q	*Komvophoron*
R	*Leibleinia*
S	*Limnothrix*
T	*Mastigocladus*
U	*Merismopedia*
V	*Microchaete*
W	*Microcoleus*
X	*Petalonema*
Y	*Placoma*
Z	*Pleurocapsa*
AA	*Pseudocapsa*
BB	*Rhaboderma*
CC	*Trichormus*

Table 1. Rare genera of cyanobacteria in Figure 3.

Just as *Nostoc* is not the only abundant symbiont genus, Nostocales is not the only abundant symbiont order. The cyanobacterial order Chroococcales constitutes about 19% of all reports of bryophyte-cyanobacterial symbiosis (Figure 4), partnering frequently with moss, rarely with liverworts. There are no reports of Chroococcales with hornworts. Chroococcales is currently defined by coccoid cells with an irregular

thylakoid structure; both unicellular and colonial forms are included (Komárek et al. 2014).

In Figure 4, a single gray line represents one report of an association between the bryophyte group and a cyanobacterial order. The wider the gray band is, the more often the cyanobacterial order and the bryophyte group are found in association with each other. Hence the width of the path leading from a given cyanobacterial order to a bryophyte group (moss, liverwort, or hornwort) is proportional to the percentage of published reports associated with that combination of cyanobacteria and bryophyte. Nostocales is the most abundant associate of the liverworts and the hornworts, but a variety of cyanobacterial orders (Table 2) are associated with the moss. Taxonomy for the cyanobacterial orders reflects Cyano.DB (August 2016).

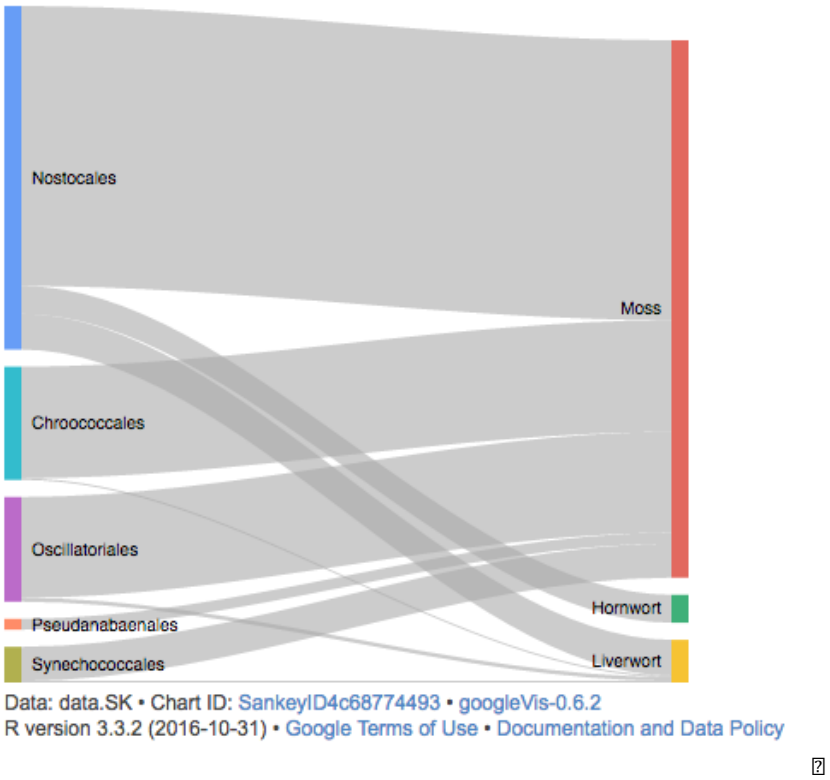

Figure 4. Sankey diagram visualizing associations between orders of cyanobacteria and bryophyte groups.

Cyanobacterial order	Mosses	Liverworts	Hornworts	Total (%) by cyanobacterial order
Nostocales	46.0%	5.9%	4.6%	56.5%
Chroococcales	18.4%	0.3%	0%	18.7%
Oscillatoriales	16.6%	0.6%	0%	17.2%
Pseudanabaenales	1.8%	0%	0%	1.8%
Synechococcales	5.6%	0.3%	0%	5.9%
Total (%) by bryophyte group	88.4%	7.1%	4.6%	

Table 2. Percentage of reports associated with various orders of cyanobacteria for moss, liverwort, and hornwort groups. Totals do not sum to 100% due to rounding.

Symbiotic genera from Chroococcales are diverse in form, including unicells such as *Cyanothece* and *Cyanobacterium,* among others (Figure 5).

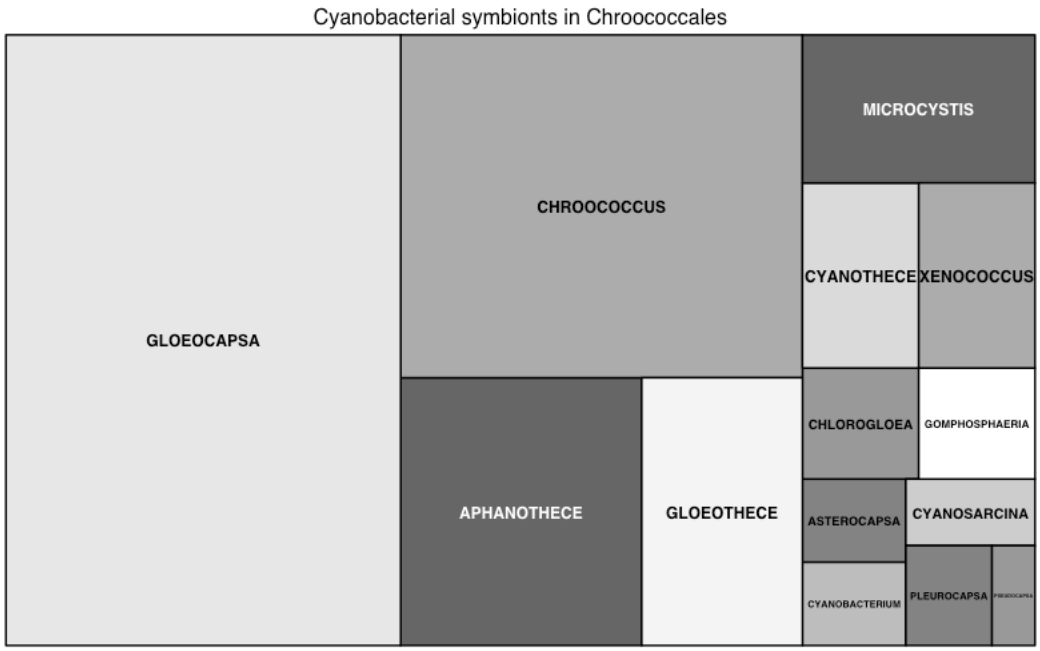

Figure 5. Area is proportional to the number of reports associated with each genus in Chroococcales, relative for all reports for the order. Color-coding is for aesthetic purposes only.

Symbionts from Chroococcales, with genera shown in Figure 5, constitute about one-fifth of all symbionts. Within Chroococcales, *Gloeocapsa* is most abundant, followed by *Chroococcus*.

The types of cyanobacteria that affiliate with mosses, hornworts, and liverworts at the level of cyanobacterial order differ (Figure 4, Table 2). Hornworts are associated solely with symbionts in Nostocales in the literature, and liverworts, primarily (but not exclusively) with Nostocales. By contrast, mosses are commonly associated with Nostocales, Chroococcales, and Oscillatoriales (Figure 4, Table 2), and rarely associated with Synechococcales and Pseudanabaenales.

DISCUSSION

Theories of bryophyte-cyanobacterial symbiosis. These findings suggest that long-standing ideas about bryophyte-cyanobacterial symbiosis, dating back to Stewart et al. (1978), may need to be re-examined. Stewart and his colleagues suggested that only multicellular heterocyst-forming cyanobacteria could be nitrogen-fixing cyanobionts, as the heterocyst provided protection against oxygen deactivation of the dinitrogenase enzyme. However, these authors were generalizing from extensive work with symbionts in *Blasia* and *Anthoceros.* In spite of the narrow focus of Stewart's evidence, these themes continue to be developed in recent research and scholarship (e.g., Adams et al. 2013, Santi et al. 2013, Ahmed et al. 2014). The results of this review affirm the importance of heterocystous cyanobacteria, as members of the heterocystous order *Nostocales* constitute about 56% of the symbiotic flora (Figure 4). But non-heterocystous forms in the orders Chroococcales, Oscillatoriales, Pseudoanabaenales, and Synechochoccocales are nearly as abundant as those in Nostocales (Figure 4); these non-heterocystous cyanobacteria constitute about 44% of all reports. While Nostocalean cyanobacteria were associated with most reports of N fixation (about 83%), non-Nostocalean cyanobacteria were associated with about 17% of the N fixation reports. Furthermore, the probability of detecting N fixation given testing was quite high for non-Nostocalean cyanobacteria: 39 of 46 samples studied, or about 85%, were associated with N fixation. This does not provide conclusive evidence of N fixation, as other cyanobacterial taxa or heterotrophic N-fixing bacteria may be responsible. But it does raise questions about potential cyanobacterial N fixation outside of Nostocales.

The diversity of non-heterocystous bryophyte cyanobionts in the literature of the past several decades, coupled with the possibility of non-heterocystous N fixation, suggest a need to expand and reinvigorate the theory guiding explorations of bryophyte-cyanobacterial symbiosis.

It is possible that some of these non-heterocystous cyanobacteria are coincidentally present as epiphytes on bryophytes, just as cyanobacteria grow on the surfaces of inert, non-living substrata such as rock and bark. But it is also possible that some or all of these relationships are more complex, and the bryophytes interact with the cyanobacteria as partners.

The most intensely-studied mechanistic models of symbiosis focus on the hornwort *Anthoceros* or the liverwort *Blasia* and the cyanobacterium *Nostoc* Vaucher ex Bornet et Flahault*,* typically *Nostoc punctiforme* (Kützing ex Hariot) Hariot. The model, reviewed in Adams & Duggan (2008), involves bryophyte-cyanobacterial chemical signaling, as well as characteristic changes in form and function in the cyanobacteria. Low levels of available N are an environmental trigger for initiation of the symbiosis; such conditions cause the bryophyte to release one or hormogonia-inducing factors. These signaling molecules cause the cyanobacterial cells to transform into a mobile form known as hormogonia. The hormogonia glide on pilli and migrate into specialized cavities on the bryophyte leaves (auricles in *Blasia,* slime pits in *Anthoceros*). Heterocysts capable of nitrogen fixation develop, typically at a much higher frequency than in free-living *Nostoc.* Further movement by the cyanobacteria is inhibited with the bryophyte's release of a

hormogonia-repressing factor. Other potential cyanobacterial changes, apparently directed by the bryophyte host, include modifications in cell shape and form, slowing growth rates, and N fixation; C fixation may be diminished or absent. The critical role of C exchange (from bryophyte to cyanobacterial cells) is highlighted by the presence of a glucose transport protein in vegetative cells of the cyanobacteria; mutations in the genes associated with this transport function make establishment of an *Anthoceros-Nostoc* symbiosis impossible (Ekman et al., 2013).

In recent years, researchers have begun to map out mechanisms of symbiosis for other bryophytes, including feather mosses such as *Hylocomium splendens* and *Pleurozium schreberi*. Comparisons of symbiotically competent and incompetent *Nostoc* strains indicate that the feather-moss symbioses are associated with novel gene families, as well as novel symbiotic traits that may be unique to these moss taxa (Warshan et al., 2017). This suggests that there may be mechanistic diversity in bryophyte symbioses with *Nostoc,* just as there is mechanistic diversity for vascular plant symbioses with other N-fixing bacteria. Most legumes that host N-fixing microbes (e.g., *Rhizobium*) release flavonoid compounds when levels of environmental N are low; these compounds interact with *nod* factors (from N-fixing bacteria) to initiate symbioses. But modified or alternative modes of signaling have been proposed for vascular plant symbioses with N-fixing bacteria such as *Frankia* (Giraud et al. 2007), and for plants such as the Caesalpinoid legumes (Sprent & James 2007) and aquatic legumes (Goormachtig et al. 2004).

At least 31 bryophyte families and 147 species (Ederer thesis, Part I, 2023) are capable of hosting cyanobacteria. If these symbioses involve chemical signaling and mutualism (e.g., nutrient exchange), can the models currently developed for *Anthoceros, Blasia,* or the feather mosses explain the initiation and maintenance of symbiosis across this wide array of bryophyte diversity? For example, is low environmental N the trigger for cyanobacterial symbioses among all symbiotically-capable bryophytes?

The formation of motile hormogonia is typically characterized as a necessary step for the recruitment of cyanobacterial symbionts (e.g., Risser et al. 2014, Risser, 2023). However, not all cyanobacteria have a morphologically distinct motile life stage; the differentiation of hormogonia is not characteristic of unicells or Oscillatorialean filaments. Yet both of these groups are notable elements of the epiphytic bryophyte flora, and some unicells and non-heterocystous filaments are capable of gliding motility (Khayatan et al., 2015). If these cyanobacterial taxa are recruited to bryophytes (as opposed to coincidentally present), the mechanisms of chemotaxis may be different for symbionts that lack hormogonia.

The potential role of non-heterocystous taxa in biogeochemical cycling is also an open question. While the importance of heterocystous cyanobacteria in bryophyte-associated N fixation is widely acknowledged in some ecosystems (e.g., boreal forests, reviewed in Rousk et al. 2013), some non-heterocystous cyanobacteria are also capable of N fixation. For example, the colonial cyanobacterium *Gloeocapsa* is characterized as one of the two most common moss cyanobionts in Antarctica (Horikawa & Ando 1967), and

is also capable of N_2 fixation (Wyatt and Silvey, 1969). Exploration of the potential role of non-heterocystous cyanobacteria in bryophyte-associated N fixation may shed light on ecosystem-scale nitrogen dynamics in areas where these symbionts are abundant.

The high diversity of cyanobionts found to associate with moss, involving five different orders of cyanobacteria, suggests the potential for mechanistic differences between the mosses and their relatives, the hornworts and liverworts. It is possible that the higher moss symbiont diversity simply reflects more robust sampling for the mosses (88% of the 784 reports of symbiosis involved moss). A broader account of taxonomic diversity in the liverworts may reveal similar levels of cyanobacterial diversity. But if inadequate sampling does not account for the differences, the nature of the relationships between moss and cyanobacteria may require further exploration. Are the signaling mechanisms that involve Nostocalean symbionts identical in all three bryophyte groups (mosses, liverworts, and hornworts)? Do moss symbionts in Oscillatoriales and Chroococcales respond to the same chemical signaling that targets Nostocales, or are other mechanisms at work?

The globally-distributed, post-fire pioneer moss *Ceratodon purpureus* might be an excellent model system for exploring such questions given the availability of genomic data (Genome Portal of the Department of Energy, 2014). This taxon is common on a global basis and typically capable of N fixation (Vlassak et al. 1973, Henriksson et al. 1987, Nakatsubo & Ino 1987, Brasell et al. 1986). *Ceratodon purpureus* is associated with a wide array of cyanobacterial symbionts, both heterocystous and non-

heterocystous. These include *Gloeocapsa* (Horikawa & Ando 1967)*, Nostoc* sp. (Vlassak et al. 1973, Brasell et al. 1986, Nakatsubo & Ino 1987, Nakatsubo & Ohtani 1991, Kanda & Inoue 1994, Kanda et al. 2004), *Lyngbya* sp. (Vlassak et al. 1973), *Oscillatoria* sp. (Vlassak et al. 1973), *Anabaena* sp. (Vlassak et al. 1973, Brasell et al. 1986), *Nostoc calcicola* Bréb in Menegh. (Henriksson et al. 1987), *Tolypothrix* sp. (Henriksson et al. 1987), *Stigonema minutum* (Nakatsubo & Ohtani 1991), and *Phormidium* sp. (Kanda & Inoue 1994). Some of these cyanobacteria develop heterocysts (*Nostoc* spp., *Anabaena* sp., *Stigonema, Tolypothrix*); others do not (*Oscillatoria*, *Lyngbya,* and colonial *Gloeocapsa*). Is the colonial cyanobiont *Gloeocapsa*, known to be capable of N fixation, involved in a genetically-regulated system of carbon-nitrogen exchange with the host plant, much like that of *Nostoc* symbionts? And what types of relationships with bryophytes (if any) are characteristic of other non-heterocystous cyanobionts?

These questions are ecologically important for *Ceratodon purpureus,* which is a pioneer of disturbed and burned sites. But they are also broadly relevant to the many bryophyte taxa (at least 147 species) known to associate with cyanobacteria.

Broader implications: mosses as model organisms. The prevalence of cyanobacterial symbiosis among the studied moss taxa also raises interesting questions about research being conducted with model bryophyte taxa. Consider the moss *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr, often used as a model organism in studies of desiccation tolerance (e.g., Oliver et al. 2000, Fernández-Marín 2013, Royles

et al. 2013). *Syntrichia ruralis* has been collected from the field both with cyanobacterial symbionts (Snyder & Wullstein 1973) and without (Opelt & Berg 2004). Some desiccation studies use fresh materials collected directly from the field, but the moss plants are not necessarily screened for cyanobacterial symbionts. Could the presence or absence of N-fixing cyanobacteria, as well as their abundance, potentially influence the ways in which *Syntrichia ruralis* responds to desiccation stress or other physiological challenges? Exploring the potential influence of such symbionts might provide deeper insights into the mechanisms and limitations of desiccation tolerance, a phenomenon of significant importance to agriculture.

The moss *Physcomitrella patens* (Hedw.) Bruch & Schimp is an important model system in genomic research; the fully-sequenced genome of *Physcomitella patens* (Rensing et al. 2008) has been employed in thousands of studies of plant physiology, plant development, and plant genome evolution (van Gessel et al. 2017). This author was unable to locate information on the symbiotic status of *Physcomitrella,* which may be difficult to study in the field—the gametophytes are fleetingly present in transient environments. *Physcomitrella* belongs to the basal moss family Funariaceae, which is capable of cyanobacterial symbiosis. *Physcomitrella* is a short-lived pioneer that often develops in flood-plain environments (Rensing et al. 2009). In Funariaceae, the widely-distributed pioneer moss *Funaria hygrometrica* Hedw. harbors cyanobacteria (Schwabe 1974, Scheirer & Brasell 1984, Brasell et al. 1986) and is capable of N fixation (Scheirer & Brasell 1984, Brasell et al. 1986). With respect to life-cycle and habitat, *Physcomitrella patens* is quite similar to *Funaria hygrometrica:* both have a fugitive

life-style, relatively rare among bryophytes, characterized by a short life-cycle in moist, unpredictably disturbed habitats (Glime 2013). Is *Physcomitrella patens,* like *Funaria hygrometica,* capable of hosting epiphytic cyanobacteria, and potentially contributing fixed N to its environment?

Given the robustness, intensity, and diversity of the research involving *Physcomitrella,* knowledge of its symbiotic capabilities (or lack thereof) may be important. If *Physcomitrella* typically receives a nitrogen supplement from cyanobacterial symbionts in the wild, or provides fixed carbon to the cyanobionts, studies of sterile plants that lack symbionts may provide a distorted view of carbon fixation, nitrogen metabolism, and other fundamental processes. If cyanobacterial symbionts are indeed a natural part of the *Physcomitrella*'s life cycle, some genomic studies may need to incorporate cyanobacterial symbionts to gain a more realistic appreciation of the plant's function. If *Physcomitrella* does not typically harbor cyanobacteria, though, a different question arises: How representative of the mosses as a whole is this experimental system? The answer will depend on what we learn in the future about the overall prevalence of moss-cyanobacterial symbioses, given that little or no information is currently available for ~ 99% of all taxa. A mismatch between the typical moss condition (symbiotic vs. non-symbiotic) and the condition of *Physcomitrella* could limit the scope of inference for studies involving this model taxon.

Regardless of whether bryophyte-cyanobacterial symbioses ultimately prove to be quasi-ubiquitous or relatively rare, expanding genomic- and transcriptomic-level studies

to some of the many mosses and liverworts that are capable of cyanobacterial symbiosis would be valuable; ideally, these studies would involve diverse cyanobacterial symbionts, as well as *Nostoc. Ceratodon purpureus* is one potential candidate, given its wide distribution and the availability of genomic data. Such research would complement intensive and ongoing work with familiar model taxa such as *Anthoceros* and *Blasia* that are better understood (e.g., Enderlin & Meeks 1983, Meeks et al. 1985, Campbell & Meeks 1989, Steinberg & Meeks 1991, Campbell & Meeks 1992, Wong & Meeks 2002, Chapman et al. 2008, Ekman et al. 2013). In like manner, fungal symbiosis may be an important element of future studies of moss function at the genomic level, as moss-fungal symbioses are being documented with increasing frequency (e.g., Read et al. 2000, Stenroos et al. 2010, Pressler et al. 2014, Maraist 2018). Although mycorrhizal-type nutrient exchange has not been documented in most fungal-bryophyte associations (Pressler et al. 2014), at least one liverwort is known to receive supplemental carbon from a vascular plant via fungus (Bidartondo et al. 2003). Genomic-level work might better illuminate the benefits (if any) of these associations, as well as highlight potential interactions among bryophytes, cyanobacteria, and fungi.

SUMMARY

In this global-scale review, 60 bryophyte families screened for cyanobacteria were categorized into groups based on their probability of hosting cyanobacteria. Cyanobacterial symbionts were categorized based on their status as members of the genus *Nostoc* or members of other taxa; taxa were also assigned to orders.

Families that did not host cyanobacteria were supported sparsely by the literature (one published report per family); these include the liverwort families Lepidoziaceae and Conocephalaceae, as well as the moss families Ptychomniaceae and Takakiaceae.

A few bryophyte families host cyanobacteria in less than half or about half of the published reports (host probability ≤ 0.5); these include the moss families Andreaeaceae, Polytrichaceae, and Pylaisiadelphaceae, and the liverwort family Lophocoleaceae. Families that host more often than not (host probability > 0.5, but $\leq$ .85) include Aulacomniaceae, Bartramiaceae, Dicranaceae, Hypnaceae, Leucobryaceae, Thuidiaceae, and Sphagnaceae, and the leafy liverwort family Scapaniaceae. For all of the other bryophyte families (46 total), more than 85% of the published reports indicated cyanobacteria were almost always or always present. Families that were relatively well-studied within this group include the mosses in Amblystegiaceae, Brachytheciaceae, Bryaceae, Calliergonaceae, Ditrichaceae, Grimmiaceae, Hylocomiaceae, Mniaceae, Pottiaceae, and Sphagnaceae as well as the liverwort family Blasiaceae and the hornwort family Anthocerotaceae.

The diversity and frequency of cyanobacterial symbiosis indicates that the role of bryophyte-cyanobacterial symbioses in nutrient cycling and community composition for diverse ecosystems needs further investigation, especially in non-boreal ecosystems. In addition, current models of plant-microbe signaling for brophyte-cyanobacteria symbiosis, which focus on *Nostoc* symbionts and the low availability of environmental N as a trigger for symbiosis, do not necessarily accommodate the diverse array of

bryophyte and cyanobacteria taxa found in the literature. Based on the abundance of published reports, *Nostoc* does not appear to be the dominant cyanobacterial symbiont for bryophytes; other cyanobacterial genera constituted at least two-thirds of all published symbioses. The globally-distributed moss *Ceratodon purpureus,* which is common in disturbed and post-fire landscapes, is one potential model taxon for the study of bryophyte-cyanobacterial symbioses. Genome data is available for this common and ecologically important moss, and it is known to associate with a wide array of cyanobacteria, including *Nostoc.* Exploration of the potential impact of cyanobacterial and fungal symbionts in well-established model moss systems such as *Physcomitrella patens* and *Syntrichia ruralis* is also proposed; while *Syntrichia* is a well-documented cyanobacterial host, it is not clear whether field populations of *Physcomitrella* typically harbor cyanobacterial or fungal symbionts.

Overall, the diversity of bryophyte families that host cyanobacteria, as well as the highly diverse forms of cyanobacterial symbionts (heterocystous filaments, non-heterocystous filaments, colonies, and unicells from at least 55 genera) suggest there are opportunities for expanding our understanding of these symbioses. Genomic and metabolic studies of *Ceratodon,* as well as other bryophytes hosting diverse microbial communities, have the potential to better illuminate plant-microbial communication in land plants, with potential applications to economically important crop plants.

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DATA ACCESSIBILITY

Data compiled from the literature (including citations and descriptions for all reports of symbiosis) are available in a spreadsheet (Supplemental Information).

Part III: A study of bryophyte-cyanobacterial biodiversity and potential microhabitat influences on the occurrence of symbiosis

Key results from Part III. How often do boreal bryophytes host cyanobacteria, and which cyanobacterial taxa are associated with bryophytes? How does the microhabitat of a given bryophyte species influence the probability of hosting cyanobacteria? In this study of a second-growth boreal forest in Baileys Harbor, Wisconsin, a grid sampling design was used to collect over 478 single-species bryophyte samples from small circular plots. Within each plot, the nature of the bryophyte microhabitat (substrate type) was characterized, as well as the height above ground level (given uneven terrain) and the presence of lichens, which may harbor cyanobacterial symbionts. Samples were collected in spring, summer, winter, and fall to illuminate the potential influence of seasonal change. Groundcover bryophyte diversity was surveyed by taking a relatively comprehensive approach, rather than focusing on a few dominant taxa, to provide information for biodiversity archives and to explore the likelihood of cyanobacterial symbiosis among less dominant taxa. Epifluorescence microscopy was used to determine whether cyanobacterial symbionts were present on bryophyte tissues, and to generate estimates of cyanobacterial abundance based on cell counts. Logistic regression was utilized to determine whether environmental variables at the scale of the microhabitat (i.e., plot) influenced the odds of hosting cyanobacteria for a given bryophyte sample.

Most microhabitat attributes were not influential, but the presence of rock substrate had a strong impact on the likelihood of hosting cyanobacteria. Bryophytes growing on rock

were approximately 17 times as likely to harbor cyanobacteria, and cyanobacterial abundances may be greater for the dominant rock-dwelling bryophytes. These included *Thuidium delicatum, Tortella tortuosa,* and various *Brachythecium* species, all of which harbored heterocystous cyanobacteria. This suggests that rocks may have the potential to function as biogeochemical 'hot spots' for N fixation in the boreal landscape.

However, logistic regression accounted for only 18% of the model deviance, indicating that controls on the formation of bryophyte-cyanobacterial associations may act at higher levels of organization, and do not have a straightforward relationship to the bryophyte microhabitat variables characterized for this study.

Among the hundreds of bryophyte samples identified, 40% of all species constituted potentially novel reports of bryophyte-cyanobacterial associations (not reported in literature accessible to the author). This represents a substantial expansion of the number of species documented as cyanobacterial hosts in Part I of this thesis (an increase of about 14%, changing the total number of known hosts from 147 species to 167), and suggests that cyanobacterial associations may be more common among bryophytes than previously understood. However, 42% of all species studied did not host cyanobacteria; most of these were relatively rare taxa (relative abundance 1-2%). More surprisingly, two of the three dominant taxa, *Pleurozium schreberi* and *Dicranum scoparium,* rarely hosted cyanobacteria. The absence of cyanobacteria on most samples of the feather mosses (*Pleurozium schreberi, Hylocomium splendens*) was particularly notable, as these taxa are strongly associated with cyanobacterial

symbionts and N fixation in the boreal forests of Canada, Alaska, and Fennoscandia. Atmospheric N deposition is one potential explanation for the sparse cyanobacterial presence, but these results also raise questions about the potential interaction of ecosystem-scale N inputs and bryophyte species identity. If N deposition is responsible for the near-total absence of cyanobacteria on the feather mosses, why are cyanobacteria robustly present on other bryophytes, including those from the families Amblystegiaceae, Brachytheciaceae, Fissidentaceae, Mniaceae, Pottiaceae, Thuidiaceae, Lophocoleaceae, and Radulaceae?

Overall this study provides evidence that bryophyte species identity may provide valuable information in studies of bryophyte-cyanobacterial associations, in accord with recent research on feather mosses at high latitudes in the boreal forest. These data support the idea that cyanobacterial associations may be relatively common among boreal bryophytes; considered in the context of the global literature, 92% of all of the species studied at this site belong to a genus known to host cyanobacteria in at least one ecosystem. More comprehensive surveys of bryophyte floras may also generate useful information for conservation purposes in light of climate change; at this site, 10% of all bryophyte species were novel records for Door County, Wisconsin.

INTRODUCTION

Bryophyte-cyanobacterial associations in the boreal forest are well-studied, with a history of observational and experimental work dating back to the 1980s (Weber & Van

Cleve, 1981; Alexander & Billington, 1986). In the last two decades ecologists have become increasingly aware of nitrogen fixation associated with the boreal bryophyte microbiome, involving either cyanobacteria or heterotrophic N fixers (reviewed in Rousk et al. 2013); recent estimates of annual N contribution are sometimes as high as 4 kg ha^{-1} yr^{-1} (e.g., DeLuca et al., 2002; Lagerström et al., 2007; DeLuca et al., 2008; Gundale et al., 2010, 2011; Jean et al. 2018; Stuart 2021). But the scientific literature indicates that bryophyte hosts of bacteria in general are understudied, with a relatively small proportion of the approximately 12,500 bryophyte species encompassed by the current literature (Alvarenga & Rousk 2022, Chen & Nelson 2022). Moreover, although boreal feather mosses such as *Pleurozium schreberi* (Willd. ex Brid.) Mitt. and *Hylocomium splendens* (Hedw.) Schimp. are found in much of the continental United States (e.g., alpine and subalpine environments), almost all of the research on cyanobacteria and these dominant moss taxa involves sites in Canada, Alaska, and northern Europe or Asia. As far as I know, only two research groups have attempted to determine whether these mosses hosted N-fixing bacteria (cyanobacterial or heterotrophic) in the continental United States. Lambert & Reiners (1979) found that *Pleurozium schreberi* was associated with symbiotic N fixation in the subalpine forest of the White Mountains in New Hampshire, but light microscopy did not reveal any epiphytic cyanobacteria. Deane-Coe & Sparks (2016) used epifluoresence microscopy to examine *Pleurozium schreberi* from a temperate forest in upstate New York, but did not find any evidence of cyanobacteria. Neither of these studies provides definitive evidence of cyanobacteria in association with *Pleurozium schreberi.* I was unable to locate any research involving moss-cyanobacterial associations in *Hylocomium*

splendens at a site within the continental United States. There is a need to better understand whether boreal bryophyte-cyanobacterial associations exist at all in these mid-temperate latitudes, and if so, what is their prevalence?

This study is an attempt to systematically categorize the presence or absence of cyanobacterial symbionts for boreal forest bryophytes in a cool habitat along the shore of Lake Michigan. This disjunct site was selected because of its position as one of the southernmost boreal forests in the Midwest, in an area likely to be sensitive to climate change; in recent years the Laurentian Great Lakes region has experienced increased lake-effect snow (Burnett et al. 2003, Kunkel et al. 2009) and increased air temperatures (Zhong et al. 2016), with warming occurring more rapidly than average within the continental U.S. (U.S. Global Climate Change Research Program 2017). Warming impacts are likely to be especially notable in winter, given that the current change in average annual minimum temperature (+ 1.75° F) is more pronounced than in other regions of the continental U.S.; only Alaska has experienced more profound winter warming (U.S. Global Climate Change Research Program 2017). Future impacts on precipitation are subject to greater uncertainty. There is evidence of greater variability and the potential for decreased precipitation and lengthier droughts (Byun & Hamlet 2018). However, more recent work dynamically downscaling global-scale General Circulation Models with three-dimensional lake models suggests that precipitation over the lake and lake runoff are likely to *increase* (Kayastha et al. 2022); ensemble averages for three lake basins (Superior, Michigan-Huron, and Erie) predict rising annual lake levels, even though individual model runs predicted both increases and

decreases in net basin supply and lake levels. Even given current uncertainty about whether mean precipitation will increase or decrease, future climate change has the potential to perturb bryophyte-cyanobacterial N fixation. Temperature and moisture are two of the prime controls on symbiotic N fixation in boreal feather mosses (Rousk et al. 2017, Rousk et al. 2018).

At the boreal forest site in Baileys Harbor, Wisconsin, a large portion of the groundcover is dominated by boreal bryophytes, including the feather mosses *Pleurozium schreberi* (Willd. ex Brid.) Mitt. and *Hylocomium splendens* (Hedw.) Schimp.; this mid-latitude boreal forest offers the researcher an opportunity to study a robust and diverse bryophyte flora. An attempt was made to characterize the groundcover bryophyte flora as a whole rather than study two or three focal taxa; the latter approach is typical in the boreal forest, where a few mosses are generally abundant and dominant (Vitt and Belland 1997). The objective was to provide baseline information on bryophyte diversity, as well as to determine whether a more comprehensive approach would yield additional insights.

At a smaller scale, the concept of the bryophyte *microhabitat* provides motivation for the study. Bryophyte ecophysiology is greatly influenced by boundary-layer conditions related to fine-scale physical features of the habitat: the type of substrate on which a given moss, liverwort, or hornwort grows, and the ways in which nearby objects (wood, rocks, other plants) may facilitate or restrict airflow, shaping the size and moisture level of the protective boundary layer (Kimmerer 2003). The microhabitat is often considered

to be a function of substrate type. For example, Vitt & Belland (1995) defined nine microhabitat categories (hummocks, hummock-sides, lawns, carpets, pools, tree bases, shaded tree-base depressions, humidified peat, and forest duff) in a study of bryophyte biodiversity in 96 peatlands in western Canada. Bryophyte biodiversity was found to be a function of microhabitat diversity: nearly half of the bryophyte diversity could be explained by the number of microhabitats present. In 1997, Belland & Vitt defined bryophyte microhabitats more specifically in the context of larger spatial units: "Microhabitats are habitat patches in which individual populations exist. Generally for mosses, this ranges in size from millimeters to centimeters. Microhabitats are arranged on the regional landscape in non-random patterns in association with localized physiographic or physiognomic forms." The latter are denoted *mesohabits* (Newcastle et al. 2005). Examples of physiographic forms that constitute mesohabitats include streams, seeps, or cliffs; examples of physiognomic forms include forests. Within this framework, the relatively homogeneous boreal-forest study site can be considered a mesohabitat, and the various substrata on which bryophyte live define microhabits nested within the forest. Restricted mesohabits may also be nested within a dominant mesohabitat, e.g., a stream threading through a forest.

Vitt & Belland's (1997) development of the microhabitat concept motivated me to focus on bryophyte microhabitat, defined by substrate type, as an explanatory variable for predicting the presence or absence of cyanobacteria for the boreal bryophyte flora. Small circular plots, scaled to the size of the microhabitat defined by Newcastle et al. 2005, were used to evaluate microhabitats for bryophyte presence. The attributes of

specific substrata helped shape my decisions about microhabitat categories. For example, cyanobacteria in general find habitats with higher pH values favorable, although there is evidence that some heterocystous forms thrive in selected low-pH conditions, such as *Sphagnum*-dominated seeps (Whitton & Potts, 2012). In many forested ecosystems, conifer litter is associated with lower soil pH in the upper horizons, and broad-leaved trees, with higher soil pH (e.g., Augusto et al. 2016, Dawud et al. 2016, Ott & Watmough 2021). These differences in leaf litter impact suggest the potential for interaction between the presence of conifer litter and the absence of bryophyte symbionts. The substrate-based microhabitats defined after preliminary surveys included coniferous litter, deciduous litter, soil, rock, grass, other bryophytes (for species growing on top of other mosses), and nutrient-rich scat (generally from white-tailed deer or rabbits).

The presence of cyanolichens at the study site, including *Peltigera* species that host N-fixing *Nostoc* cyanobacteria, also influenced the sampling design. I wondered whether the symbiotic cyanobacteria in the lichens could provide a source population for bryophyte symbionts.

This research was undertaken with an emphasis on multi-season field work, to encompass both the growing season and the snowy winter period. The temporal structure of the fieldwork is motivated by both the pronounced changes in annual minimum winter temperatures in the Midwest and an overall need for more information about winter ecology (Campbell et al. 2005, Kreyling 2010).

This study addressed the following questions:

(1) How common are bryophyte-cyanobacterial associations in a boreal forest at mid-temperate latitudes? Which bryophyte taxa host cyanobacteria?

(2) What evidence can we find of nitrogen-fixing capability among any cyanobacterial associates?

(3) Does microhabitat composition (substrate, elevation, and the presence or absence of lichens within centimeters of the bryophyte) influence the likelihood of finding cyanobacteria?

(4) Do any differences exist in the incidence of cyanobacterial associates in samples collected at different seasons (summer, fall, winter, and spring)?

Key methods for detection of cyanobacteria include light and epifluorescence microscopy; the latter highlights photosynthetic pigments that are limited to cyanobacteria, making their presence more easily discerned.

Cyanobacterial N fixation capability: What constitutes meaningful evidence?

Historically research on cyanobacterial N fixation has focused on taxa that develop heterocysts, which are characteristic of the order Nostocales (Komárek et al. 2014). These thick-walled cells exclude oxygen, which is known to irreversibly degrade the nitrogenase enzyme. Heterocysts also lack the components of PS II, meaning that the cells do not evolve oxygen and thereby incapacitate nitrogenase during photosynthesis. I have chosen to focus on cell counts of the heterocystous Nostocaleaen cyanobacteria as a minimum measure of N-fixing cyanobacterial abundance.

This is a *minimal* estimate of the incidence and abundance of N fixing-cyanobacteria because at least 17 genera of non-Nostocalean cyanobacteria possess strains that are known to fix nitrogen (Bergman et al. 1997, Tomitani et al. 2006). The non-heterocystous taxon *Microcoleus,* one of these non-Nostocalean taxa, may help to illuminate why I chose to focus solely on Nostocalean taxa. Although *Microcoleus* is known to be capable of N fixation (Bergman et al. 1997) and is an epiphytic bryophyte symbiont (e.g., Uher 2008, Arróniz-Crespo et al. 2014, Zhang et al. 2014), studies of various taxa within *Microcoleus* indicate that N fixation may not be uniformly characteristic of the genus. *Microcoleus vaginatus*, a common terrestrial member of the genus, has been found as an epiphyte on the moss *Mnium cuspidatum* Hedw. in a *Larix principis-rupprechtii* Mayr forest in Shanxi Province, northern China (Zhang et al. 2014). These researchers documented an increase in absorption of photosynthetic light wavelengths after the cyanobacterium had been in culture for three months, as well as a color change from blue-green to green (decreased phycocyanin, increased *Chl* A). This evidence suggests N fixation with C-N exchange occurred in symbiosis with *Mnium*, followed by a shift to a more autotrophic state in culture (Zhang et al. 2014). Furthermore, [5]Zhu et al (2007) have documented N fixation in *Microcoleus vaginatus*. But the genome of *Microcoleus vaginatus* FGP-2, a different strain of the cyanobacterium, provides no evidence of *nif*-related genes or N-fixation capability (Starkenburg et al. 2011, Couradeau et al. 2019). A relatively comprehensive phylogenomic study (Chen et al. 2022) involving 13 terrestrial strains of *Microcoleus* indicates that N-fixation capabilities are distributed unpredictably within the genus: 12 of

[5] I was unable to access this Chinese-language paper from my library system, but the result is presented in Zhang et al. 2014.

the 13 strains studied appear to be non-fixers based on the absence of nitrogen fixation gene clusters.

Microcoleus chthonoplastes, a cyanobacterium found in marine and hypersaline environments, has a similarly complex profile. Phylogenomic work indicates the genome contains a nitrogen fixation gene cluster in at least 8 strains of this species (Bohhuis et al. 2010), although N fixation in culture was not documented. Moreover, the organisms responsible for N fixation in environmental samples may prove difficult to identify. In a cyanobacterial mat from a saltworks complex, N fixation initially attributed to the dominant cyanobacterium, *Microcoleus chthonoplastes,* was revealed to be due to *nifH* activity in various unicellular and non-heterocystous filamentous cyanobacteria, as well as heterotrophic bacteria (Omoregie et al. 2004). The morphogenus *Microcoleus*, defined by bundles of filaments within a polysaccharide sheath, is also polyphyletic (Siegesmund 2008), with various *Microcoleus* taxa provisionally assigned to different families and orders. Strains from a new genus *Coleofasciculus,* derived from the morphotype *Microcoleus* (Siegesmund 2008), are equally unpredictable with respect to N fixation capabilities. Based on the presence or absence of nitrogen fixing gene clusters, Chen et al. (2022) inferred that 10 of 11 strains in *Coleofasciculus* studied were non-fixing cyanobacteria, including *Coleofasciculus chthonoplastes*. Overall the profile of the morphogenus *Microcoleus* (which includes both *Microcoleus* sp. and *Coleofasciculus* sp.) is still under investigation with respect to N fixation and nomenclature. It would be premature to assign cyanobacterial cells in the morphogenus

Microcoleus to a nitrogen-fixation category (present or absent) based on microscopy data alone. There is too much variation among species and strains within the genus.

These concerns have led me to focus on Nostocalean cyanobacteria as a metric for N fixation potential in symbiosis; cell counts for N-fixing taxa in this study do not include non-heterocystous cyanobacteria. These taxa would not yield cell count data that could be reliably characterized with respect to N fixation capabilities based on microscopy methods.

METHODS

The study site is located off East Cana Island Road in Moonlight Bay Bedrock Beach State Natural Area, Baileys Harbor, Wisconsin (Section 311 in Door County Wisconsin Tax Parcel Map, Appendix A). I will refer to this site by the name of adjacent roadway, Cana Island Road. The 7.5 acre property was in private ownership when the research was conducted, and is now part of a larger complex managed by the state of Wisconsin. This site is one of the southernmost boreal forest sites in Wisconsin (Figure 1), with a moist microclimate influenced by proximity to the Lake Michigan shoreline (approximately 200-300 m from border of property). Sampling to characterize bryophytes and their associated substrata occurred during four seasons (summer 2007, fall 2007, winter 2008, and spring 2008), with sampling dates given in Table 1.

	Summer 2007	**Fall 2007**	**Winter 2008**	**Spring 2008**
Dates	July 17-21, 2007	October 16-19, 2007	January 15-17, 2008	April 14-17, 2008
Code	SU07	FA07	WI08	SP08

Table 1. Sampling dates for study of bryophyte-cyanobacterial diversity.

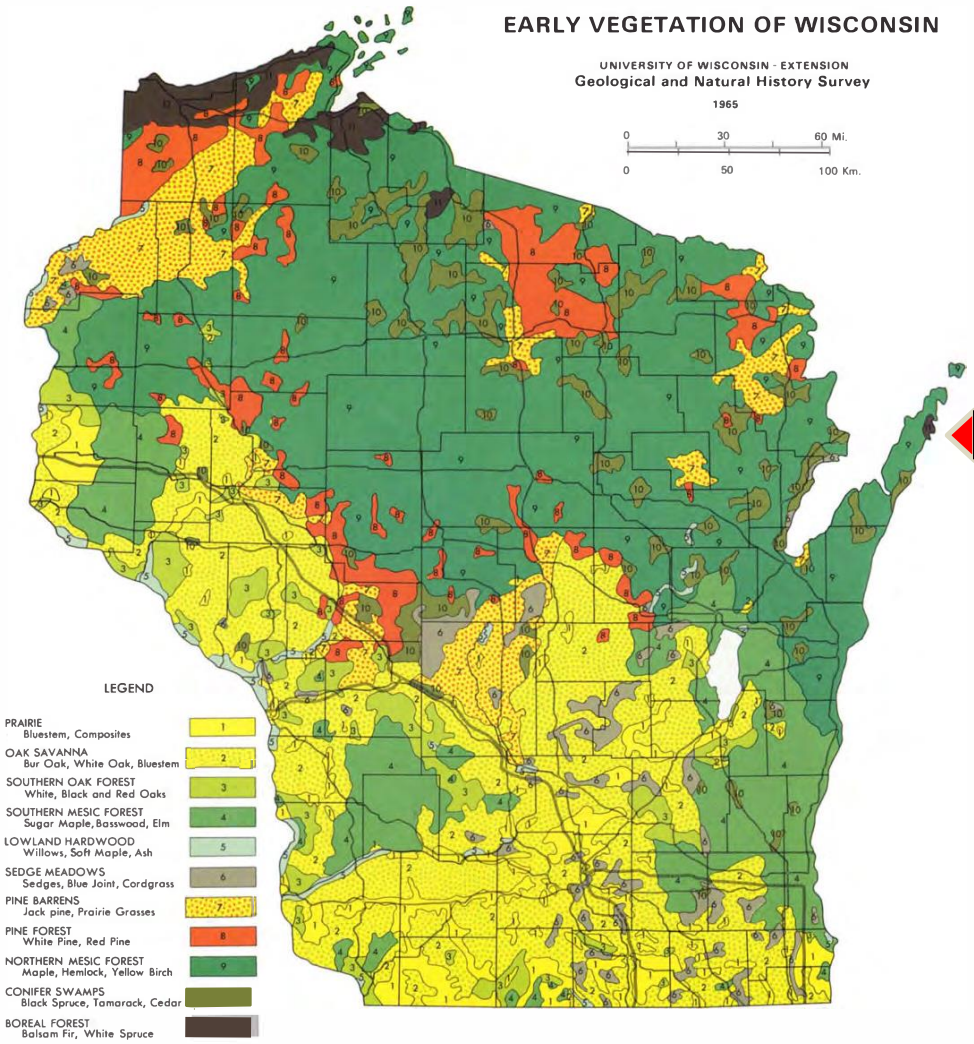


Figure 1. Early Vegetation of Wisconsin (Cottam, G., Loucks, O.L. 1965). The study site in Baileys Harbor, Wisconsin falls into the small dark brown area on the east side of the peninsula extending into Lake Michigan.

The last clear-cut logging occurred around 1957, roughly 50 years prior to the study (Ragotzie 2008, pers. comm.). Dominant trees include white spruce (*Picea glauca* (Moench) Voss), white cedar (*Thuja occidentalis* L.), and white birch (*Betula papyrifera* Marshall); also present are balsam fir (*Abies balsamea* (L.) Mill.), balsam poplar (*Populus balsamifera* L.), red pine (*Pinus rubra* D. Don), white fir (*Abies alba* Mill.), and eastern white pine (*Pinus strobus* L.). Coarse woody debris is abundant; so are fallen trees, tip-up mounds, and snags. The soils are thin (typically < 5 cm), calcareous, and rocky; large (1+ m) glacial erratics are relatively common. Vascular groundcover includes various grasses and woodland sedges, common plants of the northern Great Lakes such as thimbleberry (*Rubus parviflorus* Nutt.), baneberry (*Actaea rubra* (Aiton) Willd. and *Actaea alba* (L.) Mill.), and myriad wildflowers, including large patches of the dwarf iris (*Iris lacustris* Nutt.), designated as threatened by the federal government (U.S. Fish and Wildlife Service, 2015).

The site is notable for its dense bryophyte and lichen cover, both at the ground level and on fallen tree trunks and branches, upright trunks, and rocks and boulders (Figure 2). Overall approximately one-half of the ground is covered by bryophyte-lichen growth. Moss species include the feather-mosses typical of boreal forests, such as *Pleurozium schreberi* (Willd. ex Brid.) Mitt. and *Hylocomium splendens* (Hedw.) Schimp.; also common are *Hylocomiadelphus triquetrus* (Hedw.) Ochyra & Stebel, *Thuidium delicatulum* (Hedw.) Schimp., and the genera *Dicranum* Hedw. and *Brachythecium* Schimp. A variety of other bryophytes are also present. Lichens are abundant and diverse, including common species with green-algal symbionts such as *Flavoparmelia*

caperata (L.) Hale, *Hypogymnia physodes* (L.) Nyl., and *Phaeophyscia adiastola* (Essl.) Essl., as well as cyanolichens such as *Peltigera elisabethae* Gyelnik. On warm winter days, dense populations of snow fleas (*Collembola* sp.) emerge from the moss mat, suggesting a complex food web exists in the cryptogamic groundcover.

Figure 2. Boreal forest near Cana Island Road, Baileys Harbor, Wisconsin.

For the purposes of this study, groundcover was defined as any vegetation present at up to 25 cm from the soil or rock surface; this definition accomodates the irregularity of the surface, as well as the abundant surface area provided by fallen trees, woody debris, and rocks. The uniform sampling design involved a 10 m by 10 m grid with ten transects; a total of approximately 250 plots were sampled for each season. The

starting corner of the grid was randomly shifted by several meters each season to avoid repeat measures. The unit of sampling for substrata was defined by the edges of a 21.5 circle (embroidery hoop); bryophytes were sampled within the central region of the plot, defined by a 6-cm Petri dish. Within the 21.5 cm circle, the presence or absence of each potential substrate was recorded. I chose to record all substrates within the plot, as bryophytes can absorb nutrients via both phyllid surface and rhizoids; I assumed that the general qualities of the immediate environment, shaped by all substrates, would influence plot biochemistry. Likewise the presence/absence of bryophytes and/or lichens in the central 6 cm of the circular area was recorded. If bryophytes were present, these were collected using a sterile glove to minimize the chances of cross-contamination with cyanobacteria; each bryophyte sample was placed in its own coin envelope. Sample sizes were kept to a minimum (quarter-sized or smaller) to avoid unnnecessary impacts on the site, and leaves or spruce cones were placed in the gap if it was necessary to sample from a mat, potentially minimizing desiccation damage to the remaining plants. The substrate type (all substrates present within the boundaries of the 21.5 cm circular plot to a depth of 3 cm) was noted for all plots, regardless of whether cryptogams were present. The height of the bryophyte sample in relation to the surrounding substrate was recorded in cm.

The samples were returned to the laboratory as expediently as possible (within a day of the end of the three-to-five-day sampling period). Thereafter they were stored in the refrigerator (summer, fall) and in the freezer (winter, spring). The change in storage methods was motivated by a desire to keep the samples in cooler temperatures, similar

to those outside, as well as evidence (e.g., flourescent fecal pellets) suggesting moss-dwelling microinvetebrate grazers were consuming cyanobacteria in the refrigerator.

All samples were screened for the presence/absence of cyanobacteria using epifluorescence microscopy. For each sample, 8 drops of dd H_2O were placed in a small sterile plastic disposable dish. Only sterile instruments were used to handle the bryophyte tissues. Shoots or branches were randomly selected from the dominant bryophyte present in the sample, added to the water, and scraped with a sterile hypodermic needle for 2-3 min. The moss tissues absorbed approximately 50% of the water, leaving 4 drops of solution for screening. This liquid was placed on a slide with a 24 x 50 mm coverslip using a sterile pipette, and then examined for cyanobacteria, with intense red fluorescence indicating the presence of cyanobacteria. All such signals were examined under brightfield to ensure that cyanobacteria, rather than potential distractors (e.g., sand, fungi, green algae) were being visualized. A conservative standard was applied to determine cyanobacterial presence: fecal pellets, even those that glowed brightly and had visible cyanobacterial cells, were excluded, as were glowing materials obscured by debris or moss tissue.

The relative abundance of cyanobacteria for each sample was estimated by counting the number of cyanobacterial cells present in 15 viewfields at 100X, using a diagonal pattern starting from the upper left-hard corner. In some samples, cyanobacteria were so abundant that it was impossible to count them accurately at 100X; a count was taken at 200X for these bryophytes. The presence or absence of heterocytes, evidence of potential for N fixation, was also recorded. Cyanobacteria belonging to the form-genus

Nostoc were identified and counted as a separate subtotal. To the extent feasible, cyanobacteria from other genera and families were identified utilizing microscopy and culture methods with Bold's Basal Medium.

The variable used to characterize relative cyanobacterial abundance is based on an estimate of the total number of cells present per slide/subsample screened. This is computed based on the total number of cells present in 15 viewfields scaled in proportion to the total area of the slide cover. There are different scaling relationships for 100X and 200X, given that a much smaller area is screened for 200X; the raw cell counts for 100X were multiplied by 14.93, and for 200X, by 293.3.

After screening, the dominant bryophyte from each sample was identified utilizing *Mosses of the Great Lakes Forest* (Crum 2004). Most taxa were identified to species; a few, to genus or family due to taxonomic challenges (e.g., sterile state). Species names were updated based on currently accepted taxonomic standards per Tropicos, the database of the Missouri Botanical Garden (Tropicos 2023). For data analysis, visualization, and the discussion of results, currently valid Tropicos names are used. Although the estimation of total bryophyte species richness is not an objective of this study, species accumulation curves were created by plotting the data based on the order of sampling (similar to Scheiner's Type I curve per Scheiner 2003, Gray et al. 2004), rather than using rarefaction methods. The resulting stair-step curves cannot be used to estimate total species richness (observed + unobserved), but as the asymptotic

observed species richness is not sensitive to plot order, the curves can be used to visualize observed richness by season and year.

RESULTS

Bryophyte Diversity. A total of 1014 circular 6-cm plots were screened for the presence of bryophytes during 2007-2008. 1 plot (July 2007, 2O) fell within the trunk of of a large white cedar, and was excluded (no groundcover to sample). 491 plots (48%) contained bryophyte groundcover sufficient for sampling and cyanobacterial screening. 13 samples from July 2007 (transect 7, samples 7K, 7M, 7O, 7P, 7Q, 7R, 7S, 7T, 7U, 7V, 7W, & 7X) were contaminated due to a refrigerator malfunction; these were not screened. These samples constituted about 2.5% of the entire bryophyte collection, but the loss was non-random (samples from a specific transect and season). The remaining bryophyte samples (*n* = 478) were screened for cyanobacteria and identified to genus or species when possible. Given the difficulties of identifying largely sterile specimens, 5 samples were identified only to family (Fall 2007 1R, 9B, 10S), 2 samples to the leafy liverwort class Jungermanniopsida (Winter 2008, 1H & 7Y), and 1 to the moss class Bryopsida (Fall 2007 5A). 13 samples, all from Winter 2008, were lost after screening for cyanobacteria (1E, 1Y, 2Q, 3J, 3U, 5M, 7C, 7N, 7S, 8I, 8S, 8V, & 8W). Overall bryophyte identification data at the species/genus level is unavailable for 22 samples, less than 5% of the total sample; over 95% of the samples were identified, with 442 samples (about 92%) identified to species. Sterile specimens that could not be resolved beyond the genus level fell included mosses in Brachythecium *Schimp*, *Mnium* Hedw.,

Dicranum Hedw.*,* and *Sphagnum* L. The taxonomic level bryophyte family is best resolved, with only 17 of 478 samples (about 3.5%) missing a family identification. Data visualization focuses on largely the family level for these reasons.

The number of samples per bryophyte taxon (genus or species) is shown in Figure 1; 22 samples that could not be identified to either species or genus were excluded.

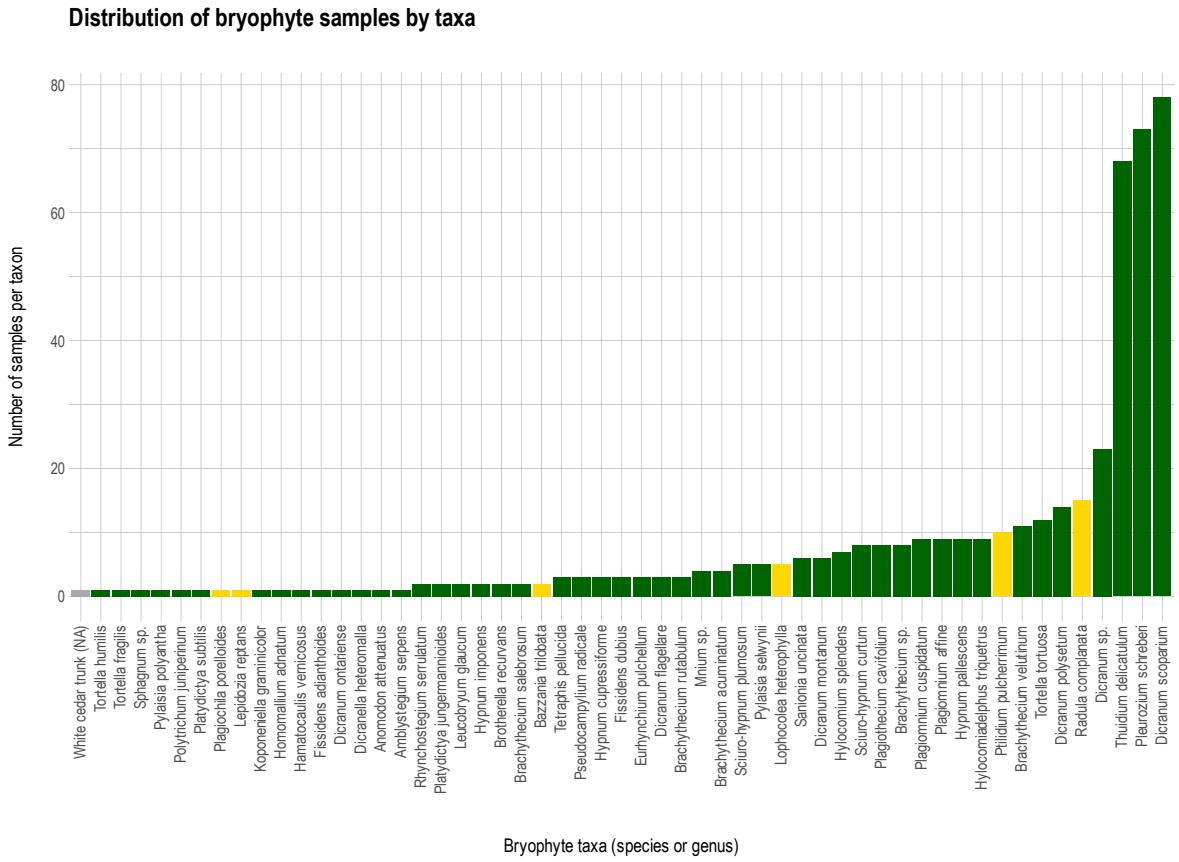


Figure 1. Number of specimens studied for various moss and liverwort specimens at Cana Island Road. Dark green bars represent moss taxa; gold bars, liverwort taxa. The smallest visible bars represent a single sample (e.g., *Tortella humilis* (Hedw.) Jenn.). The small gray bar represents a single plot excluded due to the occurrence of an upright white cedar trunk at the sample point.

Moss taxa are visualized in dark green, and liverwort taxa, in gold. *Dicranum scoparium* Hedw., *Pleurozium schreberi* (Willd. ex Brid.) Mitt., and *Thuidium delicatulum* (Hedw.) Schimp. are the most abundant bryophyte species; these strongly dominant taxa constitute almost 48% of the bryophytes studied. While the feather-moss *Pleurozium schreberi* constitutes a large proportion of the groundcover, the feather-moss *Hylocomium splendens* (Hedw.) Schimp. is far less abundant; only 7 samples (about 1.5%) were affiliated with this circumpolar boreal taxon. The bryophyte flora includes at least 50 distinct species (potentially more, given the classification to genus for some specimens). Notably, almost half of the flora consists of singletons (species that occur just once) or doubles (species that occur just twice); 23 taxa meet this criterion for rarity. About 10% of the species appear to be novel records for Door County, Wisconsin (Consortium of Bryophyte Herbaria, 2023), including *Amblystegium serpens* (Hedw.) Schimp., *Brachythecium velutinum* (Hedw.) Schimp., *Dicranella heteromalla* (Hedw.) Schimp., *Homomallium adnatum* (Hedw.) Broth., *Leucobryum glaucum* (Hedw.) Ångstr., *Plagiothecium cavifolium* (Brid.) Z. Iwats., *Platydictya subtilis* (Hedw.) H.A. Crum, *Pseudocampylium radicale* (P. Beauv.) Vanderp. & Hedenäs, *Sciuro-hypnum curtum* (Lindb.) Ignatov, and *Sciuro-hypnum plumosum* (Hedw.) Ignatov & Huttunen.

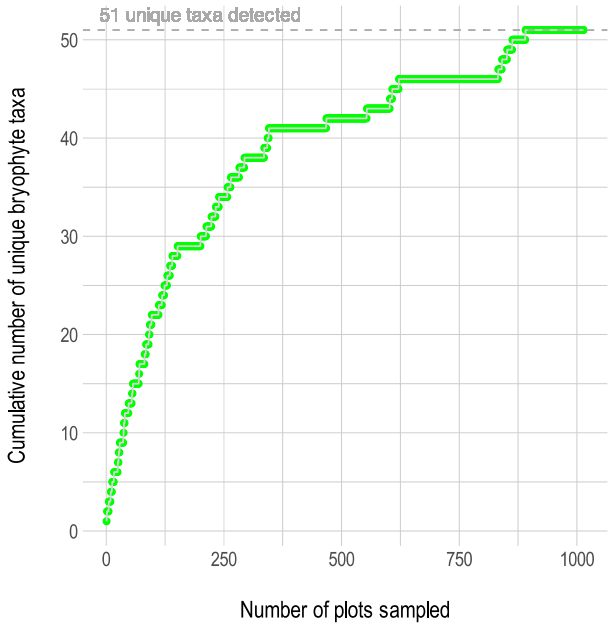


Figure 2. Species accumulation curve with pooled diversity data from all four seasons. Unique taxa include all detected species plus a single observation from the genus *Sphagnum,* which must constitute a novel taxon. Observations classified as *Brachythecium* sp., *Dicranum* sp., and *Mnium* sp. were not counted as unique taxa, given the presence of other samples determined to species within the genus.

Figure 2 shows a species-accumulation curve based on sampling from all four seasons in 2007-2008. The number of plots sampled (x-axis) includes all plots (with and without bryophytes). The rate of increase slows notably at around 375 plots, but continues to drift upward slowly throughout the sampling process. The rate of increase is sensitive to plot order; without the randomization involved in rarefaction, it is inappropriate to interpret the observed rate of increase. However, the observed species richness (51 taxa) is not sensitive to plot order.

Figure 3 visualizes the species-accumulation curve for individual seasons. The performance of the sampling design varied by season, with the lowest capture of species-level diversity occuring in winter 2008 (at least 24 distinct species).

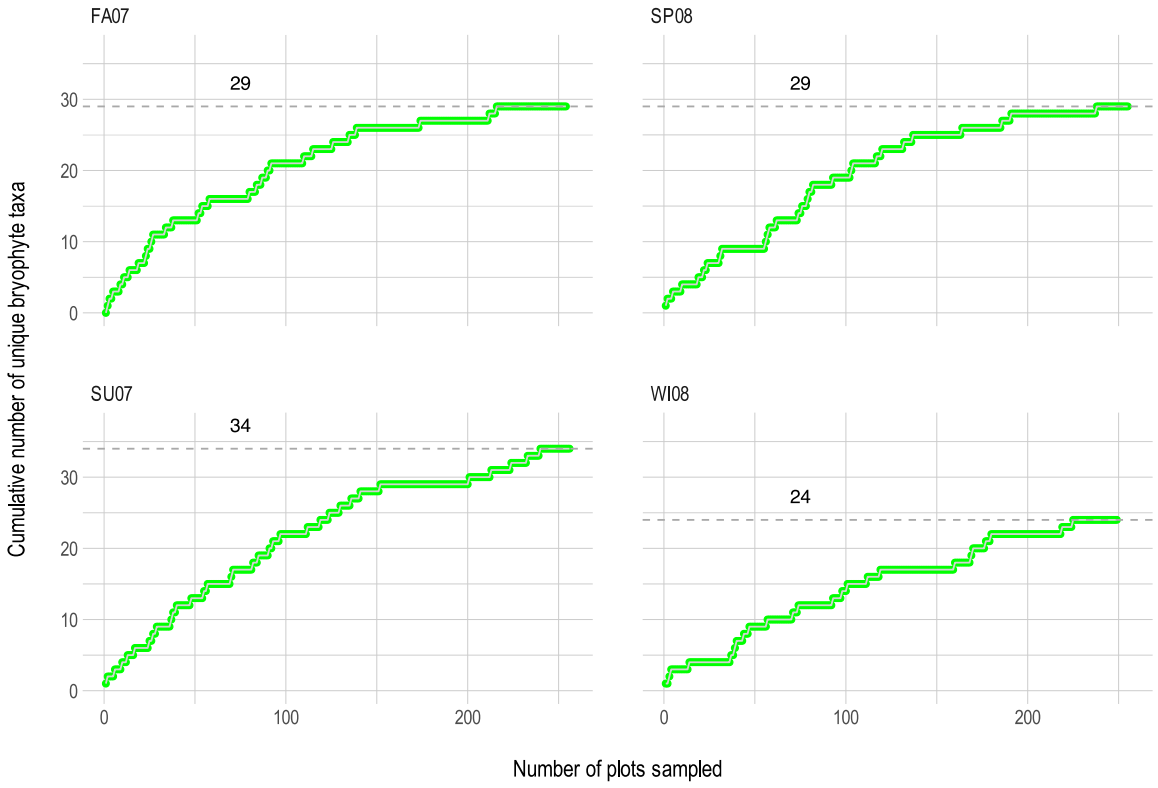


Figure 3. Species-accumulation curves organized by sampling season. As in Figure 2, observations in *Brachythecium* sp., *Dicranum* sp., and *Mnium* sp. were not counted as unique taxa, given the presence of other samples determined to species within the genus. The single observation classified as *Sphagnum* sp. was categorized as a novel taxon.

Cyanobacterial Hosts. Figure 4 visualizes bryophytes families that hosted cyanobacteria in the boreal forest at Cana Island Road based on a presence/absence criterion.

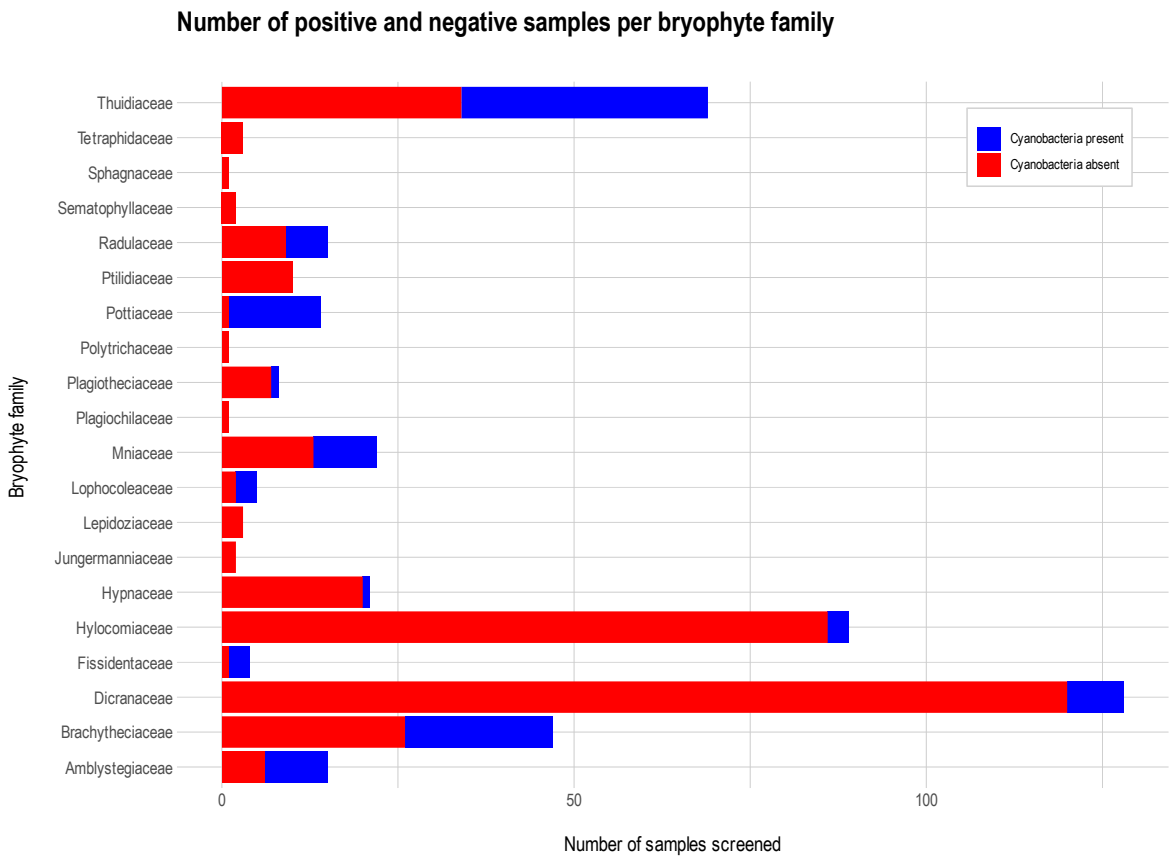


Figure 4. Proportion of samples hosting cyanobacteria organized by bryophyte family. 17 samples (about 3.5% of the total) were excluded because they could not be identified to the level of family.

Each unit of measure in an individual bar represents a single bryophyte sample, colored either red (for cyanobacterial absence) or blue (for cyanobacterial presence). Among the 20 bryophyte families studied, at least 12 are capable of hosting cyanobacteria (i.e., at least one sample was positive, and at least one unit in the family's bar is blue). All 8 non-hosting families are represented by relatively few samples ($n < 10$) due to their sparse spatial distribution. However, large sample sizes with few positive results are characteristic of the moss families Dicranaceae Schimp., Hylocomiaceae M. Fleisch., and Hypnaceae Schimp.; these families, although occasionally positive, are *typically*

free of cyanobacteria at this site. Families with a high probability of hosting cyanobacteria (> 40% of all samples positive) include the moss families Amblystegiaceae Kindb., Brachytheciaceae Schimp., Fissidentaceae Schimp., Mniaceae Schwägr., Pottiaceae Hampe, and Thuidiaceae Schimp., and the leafy liverwort families Lophocoleaceae Müll. Frib. ex Vanden Berghen and Radulaceae Müll. Frib.

Table 2 presents a complete list of all bryophytes characterized in this study, identified to either species or genus. Samples that could not be resolved to species or genus were excluded. Cyanobacteria were detected on at least one sample for 60% of the species studied (30 of 50 taxa). The observations are also contextualized with respect to published work from other sites. The fifth and sixth columns specify publications with evidence of cyanobacteria, N fixation, or both for each bryophyte species studied in Wisconsin. The last column specifies any reports that pertain to the genus. For example, the observation of cyanobacteria on *Amblystegium serpens* (Hedw.) Schimp. may be the first published observation of cyanobacteria for this species; but there is prior evidence of cyanobacteria in connection to the genus *Amblystegium* Schimp. (Priddle & Dartnell 1978). Any row with a light-green background signifies a species with evidence of cyanobacterial associates; such evidence may be from the existing literature *or* based on the flora at Cana Island Road.

The most notable finding is that almost all of the bryophyte species inventoried harbor cyanobacteria either at my boreal forest study site (based on light microscopy data) or

at another site (based on published literature). The predominantly green rows reflect this fact. Only a handful of taxa are not associated with evidence of cyanobacteria in this broader context. These species (white rows) include *Homomallium adnatum* (Hedw.) Broth., *Leucobryum glaucum* (Hedw.) Ångstr., *Pylaisia polyantha* (Hedw.) Schimp., and *Tetraphis pellucida* Hedw. Overall 92% of the species in this boreal forest groundcover flora belong to a *genus* capable of hosting cyanobacteria, if we consider the broad range of conditions and habitats inhabited by these mosses and liverworts across the globe.

Based on the literature I was able to review, about 40% of these species have *not* been reported in association with cyanobacteria before. These potentially novel symbioses involve *Amblystegium serpens, Brachythecium acuminatum* (Hedw.) Austin, *Brachythecium rutabulum* (Hedw.) Schimp., *Brachythecium salebrosum* (Hoffm. ex F. Weber & D. Mohr) Schimp., *Brachythecium velutinum* (Hedw.) Schimp., *Eurhynchium pulchellum* (Hedw.) Jenn., *Fissidens adianthoides* Hedw., *Fissidens dubius* P. Beauv., *Hamatocaulis vernicosus* (Mitt.) Hedenäs, *Koponeniella graminicolor* (Brid.) Huttunen, Ignatov, Min Li & Y.F. Wang, *Lophocolea heterophylla* (Schrad.) Dumort., *Plagiomnium affine* (Blandow ex Funck) T.J. Kop., *Plagiothecium cavifolium* (Brid.) Z. Iwats., *Platydictya jungermannioides* (Brid.) H.A. Crum, *Platydictya subtilis* (Hedw.) H.A. Crum, *Pseudocampylium radicale* (P. Beauv.) Vanderp. & Hedenäs, *Pylaisia selwynii* Kindb., *Radula complanata* (L.) Dumort., *Sciuro-hypnum curtum* (Lindb.) Ignatov, and *Sciuro-hypnum plumosum* (Hedw.) Ignatov & Huttunen.

However, about half of these potentially novel reports appear to elaborate on established prior knowledge, or involve taxa with relevant but not fully convincing published evidence of symbiosis. The genus *Brachythecium* is a well-known host of cyanobacteria; the discovery that *B. acuminatum, B. rutabulum, B*. *salebrosum,* and *B. velutinum* also host cyanobacteria at Cana Island Road is interesting, but hardly surprising. Uncertainty also arises from studies in which observations may (of necessity) involve mixed samples. For example, in an acetylene reduction study in a temperate rain forest (British Columbia, Canada), N fixation and the cyanobacterium *Scytonema* sp. were reported in connection to mixed-species samples that included *Plagiothecium undulatum* (Hedw.) Schimp. (Lindo & Whiteley, 2011; Lindo, pers. comm., 2012). Depending on which moss hosted the cyanobacteria in British Columbia, the positive report for *Plagiothecium cavifolium* at Cana Island Road may or may not be novel at the level of genus. Likewise, although there do not appear to be any clear-cut reports of cyanobacterial symbiosis for *Pylaisia selwynii*, a recent Illumina sequencing study of bacterial communities associated with *Pylaisiella polyantha* (Hedw.) Grout (Ma et al. 2017) indicates cyanobacteria are present, but they are not among 50 most common bacterial genera, and it is unclear whether some or all of the four mosses studied, including *P. polyantha,* were the cyanobacterial hosts.

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
***Amblystegium serpens* (Hedw.) Schimp.**	Amblystegiaceae Kindb.	1	1	None available to author		Priddle, J., Dartnell, J.G. 1978.
***Anomodon attenuatus* (Hedw.) Huebener**	Thuidiaceae Schimp.	1	1	Jean, M.E., Cassar, N., Setzer, C., Bellenger, J.P. 2012.	Jean et al. 2012: cyanobacteria evident (light and epifluoresecence microscopy); N fixation (acetylene reduction assay by cavity ring-down laser absorption spectroscopy)	None except Jean et al. 2012 available to author
Bazzania trilobata (L.) Gray	Lepidoziaceae Limpr.	2	0	Lambert, R.L., Reiners, W.A. 1979.	Lambert & Reiners 1979: no cyanobacteria evident (light microscopy); N fixation (acetylene reduction assay)	Brasell, H.M., Davies, S.K., Mattay, J.P. 1986. Matzek, V., Vitousek, P. 2003. Permin, A., Horwath, A.B., Metcalfe, D.B., Priemé, A., Rousk, K. 2022.
***Brachythecium acuminatum* (Hedw.) Austin**	Brachytheciaceae Schimp.	4	2	None available to author		Smith, V.R., Russell, S., 1982.

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
***Brachythecium rutabulum* (Hedw.) Schimp.**	Brachytheciaceae Schimp.	3	3	None available to author		Smith, V.R. 1984. Line, M.A. 1992. Solheim, B., Endal, A., Vigstad, H. 1996.
***Brachythecium salebrosum* (Hoffm. ex F. Weber & D. Mohr) Schimp.**	Brachytheciaceae Schimp.	2	1	None available to author		
***Brachythecium velutinum* (Hedw.) Schimp.**	Brachytheciaceae Schimp.	11	6	None available to author		
***Brachythecium* Schimp.**	* Most likely Brachytheciaceae Schimp.	8	3	NA		
Brotherella recurvans (Michx.) M. Fleisch.	Sematophyllaceae Broth.	2	0	Lambert, R.L., Reiners, W.A. 1979.	Lambert & Reiners 1979: no cyanobacteria evident (light microscopy); N fixation (acetylene reduction assay)	[6]Han, B., Zou, X., Kong, J., Sha, L., Gong, H., Yu, Z. and Cao, T., 2010.
Dicranella heteromalla (Hedw.) Schimp.	Dicranaceae Schimp.	1	0	None available to author		Line, M.A. 1992.
Dicranum flagellare Hedw.	Dicranaceae Schimp.	3	0	None available to author		Salemaa, M., Lindroos, A.J., Merilä, P., Mäkipää, R., Smolander, A. 2019.
Dicranum montanum Hedw.	Dicranaceae Schimp.	6	0	None available to author		
Dicranum ontariense W.L. Peterson	Dicranaceae Schimp.	1	0	None available to author		

[6] Multiple *Brotherella* taxa were present in mixed samples with positive acetylene reduction assays in Han et al. 2010.

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
***Dicranum polysetum* Sw.**	Dicranaceae Schimp.	14	1	Stuart, J.M. 2021. Holland-Moritz, H., Stuart, J.E., Lewis, L.R., Miller, S.N., Mack, M.C., Ponciano, J.M., McDaniel, S.F., Fierer, N., 2021.	Stuart 2021: N fixation ($^{15}N_2$ incubation) Holland-Moritz et al. 2021: Low (< 1%) relative abundance of cyanobacteria (phylogenetic analysis); N fixation ($^{15}N_2$ incubation)	Stuart, J.M. 2021. Permin, A., Horwath, A.B., Metcalfe, D.B., Priemé, A., Rousk, K. 2022. Clasen, A., L., Permin, L, Horwath, A.B., Metcalfe, D.B., Rousk, K., 2023. Holland-Moritz, H., Stuart, J., Lewis, L.R., Miller, S., Mack, M.C., McDaniel, S.F. and Fierer, N., 2018. Holland-Moritz, H., Stuart, J.E., Lewis, L.R., Miller, S.N., Mack, M.C., Ponciano, J.M., McDaniel, S.F., Fierer, N., 2021.
***Dicranum scoparium* Hedw.**	Dicranaceae Schimp.	78	6	Pentecost, A. and Whitton, B.A., 2012. Stuart, J.M. 2021. Holland-Moritz, H., Stuart, J.E., Lewis, L.R., Miller, S.N., Mack, M.C., Ponciano, J.M., McDaniel, S.F., Fierer, N., 2021. Rzepczynska, A.M., Michelsen, A., Olsen, M.A.N. and Lett, S., 2022.	Pentecost & Whitton 2012: cyanobacteria evident (light microscopy) Stuart et al. 2021: N fixation ($^{15}N_2$ incubation) Holland-Moritz et al. 2021: low (< 1%) relative abundance of cyanobacteria (phylogenetic analysis) Rzepczynska et al. 2022: N fixation (acetylene reduction assay)	
***Dicranum* Hedw.**	Dicranaceae Schimp.	23	1	NA		

Species	**Family**	**Sample size**	**Number of samples positive for cyanobacteria**	**Published reports of cyanobacteria and/or N fixation for bryophyte species**	**Nature of published evidence for species**	**Published reports of cyanobacteria and/or N fixation for bryophyte genus**
***Eurhynchium pulchellum* (Hedw.) Jenn.**	Brachytheciaceae Schimp.	3	2	None available to author		Uher, B. 2008.
***Fissidens adianthoides* Hedw.**	Fissidentaceae Schimp.	1	1	None available to author		Freytet, P., Verrecchia, E.P. 1998. Deane-Coe, K.K. 2015. Deane-Coe, K.K., Sparks, J.P. 2016. Guerra, J., Jiménez, J.A. and Cano, M.J., 2021.
***Fissidens dubius* P. Beauv.**	Fissidentaceae Schimp.	3	1	None available to author		
***Hamatocaulis vernicosus* (Mitt.) Hedenäs**	Amblystegiaceae Kindb.	1	1	None available to author		None available to author
Homomallium adnatum (Hedw.) Broth.	Hypnaceae Schimp.	1	0	None available to author		None available to author
Hylocomiadelphus triquetrus (Hedw.) Ochyra & Stebel	Hylocomiaceae M. Fleisch.	9	0	Stuart 2021 (reported as *Rhytidiadelphus triquetrus* Hedw.)	Stuart et al. 2021: N fixation ($^{15}N_2$ incubation)	None available to author
Hylocomium splendens (Hedw.) Schimp.	Hylocomiaceae M. Fleisch.	7	0	Numerous, e.g., Zackrisson, O., DeLuca, T.H., Gentili, F., Sellstedt, A. and	Zackrisson et al. 2009: cyanobacteria evident (light microscopy); N fixation (acetylene reduction assay),	None available to author except publications on *H. splendens*

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
				Jäderlund, A., 2009.		
Hypnum cupressiforme Hedw.	Hypnaceae Schimp.	3	0	Pentecost, A. and Whitton, B.A., 2012. Rousk, K., Vestergård, M. and Christensen, S., 2018.	Pentecost & Whitton 2012: cyanobacteria evident (light microscopy) Rousk et al. 2018: N fixation (acetylene reduction assay)	Sant'Anna, C.L. 1984. Brasell, H.M., Davies, S.K., Mattay, J.P. 1986. Cao, W., Xiong, Y., Zhao, D., Tan, H., Qu, J., 2020.
Hypnum imponens Hedw.	Hypnaceae Schimp.	2	0	None available to author		
Hypnum pallescens (Hedw.) P. Beauv.	Hypnaceae Schimp.	9	0	None available to author		
***Koponeniella graminicolor* (Brid.) Huttunen, Ignatov, Min Li & Y.F. Wang**	Brachytheciaceae Schimp.	1	1	None available to author		None available to author
Lepidozia reptans (L.) Dumort.	Lepidoziaceae Limpr.	1	0	None available to author		Brasell, H.M., Davies, S.K., Mattay, J.P. 1986.
Leucobryum glaucum (Hedw.) Ångstr.	Dicranaceae Schimp.	2	0	None available to author		None available to author
***Lophocolea heterophylla* (Schrad.) Dumort.**	Lophocoleaceae Müll. Frib. ex Vanden Berghen	5	3	None available to author		None available to author
***Mnium* Hedw.**	* Most likely Mniaceae Schwägr.	4	1	NA (not identified to species)		Schell, D.M., Alexander, V. 1973.

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
						Wojciechowski, M.F., Heimbrook, M.E. 1984.
Plagiochila porelloides (Torr. ex Nees) Lindenb.	Plagiochilaceae Müll. Frib.	1	0	None available to author		Sant'Anna, C.L. 1984. Xian-Meng Shi, Liang Song, Wen-Yao Liu, Hua-Zheng Lu, Jin-Hua Qi, Su Li, Xi Chen, Jia-Fu Wu, Shuai Liu, Chuan-Sheng Wu. 2017. Markham, J., Fernández Otárola, M., 2021. Fan, X., Yuan, G., Liu, W. 2022. Permin, A., Horwath, A.B., Metcalfe, D.B., Priemé, A., Rousk, K. 2022.
***Plagiomnium affine* (Blandow ex Funck) T.J. Kop.**	Mniaceae Schwägr.	9	2	None available to author		Solheim, B., A. Endal, and H. Vigstad. 1996.

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
***Plagiomnium cuspidatum* (Hedw.) T.J. Kop.**	Mniaceae Schwägr.	9	6	Lambert, R.L., Reiners, W.A. 1979. Zhang, X.-J., Feng, J., Wang, G.-H., Xie, S.-L. 2014.	Lambert & Reiners 1979: cyanobacteria absent (light microscopy); N fixation (acetylene reduction assay) Zhang et al., 2014: cyanobacteria (culture studies with 16s *r*RNA gene analysis); evidence of cyanobacterial pigment changes in symbiosis (spectroscopy)	
***Plagiothecium cavifolium* (Brid.) Z. Iwats.**	Plagiotheciaceae M. Fleisch.	8	1	None available to author		None available to author
***Platydictya jungermannioides* (Brid.) H.A. Crum**	Amblystegiaceae Kindb.	2	2	None available to author		None available to author
***Platydictya subtilis* (Hedw.) H.A. Crum**	Amblystegiaceae Kindb.	1	1	None available to author		
***Pleurozium schreberi* (Willd. ex Brid.) Mitt.**	Hylocomiaceae M. Fleisch.	72	3	Numerous, e.g., DeLuca, T.H., Zackrisson, O., Nilsson, M.C., Sellstedt, A., 2002.		None available to author except publications on *P. schreberi*
***Pseudocampylium radicale* (P. Beauv.) Vanderp. & Hedenäs**	Amblystegiaceae Kindb.	3	2	None available to author		None available to author

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
Ptilidium pulcherrimum (Weber) Vain.	Ptilidiaceae H. Klinggr.	10	0	Maass, W., Yetman, D. 2002.	Unpublished data from Tomas Hallingbäck in Maass & Yetman 2002: cyanobacteria evident (nature of evidence observational, otherwise unspecified)	Solheim, B., Endal, A., Vigstad, H. 1996. Holland-Moritz, H., Stuart, J.E., Lewis, L.R., Miller, S.N., Mack, M.C., Ponciano, J.M., McDaniel, S.F., Fierer, N., 2021.
Pylaisia polyantha (Hedw.) Schimp.	Hypnaceae Schimp.	1	0	None available to author		None available to author
***Pylaisia selwynii* Kindb.**	Hypnaceae Schimp.	5	1	None available to author		None available to author
***Radula complanata* (L.) Dumort.**	Radulaceae Müll. Frib.	15	6	None available to author		
Rhynchostegium serrulatum (Hedw.) A. Jaeger	Brachytheciaceae Schimp.	2	0	None available to author		Uher, B. 2008.
***Sanionia uncinata* (Hedw.) Loeske**	Amblystegiaceae Kindb.	6	1	Numerous, e.g., Solheim, B. Wiggen, H. Røberg, S., Spaink, H.P. 2004.	Solheim et al. 2004: cyanobacteria evident (light microscopy); N fixation (acetylene reduction assay)	None available to author except publications on *S. uncinata*
***Sciuro-hypnum curtum* (Lindb.) Ignatov**	Brachytheciaceae Schimp.	8	1	None available to author		None available to author

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
***Sciuro-hypnum plumosum* (Hedw.) Ignatov & Huttunen**	Brachytheciaceae Schimp.	5	2	None available to author		
Sphagnum L.	Sphagnaceae Dumort.	1	0	NA		Numerous; see review by Kostka, J.E., Weston, D.J., Glass, J.B., Lilleskov, E.A., Shaw, A.J. and Turetsky, M.R., 2016.
Tetraphis pellucida Hedw.	Tetraphidaceae Schimp.	3	0	None available to author		None available to author
***Thuidium delicatulum* (Hedw.) Schimp.**	Thuidiaceae Schimp.	68	34	Deane-Coe, K.K., 2015.		Markham, J., Fernández Otárola, M., 2021. Fan, X., Yuan, G., Liu, W. 2022. Permin, A., Horwath, A.B., Metcalfe, D.B., Priemé, A., Rousk, K. 2022. Clasen, A., L., Permin, L, Horwath, A.B., Metcalfe, D.B.,

Species	Family	Sample size	Number of samples positive for cyanobacteria	Published reports of cyanobacteria and/or N fixation for bryophyte species	Nature of published evidence for species	Published reports of cyanobacteria and/or N fixation for bryophyte genus
						D.B., Rousk, K., 2023.
Tortella fragilis (Drumm.) Limpr.	Pottiaceae Hampe	1	0	None available to author		Wojciechowski, M.F., Heimbrook, M.E. 1984. Brasell, H.M., Davies, S.K., Mattay, J.P. 1986.
***Tortella humilis* (Hedw.) Jenn.**	Pottiaceae Hampe	1	1	Oliveira, M.F., Figueredo, C.C., Konell, A.H., Maciel-Silva, A.S. 2023.	Oliveira et al. 2023: cyanobacteria evident (light microscopy)	
***Tortella tortuosa* (Schrad. ex Hedw.) Limpr.**	Pottiaceae Hampe	12	12	Pentecost, A., Whitton, B.A. 2012.	Pentecost & Whitton 2012: cyanobacteria evident (most likely light microscopy, methods unspecified)	

Table 2. Bryophyte taxa and evidence of cyanobacterial associates or bacterial N-fixation. Evidence summarized is drawn from the present study (Cana Island Road, Baileys Harbor, Wisconsin) and from published scientific literature. **Bold-faced taxa** hosted cyanobacteria in this study. The light green background signifies that at least one source (Wisconsin field data or publications) provides evidence of symbiotic capability.

For about 18% of the bryophytes studied (9 of 50 species), I was unable to locate reports of cyanobacteria for either the species *or* genus. These species (a subset of the taxa noted previously) include the mosses *Hamatocaulis vernicosus*, *Koponeniella graminicolor*, *Platydictya jungermannioides*, *Platydictya subtilis*, *Pseudocampylium radicale*, *Pylaisia selwynii*, *Sciuro-hypnum curtum*, and *Sciuro-hypnum plumosum*, as well as the liverwort *Lophocolea heterophylla*.

A substantial portion of the bryophyte species at Cana Island Road (about 42%) do not host cyanobacteria at this particular boreal forest site. The majority of these 21 species are relatively rare (constitute less than 1% of the entire bryophyte flora) with small sample sizes ($n \leq 4$) that reflect their sparse spatial distribution. Only four bryophytes have moderately larger sample sizes and negative status: mosses *Hylocomiadelphus triquetrus* (Hedw.) Ochyra & Stebel ($n = 9$), *Hylocomium splendens* (Hedw.) Schimp. ($n = 7$), and *Hypnum pallescens* (Hedw.) P. Beauv. ($n = 9$), as well as the liverwort *Ptilidium pulcherrimum* (Weber) Vain ($n = 10$).

Abundance of Cyanobacterial Epiphytes. The estimated total number of cyanobacterial cells per slide of bryophyte tissue screened varied by orders of magnitude, from a minimum of 0 to a maximum of over 1 million associated with *Thuidium delicatulum* (Hedw.) Schimp. The distribution of counts is strongly right-skewed and zero-inflated, with a median value of 0. The radically different magnitudes and the large number of zero observations make it difficult to visualize the data. In

Figure 5, the histogram shows the data with a discontinuous scale along the x-axis, but a consistent bin width throughout.

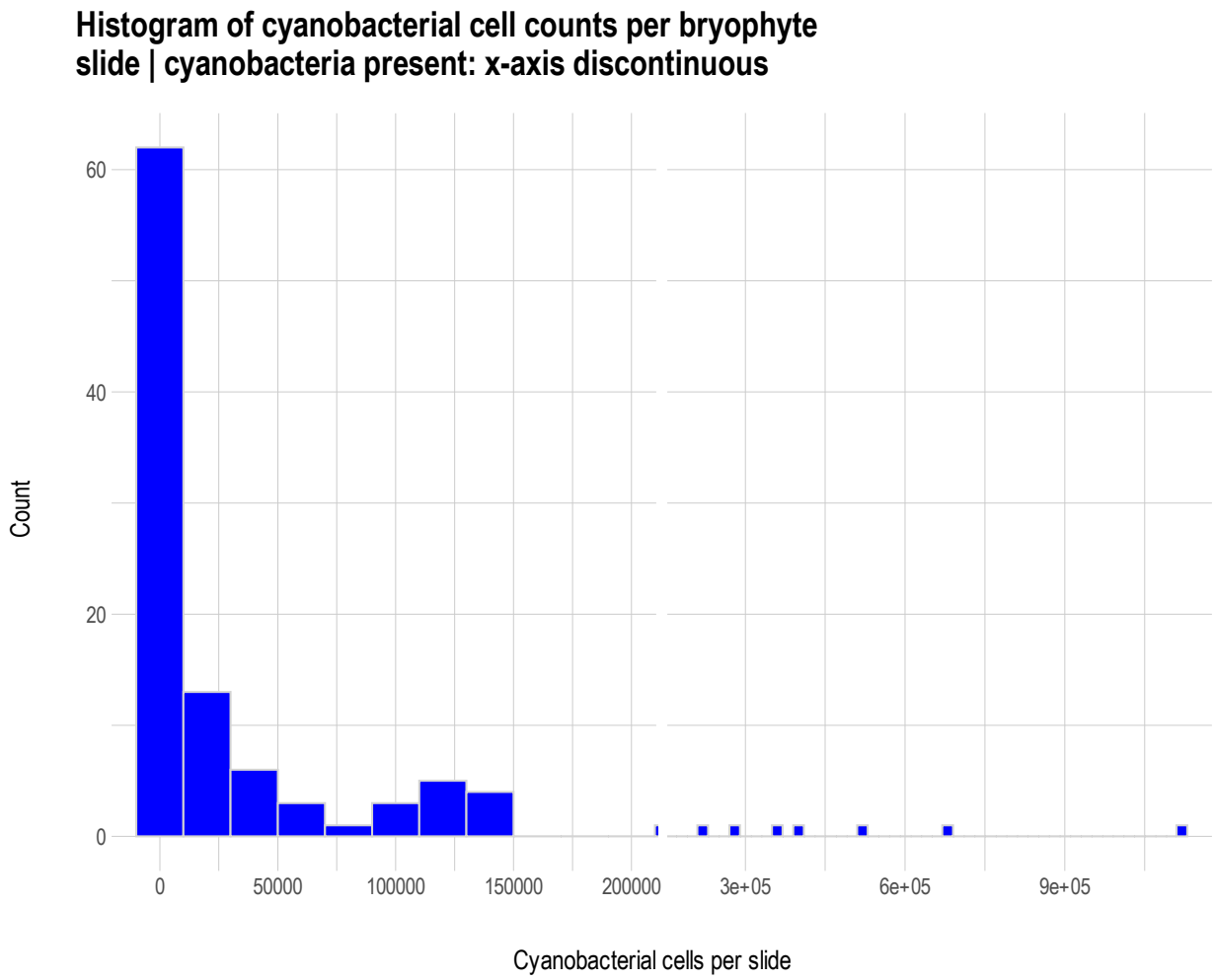


Figure 5. Histogram of estimated cyanobacterial cell counts for bryophyte samples that hosted cyanobacteria (non-zero counts only). Note the discontinuity in scale (break at 200,000) to illuminate structure of strongly skewed data.

Count observations of 0 were excluded to minimize difficulties related to scale; the histogram visualizes count distributions specifically for bryophyte samples with

cyanobacteria. Given a binwidth of 20,000[7], this first and largest bar represents cyanobacterial count estimates between 1 and 10,000.

A subset of the positive samples represented by that first bar is visualized in a histogram in Figure 6.

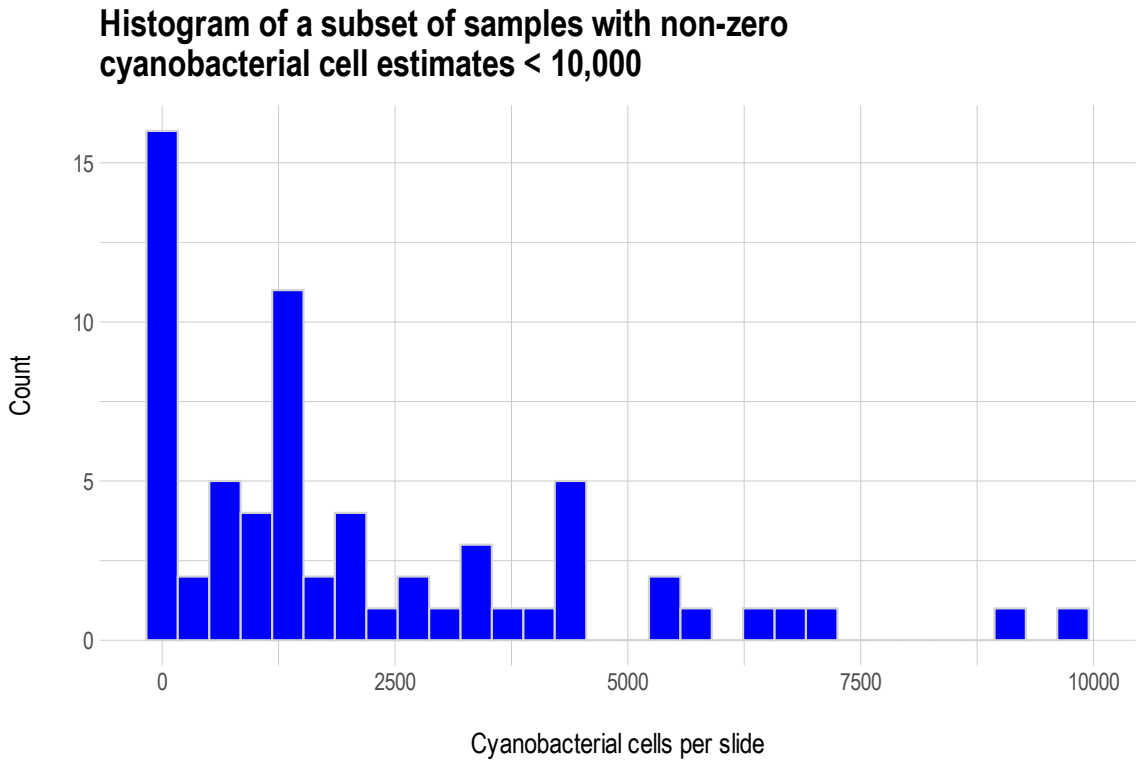


Figure 6. Histogram of estimated cyanobacterial cell counts for bryophyte samples that hosted cyanobacteria (non-zero counts only, < 10,000 cells).

[7] Even though lower limit for the data range is zero, *ggplot* graphics set the first histogram bar over a range of positive and negative values, spanning (-10,000, 10,000). The specified range constitutes a misrepresentation of the count data, as values for cyanobacterial cell counts cannot be less than zero.

This subset of the data (66 of the 104 positive samples) indicates that the distribution is strongly non-normal and multimodal, even when we exclude the extreme count estimates (> 50,000 cells per slide) visualized in Figure 5.

Figure 7 illuminates the structure of the count estimates associated with all of the bryophyte families that hosted cyanobacteria; families with no positive samples were excluded. These histograms, unlike Figures 4 and 5, do include zero observations; the bar nearest the y-axis represents the number of samples with zero or close-to-zero counts per family. Note that the scale is specific to each family on both the x-axis (cell count estimates) and the y-axis (number of samples).

[Figure 7 on next page.]

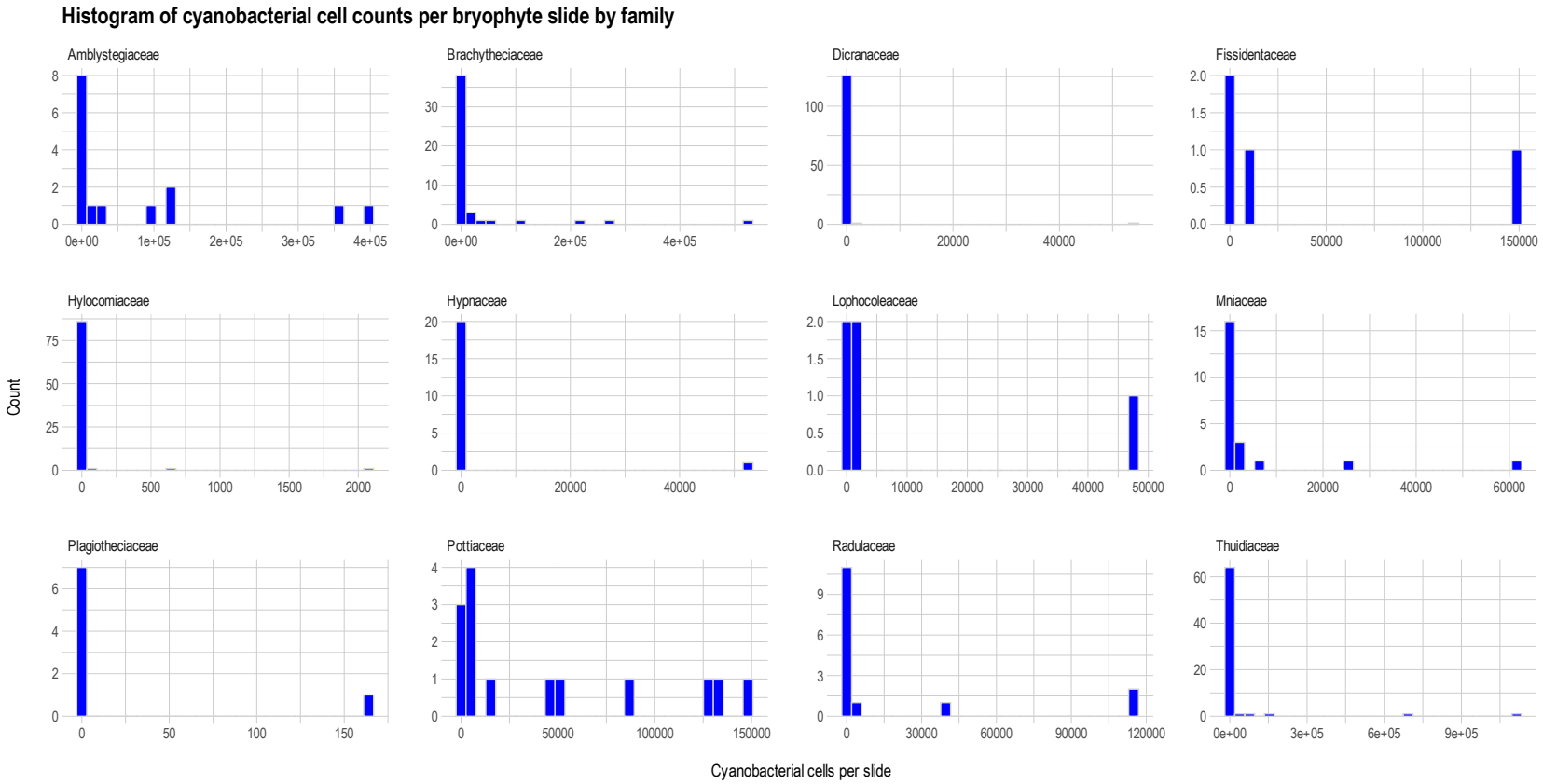


Figure 7. Histograms of cyanobacterial cell counts by bryophyte family. Only families with at least one sample containing cyanobacteria are visualized. The families Jungermanniaceae, Lepidoziaceae, Plagiochiliaceae, Polytrichaceae, Ptilidiaceae, Sematophyllaceae, Sphagnaceae, and Tetraphidaceae had zero counts for all samples, and were not visualized.

The families Amblystegiaceae, Brachytheciaceae, Pottiaceae, and Thuidiaceae are responsible for many of the relatively large abundance estimates, suggesting a strong capacity for hosting cyanobacteria at this site. Most of the other families have at least one or two large estimates for cyanobacterial cell count, although the number of samples is relatively small. For example, the family Fissidentaceae has a sample with an estimate around 150,000, but a sample size of $n = 4$. There is no way to know whether Fissentaceae typically hosts relatively large numbers of cyanobacterial cells.

Figure 8 visualizes the cell count estimates by family and by bryophyte species nested within family. In this figure, the number of negative samples and the number of samples with small or near-zero counts is not clearly visualized due to overplotting (one dot near the y-axis may represent multiple samples with these attributes). Interpretation requires caution. For *Dicranum scoparium*, for example, the single point associated with the 0 counts represents 67 samples; for *Sphagnum* sp., the single point associated with the 0 counts represents one sample. But the distribution of extreme values, and the species associated with these robust cyanobacterial communities, is shown more clearly. *Thuidium delicatulum* (Thuidiaceae) and *Tortella tortuosa* (Schrad. ex Hedw.) Limpr. (Pottiaceae) are two well-sampled species with relatively large cyanobacterial cell count estimates, suggested a strong propensity for symbiosis.

Many extreme values in Figure 8 are associated with species that may be novel cyanobacterial hosts (i.e., not studied in previously published literature). In the moss family Amblystegiaceae, *Platydictya jungermannioides* and *Platydictya subtilis,* as well

as *Pseudocampylium radicale,* three of the potentially novel symbioses reported at this site, host relatively large cyanobacterial communities. In the moss family Brachytheciaceae, potentially novel symbioses associated with *Brachythecium velutinum* and *Eurhynchium pulchellum* have large estimated cell counts. Other potentially novel taxa in this family (*Sciuro-hypnum curtum* (Lindb.) Ignatov and *Sciuro-hypnum plumosum* (Hedw.) Ignatov & Huttunen) have far less abundant cyanobacterial cell count estimates, and are more typical of the bryophyte flora studied.

Most of the species associated with Dicranceae, Hylocomiaceae, and Hypnaceae do not host cyanobacteria, or host with low cell count estimates that are poorly visualized here (overplotting). Among the leafy liverworts, most species do not host; the exceptions appear to be *Radula complanata* (L.) Dumort. (Radulaceae Müll. Frib.) and *Lophocolea heterophylla* (Schrad.) Dumort. (Lophocoleaceae Müll. Frib. ex Vanden Berghen). These liverwort-cyanobacterial symbioses are also potentially novel.

[Figure 8 on next page.]

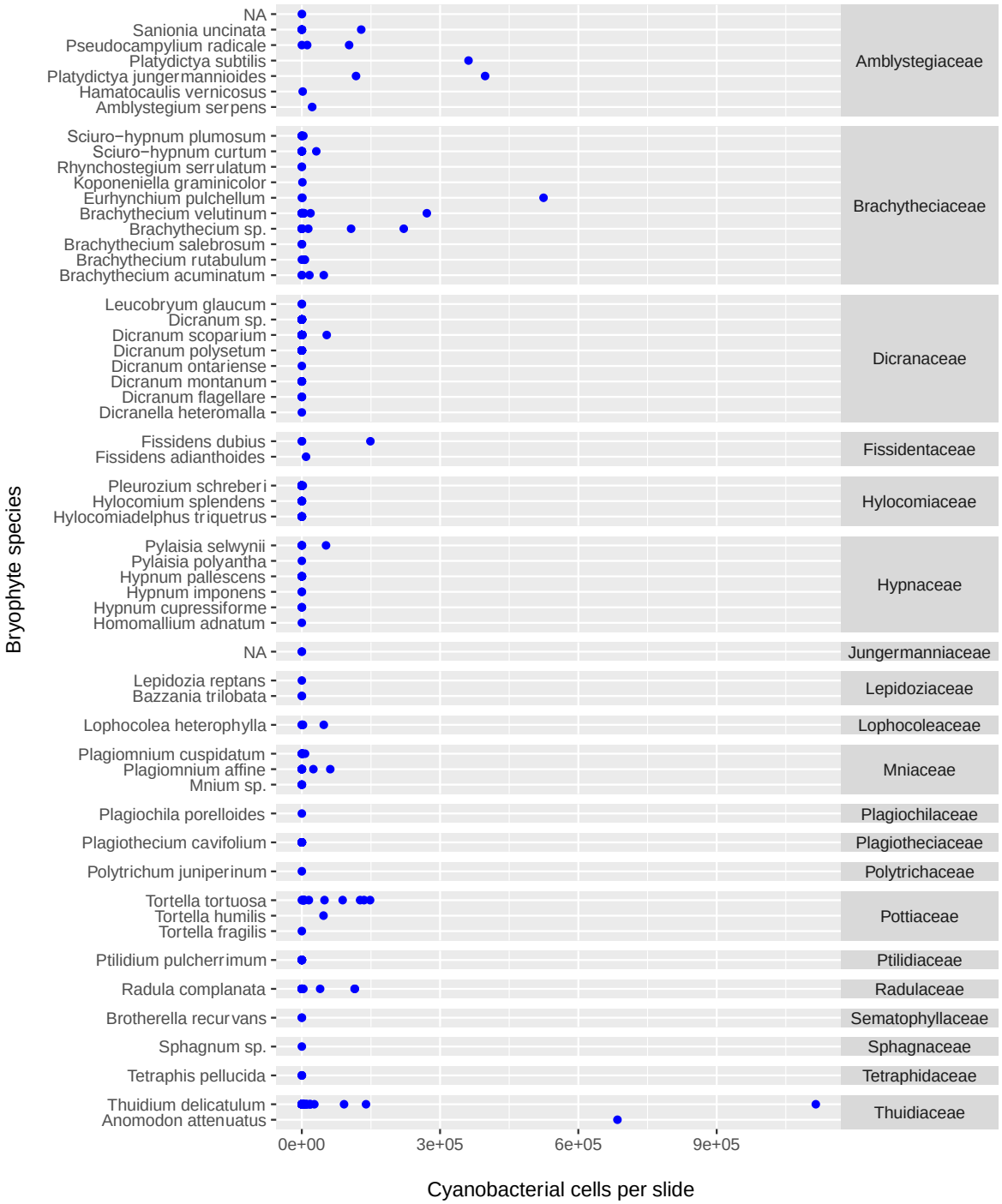


Figure 8. Estimated cyanobacterial cell counts by bryophyte species nested within family. Given zero-inflated data, the point near the x-axis value of 0 (no cyanobacteria present) should be interpreted as "at least one sample has no cyanobacteria." Due to overplotting, this single point may represent multiple samples. The larger values are more clearly differentiated.

Figure 9 visualizes the median values of cyanobacterial cell count estimates by family.

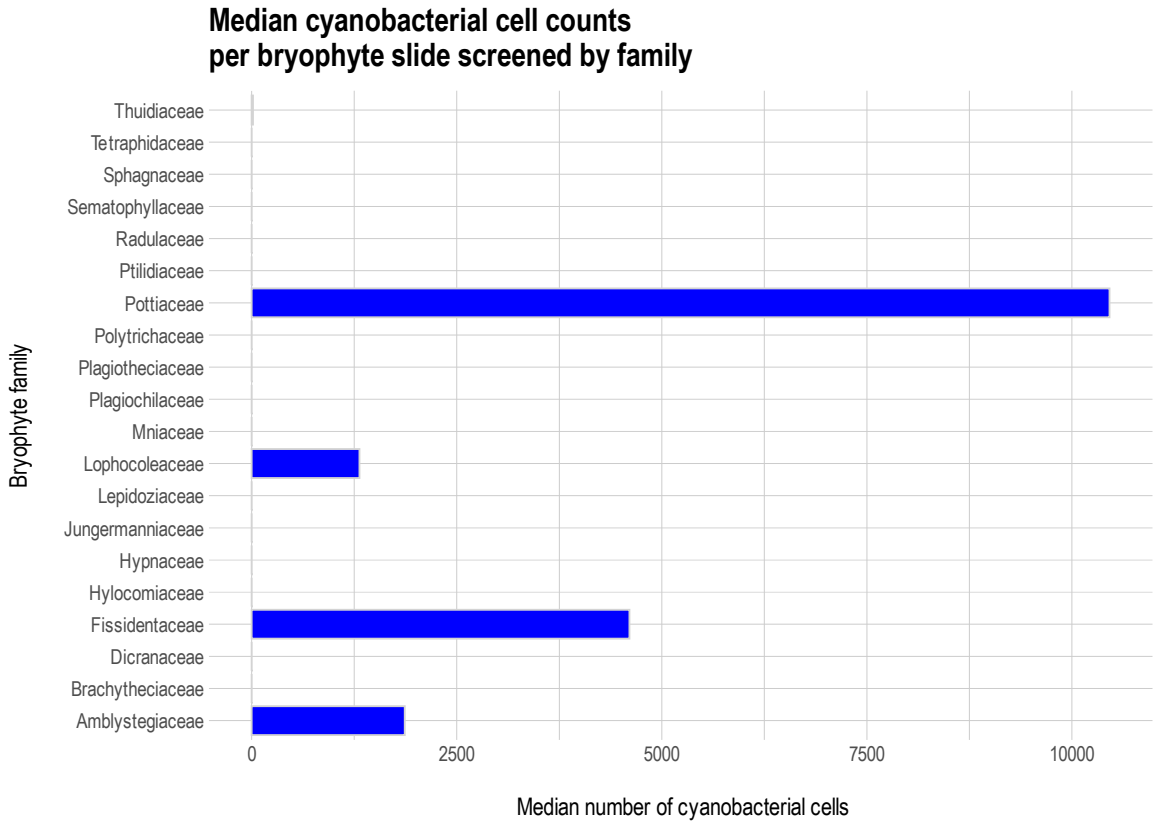


Figure 9. Median number of cyanobacterial cells per estimate by family.

The families Amblystegiaceae, Fissidentaceae, Lophocoleaceae, Pottiaceae, and Thuidiaceae have non-zero median values. The median value for Thuidiaceae is small but non-zero (15); the scale differences make the bar for Thuidiaceae rather hard to see. The median values for the other families are: Amblystegiaceae (1866), Fissidentaceae (4606), Lophocoleaceae (1314), and Pottiaceae (5599).

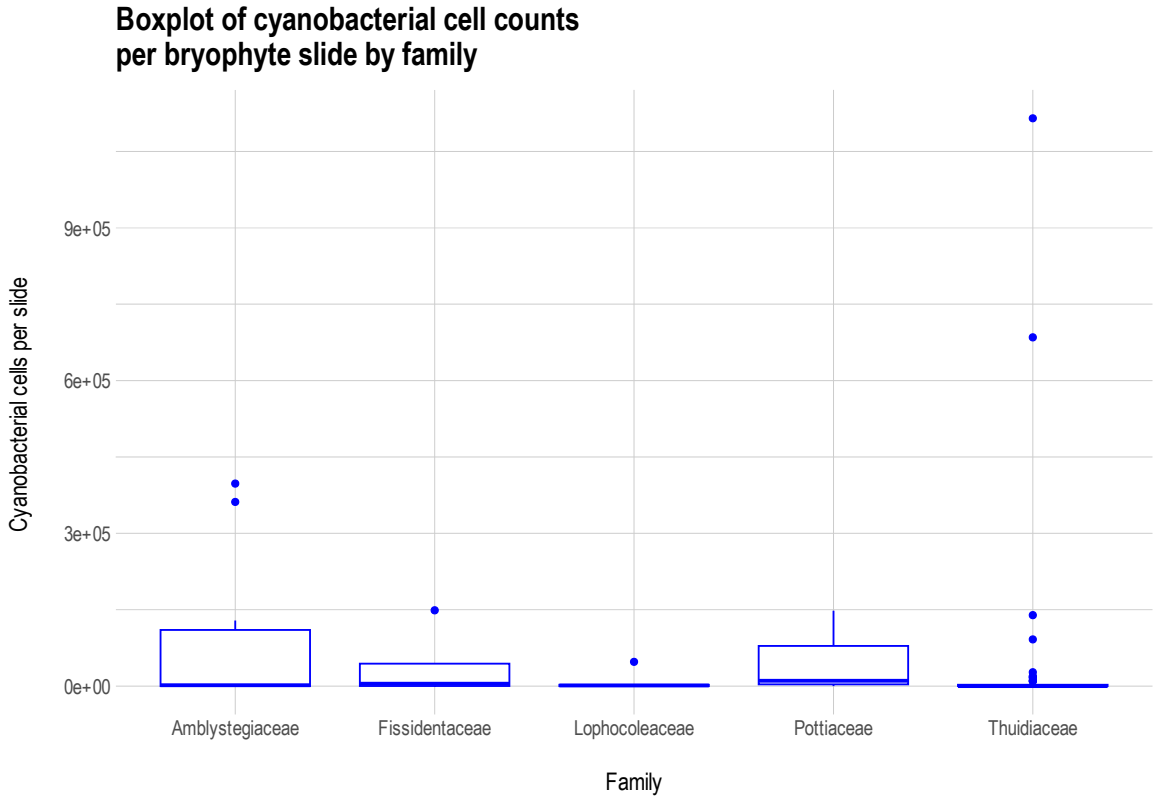


Figure 10. Boxplot of estimated cyanobacterial cell count per slide by family.

Figure 10 visualizes the data with a boxplot and a single scale for units along the y-axis (estimated cell counts). There is a high concentration of zero or near-zero results for Lophocoleaceae and Thuidiaceae (strong skewness), but the extreme values make it difficult to differentiate the median values for each family.

Figure 11 visualizes the estimated cell counts for these five families with a break in scale at 200,000 cyanobacterial cells, allowing for finer resolution near the median.

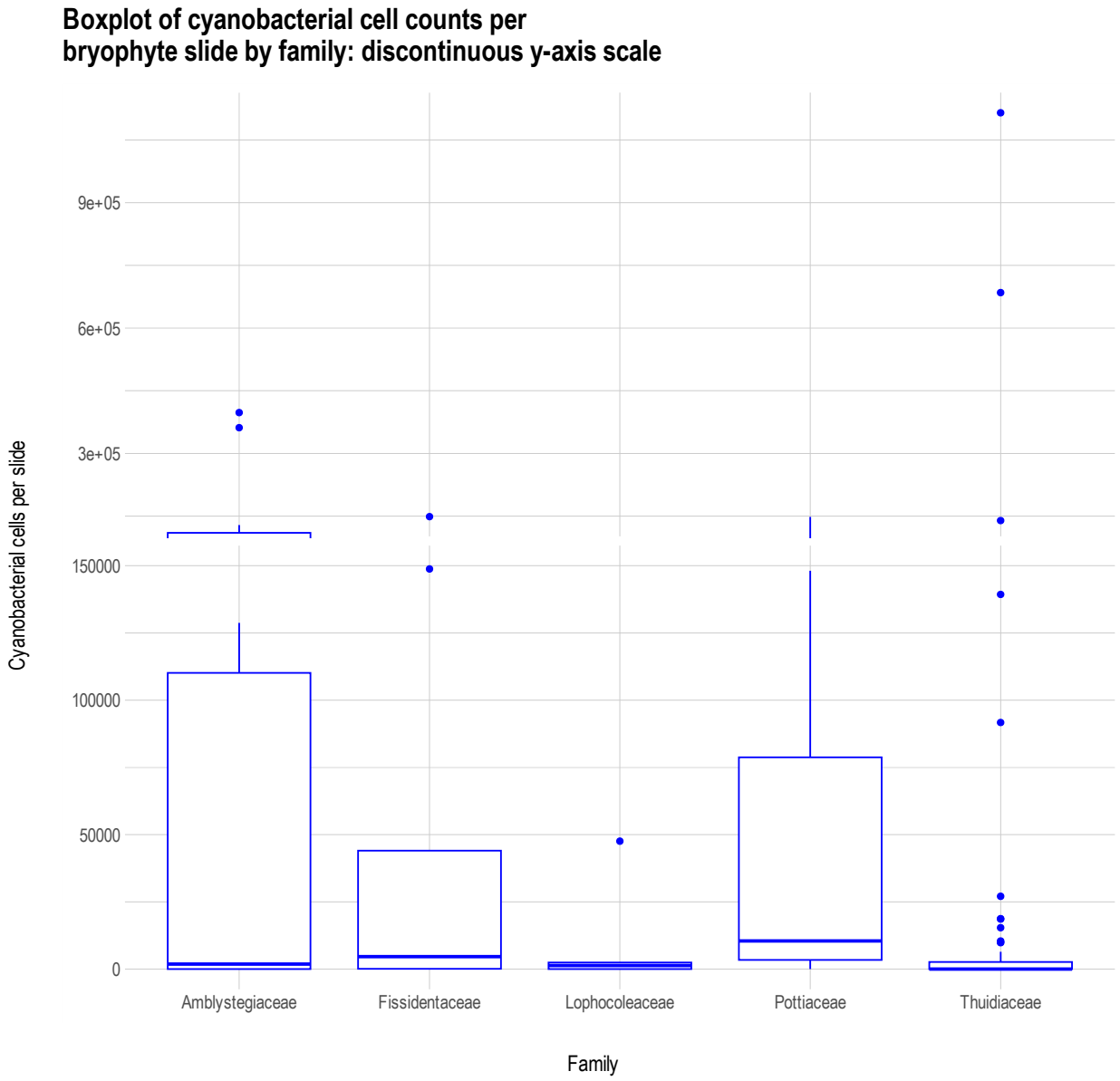


Figure 11. Boxplot of estimated cyanobacterial cell count per slide by family. Bryophyte families with median values of zero are excluded from this visualization. Note the discontinuity in y-axis scale.

In Figure 11, Pottiaceae has the greatest median value, followed by Fissidentaceae, Amblystegiaceae, Lophocoleaceae, and Thuidiaceae (estimated median cell count = 15).

Logististic regression model for prediction of cyanobacterial presence

Logistic regression models were used to address the following questions:

(1) Does the presence or absence of various substrata within the 21.5-cm-diameter circular plot, or the elevation of the plot with respect to ground level, influence the probability of detecting cyanobacteria with epifluorescence microscopy, given the presence of bryophytes? Do season or lichen presence have explanatory power?

(2) Does the presence or absence of various substrata within the 21.5-cm-diameter sample plot, or the elevation of the plot with respect to ground level, influence the probability of detecting Nostocalean cyanobacteria with epifluorescence microscopy, given the presence of bryophytes? Do season or lichen presence have explanatory power?

The probability of detecting cyanobacteria is studied conditional on the presence of bryophytes, because a great of research and informal field study tell us whether bryophytes are likely to occur in specific habitats. Generalists may be found in a wide range of habitats; but many species have specific habitat preferences that are well-known. For example, *Tetraphis pellucida,* one of the forest-floor mosses at Cana Island Road, is typically abundant on rotting conifer logs and other moist, woody debris in northern forests (e.g., Kimmerer, 1993). As there is no need to re-create existing knowledge with respect to bryophyte ecological niche, this analysis focuses on a subset of the plot data: plots with sufficient bryophyte tissue present for screening via microscopy. Given the presence of conditions suitable for bryophytes, I am interested in

whether substrata present within the plot (conifer leaf litter, decidous leaf litter, wood, soil, rock, grass, moss mat), lichen present within the plot, season, or elevation might influence the probability of detecting cyanobacteria via microscopy.

This line of inquiry implies that the first step in analysis should be to subset the data, focusing specifically on plots with sufficient bryophyte material for screening. This yields a dataframe with $n = 478$ for all four seasons for question (1). Applying the same logic, I subset the data for question (2) to include only plots with both bryophytes and cyanobacteria present ($n = 120$). The modeling process and results for each question are presented sequentially below. A brief overview of the rationale for selection of logistic regression as the framework for analysis precedes the results.

Method of choice: logistic regression

Logistic regression involves a general linear model and is suitable for data that meet several conditions (Midi et al. 2010). The response variable must be a binary categorical variable, in which each observation has one of two mutually exclusive outcomes. The logistic model for the data involves the log of the odds ratio, where the odds ratio is defined as the probability of success (π) divided by the probability of failure ($1 - \pi$). In my analysis:

1. The probability of detecting cyanobacteria via epifluoresence microscopy, P(cyanobacteria), is denoted π, and

2. The probability of *not* detecting cyanobacteria via epifluorescence microscopy, *P*(no cyanobacteria), is denoted $1 - \pi$.

The explanatory variables, which may be quantitative or categorical, must include all potentially influential variables and be measured without notable error, and may not be strongly correlated with each other. The observations must be independent.

The binary response variable, typically encoded as 0 = failure and 1 = success, yields residuals with a non-normal distribution due to its discrete nature; such data are not suitable for multiple linear regression, which require a more or less normal residual distribution. The resulting residual distribution is binomial. To generate residuals suitable for analysis by a general linear model, a link function is used to transform the scale of all variables (response and predictors). This accommodates the binomial error structure, which has a non-constant variance, and allows us to conduct inference with respect to the significance of the model coefficients under the assumption of a χ^2 distribution for the coefficient estimates.

Various functions can be applied to transform the raw observational data. The logit link function is the canonical link function for models with a binary response, and has been applied to the data on cyanobacterial occurrence. Hence the response variable for the logistic model is the *log* of the odds ratio, rather than the odds ratio itself.

To interpret the logistic model on a more accessible scale, the estimates for model coefficients and the model response (i.e., the log-odds ratio) must be exponentiated (back-transformed) for interpretation in a realistic context. With back-transformation, the predicted response reverts to the odds ratio, $\pi / (1 - \pi)$. In general, an increase in the value of the odds ratio is associated with an increased probability of cyanobacterial detection, and a decrease in the odds ratio is associated with a decreased probability of cyanobacterial detection. The impact of the back-transformed model coefficients on the odds ratio is multiplicative, rather than additive, due to exponentiation.

Logistic regression model focusing on microhabitat variables and their potential relationship to the probability of cyanobacteria

The variables defined by the sampling design are characterized in Table 3. Presence/absence of substrate is determined by occurrence within the 21.5-cm-diameter circular plot, not specifically by whether the moss sampled was growing on a given substrate within the plot. This is because often moss samples were associated with more than one substrate.

Name	Code	Description	Type
Season	SEASON	Season during which sampling occurred. Levels = Fall for intercept, Summer, Winter, Spring.	Categorical
Conifer litter	CONL	Leaf litter from coniferous trees and shrubs. Levels = 0 (absence) for intercept, 1 (presence)	Categorical
Deciduous litter	DECL	Leaf litter from deciduous trees and shrubs. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Wood	WOOD	Woody debris, downed branches, and logs. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Soil	SOIL	Exposed soil. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Rock	ROCK	Rock. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Grass	GRASS	Grass. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Moss mat	MOSS.MAT	Extensive moss growth providing a substrate for the moss sampled and studied (e.g., mosses covering a glacial erratic that provide a substrate for the bryophyte sampled). Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Scat	SCAT	Any form of animal waste. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Lichen	LICHEN	Any form of lichen, including but not limited to cyanolichens. Levels = 0 (absence) for intercept, 1 (presence).	Categorical
Elevation	ELEVATION	Elevation of the plot with respect to surrounding groundlevel (within 1 m), measured in cm. Maximum value 25 cm.	Continuous quantitative variable

Table 3. Variable definitions based on the sampling design.

Exploratory data analysis was conducted prior to model fitting. For the quantitative variable ELEVATION, summary statistics and a histogram revealed the presence of 7 observations with a height > 25 cm, outside the bounds of “groundcover” initially defined by the researcher. Given uneven terrain due to the presence of glacial erratics and downed logs, these observations were not removed arbitrarily, but flagged for further investigation with respect to influence after fitting the full model. Linear correlation

among quantitative predictors was not possible given the presence of just one quantitative predictor.

For the categorical variables, contingency tables characterizing the co-occurrence of levels for each factor variable in relation to the presence and absence of cyanobacteria (the response) were used to explore adequacy of sample size. The contingency table (Appendix C) indicated that the variable SCAT did not occur in any plot with bryophyte material sufficient for screening. Given a lack of co-occurrence with cyanobacteria, this variable has no predictive power and was dropped from the model. The variable MOSS.MAT also co-occurred with cyanobacteria rarely (2 of 478 samples). To ensure adequate sample size for convergence to a χ^2 distribution, merging with another category was necessary. From a biological perspective, the potentially similar categories relate to plant tissues: conifer leaf litter, deciduous leaf litter, and grass. Conifer leaf litter was excluded from consideration due to its robust sample size and its acidity, which could provide unsuitable habitat for cyanobacteria; exploration of the impacts of conifer leaf litter as an individual predictor is well-justified by existing scientific knowledge (Augusto et al. 2016). However, deciduous leaf litter (8 of 478 samples) and grass (12 of 478 samples) also co-occurred rarely with cyanobacteria. Less than 2% of the total observations yielded a positive response for both cyanobacterial presence and the factor variable DECL; less than 3%, for the factor variable GRASS. From a statistical perspective, these two variables were also not well-represented, even though they did fulfill the “rule of thumb” of $n \geq 5$ for each cell. I considered both for merger with the moss mat variable. I am not aware of any potential

mechanism associated with either deciduous litter or grass that would make me prioritize one over the other; all three (DECL, GRASS, and MOSS.MAT) include biochemically different plant tissues derived from plants with different habits and patterns of growth. Ultimately I decided to merge all three categories, motivated by the deficits of small sample size; none of these were likely to be informative as individual variables. A new variable, non-conifer plant matter, was created, with 0 = no occurrence of deciduous leaf litter, grass, or moss mat, and 1 = occurrence of at least one of these three substrates.

This decision appears to be statistically sound, but it does have costs with respect to interpretability. The biological dissimilarity of the non-conifer plant materials means that potentially opposing effects may be included within this category. For example, the composition of grass tissues, generally high in silicon (Hodson et al. 2005, Piperno 2006), is quite dissimilar to that of moss, often highly recalitrant and more resistant to decomposition than deciduous leaves (Cornelissen et al. 2007). This biologically artificial and “noisy” variable is unlikely to yield biologically coherent interpretations, but its inclusion limits distortion of the regression standard errors and inference due to inadequate sampling across all levels of the factor variables prior to merging.

An updated continency table summarizing the occurences of cyanobacteria in relation to the factor variables, including the variable non-conifer plant matter (abbreviated NCPM), is found in Appendix D.

Mosaic plots and contingency tables at finer levels of nesting were used to explore the potential for modeling interactions. As a starting point, three-way tables involving the predictor SEASON and two of the substrate predictors (e.g., ROCK and WOOD) were created. Several of these three-way tables had empty cells or values ≤ 5 within one or more cells. I concluded that the sample size was insufficient to model interactions, even after merging data for three of the plant material variables. This is unfortunate; generally we prioritize investigating interactions, and study lower-level effects (e.g., the impact of individual variables) only after interactions have been characterized. In this case, what appeared to be a relatively ambitious sampling plan for a novel system (> 1000 plots) did not yield enough information for a model including interactions, because only about half of the plots generated useful bryophyte samples, and some substrata were relatively rare.

Based on the exploratory data analysis, I chose to focus on a model that would provide information on the predictive power of individual variables conditional on the presence of other explanatory variables in the model, but no information on potential interactions. The categorical predictors included the variable non-conifer plant matter (NCPM) and excluded the variable SCAT. The model was specified in R using the function *glm*:

```
log_merge_full <-glm(POSITIVE.CYANO ~ SEASON + CONL + NCPM + WOOD +
SOIL + ROCK + LICHEN + ELEVATION, family = "binomial", data =
        cirw_merge_bryo)
```

The regression table for the full model, a first step in analysis, is given in Table 4.

Model: glm(formula = POSITIVE.CYANO ~ SEASON + CONL + NCPM + WOOD + SOIL + ROCK + LICHEN + ELEVATION, family = "binomial", data = cirw_merge_bryo)				
Variable: level	**Coefficient estimate**	**Standard error**	**z value**	**Pr(>|z|)**
Intercept	-1.251	0.438	-2.860	0.004 **
SeasonSP08	-0.407	0.355	-1.145	0.252
SeasonSU07	-0.211	0.333	-0.635	0.526
SeasonWI08	-0.186	0.352	-0.527	0.598
CONL1	-0.323	0.257	-1.260	0.208
NCPM1	-0.061	0.315	-0.193	0.847
WOOD1	0.240	0.323	0.741	0.459
SOIL1	-0.093	0.329	-0.283	0.777
ROCK1	2.709	0.407	6.657	2.790e-11 ***
Lichen1	-0.398	0.309	-1.288	0.198
Elevation	-0.000	0.021	-0.018	0.986
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				
Dispersion parameter for binomial family taken to be 1				
Number of Fisher Scoring iterations: 4				
Null deviance: 538.69 on 477 degrees of freedom				
Residual deviance: 439.28 on 467 degrees of freedom				
AIC: 461.28				

Table 4. **Initial logistic regression model including all predictor variables and all observations**. Note that the coefficient for elevation rounded to zero, but was not originally equal to zero.

The standard errors in this initial model are large relative to most of the coefficients, and the *p*-values provide no substantial evidence that the coefficients are non-zero. This uncertainty is reflected by the odds ratio estimates for individual coefficients for most predictor variables, which are very close to 1, and the confidence intervals for the odds ratios—most of these span 1 (Table 5).

Model: glm(formula = POSITIVE.CYANO ~ SEASON + CONL + NCPM + WOOD + SOIL + ROCK + LICHEN + ELEVATION, family = "binomial", data = cirw_merge_bryo)		
Variable: level	**Odds ratio**	**CI for odds ratio**
SeasonSP08	0.666	(0.332, 1.335)
SeasonSU07	0.809	(0.421, 1.555)
SeasonWI08	0.830	(0.416, 1.657)
CONL1	0.724	(0.438, 1.197)
NCPM1	0.941	(0.508, 1.744)
WOOD1	1.271	(0.674, 2.396)
SOIL1	0.911	(0.478, 1.735)
ROCK1	15.0135	(6.763, 33.331)
Lichen1	0.672	(0.367, 1.231)
Elevation	1.000	(0.959, 1.042)

Table 5. Odds ratios and 95% confidence intervals for the model with all predictors and all observations.

With these data and this model, it is rarely possible to determine whether the presence or absence of a specific substrate has a positive, neutral, or negative impact on the odds of detecting cyanobacteria. The sole exception is ROCK, which has a large multiplicative effect (about 15) on the odds of detecting cyanobacteria when bryophytes are present. The effects of the only quantitative variable, ELEVATION with respect to ground level, are likewise difficult to characterize.

The potential importance of rock substrates is highlighted by the added-variable plot shown in Figure 12. Added-variable plots help us visualize the impacts of a specific variable in a regression model, considering the predictive power of all other explanatory variables (Fox & Weisberg, 2011). The points displayed in the plot represent residuals rather than actual data. Specifically:

• The vertical axis represents residuals from the regression of the response variable (log-odds of detecting cyanobacteria) on all predictor variables except the variable named on the x-axis.

• The horizontal axis represents residuals from the regression of the predictor variable that is the focus of the plot against all of the other predictor variables.

The y-axis trend in the added-variable plot represents the portion of variability in the response (log-odds of detecting cyanobacteria) that is *not* explained by all of the other predictors. Horizontal or near-horizontal trend lines suggest that essentially none of the variability in cyanobacterial detection can be explained by the predictor of interest. With this model, we see a modest positive trend associated with ELEVATION, and a notable positive trend associated with ROCK. The other variables appear to have little explanatory power when their impact is examined relative to other predictors.

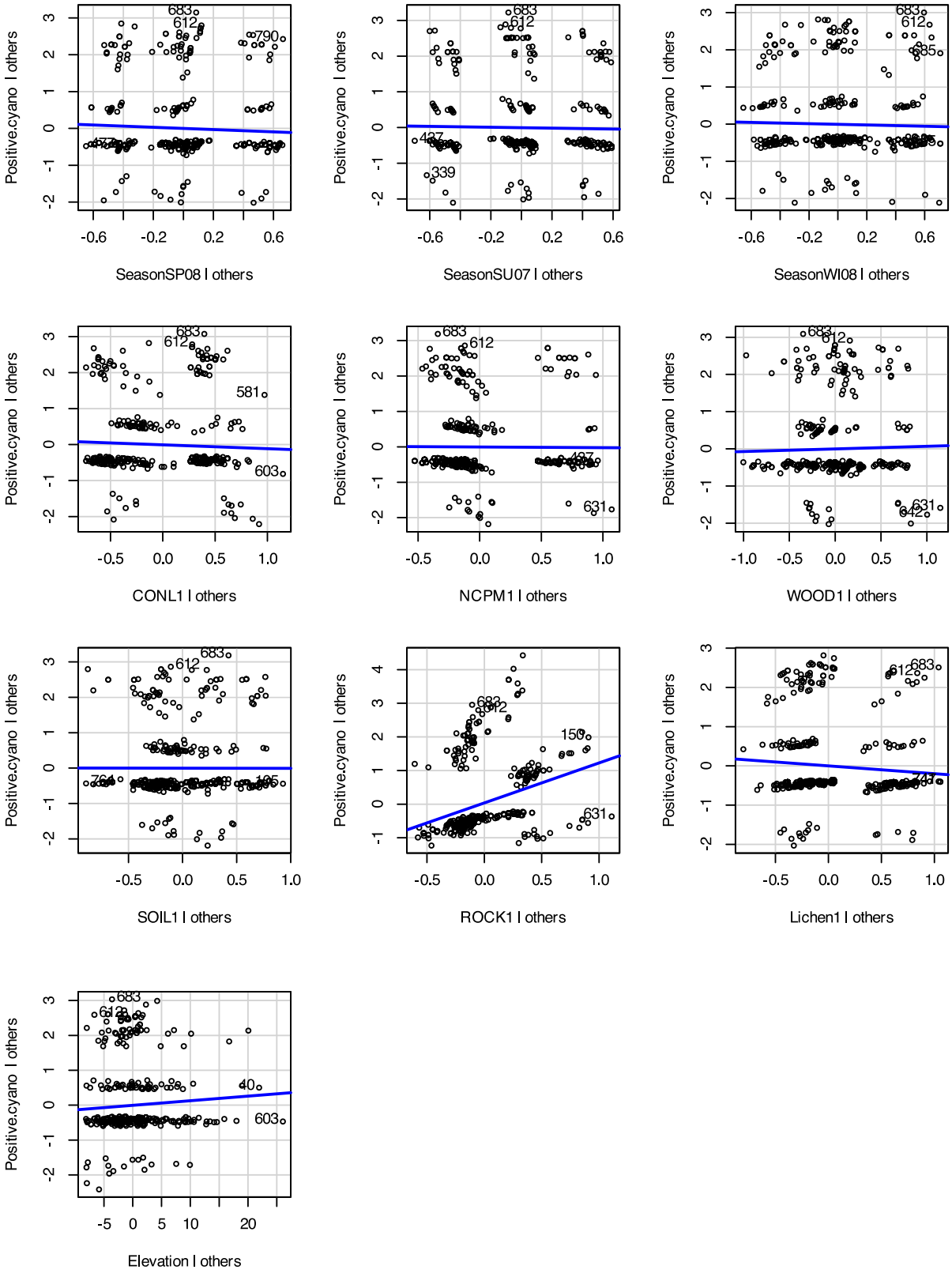

Positive.cyano | others
SeasonSP08 | others
SeasonSU07 | others
SeasonWI08 | others
CONL1 | others
NCPM1 | others
WOOD1 | others
SOIL1 | others
ROCK1 | others
Lichen1 | others
Elevation | others

Figure 12. Added variable plots for the full model with all predictors and all observations. Slopes for a given plot represent the partial-regression effects of the predictor variable, conditional on the presence of all other predictor variables. The observations flagged represent points that are extreme with respect to the mean (along the x-axis), and points that are extreme with respect to the absolute value of the residual (along the y-axis).

The strong impact of ROCK substrates in the added-variable plot hints at potential regression results, but model diagnostics and model selection may modify these conclusions. We will explore model diagnostics first.

Potential multicollinearity was invested using generalized variance inflation factors (Fox & Monette, 1992) implemented in the package *car*. The values of the generalized variance inflation factors (GVIF) are given in Table 6.

	GVIF	Degrees of freedom	GVIF^(1/(2*Df))
SEASON	1.194	3	1.030
CONL	1.127	1	1.062
NCPM	1.122	1	1.059
WOOD	1.776	1	1.333
SOIL	1.699	1	1.303
ROCK	1.771	1	1.331
LICHENa	1.185	1	1.089
ELEVATION	1.283	1	1.133

Table 6. **Generalized variance inflation factors for the full model (all possible predictor variables).**

Multicollinearity appears to be mild for the full model. Generally variance inflation factors motivate further investigation of multicollinearity with values > 4; with GVIF values based on GVIF^(1/(2 * DF), we square the values to apply this rule of thumb (Fox 2016). None of the values from the last column of Table 6 exceed the threshold of concern.

Potentially influential observations were investigated by computation of hat values (a measure of leverage), an outlier test, and study of Cook's distance (a measure of

influence). The overall goal was to ensure that unusual observations would not distort the model deviance and inference with respect to coefficients. To test for leverage, the command *hatvalues* (package *stats*) was used to extract hat values (h_i) from the hat matrix associated with *log_merge_full.* Extreme hat values are greater than $2p/n$, where p = number of parameters and n = sample size. For the full model, p = 11 and n = 478, so the threshold of concern is (2 x 11) / 478 = 0.046. Some of the observations (15 of 478) are high-leverage points. With a large dataset, it is not surprising that a small number of observations (about 3%) have high leverage.

[Figure 13 on next page.]

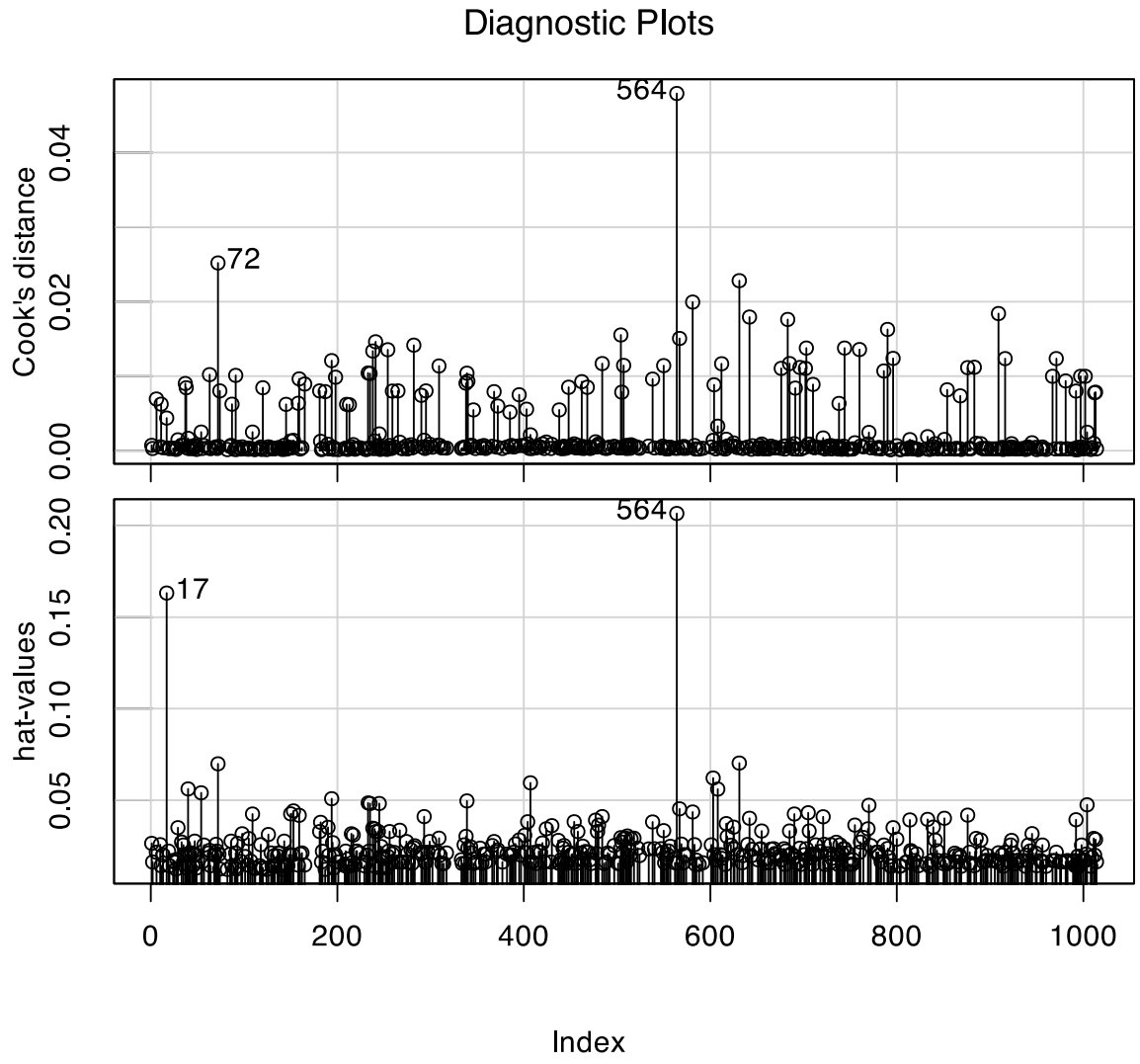


Figure 13. Visualization of hat values and Cook's distance for each observation in the full dataset. The x-axis represents index values associated with each observation. The number of plots with bryophyte material sufficient for screening has 478 observations. Each plot in the plot retains its index number from the original dataset (plots with and without bryophytes), with *n* = 1014; but only the observations used to build the model (*n* = 478) are visualized. The y-axis in the lower graph represents the magnitude of the hat-value (leverage measure); the y-axis in the upper graph represents the magnitude of Cook's distance (influence measure).

To test for outlier status, the command *outlierTest* (package *car*) was used to test whether any observations were extreme enough to qualify for outlier status. The

Bonferroni correction is applied to compensate for multiple testing (inference in model as well as in outlier test). No observations had a studentized residual (computed with *rstudent* in *stats*) large enough to qualify for outlier status after the Bonferroni correction.

Influence is often measured with Cook's distance (Cook 1977), a measure incorporating both leverage and the magnitude of the residuals. Both the hat values and the squared standardized residuals are used to compute Cook's distance, denoted C_i; it incorporates information related to both leverage and error. The *F*-distribution can be used to identify extreme values of Cook's distance (Spector 2011), with values exceeding the 10^{th} percentile of the appropriate *F*-distribution of potential concern. The value of Cook's distance associated with the 10^{th} percentile of *F* on 11 and 467 degrees of freedom (derived from *p,* the number of model parameters, and the value for the residual degrees of freedom) is 0.505, approximately equal to the 0.5 measure commonly used as a "rule of thumb" criterion. There were no observations with a value of Cook's distance that exceeded this threshold.

These diagnostic measures suggest that influential observations may not be a concern for the logistic regression under investigation. However, while Cook's distance is a useful guideline, it does not actually dictate whether regression coefficients and standard errors will respond disproprotionately to the presence or absence of a particular observation. I also investigated model performance with and without observations that had relatively large values for Cook's distance—unusual in comparison to the remainder of the data. The measures of leverage (hat values) and

influence (Cook's distance) are summarized visually in Figure 13, with the most extreme values flagged (observations #564 and 72 for C_i, and observations #564 and 17 for h_i).

In this exploration, I focused primarily on the odds ratios associated with each coefficient term, which are accessible after back-transformation. A shift from an odds ratio < 1 to a value > 1 (or vice versa) with removal of a single observation would indicate fundamental instability, i.e., uncertainty about whether a change in a specific predictor is associated with a lower, neutral, or higher odds ratio for cyanobacterial detection (conditional on observed values of other predictors). This is one potential outcome of undue influence in the context of a logistic regression model; I also looked for changes in the results of inference with respect to a coefficient (from significant to non-significant, or vice versa), or notable changes in the AIC value (> 2).

I fit a new linear regression model after removing the observation with the highest value of C_i, #564. There was nothing obviously unusual about this observation except for the value of elevation (44 cm), greater than the limit of 25 cm set in the sampling design. Removing this observation did not result in any changes in coefficient tests (*p*-values did not change from significant to non-significant, or vice versa). But the coefficient estimates for NCPM and ELEVATION shifted from negative to positive, and for SOIL, from positive to negative. The changes were small in magnitude, but impacted the nature of the impact on log-odds of detecting cyanobacteria for NCPM. The value of the odds ratio for NCPM shifted from slightly negative (0.941) to essentially neutral (1.017); for ELEVATION, the odds ratio was very close to neutral (0.999 with all observations,

1.011 after the deletion of #564); and for SOIL, the odds ratio remained slightly negative (0.941 for all observations, and 0.939 after deletion). The AIC value also decreased by slightly more than 2, suggesting improved model fit.

Although these changes were not dramatic, the perturbation seemed more substantial than one might expect with deletion of a single observation. Executing a similar model diagnostic procedure after the deletion of high-influence observation #72 and high-leverage observation #17 (sequentially, not simultaneously), I settled on a dataset that excluded two of these three observations, #564 and #72. These two plots had the highest values of ELEVATION (50 and 44 cm, respectively), notably greater than the height of 25 cm defined as a maximum for groundcover. Measures of model efficacy for each of these models are summarized in Table 7.

	Logistic model: no deletions	**Logistic model: one deletion**	**Logistic model: two deletions**	**Logistic model: three deletions**
Sample size: *n*	478	477	476	475
Observations deleted	None	# 564	# 72, 564	# 17, 72, 564
AIC	461.28	458.95	455.2	454.07
Percent deviance explained	0.185	0.188	0.195	0.195

Table 7. Model efficacy in relation to deletion of extreme observations on ELEVATION. Changes in AIC values suggest observations #564 and 72 were responsible for unusually large amounts of deviance and changes in coefficient sign. The deletion of observation #17 did not notably impact model efficacy or stability.

Model selection using an information theory approach (AIC) was applied to the reduced dataset (after deletion of #564 and 72). The AIC is based on model deviance, with a penalty exacted for the number of parameters. The command *step* in package *stats* was used to determine which model was most efficacious based on a backwards selection

process given the models examined. (It does not necessarily guarantee an optimal model, as the efficacy is path-specific.) The results of model selection with AIC are given in Table 8.

	Model: glm(formula = POSITIVE.CYANO ~ ROCK, family = "binomial", data = cirw_merge_bryo)		
	Coefficient estimate	**Odds ratio**	**95% confidence interval for odds ratio**
Intercept	-1.624		
ROCK	2.822	16.810	(9.066, 31.167)
AIC	442.8		
	Degrees of freedom		**Deviance**
Null	475		537.530
Residual	474		438.800
Percent deviance explained	18.360		

Table 8. Model for Log Odds-Ratio of Detecting Cyanobacteria | Bryophyte Presence.

Model diagnostics (*vif* in package *car*, *hatvalues* and *cooks.distance* in *stats*) indicate this model has no notable problems with multicollinearity or influential observations.

The first key conclusion is that the presence of a rock substrate increases the odds ratio (probability of detecting cyanobacteria | bryophyte) / (probability of not detecting cyanobacteria | bryophyte) by a factor of approximately 17. This is not suprising, given the probability of detecting cyanobacteria is notably high: 53 of 70 plots with bryophytes and rock substrate (about 76%) yielded evidence of cyanobacteria. The second key conclusion is that most of the variability with respect to the odds ratio remains unexplained: the model explains a little less than 20% of the overall variability in outcomes.

Logistic regression model focusing on microhabitat variables and their potential relationship to the probability of Nostocalean cyanobacteria | *presence of cyanobacteria*

This model, as mentioned previously, utilizes a subset of the dataset: the observations in which both bryophytes and cyanobacteria were present (*n* = 120). A logistic model with the same set of predictor variables used in the previous section (season, conifer litter, non-conifer plant matter, wood, soil, rock, lichen, and elevation) was fit to all observations using *glm* in R:

```
log_BC_full <-glm(Heterocysts.present ~ SEASON + CONL + NCPM + WOOD + SOIL + ROCK + LICHEN + ELEVATION, data = cirw_BC, family = "binomial")
```

Multicollinearity, potential outlier status, leverage, and influence were investigated with *vif* and *outlierTest* in package *car,* as well as *hatvalues* and *cooks.distance* in *stats*. No evidence of multicollinearity, outliers, high-leverage observations, or unduly influential observations was detected.

The observation with the largest C_i value, #581, was also deleted from the dataset to examine the stability of coefficient estimates, odds ratios, and the 95% CIs for odds ratios. This may have been excessively cautious, as the observation did not appear to be notably elevated in the influence index plot (Figure 14).

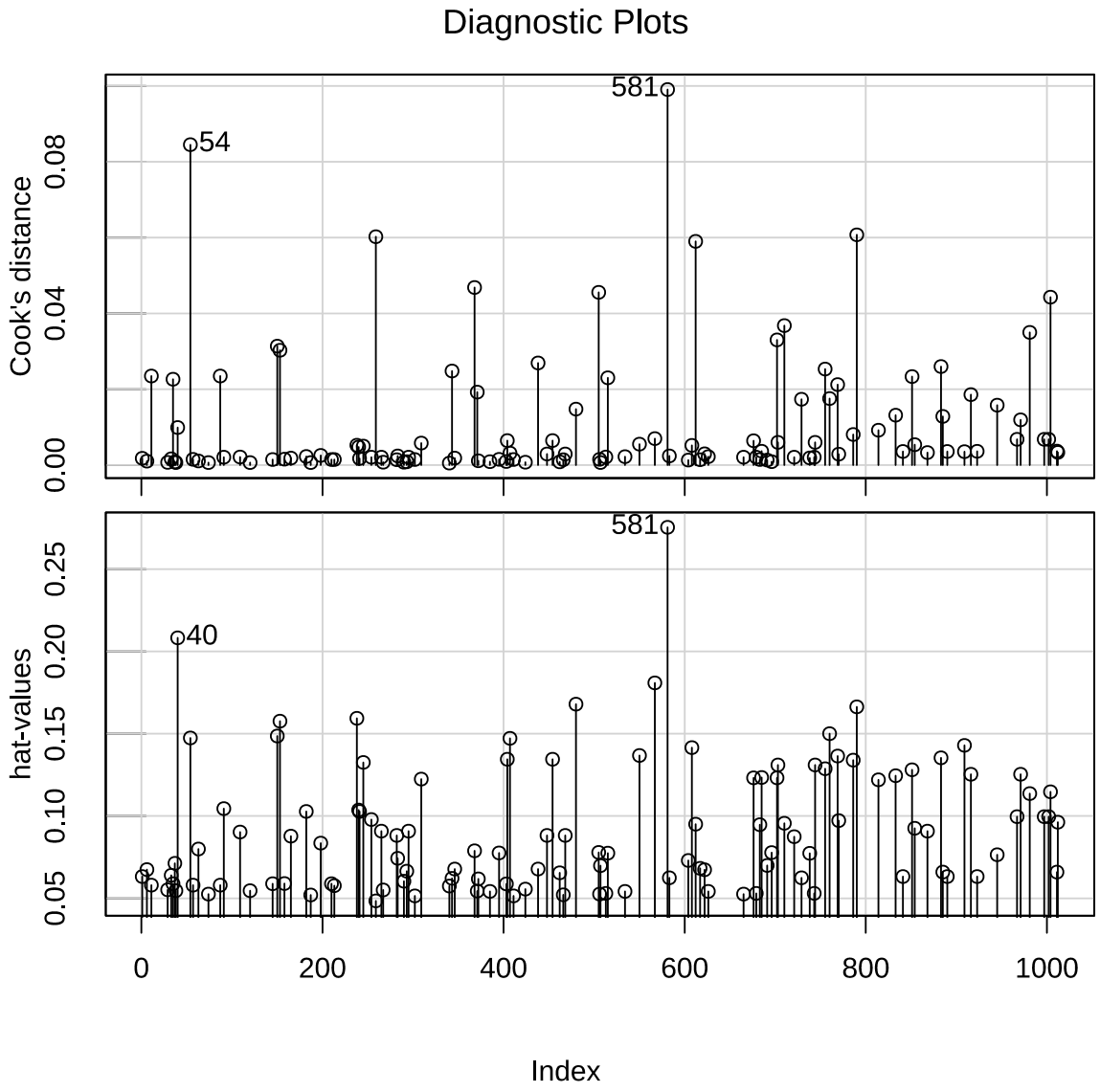


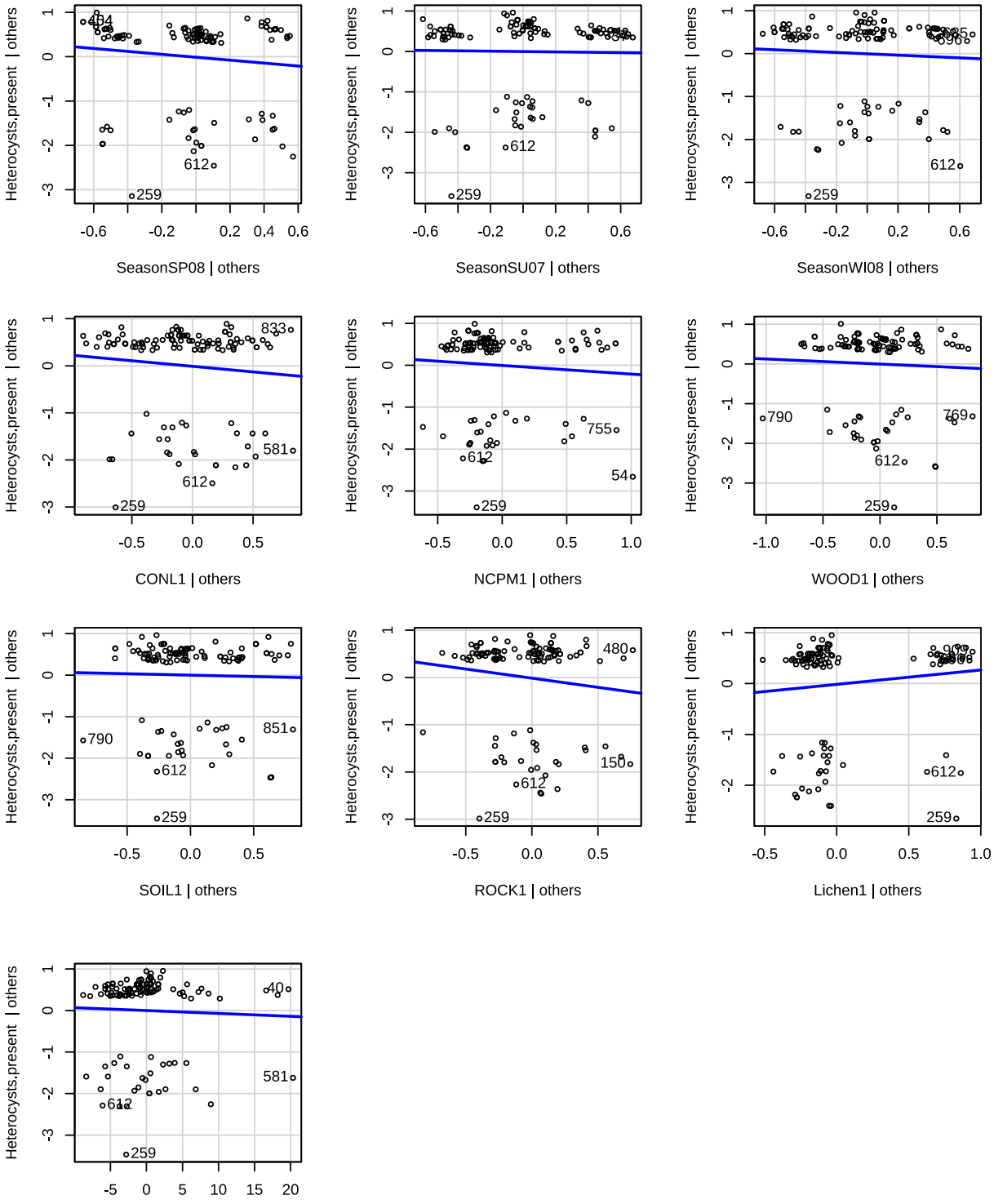
Figure 14. Added-variable plots for the full model of the log-odds ratio of P(heterocysts present) / *P*(heterocysts absent), given cyanobacteria and bryophytes present in the plot. Sample size (*n*) = 120, even though index numbers (e.g., 40, 54, 581) range from 1 to 1014; the model utilizes only a subset of the observations.

The AIC values and percentage of deviance explained were similar for the two models (with and without #581). There were no changes in the sign of the coefficients, nor in

the impact of the coefficient odds ratios after deletion. Both models explained a small proportion of the total deviance, just under 5%. Overall there was no evidence to suggest multicollinearity or extreme values were distorting the regression coefficients, so I chose to focus on the full dataset with all observations.

Contingency tables (not included in this thesis) exploring the number of presence and absence observations for each categorical predictor in relation to the presence or absence of heterocysts indicated that sample size was largely adequate, with $n > 5$ for all cells except one. Few observations ($n = 4$) were made for the co-occurrence of lichens and heterocystous cyanobacteria, given the presence of bryophytes and cyanobacteria. The sample size was not adequate to model three-way interactions.

The added-variable plot for the full model provided no strong evidence of structure within the data (Figure 15). The trend lines are mostly near zero or slightly negative; only the ROCK and LICHEN plots suggest a potential for impacts on the log-odds ratio (negative for ROCK, positive for LICHEN).


Heterocysts.present | others
SeasonSP08 | others
SeasonSU07 | others
SeasonWI08 | others
CONL1 | others
NCPM1 | others
WOOD1 | others
SOIL1 | others
ROCK1 | others
Lichen1 | others
259
612
581
790
769
755
480
851
150
54
40

With a reasonable sample size for main effects and no evidence of unsuitable data, I chose to apply AIC model selection with *step* (direction backward) to the full model. The intercept-only model supported by AIC selection is shown in Table 9.

	Model: glm(formula = HETEROCYSTS.PRESENT ~ 1, family = "binomial", data = cirw_merge_bryo)		
	Coefficient estimate	**Odds ratio**	**95% confidence interval for estimate**
Intercept	1.19	--	(0.767, 1.613)
AIC	132.4		
	Degrees of freedom		**Deviance**
Null	119		130.4
Residual	119		130.4
Percent deviance explained	0%		

Table 9. Logistic Model for Odds of Detecting Heterocysts | Cyanobacteria & Bryophyte Presence. AIC model selection does not support inclusion of *any* predictor variables. The intercept-only model appears to be reasonable.

Given that AIC selection supports a null model (intercept only), there is no possibility of distortion due to multicollinearity. No observations were characterized as outliers by *outlierTest* (package *car*). The AIC for this model is lower than that of the full model (143.7), indicating lower information loss with the null model. But there is not really any meaningful structure here; a model in which observations for the response cluster around the intercept at random distances appears to have lowest information loss. We can conclude that none of the microhabitat variables studied (neither substrate nor elevation nor lichen presence) influence the log-odds of detecting heterocystous cyanobacteria for these data. Likewise the season does not notably influence the probability of detecting heterocystous cyanobacteria, possibly because they are

relatively ubiquitous—found in about 77% of all samples, given the presence of cyanobacteria and bryophyte tissue.

DISCUSSION

The results suggest that survey methods geared towards capturing a substantial portion of the boreal groundcover bryophyte flora, rather than two or three focal taxa, may play a useful role in illuminating the prevalence of bryophyte-cyanobacterial symbioses. This relatively comprehensive survey indicated that 30 of the 50 bryophyte species present are capable of hosting cyanobacteria, hinting at the potential for more substantial impacts on ecosystem-scale function with respect to N fixation. Furthermore, a substantial proportion of these species (40%) may constitute novel reports of symbiosis, although that must be framed cautiously—the literature on bryophyte-cyanobacterial associations is multidisciplinary and diverse with respect to language, and no institution or individual has complete access to the global scientific literature. However, the potential novelty of these symbioses is supported by a review of over 250 publications that assess bryophytes for cyanobacterial presence, or characterize the bryophyte microbiome in general.

The framework developed in Part I (review of bryophyte-cyanobacterial literature from 1955-2016) indicates that at least 147 different bryophytes are capable of hosting cyanobacteria. The addition of 20 species to the list of known hosts represents a substantial expansion (+ 14%), although the number of bryophytes known to host

cyanobacteria is changing rapidly. Several decades ago, bryophyte-cyanobacterial associations were considered rare; the discovery of *Nostoc* on three species of the leafy liverwort *Porella* was considered notable (Dalton & Chatfield 1985). Since 2016, research on this topic has intensified, encompassing a much broader array of methods (primarily genomic) and geographic areas. Studies of bryophyte-cyanobacterial associations involving a survey of many bryophyte species are not new (e.g., Brasell et al., 1986), but they have become more common, especially in the boreal forest (e.g., Holland-Moritz et al. 2018, Stuart 2021, Holland-Moritz et al. 2021). These three Alaskan studies examined a relatively large number of boreal bryophyte taxa, and their results highlight the potential importance of species identity. Holland-Moritz et al. (2018) undertook a more comprehensive survey of boreal bryophyte diversity before focusing on seven common taxa; these researchers found that moss species identity was responsible for 63% of the variation in epiphytic bacterial community composition based on Permanova analysis. Stuart et al. (2021) studied N fixation in 35 boreal moss species, finding that N fixation was nearly ubiquitous (detected in 34 of 35 species) and rates differed by moss genera; moss family was found to be the most important predictor of N fixation rates across mutiple sites. In an expanded regional-scale study, Holland-Moritz (2021) studied 26 boreal moss species to examine the potential roles of site environmental conditions, host moss identity, and host phylogeny (based on genetic distance between moss hosts) in structuring moss microbial communites. Even with greater site diversity in the larger study area, host identity and host phylogeny were the strongest predictors of microbial community composition. Taken cumulatively, this evidence suggests the more information we have about bryophyte species identity, the

more leverage we may have with respect to questions of ecosystem biogeochemistry, host-microbe biodiversity, and host-microbial evolution.

This study also utilizes extensive sample collection (*n* = 478) to enhance our knowledge of bryophyte biodiversity for conservation and land management. Bryophyte biodiversity is poorly known in many regions of the world, including the circumpolar north (Lewis et al. 2017). About 25% of all vascular plants have been assessed for conservation status by a Red List (regional or global); but only about 12% of all bryophtes (Mounce et al., 2018). The bryophytes of the Great Lakes region are largely well-characterized with respect to taxonomy (e.g., Crum 1991, Crum 2004, Flora of North America Editorial Committee 2007, 2014), and Wisconsin does not fall into a region known for high moss[8] endemism (Carter et al. 2016). But there is still a need for more information on the distribution and conservation status of bryophyte species, particularly in light of climate change. Declines in bryophyte diversity are expected to accompany changes in the boreal forests of the Great Lakes region, as fires and blowdown events become more frequent (Apfelbaum & Haney 2023). At this site, repeated multi-season sampling yielded multiple novel records for Door County, Wisconsin, underscoring the degree to which bryophyte taxa may be underrepresented in digital herbarium records.

The multi-season sampling design involved intense sampling with small circular plots for bryophyte collection (6 cm diameter); these plots were employed to match the scale of the sampling unit to the scale of the microhabitat as defined by Belland & Vitt (1997).

[8] The biogeography of endemism in other North America bryophytes (hornworts, liverworts) is not well characterized at this time.

This allowed for straightforward inference with respect to the formulated hypotheses, and will make replication in any future studies relatively simple. The sampling methods captured sufficient diversity to generate a plateau in season-based species-accumulation curves, but captured a relatively modest proportion of the total sampled diversity within a given season. Even with a large number of plots per season (~ 250), data from an individual season captured between 47% and 67% of the 51 unique taxa. This is not surprising, as plot-based sampling methods for bryophytes do not capture as many species as floristic habitat sampling, which is stratified with respect to microhabitats and mesohabits (Newmaster et al. 2005). A loss of samples (2-3%) in two seasons may have also suppressed seasonal species richness, although these impacts are likely to be modest. On average, roughly ten plots are associated with one unique taxon; the amount of information lost probably amounts to 1-2 species per season (2-4 species total). Vanderpoorten et al. (2010) suggests repeat sampling of bryophytes is useful given habitat instability over time (creation of new microhabitats) and greater ease of identification during certain times of the year (e.g., sporophytic phase). In this case, repeated sampling on a 10-m grid with different plot locations each season clearly improved the capture of bryophyte diversity; sampling at just a single point in time would have suppressed the sample species richness by 30-50%. This could be due to the larger sample size captured with multi-season sampling and/or differential detection probabilities by season (e.g., leaf litter may obscure some species in fall).

The potential benefits of a relatively comprehensive, multi-season survey of bryophyte diversity are clear: more information with respect to taxonomic identity, which is clearly

an important predictor of microbial community structure and function in the boreal forest (Holland-Moritz et al. 2018, Stuart 2021, Holland-Moritz et al. 2021, Renaudin et al. 2022), and more information with respect to understudied cryptogamic diversity. That said, studying two or three dominant bryophyte species intensively might be equally appropriate for some questions. Estimates of the prevalence of bryophyte-cyanobacterial symbioses are fairly similar regardless of whether one focuses on the dominant taxa (*Dicranum scoparium*, *Pleurozium schreberi*, and *Thuidium delicatulum*) or the more comprehensive sample (at least 50 species for this site). The three dominant mosses constitute about 46% of the sampled biodiversity, and about 43 of 218 samples (20%) hosted cyanobacteria. For the entire collection of mosses and liverworts (100% of the sampled biodiversity), 120 of 475 samples (after deletion of the three highest observations for elevation) hosted cyanobacteria (25%). In this case, the extra sampling effort does not appear to yield a substantially different conclusion: we could assume that one in four bryophyte samples, or maybe one in five, will host cyanobacteria. Whether this conclusion regarding sampling methods is generalizable to other sites, or potentially sensitive to the identity and hosting status of the dominant mosses, is unclear.

The focal taxa also highlight questions relevant to the study as a whole. Among the dominant moss species, only *Thuidium delicatulum* hosts cyanobacteria frequently, sometimes with high abundance. *Thuidium delicatulum* appears to have a highly variable response to environmental conditions at this boreal forest site: half of the samples host cyanobacteria, and half do not. Furthermore, the abundance of

cyanobacterial cells varies by several orders of magnitude, given presence. By contrast, *Pleurozium schreberi* and *Dicranum scoparium* rarely host cyanobacteria; about 8% and 4% of the samples at this site yielded evidence of cyanobacterial symbiosis. When cyanobacteria are present, their abundance is low. At an ecosystem scale, all three mosses are likely subject to similar inputs of atmospheric nitrogen deposition, precipitation, and other control factors, e.g., temperature, yet their responses with respect to cyanobacterial symbiosis differ dramatically. The conditions are suitable for symbiosis for about half of the *Thuidium* samples, and yet unfavorable for *Pleurozium schreberi* and *Dicranum scoparium*. Moreover, both of these species are known to host heterocystous cyanobacteria in other ecosystems (see Table 2 for references). This suggests that (a) the bryophytes respond to environmental filters that act at the mesohabitat scale in a taxon-specific manner, (b) the bryophytes respond to environmental filters at the microhabit scale in a taxon-specific manner; or both (potentially with interactions).

For example, atmospheric N deposition is known to suppress symbiotic N-fixation in boreal bryophytes; a substantial body of evidence documents this phenomenon in *Pleurozium schreberi*, *Hylocomium splendens,* and *Dicranum* species (e.g., Zackrisson et al. 2009, Leppanen et al. 2013, Salemaa et al., 2018). There is also evidence of species-specific thresholds for inactivation of N fixation: *Pleurozium schreberi* and *Dicranum* sp. are typically more sensitive to atmospheric N deposition than *Hylocomium splendens* (Zackrisson et al. 2009, Salemaa et al., 2018), with evidence of inactivation at around 4-5 kg ha^{-1} yr^{-1} for the more sensitive taxa. The absence of cyanobacteria on

> 90% of the feather-moss samples is potentially consistent with inactivation by N deposition, although other causes are also possible. According to 30-m resolution maps of total N deposition (wet and dry) produced for the Great Lakes region (Hamlin et al., 2020), deposition in the Door County area is less than 7 kg N ha^{-1} yr^{-1}; the source data (Schwede & Lear, 2014) for these maps encompasses the year of my study.

My working assumption is that exposure to atmospheric N deposition is similar for bryophytes throughout the study site. If atmospheric N deposition is indeed responsible for the notable scarcity of cyanobacteria on feather mosses such as *Pleurozium schreberi* and *Hylocomium splendens,* as well as on *Dicranum* mosses, how do we reconcile this with the robust cyanobacterial presence associated with taxa in Amblystegiaceae, Brachytheciaceae, Fissidentaceae, Mniaceae, Pottiaceae, Thuidiaceae, and Lophocoleaceae? In all of these families, at least 40% of the affiliated samples host cyanobacteria. In the family Pottiaceae, almost every sample studied for the genus *Tortella* (93%) hosted a robust and diverse population of cyanobacteria. One possible explanation is species-, genus-, or family-specific responses to control mechanisms such as atmospheric N deposition; perhaps species such as *Tortella tortuosa* (Pottiaceae), and *Thuidium delicatulum* (Thuidiaceae), are relatively resistant to the disruptive effects of excessive N.

Another possible explanation is that the impacts of these large-scale control factors are mediated in some manner by microhabitat attributes. This study was designed to explore whether microhabitat attributes (substrate type and elevation with respect to

ground level) might explain variations in the odds detecting of cyanobacterial symbionts among bryophyte samples. Considering all bryophytes without regard for species, most substrates have no predictive power—only rock appears to be associated with an elevated probability of cyanobacteria. And the presence or absence of rock explains a relatively modest proportion of the deviance in the logistic regression model (< 20%). Still, it is somewhat surprising that one substrate would be so influential.

25 bryophyte species were collected from plots with rock substrate; most species were observed less than five times. The dominant moss species associated with rock are *Thuidium delicatulum* (*n* = 20), *Tortella tortuosa* (n = 13), and taxa in *Brachythecium* (*n* = 10). Rock substrate is not associated with a significantly higher-than-expected incidence of Nostocalean cyanobacteria, but heterocystous cyanobacteria do live on the rock-dwelling bryophytes. This suggests that N fixation may be associated with these bryophytes. The presence of non-heterocystous cyanobacterial N fixers (sometimes difficult to identify via microscopy) or heterotrophic N-fixers (not studied) is also possible. If N fixation is associated with bryophytes on these rock substrata, it may be interesting to investigate the prevalence and N-fixation capabilities of bryophytes in rocky environments such as cliffs, boulder fields, and forests with many glacial erratics. Rocks in the Door County area often bear evidence of low N availability; it is fairly common to see bright-orange *Trentepohlia* present on rocks near water, both inland and along the coast (Figure 16).

Figure 16. Some rock provide visual evidence of low nitrogen availability. This rock, found along the shore of Lake Michigan at nearby Toft's Point in Baileys Harbor, Wisconsin, provides a substrate for a variety of mosses, lichens, and algae, including the moss *Tortella tortuosa* and the green alga *Trentepohlia.*

Figure 16 shows one of the dominant rock-dwelling mosses found at the study site, *Tortella tortuosa* in Pottiaceae. The bright-green gametophytes of *Tortella* have tightly coiled phyllids, which are associated with desiccation tolerance. *Trentepohlia* is a subaerial green alga, but under low-nitrogen conditions, it is capable of intensified production of carotenoid pigments (Chen et al., 2016). The accumulation of carotenoids, in conjunction with a decline in chlorophyll concentrations, generates bright orange filaments in low-nitrogen habitats. Carotenoid accumulation is also intensified by high-light environments (Chen et al. 2016), which may be present in some boreal forest gaps. This made me wonder whether *Tortella tortuosa* on rock might be experiencing a nitrogen deficit that triggers symbiosis formation. Assays to study N fixation, as well as

genomic studies of the microbiome of *Tortella tortuosa*, might yield additional insights into this symbiotic system. The family Pottiaceae is strongly represented in data on the biodiversity of bryophyte-cyanobacterial symbioses, but information on mechanisms of symbiosis formation is much more limited than for mosses such as *Sphagnum* or *Pleurozium*.

Some of the larger rocks are glacial erratics, which are often dominated by *Thuidium delicatulum* (Figure 17). In shady habitats, these rocks are densely covered by groundcover moss. Rock-dwelling *Thuidium delicatulum,* like rock-dwelling *Tortella tortuosa,* frequently hosts heterocystous cyanobacteria. About 65% of the rock-dwelling *Thuidium delicatulum* samples host cyanobacteria, and about 50% host heterocystous cyanobacteria. As noted previously, the estimated abundance of cyanobacterial cells for *Thuidium delicatum* in all plots (not just those with rock) is highly variable. Informally, the mean estimated cell count for rock-dwelling *Thuidium* (~ 63,000 cells per slide) is much higher than that for *Thuidium* on other substrates (~ 4800 cells per slide).

If N fixation occurs at relatively high rates in association with these rock-dwelling bryophytes, stable isotope studies might determine whether the rock communities are a net source of N to the surrounding vascular plants. We can speculate that rocks might even serve as N fixation “hotspots,” areas in which N fixation rates are notably elevated with respect to background rates (McClain et al. 2003).

Figure 17. Glacial erratic dominated by *Thuidium delicatulum.* Common vascular plants such as baneberry (*Actaea alba* (L.) Mill., *Actaea rubra* (Aiton) Willd.) and big-leafed aster (*Eurybia macrophylla* (L.) Cass.) surround the boulder.

What about the other substrates? These results do not necessarily rule out a potential role for microhabitat attributes, but they do indicate the variables defined and measured for this study are (for the most part) not influential, including most substrates, SEASON, and LICHEN (presence or absence). It is possible that the substrate categories here are too broadly defined and do not reflect the nuanced differences among substrata. For example, the category WOOD includes coniferous wood and deciduous wood in the form of fine woody debris, coarse woody debris, logs, stumps, humus, and rotten logs and stumps. These potential habitats have various moisture-holding capacities, pH values, and nutrient availabilities. Some studies of bryophyte community structure (e.g., Newmaster 2000, Ódor & van Hees 2004, Cole et al. 2008) have found that characterizing logs by decay stage provided insights into bryophyte distribution and

richness; but others have found fine-scale distinctions among wood substrate types held little explanatory power. For example, Goia & Gafta's (2019) study of montane bryophytes found that most moss species (150 of 153) had no preference with respect to deadwood based on tree species (beech vs. spruce).

A study with more narrowly-defined substrate types may or may not reveal some additional structure that is not evident here. The merger of sparsely represented substrates into the awkward "non-conifer plant material" category certainly did not enhance the model's ability to detect meaningful associations. But a study with more microhabitat types, or more levels for a given microhabitat, would be difficult to execute from a statistical perspective. Even the relatively broad categories used in this study did not provide enough degrees of freedom to test a full model with interactions; a much larger sample (> 1000 plots) would be required to explore the influence of additional microhabitat variables, especially since narrowly-defined substrate categories are likely to be rare. Landscape-scale studies, such as Vitt & Belland's (1997) study of bryophyte diversity in Alberta, Canada, might make a larger number of microhabitats tractable; these researchers had over 20,000 vouchered herbarium specimens to work with in their analysis. However, the number of substrate categories ultimately used in this study (5) provided a sufficient sample size for each substrate, and did not preclude detection of a strong effect associated with rock. Measuring chemical microhabitat attributes directly (particularly those known to influence the distribution of cyanobacteria, such as pH, and the synthesis of the nitrogenase enzyme, such as P, Fe, Mo, or V) might be a more promising approach than elaborating on microhabitat categories at this site.

Another potential limitation of the study was the decision to record all substrates within the 21.5-cm plot. Most contained multiple substrates (e.g., a rock with soil and dried white cedar leaves on its surface), and my study design was based on the assumption that all could influence symbiotic status. An alternative approach might be to record only the substrate that held bryophyte rhizoids, under the assumption that the substrate providing a physical anchor is the most critical influence.

Could mesohabitat attributes, unexplored in this study, explain variability in the presence or absence of cyanbacteria on bryophytes? My study explored the potential impacts of forest structure and composition indirectly, via leaf-litter inputs. More recent work has tested the potential influence of forest composition directly. In Alaska, Jean et al. (2020) found that cyanobacterial abundances and N fixation rates for feather mosses were notably higher in boreal conifer forests than in boreal broadleaf forests dominated by *Betula neoalaskana.* By contrast, Rodríguez-Rodríguez et al. (2022) found that bacterial community composition differed significantly in boreal black spruce forests and boreal trembling aspen forests of northeastern Canada, and cyanobacteria were more strongly associated with the broadleaf forest—particularly for Nostocaceae. In both cases, tree composition, attributes of the mesohabitat, greatly influenced the structure and function of cyanobacterial communities and other moss-associated bacteria. Likewise, Renaudin et al. (2022) ascertained that tree density explained about 10% of the variability in relative abundance of *nifH* sequences for *Stigonema* and *Nostoc* associated with boreal feather mosses in North America. However, the Renaudin et al.

(2022) study was conducted across a large gradient in latitude. Mesohabit structure is less likely to vary notably within a single 7.5-acre site; as far as I know, the history of logging and disturbance is similar throughout my site. Expanding the study to multiple sites in the southern boreal zone of North America and examining tree community structure and composition might prove fruitful, as density and dominant tree species would vary more in a multi-site design.

SUMMARY

Overall, there is a wide range of variables, both biotic (e.g., moss identity and phylogenetic history) and abiotic, that may explain the differential association of cyanobacteria with various bryophyte taxa at this boreal forest site. These unidentified control factors are responsible for a large percentage of the variability in bryophyte host status (i.e., affiliated or unaffiliated with cyanobacteria). But rock substrates appear to have a strong affiliation for bryophytes that host cyanobacteria, explaining about 18% of the variability in symbiotic status; future research might help to clarify the mechanisms and functions of these symbioses, especially with respect to ecosystem-scale N fixation. The bryophyte taxa *Thuidium delicatum, Tortella tortuosa,* and various *Brachythecium* species are dominant species on rock at this site, with strong potential for heterocystous N fixation. Inhibition by atmospheric N deposition may explain the lack of cyanobacterial symbionts for the feather-mosses *Pleurozium schreberi* and *Hylocomium splendens,* as well as the dominant species *Dicranum scoparium*, but this is speculative; direct measurements of N inputs, as well as characterization of N fixation, might be necessary

to confirm this hypothesis. Moreover, if N deposition is the reason for the absence of cyanobacteria on taxa known as hosts (e.g., *Pleurozium schreberi, Hylocomium splendens*), species-specific thresholds for inhibition, previously documented in other areas (Zackrisson et al. 2009, Leppanen et al. 2013, Salemaa et al., 2018), may be responsible. At the level of bryophyte family, there is a great deal of variation in symbiotic status that cannot be predicted with the microhabit substrate variable, lichen presence, or season. Determining why Thuidiaceae, Pottiaceae, and the other highly symbiotic bryophyte families are not sensitive to the levels of N deposition that potentially incapacitate the feather-moss symbioses could be a useful next step in conjunction with a broader exploration of biotic and environmental controls.

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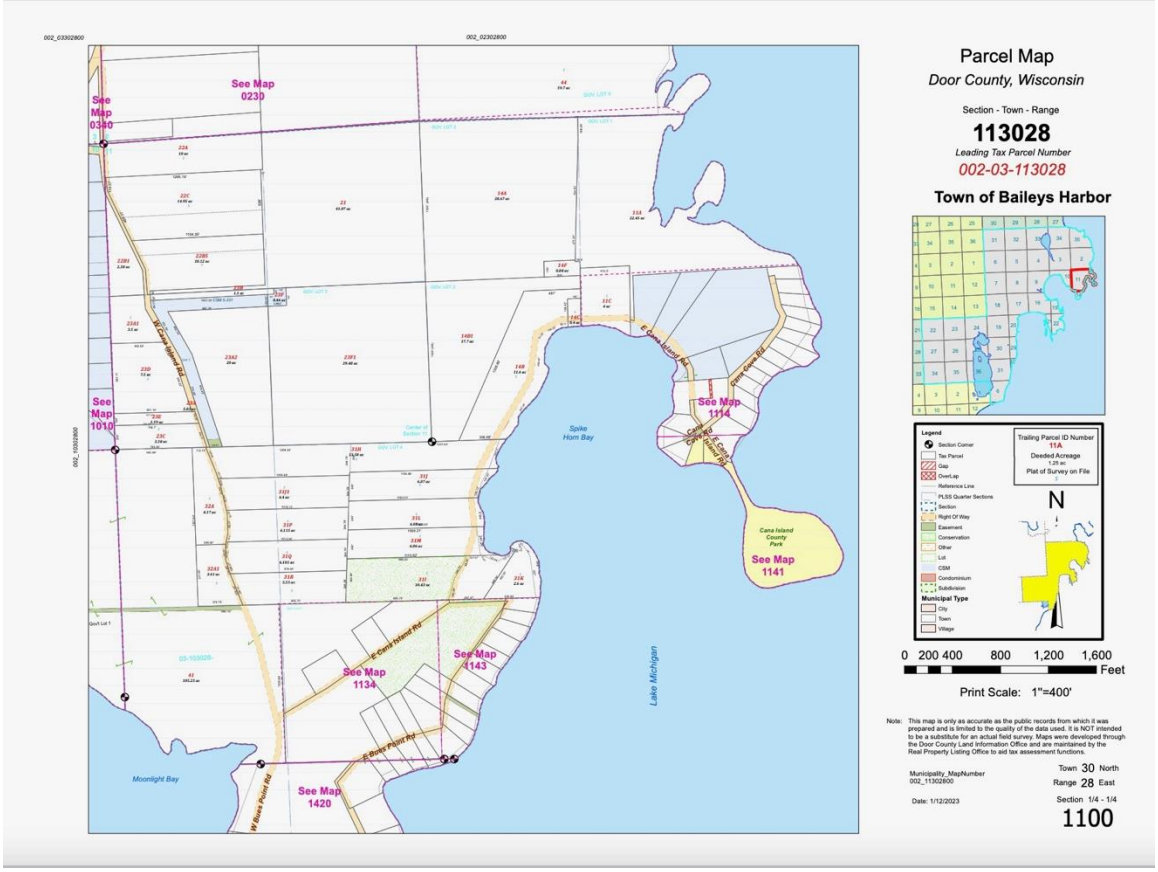
Appendix A: Baileys Harbor Parcel Map with Study Site

The study was conducted on Parcel 311, located in T30N-R28E, Section 11 SW ¼ of Bailey's Harbor, Wisconsin.



Source:

Door County Tax Parcel Maps. 2023.
https://www.co.door.wi.gov/DocumentCenter/View/5587/T_Baileys-Harbor-30-28
Accessed online July 20, 2023.

Appendix B: Substrate Data

Microhabitat categories

1. Conifer litter (CL)
2. Deciduous litter (DL)
3. Wood (WO)
4. Rock (RO)
5. Soil (SO)
6. Grass (GR)
7. Scat (SC)
8. Moss mat (M)

Categories (1)-(8) were used in analysis with logistic regression. The table below shows the codes that were used the field, the substrate asssociated with each code, and the categories to which each code was assigned for analysis.

Code	Substrate	Microhabitat category
BARK	Bark	Wood
BR	Branch	Wood
CL	Cedar Litter	Conifer Litter
DL	Deciduous Litter	Deciduous Litter
CWD	Coarse Woody Debris	Wood
FWD	Fine Woody Debris	Wood
GR or G	Grass	Grass
LO	Log	Wood
M	Moss	Moss mat
PL	Pine Litter	Conifer Litter
SO or S	Soil	Soil
RC	Root Collar	Wood
RL	Rotten Log	Wood
SOR	Soil on Rock	Soil
TW	Twig	Wood
WC	Wood Chip	Wood

SOR: Soil counted as potential substrate; rock is not, unless given a separate entry as RO.

Appendix C: Exploratory data analysis for predictor variables

Contingency tables: substrate count data

Conifer litter		**0**	**1**
Cyanobacteria	**0**	155	203
	1	73	47

Deciduous litter		**0**	**1**
Cyanobacteria	**0**	328	30
	1	112	8

Wood		**0**	**1**
Cyanobacteria	**0**	150	208
	1	67	53

Soil		**0**	**1**
Cyanobacteria	**0**	199	159
	1	90	30

Rock		**0**	**1**
Cyanobacteria	**0**	340	18
	1	67	53

Grass		**0**	**1**
Cyanobacteria	**0**	313	45
	1	108	12

Moss mat		**0**	**1**
Cyanobacteria	**0**	339	19
	1	118	2

Scat		**0**	**1**
Cyanobacteria	**0**	358	0
	1	120	0

Contingency table: lichen data

Lichen		**0**	**1**
Cyanobacteria	**0**	266	92
	1	92	28

These tables reflect the distribution of data across all plots with bryophyte material sufficient for sampling (*n* = 478).

Appendix D: Exploratory data analysis for predictor variables

Contingency tables: substrate count data with merged plant materials

Non-conifer plant materials (NCPM):

1 = at least one observation of deciduous litter, grass, or moss mat within the plot of interest

0 = no observations of deciduous litter, grass, or moss mat within the plot of interest

Conifer litter		**0**	**1**
Cyanobacteria	**0**	155	203
	1	73	47

Non-coniferous plant matter		**0**	**1**
Cyanobacteria	**0**	270	88
	1	99	21

Wood		**0**	**1**
Cyanobacteria	**0**	150	208
	1	67	53

Soil		**0**	**1**
Cyanobacteria	**0**	199	159
	1	90	30

Rock		**0**	**1**
Cyanobacteria	**0**	340	18
	1	67	53

Contingency table: lichen data

Lichen		**0**	**1**
Cyanobacteria	**0**	266	92
	1	92	28

These tables reflect the distribution of data across all plots with bryophyte material sufficient for sampling (*n* = 478) after merging observations in the categories deciduous litter, grass, and moss mat.

Part IV: The influence of moisture limitation and wetland habitat on bryophyte-associated N fixation in a beach-ridge ecosystem along Lake Michigan

Key results from Part IV. The shores of the Laurentian Great Lakes have long been a laboratory for ecologists seeking to understand how newly-deposited sand is changed by nutrient cycling and ecosystem development over time. In Baileys Harbor, Wisconsin, the alternating wetland swales and sandy upland dunes of a beach-ridge complex along Lake Michigan are characterized by bryophyte-dominated groundcover; this diverse collection of microhabitats harbors a rich array of bryophyte species. This study focuses on two potential controls of bryophyte-associated N fixation in the beach-ridge ecosystem: (1) site status (upland ridge versus wetland swale), and (2) the degree of saturation for a given bryophyte sample, identified to species. Paired bryophyte samples (two from each randomly-located sample plot) were collected at 10 sites on multiple dates along a substrate-age gradient extending from young, sandy beach sediments to older sediments inland. In each pair of bryophyte samples, both were subjected to the acetylene reduction assay, one at ambient moisture levels and one at saturated moisture levels, to determine whether dry environmental conditions might be limiting N fixation potential. Sites were also paired by substrate age (one wetland swale site, one upland ridge site).

A general linear model utilizing the Likelihood Ratio Test provided strong evidence that wetland status is associated with a higher probability of detecting symbiotic N fixation. The water saturation treatment increased the probability of detecting N fixation by a small but statistically significant amount, implying that short-term moisture limitations

may decrease annual N fixation rates in about 12% of all samples. A quantitative “pilot study” based on limited seasonal data and strongly simplifying assumptions suggests the losses may be up to 0.6 kg N ha^{-1} yr^{-1} (upland ridges) and 1.6 kg N ha^{-1} yr^{-1} (wetland swales); more detailed seasonal data on N fixation in the wetlands could better resolve any error associated with these tentative estimates. Overall the evidence suggests moisture limitation plays a minor but measurable role in determining whether bryophyte-associated N fixation will occur on any given day, and raises questions about the potential impact of moisture limitations on longer time scales, e.g., seasonal, annual, or decadal.

Within the context of ecosystem development across the substrate-age gradient, the wetland beach sites were dominated by mosses likely to harbor active N-fixing microbes, primarily from the bryophyte families Amblystegiaceae, Bartramiaceae, Bryaceae, and Hypnaceae. The estimated probability of detecting N fixation in wetland bryophyte samples decreased with swale age, ranging from 0.70-0.97 (beach sites) to 0.17 (oldest wetland along gradient) based on pooling all samples by site. However, the probability of detecting N fixation at any of the upland ridge sites was low; pooling upland samples across all sites and dates yielded an estimated probability of 0.05. These negative results were strongly associated with dominant upland mosses in Dicranaceae and Hylocomiaceae; less than 25% of the mosses in Dicranaceae produced measurable N fixation, and less than 5% of the feather mosses in Hylocomiaceae.

In spite of limited N fixation in upland sites, almost half of the bryophyte taxa studied (20 of 46 taxa) were associated with measurable N fixation in at least one sample. Over 70% of the bryophyte genera studied (26 of 32 genera) have demonstrated symbiotic N fixation activity in either the beach-ridge system in Baileys Harbor, Wisconsin or in an existing publication. 20% of the bryophyte species studied generated potentially novel positive results for N fixation, despite a bias towards negative results due to logistical difficulties with *in-situ* incubation for the acetylene reduction assay.

The high probability of detecting N fixation at wetland beach sites suggests that bryophyte-microbe associations may provide substantial inputs of fixed N to sandy beach soils and vascular plants. However, the near-total absence of N fixation on the upland ridges is harder to explain; the minimal impact of the water treatment on bryophytes from these areas indicates that short-term water limitations play a minor role in the suppression of N fixation on the upland ridges. Investigations of other control factors are needed to understand why so many of the mosses and liverworts in the upland areas of this beach-ridge ecosystem do not harbor active N-fixing microbes, especially with respect to the feather mosses in Hylocomiaceae and various species of moss in Pottiaceae.

INTRODUCTION

One of the earliest studies of biotic nitrogen fixation was conducted by W.D.P. Stewart (1967) in a dune system in Blakeney Point, Norfolk, England. Stewart focused on soil

samples from the moist regions of system, known as dune slacks. These low-lying areas, formed by wind erosion, harbor moist soils and abundant cyanobacteria. Using stable isotope methods, Stewart documented high rates of N fixation in soil samples, as well as the transfer of fixed N from cyanobacteria to dune plants including the moss *Bryum pendulum*[9] and three dune grasses. Stewart (1967) argued that the cyanobacteria, as early colonizers of the sand dunes, provide fixed nitrogen to higher plants and may make colonization by vascular plants more feasible, helping to physically stabilize the dune systems. But he did not directly study the N fixation potential of epiphytic bacteria in dune bryophytes.

Plant-microbial associations are a potentially critical source of organic nitrogen in many ecosystems. Among the plants that associate with N-fixing bacteria, the bryophytes (mosses, liverworts, and hornworts) may respond with unusual speed to environmental change, given the lack of water-conservation mechanisms (e.g., roots, stomata on leaves, multistratose tissues) characteristic of vascular plants. Bryophyte groundcover in a beach-ridge ecosystem in Bailey's Harbor, Wisconsin was used to test the following hypotheses:

[9] The name *Bryum pendulum* is no longer valid; Stewart did not cite an authority. The taxa formerly recognized as *Bryum pendulum* (under several different authorities) are now assigned to at least three different legitimate names, all members of Bryaceae (Tropicos 2023). One of these species, *Bryum algovicum* Sendt. ex Müll Hal., was documented along shores of the Great Lakes by Howard Crum and is similar to the sterile specimens assigned to *Bryum* sp. from the two beach sites (Baileys Harbor, Wisconsin) in this study.

(1) **Bryophytes of wetland swales harbor active N-fixing bacteria more often than bryophytes of adjacent dune ridges**; and

(2) **Lack of water on short time scales (e.g., within a given day) limits the activities of any N-fixing bacteria associated with beach-ridge coastal bryophytes**.

METHODS

Site Description: The Ridges Sanctuary. The Ridges Sanctuary (Figure 1), founded in 1937, is a private, non-profit organization charged with the protection of over 400 ha of land in Baileys Harbor, Wisconsin. The sanctuary prioritizes conservation and informal education, with an emphasis on the rich vascular flora and historical value of the landscape. 475 species of vascular plants have been documented within the sanctuary borders (Trick, 1983), including over two dozen types of orchids. Some of these plants are rare or endangered, such as the federally-endangered dwarf lake iris (*Iris lacustris* Nutt.) and the small round-leafed orchid (*Orchis rotundifolia* Banks ex Pursh). Wetland areas are home to a significant population of the federally-endangered Hine's emerald dragonfly (*Somatochlora hineana* Williamson). There is also an abundant cryptogam community, including a diverse array of mosses, lichens, and liverworts. The unusually cool and moist conditions at The Ridges enable plants typical of climates farther north to thrive, including trailing arbutus (*Epigaea repens* L.), creeping snowberry (*Gaultheria hispidula* (L.) Muhl. ex Bigelow), blue-bead lily (*Clintonia borealis* (Aiton) Raf.), twinflower (*Linnaea borealis* L.), bunchberry (*Cornus*

canadensis L.), and the arctic primrose (*Primula mistassinica* Michx.). These diverse vascular and nonvascular plants comprise at least 15 distinctive plant communities (Trick 1983).

The sanctuary is named for its distinctive topography: it is a *beach-ridge complex,* a landscape composed of " . . . relict, semiparallel, multiple wave- and wind-built landforms that originated in the inter- and super-tidal zones . . . " (Otvos, 1999). In the Great Lakes area, the ridges typically consist of sand and an upper layer of eolian sediment (Olson, 1958; Thompson, 1992; Thompson and Baedke, 1997). Wetland swales, some of which may persist year-round, often form in the low-lying areas between ridges. Approximately 95 beach-ridge complexes have been found along the shores of the Great Lakes (Michigan Natural Features Inventory, 2007); these are often rich repositories of geological, ecological, and cultural history.

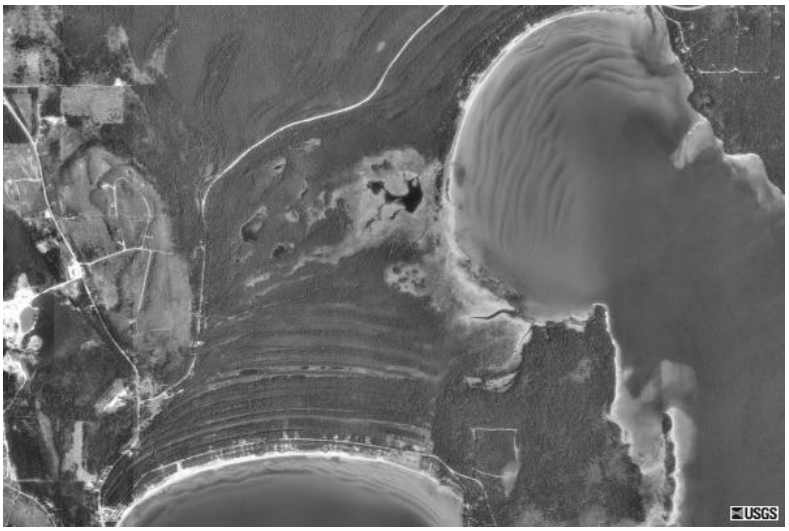


Figure 1. An aerial view of The Ridges Sanctuary in Baileys Harbor, Wisconsin.

Source: USGS.

A subset of 40 sites found in Michigan has an area of 28,370 ha (Michigan Natural Features Inventory, 2007); if these sites were representative with respect to area, beach-ridge complexes may have occupied approximately 60,000 ha[10] along the Great Lakes shoreline. The broad distribution of beach-ridge complexes in the upper Midwest, coupled with their potentially extensive area, suggests that a better understanding of bryophyte-cyanobacterial associations at The Ridges has the potential to contribute to our understanding of the Great Lakes region as a whole. Furthermore, the Nature Conservancy has designated such ecosystems as *globally rare* (Comer and Albert, 1993), suggesting conservation of beach-ridge complexes may be a global priority.

In the Great Lakes region, paleoecological and geological evidence indicates that the formation of individual ridges is associated with falling lake levels and climate change, particularly periods of drought (Olson, 1958; Lichter, 1995; Thompson and Baedke, 1997). During these dry periods, sediments accumulated in the foreshore area, as illustrated in Figure 2 (Thompson and Baedke, 1997). Several decades after the original ridge formed, a newer, younger ridge would eventually develop along the shoreline, parallel to the first. Water trapped between the two ridges, in what was originally the backshore area, sometimes developed into a low-lying wetland swale. The Ridges, like many other Great Lakes beach-ridge complexes, contains evidence suggesting that individual ridges may have migrated and merged with one another, or been destroyed after their formation (Johnson & Stieglitz, 1990).

[10] As far as I know, no comprehensive estimate of area exists for all beach-ridge complexes in the Great Lakes area.

The interval between ridge-formation events at Baileys Harbor has been estimated at approximately 31 yr, with an error of +/- 10 yr (Thompson & Baedke, 1997). These estimates were based on radiocarbon age estimates of 10 different ridges, and are similar to estimates based on other Great Lakes beach-ridge complexes (30 yr, Olson, 1958; 29-38 yr, Thompson and Baedke, 1997; 33 yr, Lichter, 1995). However, these average values cannot be interpreted as evidence of ridge formation at regular intervals. J. Lichter's work with accelerator mass spectrometry dates of plant macrofossils at Wilderness Park, Michigan (1997) revealed great differences in the rate of ridge formation over time: estimated intervals ranged from 17-135 yr.

Thompson & Baedke (1997) note that their radiocarbon dates for The Ridges are poorly constrained, with a laboratory error of ± 65 yr for the average time period between the formation of successive ridges. In addition, there is the potential for contamination by hard groundwater in dates from The Ridges. However, these issues may not be critical; the dates for Baileys Harbor correlate well with dates and lake-level histories from other beach-ridge complexes.

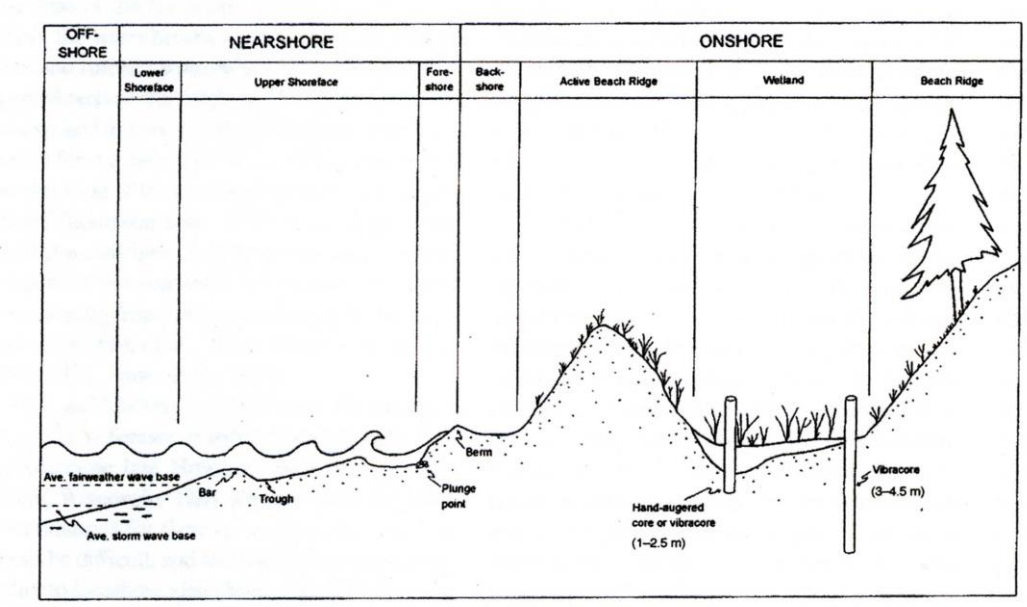


Figure 2. Ridge formation. A cross-section of a beach-ridge complex, showing the formation of a ridge in the foreshore area and two dune ridges further inland. From Thompson & Baedke, 1997.

More precise dates for the ridge sediments were generated using optically-stimulated luminescence techniques (Argyilan et al., 2010); these are given below in Table 1. Distances from the shoreline were graciously provided by Erin Argyilan (pers. comm., 2009).

Ridge number	Ridge distance (meters landward)	Optically-stimulated luminescence (OSL): years before 2000 A.D.	Age calendar year A.D.
2	166.7	80 ± 20	1900-1940
8	700.0	410 ± 45	1550-1645
12	1366.7	595 ± 60	1435-1465
24	2583.3	830 ± 80	1090-1250
28	3166.6	930 ± 95	975-1165

Table 1. Optically-stimulated luminescence dates for selected ridges at Baileys Harbor.

Sediments from near-shore areas have basal dates of approximately 100 yr; sediments from the innermost and oldest ridges have basal dates of approximately 1000 yr; and sediments from intermediate locations have age estimates that fall between these two extremes, although it is difficult to match exact coring sites from Argyilan et al. (2010) to bryophyte plot locations given complex topography and dense vegation. Overall strong evidence indicates that there is a substrate-age gradient at The Ridges, even if the ages of certain individual ridges have not been determined.

The timing of individual ridge-forming events is not the only evidence of fluctuations in the Lake Michigan basin recorded in the geomorphology of the beach-ridge complex. Superimposed on this pattern of short-periodicity climate change (with fluctuations related to ridge formation occurring every 30 yr or so) is a long-periodicity pattern of relatively large-magnitude climate change, evident in groups of ridges within which the foreshore elevation rises and falls (Thompson & Baedke, 1997). Typically these groups consist of four to six ridges, often separated from those in other groups by an unusually wide swale. Such groups were identified at The Ridges by multiple research teams (Johnson & Stieglitz, 1990; Thompson & Baedke, 1997), and at other sites throughout the Great Lakes, with elevational peaks and dates that correlated well across sites (Thompson & Baedke, 1997). Assuming that basin-wide events resulted in delineation of the groups, the initial ridge in a group most likely formed after a major change in climate and lake level. However, like the ridge-formation events, group-delineation events were not strictly periodic; estimates of the time between formation of groups range from 120 to 180 yr (Thompson & Baedke, 1997). On average, such events

occurred at intervals of 160 yr over the past 4500 yr (Thompson & Baedke, 1997), dating back to the Nipissing high-stand of Lake Michigan.

Topographic relief within the Baileys Harbor complex is modest, with a total change in elevation of approximately 2.4 m from the shore to the innermost ridges (Johnson & Stieglitz, 1990); individual ridges have heights of 0.5-2.0 m (Thompson & Baedke, 1990). Given the modest elevation of the ridges, determining what constitutes an individual ridge is somewhat subjective; estimates of the number of ridges based on aerial photographs vary between 17 and 30 (Johnson & Stieglitz, 1990; Thompson & Baedke, 1997). The topography creates linear wetland areas parallel to the ridges, meaning that there is a gradient in soil moisture that fluctuates quasi-periodically over distances of approximately 30 m. This spatial pattern in soil moisture is characteristic of many beach-ridge systems in the Great Lakes area, and typically influences the development of soils over time. Generally the drier ridge-tops have relatively little organic soil, often less than 3 cm, whereas the moister swales typically accumulate more organic soil, often 4-25 cm (Michigan Natural Features Inventory, 2007). No detailed studies of soil nutrients across the substrate-age gradient have been conducted at The Ridges.

Three soil types are found in the central areas of The Ridges where sampling occurred. The shoreline areas are dominated by wet beach deposits, characterized as " . . . somewhat poorly drained . . . . Deep, sandy, and cobbly soil . . . " (Door County, Wisconsin Soil and Water Conservation District, 1972). Approximately 100 m inland one finds a thin band of Wainola loamy fine sand; and the inland ridges have soils assigned

to the Rousseau-Wainola-Roscommon complex (Door County, Wisconsin Soil and Water Conservation District, 1972). Niagara dolomite, a dominant bedrock form in Door County, lies underneath these soils.

Sampling Design. The hypotheses on wetland status and water limitation were tested in paired upland ridge/wetland swale sites in Baileys Harbor, Wisconsin. Most of the sites are concentrated in and near The Ridges Sanctuary, encompassing progressively older sediments as distance from the lakeshore increases. Additional sites are located on the beach at nearby Moonlight Bay, off Stone Mill Lane in Baileys Harbor. Moonlight Bay also has ridge-swale topography, but the topography is much less pronounced (just two or three ridges are evident). The beach sites on Moonlight Bay were selected to increase the area available for sampling the coastal bryophyte communities; the lakeshore is heavily developed (e.g., vacation homes and condominiums) and only a few small sites have relatively undisturbed bryophyte groundcover suitable for study.

For the purposes of this study, a *site* is defined as a sampling area encompassing 1275 m^2. Each 85 m x 15 m site characterizes either an upland ridge or an adjacent wetland swale. Attributes of sites are summarized in Table 2.

Code	Name	Status	Dimensions	Sediment Age (relative)	Number of bryophytes sampled per date
1 SM	Stone Mill Lane Beach	Wetland	85 m x 15 m	Youngest: < 100 yr	5
1 SM	Stone Mill Lane Beach	Upland	85 m x 15 m	Youngest: < 100 yr	5
1 TB	Baileys Harbor Town Beach	Wetland	85 m x 15 m	Youngest: < 100 yr	5
1 TB	Baileys Harbor Town Beach	Upland	85 m x 15 m	Youngest: < 100 yr	5
2 SS	The Ridges Sanctuary: South Sandy Ridge	Wetland	85 m x 15 m	Approximately 100 yr	5
2 SS	The Ridges Sanctuary: South Sandy Ridge	Upland	85 m x 15 m	Approximately 100 yr	5
3 DL	The Ridges Sanctuary: Deerlick Ridge	Wetland	85 m x 15 m	Approximately 400 yr	5
3 DL	The Ridges Sanctuary: Deerlick Ridge	Upland	85 m x 15 m	Approximately 400 yr	5
7 HU	The Ridges Sanctuary: unnamed ridge	Wetland	85 m x 15 m	Approximately 800 yr	5
7 HU	The Ridges Sanctuary: unnamed ridge	Upland	85 m x 15 m	Approximately 800 yr	5
Total number of samples per date					50

Table 2. Attributes of the sampling sites in Baileys Harbor, Wisconsin.

The rectangular site defines a general area (upland or wetland) to be characterized by subsampling with 21.5 cm circular *plots* for bryophytes. Random coordinates were generated to locate circular plots within each site for each sampling day. The dominant species of bryophyte found in each plot was collected, if bryophytes were present. If bryophytes were not present within a given plot, the nearest location with bryophyte cover was utilized (selecting direction randomly and checking for cover at 1 m intervals). This plot-based sampling procedure was repeated until 5 bryophyte samples had been collected for each rectangular site on an individual sampling date.

Acetylene Reduction Assay. The acetylene reduction assay (Stewart et al. 1967), an indirect measure of nitrogen-fixing activity in biological systems, was applied to 300

bryophyte specimens collected from plots in the summer of 2011. Acetylene reduction assays (ARAs) were conducted during three different weeks: July 27-August 1, and August 25-31, and October 21-24. In the field, three ARA samples were prepared for each plot: a sample with bryophyte tissue at ambient moisture levels, a sample with bryophyte tissues at saturated moisture levels, and a control vial. All samples utilized 10 mL glass vials sealed with a rubber stopper and an aluminum crimp seal to minimize leakage of gases. For the ambient moisture bryophyte sample, green shoots encompassing approximately 20% by volume were sealed into a glass vial (headspace approximately 8 mL). Detritus and senescent tissue (e.g., older increments of *Dicranum* sp.) were removed prior to the assay. After sealing the bryophyte tissue into the vial, 0.8 mL of air was withdrawn from each vial with a glass syringe, and then 0.8 mL of high-purity acetylene gas was introduced into the sealed vial by syringe. A similar process was applied to the saturation treatment sample, the notable difference being the addition of water to the bryophyte sample (to the point at which all tissues appeared saturated and dripped with moisture). The control vial was prepared with acetylene but without bryophyte tissue.

Each bryophyte ARA sample was allowed to incubate *in-situ* for 24 hr, subject to environmental variation with respect to light and temperature. The two bryophyte samples from each plot were incubated with a control vial consisting of 10% acetylene, to determine whether ethylene produced by environmental sources (or contaminants in the acetylene) were distorting the signal. After 24 hr, the assay was terminated by transferring gases from the headspace of the bryophyte vial to a rubber-stoppered and

crimp-sealed 10 mL vial to which a vacuum of -29 psi had been applied for 30 s, creating negative pressure within the vial.

All bryophyte samples for a given plot (ambient moisture and saturated) involved the same bryophyte species, although occasionally a small number of shoots from a second species was also present.

After the 24-hour incubation period had been terminated, the samples were transported to Madison, Wisconsin for analysis by gas chromatography as soon as possible (1-5 days). The gas chromatograph utilized N_2 (g) at 200 psi, air at 50 psi, and H_2 (g) at 75 psi with a sample volume of 200 µL. A standard calibration curve was utilized to estimate the area under the curve associated with the ethylene peak.

Determination of the Method Detection Limit. The threshold for a positive ARA result (i.e., amount of ethylene that provides evidence of N fixation) is specified by the Method Detection Limit (MDL). Values equal to or greater than the Method Detection Limit constitute a positive response. According to Guidelines for Quality Management in Plant and Soil Laboratories (van Reeuwijk & Houba, 1998), a standard definition of a positive response is specified by “the value of the blank plus three times the standard deviation of the blank.” The threshold concentration of ethylene for a positive result was determined based on the distribution of the area under the curve for ethylene in 251 control vials (10% acetylene, no bryophyte tissue). In my study, the control vials function as blanks. Summary statistics for the control vials are given in Table 3.

Number of blanks	Mean area under curve for ethylene peak	Standard deviation of area under curve
251	0.031861	0.034087

Table 3. Blank attributes (control vials with 10% acetylene, no bryophyte tissue).

The computed value of the method detection limit is 0.13412, suggesting that values for the area of the ethylene peak ≥ 0.13412 constitute positive responses (ethylene present and detectable, N fixation evident).

The distribution of values for the ethylene peak based on 251 control vials is shown in Figure 3, along with the value of the computed method detection limit.

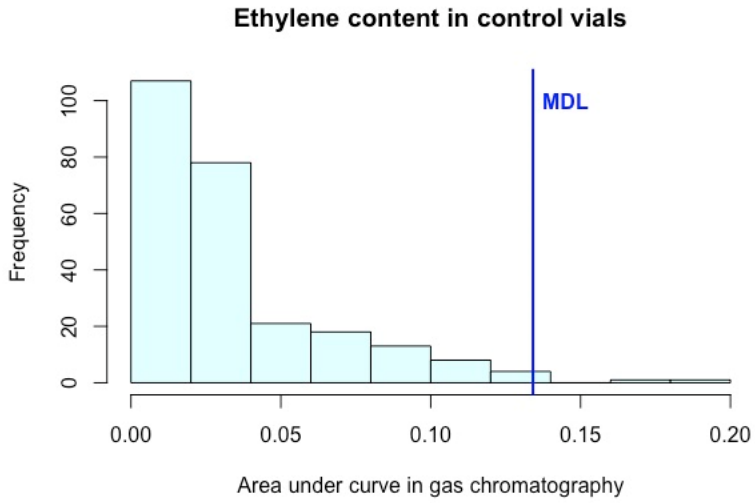


Figure 3. Computed value of Method Detection Limit (MDL). Only a handful of the control samples (10% acetylene, no bryophyte tissue) contained trace amounts of ethylene contamination greater than the Method Detection Limit of 0.13412.

Given that only 3 of the 251 controls would be defined as positive results under the computed Method Detection Limit, the rate for false positives would be approximately 1.2%. I used bootstrapping methods to characterize the uncertainty associated with this estimate, as well as with a range of plausible alternatives for the Method Detection Limit

(0.1000, 0.12500, 0.15000, 0.17500, 0.12000). The distribution of false positive values (measured as a percentage of all observations) for each MDL is visualized in Figure 4. No histogram is shown for the MDL = 0.200, because no false positives occur at this limit.

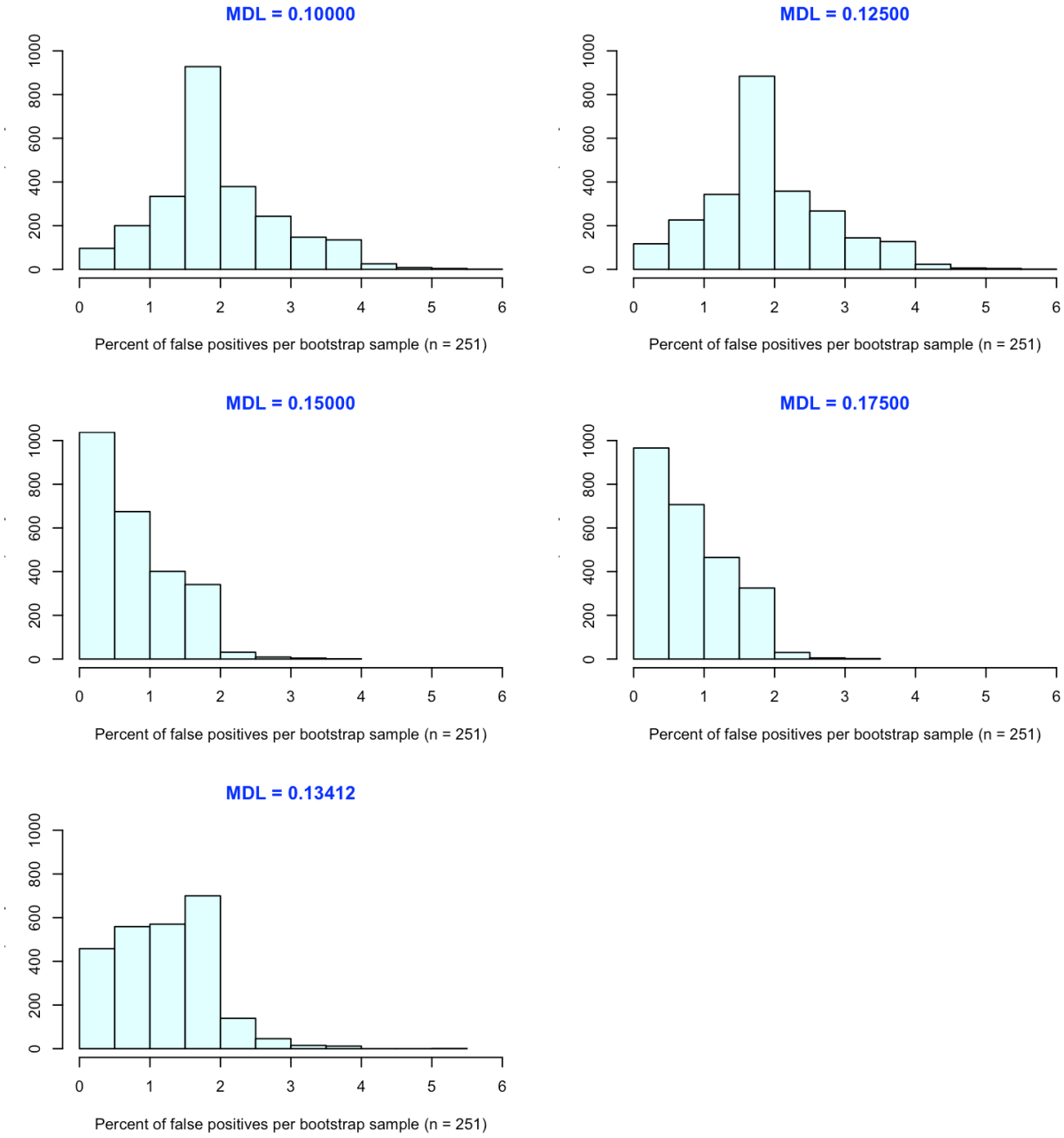


Figure 4. Uncertainty in false positive error based on bootstrap ped data. The number of bootstrapped replicates = 2500, sampling without replacement from blank data (10% acetylene).

Summary statistics for false-positive error rates based on the bootstrapped data are given in Table 4.

Method Detection Limit	Mean of false positive observations (%)	Standard deviation of false positive observations (%)	Standard error of false positive observations (%)	99% confidence interval
0.10000	1.99299	0.87956	0.055517	(1.82644, 2.15954)
0.12500	1.95936	0.89489	0.056485	(1.78991, 2.12882)
0.15000	0.79984	0.57220	0.036117	(0.69149, 0.90819)
0.17500	0.80829	0.55559	0.035069	(0.70308, 0.91349)
0.13412	1.21562	0.68999	0.043552	(1.08496, 1.34627)

Table 4. False-positive error rates and confidence intervals based on bootstrapped data (blanks: 10% acetylene, no bryophyte) with 2500 bootstrap replicates, n = 251**.**

The lowest mean values for false positive observations based on the percentage of misclassified observations (approximately 0.8%) were associated with Method Detection Limit Values of 0.150 and 0.175. The width of the confidence interval was almost identical for 0.150 and 0.175. I chose to use 0.150 as the Method Detection Limit in analysis given its relatively low rate of false-positive errors (~ 0.8%), although 0.175 would be reasonable, too.

It is not possible to characterize the rate of false negative results associated with this limit. Even if I can ascertain via microscopy that a bryophyte sample hosts N-fixing cyanobacteria, that does not necessarily mean that the microbes are actively fixing nitrogen at the time of the assay. With the methods accessible to me, there is no reliable method of characterizing false-negative errors. Thus my approach to selecting the Method Detection Limit is fundamentally conservative, and focuses on minimizing false-positive errors.

General Linear Models for Inference. A generalized linear model with a binomial error structure was applied using the *glmer* function in the R package *lme4* (version 1.1-27) The ARA results constituted the binomial response variable (0 = absence of N-fixing activity, 1 = presence of N-fixing activity). Predictors included two fixed-effects terms (wetland status and water treatment) and two random-effects terms (site and sampling date).

Given that only 5 samples per treatment were assayed for each wetland or upland site on a given date, the potential values of $\hat{p}$ are 0.0, 0.2, 0.4, 0.6, 0.8, 1.0. The complementary log-log link function was specified given its suitability for potentially extreme values of $\hat{p}$ (e.g. 0.2, 1.0) (Zuur et al., 2009). Variables in the model are characterized in Table 5.

Variable name	Definition	Type
Nfix	Characterizes evidence of N fixation associated with a given bryophyte sample (0 = evidence not detected, 1 = evidence detected) based on the area of the ethylene peak and the Method Detection Limit (0.015) for sample	Binomial
Wetland	Characterizes status of a given ridge site (0 = upland, 1 = wetland).	Binomial
Water	Characterizes nature of treatment applied to a given bryophyte sample (0 = ambient moisture conditions, 1 = bryophyte saturated with water) prior to the acetylene reduction assay.	Binomial
Date	Identifies sampling dates (0 = July 27 – August 1, 1 = August 25 – 31, 2 = October 21-24) in 2011.	Categorical
Site	Identifies ridge location in relation to lakeshore (1TB, 1SM = beach site, 2, 3, and 7 are inland ridges at increasingly greater distances from the lake).	Categorical

Table 5. Attributes of model variables.

Nitrogen fixation (*Nfix*) is the response variable. *Wetland* and *Water* are predictor variables treated as fixed effects. *Date* and *Site* are treated as random effects given my focus on inference with respect to (1) the influence of upland/wetland status for a given

ridge, and (2) water limitation for individual bryophyte samples. This also conserves degrees of freedom given a modest sample size (n = 300 total, with n = 5 for each unique combination of site, date, and treatment status (wetland status + water)).

The random effects have a crossed structure: date is not nested within time, nor is time nested within date (Figure 5).

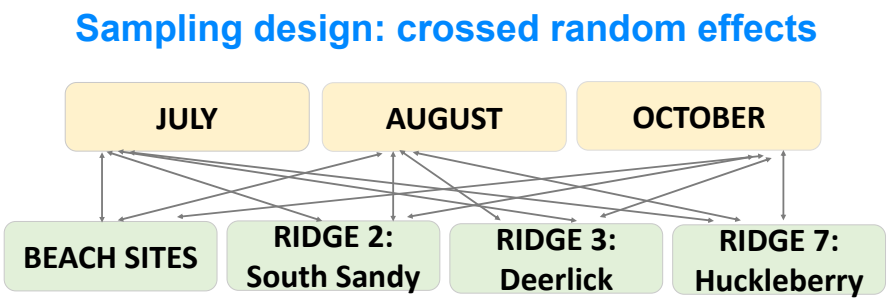


Figure 5. Crossed Effects Structure. The random effects do not have a hierarchical structure; each date is associated with more than one site, and vice versa.

Given crossed random effects, the model specified in R is

```
cross_model <-glmer(Nfix ~ Wetland + Water + (1|Date) + (1|Site), data = sat_data,
                    family = binomial(link = "cloglog"))
```

The default mode of inference for fixed effects in *glmer* is the Wald test, which provides results equivalent to those of the Likelihood Ratio Test under conditions of asymptotic convergence, *i.i.d*. residuals, and uncorrelated predictors for the fixed-effect variables (Social Science Computing Collaborative, University of Wisconsin-Madison, 2023). Given that we have a binomial response variable (*Nfix*) and relatively small subgroups (n = 5), it is unlikely that distributions of parameter estimates converge to parametric forms. I chose the Likelihood Ratio Test, rather than the Wald Test, to examine the potential impacts of the fixed effects (wetland versus upland status for a given ridge,

and the presence or absence of saturated moisture conditions for a given sample). The test was conducted with the command *drop1* in R package *stats* (version 4.1.0).

Computation of variance inflation factors (Monette & Fox, 1992) was executed with *vif* in R package *car* (version 3.0-10). The command *confint* in the R package *stats* (version 4.1.0) was used to compute confidence intervals for both fixed and random effects.

Bryophyte Diversity. The diversity of the bryophytes assayed for N fixation activity was characterized. Bryophyte identifications are based on Crum (2004) for mosses, and Crum (1991) for liverworts. No hornworts were captured in the sample. Tropicos (Missouri Botanical Garden, 2023) was used to assign currently legitimate names to taxa. Most samples were identified to species, although sterile specimens made it impossible to differentiate among species within a genus in a few cases. Diversity analysis focused on bryophyte families, rather than species or genera, due to the rapidly evolving state of bryophyte phylogenetics. For example, the species *Bryum algovicum* Sendt. ex Müll Hal., documented on Great Lakes beach sites by Howard Crum, was characterized as *Plagiobryum algovicum* (Sendt. ex Müll Hal.) Pederson, comb. nov. (Pederson & Hedenäs 2005) over a decade ago; but Tropicos currently recognizes both *Bryum algovicum* Sendt. ex Müll Hal. and *Plagiobryum algovicum* (Sendt. ex Müll Hal.) Pederson as legitimate names. Furthermore, Holyoak (2021) has assigned the taxon to a new genus, *Ptychostomum compactum* Hornsch. var. *compactum;* this third name is also deemed legitimate by Tropicos. Three legitimate names are currently associated with the morphotype taxon defined by Crum (2004); but all three taxa belong to the

family Bryaceae. Similar inconsistencies, a function of intense phylogenetic research and revision in recent years, exist for several other species names. For these reasons I have chosen to work with family, rather than species or genera, as the primary unit of analysis for diversity.

RESULTS

Frequency of N fixation at upland and wetland sites in the beach-ridge complex. At paired upland and wetland sites with similar substrate age, wetland sites were more likely to harbor N-fixing symbioses. Pooling samples across all dates, the mean frequency of N-fixing activity in wetland sites was approximately 45%; the mean frequency in upland sites was approximately 5%. Figure 6 shows site-specific seasonal frequencies for N fixation, based on pooling data across all dates for a given site.

Site	Seasonal Frequency: UPLAND	Seasonal Frequency: WETLAND	Relative Substrate Age
CLOSE TO LAKE MICHIGAN (YOUNGEST)			
1A: Lakeshore site (Stone Mill Road). Wet sedge meadow and sandy dune ridge.	3.3%	96.7%	Youngest
1B: Lakeshore site (Town Beach). Wet sedge meadow and sandy dune ridge.	13.3%	70.0%	Youngest
2: Sandy upland ridge with juniper shrubs and cedar tree; **wetland swale** with sedge tussocks.	10.3%	40%	80 ± 20 yr
3: Upland ridge with mixed conifer and hardwood forest; **wetland** cedar swamp.	0%	10%	~ 400 yr
7: Upland ridge with ericacad shrubs and conifers; **wetland** cedar swamp.	0%	16.7%	830 ± 80 yr
FAR FROM LAKE MICHIGAN (OLDEST)			

Figure 6. Seasonal N Fixation Frequency Across the Substrate-Age Gradient.

Frequencies for each unique combination of site, status (wetland vs. upland), treatment (ambient moisture vs. saturation), and date are visualized in Figure 7. Note that N fixation is typically absent or sparse at upland ridge sites (yellow), regardless of whether a saturation treatment was applied. N fixation occurred most frequently in wetland sites (blue bars) with the saturation treatment.

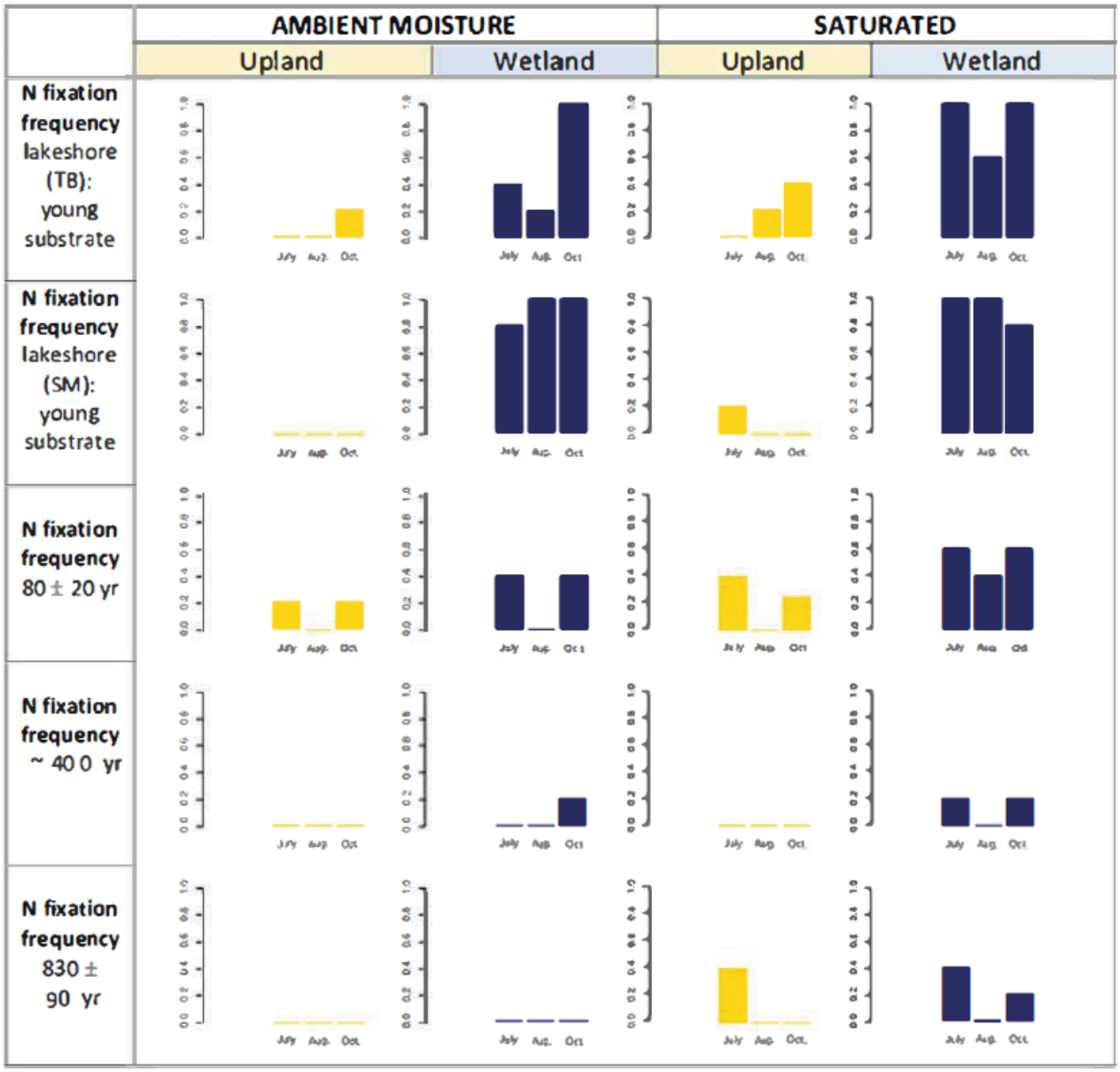


Figure 7. N fixation frequencies by site, date, and treatment (ambient moisture vs. saturation) across wetland and upland ridges in the beach-ridge complex. Each frequency is based on 5 samples, with the exception of October for Deerlick Ridge (80 ±20 yr), where n = 4 due to missing data.

Crossed mixed-effects model: diagnostics. Low variance inflation factors indicate that the fixed-effect variables for the model are not notably correlated (Figure 8a). The residuals do not closely approximate a normal distribution (Figure 8b), but they are sufficiently normal given the relatively robust nature of mixed-effects linear models (Schielzeth et al., 2020).

(a)	Wetland	H_2O Treatment
VIF	1.004718	1.004718

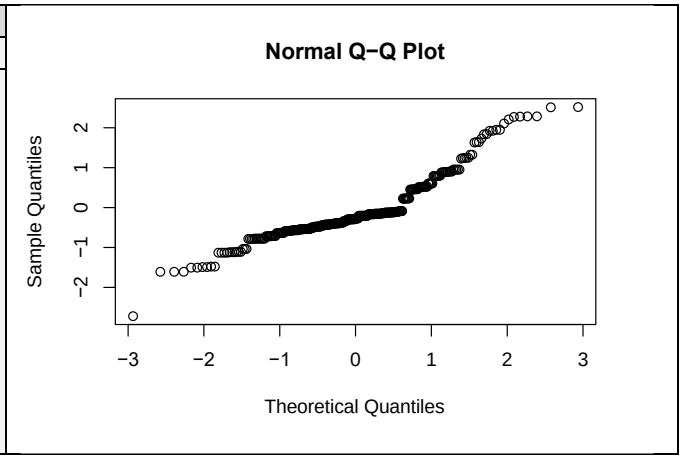


Figure 8. (a) Variance inflation factors. (b) QQ plot to evaluate normality of residuals.

It is also reasonable to assume that the observations within levels of the random effects (date, site) are independent of observations within another level. Bryophytes respond rapidly to changing environmental conditions, especially the moisture levels known to be critical controls on N fixation, within the scale of minutes or hours; observations taken a month or more apart are likely be independent. The bryophyte colonies are also small enough and discontinuous enough to make the assumption of independence among plots that are 10 m apart reasonable. There is only one missing observation; the data meet model assumptions for missing data (i.e., occurring with complete randomness).

Crossed mixed-effects model: results of Likelihood Ratio Test for fixed effects.

	Number of parameters	AIC	Likelihood Ratio Test	Pr(Chi)
<none>		225.47		
Wetland	1	312.49	89.022	< 2e-16 ***
Water	1	228.51	5.042	0.02474 *
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

Table 6. Summary of Likelihood Ratio Test for fixed effects

The Likelihood Ratio Test (Table 6) indicates that both wetland status and the water treatment have a significant impact on the log-likelihood of the response variable. Bryophytes in wetland areas are more likely than bryophytes in upland areas to harbor active N-fixing bacteria, and bryophytes that were saturated with additional water were more likely to provide evidence of active N fixation. These results were confirmed by the computation of 95% confidence intervals for the estimates of the fixed-effect parameters (Table 7).

	Estimate	95% CI
Intercept	-3.6241	(-5.2840, -2.1057)
Wetland	2.6639	(2.0087, 3.4251)
Water	0.5749	(0.0728, 1.0906)
Degrees of freedom for model	294	

Table 7. Estimates of the intercept and slope for fixed effects in the generalized linear model.

The random effects estimates of variance and the 95% CI are reported in Table 8.

Groups	Name	Variance	Std. deviation	95% CI
Site	Intercept	1.5016	1.2254	(0.6592, 2.8048)
Date	Intercept	0.1977	0.4446	(0.0956, 1.642559)
	Number of observations: 299. Site (5 groups), Date (3 groups).			

Table 8. Estimates of variance associated with random effects in the generalized linear model.

All of the confidence intervals exclude zero, providing evidence that the parameters estimated are unlikely to be equal to zero. But the lower limit of the confidence interval for the slope of *Water* (fixed effect based on addition of H_2O to individual samples) is very close to zero. The *p*-value from the Likelihood Ratio Test for *Water* is significant (0.02474), but weakly so—somewhat close to $\alpha = 0.05$. This raises the possibility that this test result may be fairly sensitive to the value of the Method Detection Limit (0.150).

A sensitivity analysis was conducted by executing the Likelihood Ratio Test with two alternate plausible values of the Method Detection Limit, 0.17500 and 0.13412. The results were significant for all fixed effects (*Wetland* and *Water*) in both models. More extreme values of the Method Detection Limit (e.g., 0.20) were not tested due to the higher prevalence of false-positive results in the bootstrap studies.

Overall the general linear model provides strong evidence that bryophytes in the wetland areas of the beach-ridge complex are more likely to foster N fixation detectable with the acetylene reduction assay. The evidence for a short-term water limitation (e.g., lack of water in bryophytes from dry, sandy upland ridges) is also statistically significant, but less robustly so.

18 of the 150 paired samples (1 sample pair for a given bryophyte = 1 at ambient moisture, 1 at saturated moisture) had discordant results (negative ARA result became positive with water addition, or vice-versa). The majority of the discordant results (16/18) had *larger* ethylene peaks in vials saturated with water; these bryophytes were collected

from both wetland and upland sites. (During hot, dry conditions some of the wetland areas may become relatively dry.) These samples suggest that additional moisture (e.g., input from fog, rain, etc.) may activate N fixation in about 12% of the bryophytes sampled. For discordant samples, the mean value of the difference in ethylene peak area is 0.92950.

Positive response to the water treatment was concentrated in mosses from the family Amblystegiaceae, dominated by wetland taxa, and in *Thuidium delicatulum* (Hedw.) Schimp., a moss in Thuidiaceae Schimp. capable of growth in diverse habitats, including wetlands and sandy upland ridges. Mosses or liverworts in other families rarely responded to the water treatment. Overall the discordant results indicate that short-term water limitations exist for about 12% of the sampled bryophytes. The short-term water limitations affect a modest number of samples, but have a statistically significant impact on overall N fixation.

Only two of the discordant results involved larger ethylene peaks at ambient moisture levels, and smaller ethylene peaks in vials saturated with water. These biologically counterintuitive results involved the species *Thuidium delicatulum* (Hedw.) Schimp. and *Hamatocaulis vernicosus* (Mitt.) Hedenäs.

Bryophyte diversity and N fixation across the gradient. The prevalence of N fixation among samples from diverse bryophyte families varies widely (Figure 9), although only

a handful of families are represented by larger sample sizes (> 30 assays conducted). The plants sampled included 22 different bryophyte families.

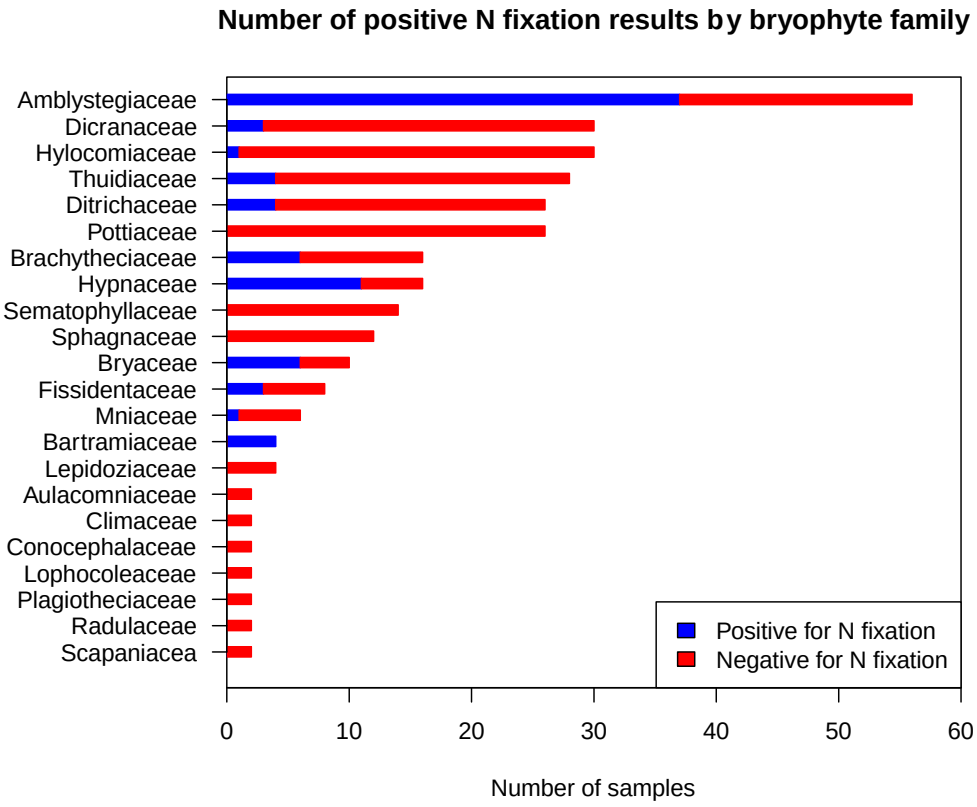


Figure 9. Acetylene reduction results by bryophyte family.

Overall the bryophyte flora was dominated by mosses (288 moss samples, 12 liverwort samples). Mosses in the family Amblystegiaceae Kindb., characteristic of wetland habitats, provided the strongest evidence of frequent N fixation: 37 of 56 samples (66%) yielded positive ARA results. Saturation with H_2O allowed some of the samples that were negative at ambient moisture levels to reduce acetylene, suggesting that N fixation is occasionally water-limited in Amblystegiaceae at these sites. Mosses in Hypnaceae Schimp. also provided strong evidence of N fixation capacity, but with a smaller sample

size: 11 of 16 samples (69%) were positive. As in Amblystegiaceae, the species associated with positive ARA results were from wetland habitats.

The moss families Bartramiaceae Schwägr. and Bryaceae Rchb. were also associated with high ARA positivity rates (100% and 60%), but sample sizes were modest (*n* = 4 for Bartramiaceae, *n* = 10 for Bryaceae). The species present were also characteristically from wetland habitats.

Mosses in Brachytheciaceae Schimp. and Fissidentaceae Schimp. generated positive ARA results fairly often, but less consistently. For Brachytheciaceae, 6 of 16 samples (38%) were associated with evidence of N fixation; for Fissidentaceae, 3 of 8 (38%).

Moss families with low frequencies (10-25%) of positive ARA results include Dicranaceae Schimp., Ditrichaceae Limpr., Mniaceae Schwägr., and Thuidiaceae Schimp.

Over half of the bryophyte families studied (12 of 22) provided little or no evidence of N fixation activity (frequency of positive ARA results: 0-5%). These families included mosses in Aulacomniaceae Schimp., Climaciaceae Kindb., Hylocomiaceae M. Fleisch Plagiotheciaceae M. Fleisch., Pottiaceae Hampe, Sematophyllaceae Broth., and Sphagnaceae Dumort., as well as liverworts in Conocephalaceae Müll. Frib. ex Grolle, Lepidoziaceae Limpr., Lophocoleaceae Müll. Frib. ex Vanden Berghen, Radulaceae Müll. Frib., and Scapaniaceae Mig. Numerous families (9) were represented by less

than 5 samples; negative ARA results for these taxa could be interpreted as evidence of symbiotic incapacity, symbiosis with inactive N-fixers, or insufficient sample size to detect N fixation in the family studied.

Figure 10 visualizes the diversity of bryophyte families by site within the complex. The length of the color-coded rectangle represents the proportion of bryophyte samples found within the specified family for each site. The liverworts are treated collectively.

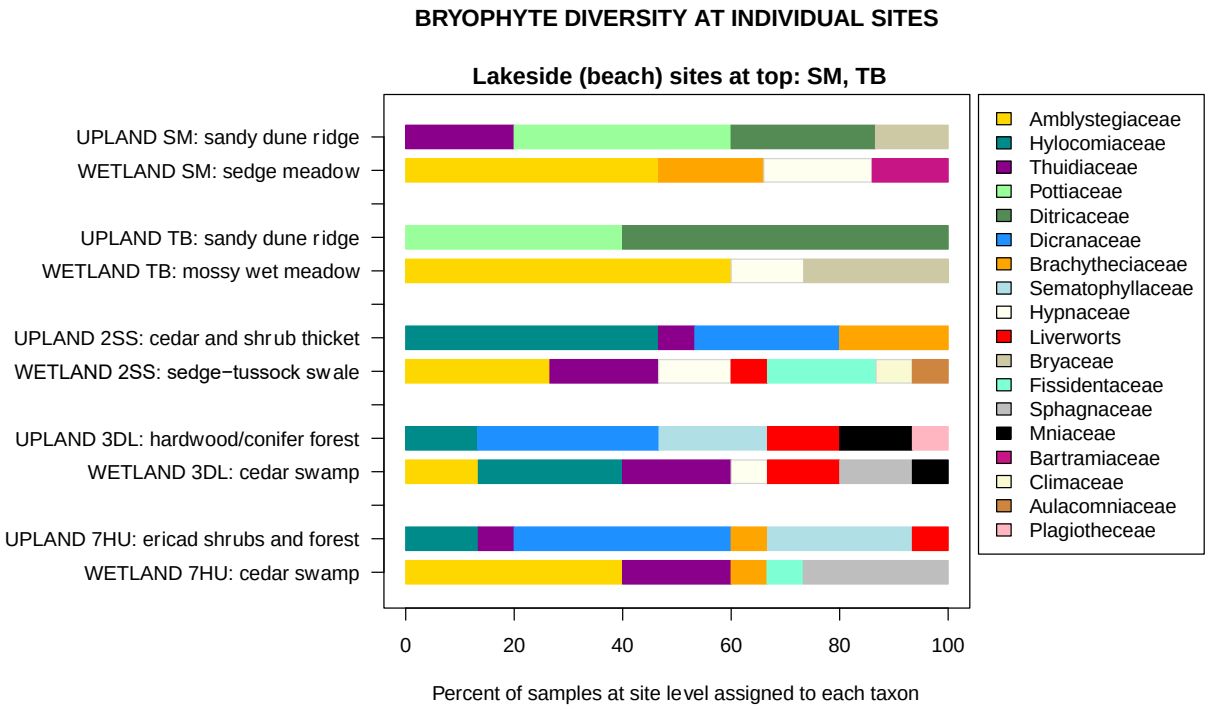


Figure 10. Distribution of bryophyte diversity by family across the beach-ridge complex. The two youngest sites (lakeside) are represented by the sandy dune ridges and wetlands at the top of the figure. The inland sites become progressively older (and farther from the lake) towards the base of the figure.

Figure 10 indicates that bryophyte communities for wetland swales and upland ridges differ greatly, with wetlands dominated by the families Amblystegiaceae and Hypnaceae. The family Amblystegiaceae (gold) is restricted to wetland sites. The family

Hypnaceae (white) is typically found in wetland sites, although at least one species (*Hypnum fertile* Sendtn.) is found in upland ridges. A greater diversity of bryophyte families is evident in the older, inland ridges (both wetland and upland). *Thuidium delicatulum*, a member of the family Thuidiaceae (purple), is one of the most widely dispersed bryophytes, present in wetland and upland sites across the substrate-age gradient. In beach sites along the lakeshore, *Thuidium delicatulum* survives storms and blowing sand, which carries the risk of burial. At inland sites *Thuidium delicatulum* is present in diverse habitats, including cedar swamp, forested upland ridge, and upland areas dominated by ericaceous shrubs. Common mosses in the family Brachytheciaceae Schimp. (orange) are also distributed in wetland and upland areas throughout the beach-ridge system.

Mosses in the families Potticeae Hampe (light green) and Ditrichaceae (dark green) are restricted to the lakeside beach sites, specifically to sandy upland ridges.

Mosses in Hylocomiaceae M. Fleisch (aqua), commonly known as feather mosses, are present at inland sites. These taxa may be dominant in forested upland ridges, but they are also present in one of the wetland swales. The families Dicranaceae Schimp. (dark blue) and Sematophyllaceae Broth. (light blue) are also dominant elements of the upland bryophyte flora for inland sites. By contrast, Fissidentaceae (turqoise) and Sphagnaceae Dumort. (gray) are restricted to the inland wetland sites.

Figure 11 visualizes the frequency of N fixation activity for bryophyte families across the beach-ridge complex. The figure includes the same taxonomic groups and site order (youngest soils/beach at top, oldest soils/inland at base) as Figure 10, but each taxon is color-coded based on the frequency of positive ARA results at that site (i.e., probability of N fixation detection). The length of the rectangle represents the proportion of bryophytes in that family for a given site, as in Figure 10. The smallest rectangles visualize two bryophyte samples for a given family. Frequencies for these small shapes should be interpreted with caution, as these are based on only two samples.

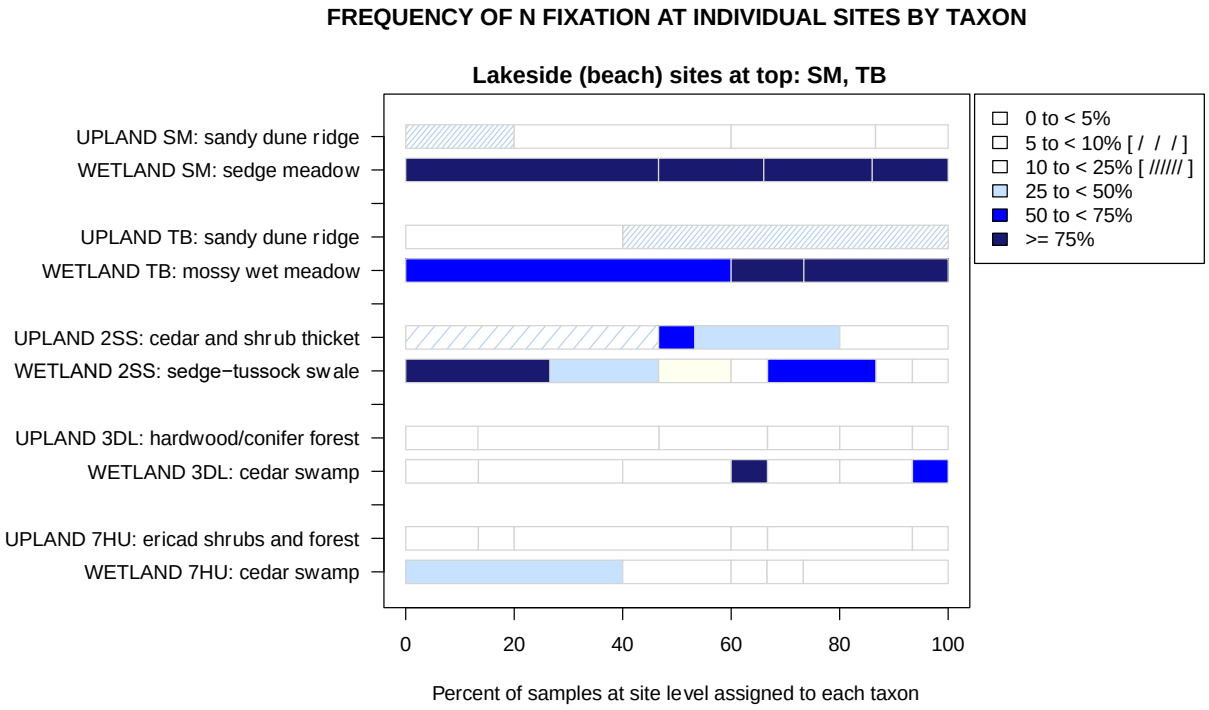


Figure 11. Color-coded frequency of positive ARA results visualized by site and bryophyte family. The organization of the rectangles is identical to that in Figure 10, to facilitate comparison between the two figures. The top row, for example, represents the frequency of N fixation by family for mosses in Thuidiaceae, Pottiaceae, Ditrichaceae, and Bryaceae (compare to key for Upland SM site in Figure 10).

The darkest blue represents families with strong evidence of symbiotic N fixation (ARA results positive in ≥ 75% of all samples for a given site). Mosses in Amblystegiaceae at two of five wetland sites, and mosses in Hypnaceae at three of five wetland sites provided strong evidence of N fixation. Highly frequent N-fixing activity for Amblystegiaceae is concentrated at one of the two beach wetlands (Stone Mill Road: SM), and in the wetland swale adjacent to the beach (2 South Sandy: 2SS). Highly frequent N-fixing activity for Hypnaceae occurs at both of the beach wetland sites (SM and Town Beach: TB), as well as in one of three inland wetland sites (Deerlick Ridge: DL). Mosses in Bartramiaceae, Brachytheciaceae, and Bryaceae are also associated with high frequencies of N fixation at the beach wetland sites, albeit with smaller sample sizes per site (n = 4 for Bartramiaceae, n = 6 for Brachytheciaceae, n = 8 for Bryaceae). Overall high-frequency N fixation is limited to taxa in wetland sites, and most concentrated in the youngest sites along the lakeshore.

Moderately high-frequency N fixation (ARA positive for 50 to ≤ 75% of all samples for a given site) is represented by royal blue in Figure 11. Moss families exhibiting moderately high-frequency N fixation include Amblystegiaceae in one of the two beach wetland sites (Town Beach), Thuidiaceae for the upland site and Fissidentaceae for the wetland site of South Sandy (inland), and Mniaceae in the wetland site of Deerlick Ridge (inland). Moderately high-frequency N fixation occurs in families found primarily but not exclusively in wetland sites (3/4 sites = wetland).

Light blue visualizes modest evidence of symbiotic N fixation (ARA results positive in 25 to < 50% of all samples for a given site). Amblystegiaceae in the wetland site of the Town Beach is associated with modest frequencies of N fixation. Otherwise the light-blue rectangles are associated with moss families found on inland ridges, including Dicranaceae (upland South Sandy), Thuidiaceae (wetland South Sandy), and Amblystegiaceae (wetland Huckleberry Ridge).

The dense blue diagonal lines represent families with low frequencies of symbiotic N fixation (10 to < 25% of all samples for a given site). Thuidiaceae and Ditrichaceae on the upland beach sites provided evidence of low-frequency N fixation.

The sparse blue diagonal lines represent families with very low frequencies of symbiotic N fixation (5 to < 10% of all samples). Hylocomiaceae on the upland site of South Sandy ridge (inland) is the only family in which N fixation occurs so rarely.

White represents families associated with negligible evidence of symbiotic N fixation (ARA positive for 0 to < 5% of all samples for a given site). At the upland beach sites, this includes Ditrichaceae and Bryaceae at Stone Mill Lane beach, and Pottiaceae at Baileys Harbor Town Beach. Numerous families associated with the inland ridges also fail to provide compelling evidence of N fixation. Given the plethora of bryophyte families involved, I will not specify all relevant combinations of site and family for such results. Let it suffice to note that the families Amblystegiaceae, Aulacomniaceae, Brachytheciaceae, Climaceae, Conocephalaceae, Dicranaceae, Fissidentaceae,

Hylocomiacea, Hypnaceae, Lepidoziaceae, Lophocoleaceae, Mniaceae, Plagiotheciaceae, Radulaceae, Scapaniaceae, Sematophyllaceae, Sphagnaceae, and Thuidiaceae all fail to provide meaningful evidence of N fixation in at least one of the inland sites (wetland or upland). Many of these families yielded no positive ARA results in one or more sites.

Overall the site-specific portrait of N fixation (Figure 11) indicates that frequencies vary notably by site, even for a well-studied family with many samples. Amblystegiaceae (*n* = 56) is a high-frequency N fixer at the youngest sites (beach and first inland ridge), but fixation occurs sporadically or not at all in the older wetland sites. Dicranaceae (*n* = 27) is not associated with N fixation anywhere except at the first inland ridge (South Sandy). Thuidiaceae (*n* = 28) is associated with various frequencies of N fixation (negative/neglible, low, and moderate) in a wide range of habitats, including sandy beach sites and the older wetland and upland sites found inland. In general, the probability of detecting N fixation for a given family of bryophytes is spatially heterogeneous, and dependent on the spatial unit of analysis.

Figure 11 provided *site-specific frequencies* for positive ARA results; Table 9 (next page) provides frequency data by family based on results across *all* sites. Sample size is used to characterize the strength of evidence. The descriptions pertain specifically to species for a given family present at the Baileys Harbor sites, not to the family as a whole.

Bryophyte family	Description	Estimated frequency of positive ARA results	Strength of evidence
Bartramiaceae	Moss of wetland sites	Very high (≥ 75%)	Inadequate ($n \leq 4$)
Amblystegiaceae	Mosses of wetland habitats	High (50 - < 75%)	Strong ($n > 30$)
Bryaceae	Mosses of wetland and upland habitats	High (50 - < 75%)	Modest ($10 < n \leq 20$)
Hypnaceae	Mosses of wetland and upland habitats	High (50 - < 75%)	Modest ($10 < n \leq 20$)
Brachytheciaceae	Weedy mosses of wetland and upland habitats	Moderate (25 - < 50%)	Modest ($10 < n \leq 20$)
Fissidentaceae	Mosses of wetland swales in older inland sites	Moderate (25 - < 50%)	Weak ($4 < n \leq 10$)
Dicranaceae	Moss of forest floor on older inland ridge sites	Low (10 - < 25%)	Strong ($n > 30$)
Ditrichaceae	Mosses of sandy, disturbance-prone beach ridges along lakeshore	Low (10 - < 25%)	Adequate ($20 < n \leq 30$)
Thuidiaceae	Mosses of diverse habitats: sandy upland beach ridges, lakeshore wetlands, inland forested ridges, inland wetlands	Low (10 - < 25%)	Adequate ($20 < n \leq 30$)
Mniaceae	Mosses of wetlands and upland ridges in older inland sites	Low (10 - < 25%)	Weak ($4 < n \leq 10$)
Hylocomiaceae	Feather mosses of forest floor on older inland ridge sites; sometimes found in older inland wetlands	Negligible (0 - < 5%)	Strong ($n > 30$)
Pottiaceae	Mosses of sandy, disturbance-prone beach ridges along lakeshore; occasionally found in open sandy areas inland	Negligible (0 - < 5%)	Adequate ($20 < n \leq 30$)
Sematophyllaceae	Mosses of forested upland ridges in older inland sites	Negligible (0 - < 5%)	Modest ($10 < n \leq 20$)
Sphagnaceae	Mosses of wetlands in older inland sites	Negligible (0 - < 5%)	Modest ($10 < n \leq 20$)
Aulacomniaceae	Moss of wetland sites	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Climaceae	Moss of forest floor on older inland ridge sites	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Conocephalaceae	Liverwort of young lakeshore wetlands	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Lepidoziaceae	Liverwort of forested inland ridges	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Lophocoleaceae	Liverwort of forested inland ridges	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Plagiotheciaceae	Mosses of forested inland ridges	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Radulaceae	Liverwort of forested inland ridges	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)
Scapaniaceae	Liverwort of forested inland ridges (wetland)	Negligible (0 - < 5%)	Inadequate ($n \leq 4$)

Table 9. Characteristic frequencies of positive ARA results for all observed bryophyte families in the beach-ridge complex.

Overall the evidence indicates that mosses in the families Amblystegiaceae, Bryaceae, and Hypnaceae are associated with high-frequency N fixation and are important constituents of the wetland bryophyte flora, especially at the young wetland beach sites. Likewise there is strong evidence that Hylocomiaceae (inland sites) and Pottiaceae (beach sites) are rarely involved in N fixation, even though these families are notable elements of the upland bryophyte flora. Other upland and wetland families (5 of 22) are sometimes associated with symbiotic N fixation, although over half of the bryophyte families (12 of 22) are rarely associated with fixation or provided no positive ARA results.

Table 10 summarizes the bryophyte species studied at the beach-ridge complex and their potential for N fixation or symbiosis with cyanobacteria based on (a) ARA results in my study, and (b) literature review.

Almost half of the taxa in Table 10 (20 of 46) are associated with at least one positive ARA result at the beach-ridge complex. Tropicos recognizes a valid species name for 41 of the 46 taxa studied. Among these, 8 species (almost 20%) represent potentially novel results—i.e., symbiotic N fixation in bryophytes that have not demonstrated such potential elsewhere, either because they have not been studied or because published reports are negative. These species include *Calliergonella lindbergii* (Mitt.) Hedenäs, *Campylium chrysophyllum* (Brid.) Lange, *Campylium protensum* (Brid.) Kindb.,

Fissidens adianthoides Hedw., *Fissidens osmundioides* Hedw., *Hamatocaulis vernicosus* (Mitt.) Hedenäs, *Hypnum pratense* W.D.J. Koch ex Spruce, and *Philonotis marchica* (Hedw.) Brid. *Drepanocladus aduncus* var. *polycarpus,* which is not associated with a valid name in Tropicos, may also represent a novel result.

[Table 10 on next page.]

Bryophyte species	**Number of positive ARA results ≥ 1 in beach-ridge complex**	**Sample size (*n*)**	**N fixation previously reported**	**Cyanobacterial symbionts previously reported**
Aulacomnium palustre (Hedw.) Schwägr.	No	2	Yes: Solheim et al. 1996, Gavazov et al. 2010.	Yes: Solheim et al. 1996, Gavazov et al. 2010.
Bazzania trilobata (L.) Gray	No	4	Yes: Lambert & Reiners, 1979.	No: Lambert & Reiners 1979, Deane-Coe & Sparks 2016.
Brachythecium acuminatum (Hedw.) Austin	No	2	No	No
Brachythecium rutabulum (Hedw.) Schimp.	No	2	No	No
Brachythecium turgidum (Hartm.) Kindb.	Yes	6	No: Solheim et al. 1996.	Yes: Solheim et al. 1996.
Brachythecium velutinum (Hedw.) Schimp.	No	4	No	No
Brachythecium Schimp.	No	2	Yes: Line 1992, Solheim et al. 1996.	Yes: Smith 1984, Solheim et al. 1996.
Brotherella recurvans (Michx.) M. Fleisch.	No	14	Yes: Lambert & Reiners 1979.	No: Lambert & Reiners 1979.
Calliergon giganteum (Schimp.) Kindb.	No	2	No	No
Calliergon richardsonii (Mitt.) Kindb. ex G. Roth	No	2	Yes: Solheim et al. 1996; Liengen & Olson, 1997; Solheim et al. 2004.	Yes: Solheim et al. 1996; Liengen & Olson, 1997; Solheim et al. 2004.
***Calliergonella lindbergii* (Mitt.) Hedenäs**	Yes	14	No	No
***Campylium chrysophyllum* (Brid.) Lange**	Yes	2	No	No
***Campylium protensum* (Brid.) Kindb.**	Yes	2	No	No
Campylium stellatum (Hedw.) Lange & C.E.O. Jensen	Yes	2	No	Yes: Garbary et al. 2008.
Ceratodon purpureus (Hedw.) Brid.	Yes	16	Yes: Vlassak et al. 1973; Brasell et al. 1986; Henriksson et al. 1987; Nakatsubo & Ino, 1987; Nakatsubo & Ohtani, 1991.	Yes: Fukushima, 1959 as cited in in Matsuda, 1968; Takagi, 1962 as cited in Matsuda, 1968; Horikawa & Ando, 1967; Vlassak et al. 1973; Brasell et al.

Bryophyte species	**Number of positive ARA results ≥ 1 in beach-ridge complex**	**Sample size (*n*)**	**N fixation previously reported**	**Cyanobacterial symbionts previously reported**
				1986; Henriksson et al. 1987; Nakatsubo & Ino, 1987; Nakatsubo & Ohtani, 1991; Kanda & Inoue, 1994; Kanda et al., 2004.
Climacium dendroides (Hedw.) F. Weber & D. Mohr	No	2	No	No
Conocephalum conicum (L.) Dumort.	No	2	No	No
Dicranum montanum Hedw.	No	2	No	No
Dicranum polysetum Sw.	Yes	12	Yes: Stuart et al. 2021.	Yes: Stuart et al. 2021.
Dicranum scoparium Hedw.	No	6	No	Yes: Stuart et al. 2021.
Ditrichum flexicaule (Schwägr.) Hampe	Yes	10	No	No
Drepanocladus polygamus (Schimp.) Hedenäs	Yes	4	No	No
Drepanocladus (Müll. Hal.) G. Roth	No	2	Yes: Schell & Alexander 1973, Basilier et al. 1978, Jordan et al. 1978, Basilier 1979, *Gavazov et al. 2010, Stewart et al. 2013.	Yes: Schell & Alexander 1973, Basilier et al. 1978, Jordan et al. 1978, Priddle & Dartnell 1978, Basilier 1979, Wojciechowski & Heimbrook 1984, McIntire et al. 1994, Solheim et al. 1996, *Gavazov et al. 2010, Stewart et al. 2013.
***Fissidens adianthoides* Hedw.**	Yes	6	No	No
***Fissidens osmundioides* Hedw.**	Yes	2	No	No
***Hamatocaulis vernicosus* (Mitt.) Hedenäs**	Yes	10	No	No
Hylocomiadelphus triquetrus (Hedw.) Ochyra & Stebel	No	2	Yes: Calabria et al. 2020.	Yes: Calabria et al. 2020.

Bryophyte species	**Number of positive ARA results ≥ 1 in beach-ridge complex**	**Sample size (*n*)**	**N fixation previously reported**	**Cyanobacterial symbionts previously reported**
Hylocomium splendens (Hedw.) Schimp.	No	8	Yes: Solheim et al. 2002; Solheim et al, 2004; Houle et al. 2006; Zackrisson et al. 2009; Gavazov et al. 2010; Ininbergs et al. 2011; Gundale et al. 2012; Bay et al. 2013; Leppänen et al. 2013; Giundale et al. 2016; Rousk et al. 2016, Warshan et al. 2016, Rousk et. al 2017, Warshan et al. 2017a, Warshan et al. 2017b, Jean et al. 2018, Salemaa et al. 2019, Goth et al. 2019, Jean et al. 2020, Alvarenga & Rousk, K. 2021; Permin et al. 2022; Kubota et al., 2023; Alvarenga et al., 2023.	Yes: Solheim et al. 2002; Solheim et al, 2004; Zackrisson et al. 2009; Gavazov et al. 2010; Ininbergs et al. 2011; Gundale et al. 2012; Leppänen et al. 2013; Giundale et al. 2016; Rousk et al. 2016, Warshan et al. 2016, Rousk et. al 2017, Warshan et al. 2017a, Warshan et al. 2017b, Jean et al. 2018, Salemaa et al. 2019, Goth et al. 2019, Jean et al. 2020, Alvarenga & Rousk, K. 2021; Permin et al. 2022; Kubota et al., 2023.
***Hypnum pratense* W.D.J. Koch ex Spruce**	Yes	2	No	No
Leucobryum glaucum (Hedw.) Ångstr.	No	10	No	No
Lophocolea heterophylla (Schrad.) Dumort.	No	2	No	No
No equivalent taxon recognized in Tropicos (2023); *Drepanocladus aduncus* var. *polycarpus* per Crum (2004)	Yes	12	Unable to assess for variety given taxonomic uncertainty. Yes at species level: Solheim et al. 1996.	Unable to assess for variety given taxonomic uncertainty. Yes at species level: McIntire et al. 1994.
No equivalent genus recognized in Tropicos (2023); various *Bryum* species possible per Crum (2004), difficult to assess with sterile material.	Yes	10	Unable to assess at species level given taxonomic uncertainty. Yes at genus level: Reddy & Givens 1981, Kanda & Inoue 1994, Henriksson et al. 1987, Nakatsubo & Ino 1987, Nakatsubo & Ohtani 1991, Solheim et al. 2004, Solheim et al. 1996, Egorov et al. 2007.	Unable to assess at species level given taxonomic uncertainty. Yes at genus level: Horikawa & Ando 1967, Matsuda 1968, Light & Heywood as cited in Priddle & Dartnell 1978, Reddy & Givens 1981, Davey 1982, Wojciechowski & Heimbrook 1984, Emeis et al. 1987, Henriksson et al. 1987, Nakatsubo & Ino 1987,

Bryophyte species	**Number of positive ARA results ≥ 1 in beach-ridge complex**	**Sample size (*n*)**	**N fixation previously reported**	**Cyanobacterial symbionts previously reported**
				Nakatsubo & Ohtani 1991, Broady as cited in Schwarz et al. 1992, Kanda & Ohtani 1991, Kanda & Inoue 1994, Solheim et al. 1996, Alfinito et al. 1998, Solheim et al. 2004, Kanda et al. 2004, Egorov et al. 2007, Nakai et al. 2012, Rankin et al. 2017, Castter et al. 2019, Raymond 2016, Singh 2022.
***Philonotis marchica* (Hedw.) Brid.**	Yes	2	No	No
Plagiomnium affine (Blandow ex Funck) T.J. Kop.	No	2	No	No
Plagiomnium cuspidatum (Hedw.) T.J. Kop.	Yes	4	Yes: Lambert & Reiners 1979.	Yes: Zhang et al. 2014.
Plagiothecium laetum Schimp.	No	2	No	No
Pleurozium schreberi (Willd. ex Brid.) Mitt.	Yes	20	Yes: Lambert & Reiners 1979; Alexander & Billington 1986, DeLuca et al., 2002; Zackrisson et al. 2004; Gentili et al. 2005; DeLuca et al. 2007; DeLuca & Zackrisson 2007; Lagerström et al., 2007; DeLuca et al., 2008; Gundale et al. 2009; Zackrisson et al. 2009; Gavazov et al. 2010; Gundale et al., 2010, 2011; Ininbergs et al. 2011; Ackermann et al. 2012; Gundale et al. 2012; Leppänen et al. 2013; Kardol et al. 2016; Warshan et al. 2016; Whiteley-Gonzalez 2016; Rousk et al. 2017; Jean et al. 2018; Salemaa et al. 2019; Calabria et al. 2020; Stuart 2021; Arróniz-Crespo et al. 2022;	Yes: Alexander & Billington 1986, DeLuca et al., 2002; Zackrisson et al. 2004; Gentili et al. 2005; Houle et al. 2006; Deluca et al. 2007; DeLuca & Zackrisson 2007; DeLuca et al., 2008; Gundale et al. 2009; Zackrisson et al. 2009; Gavazov et al. 2010; Gundale et al., 2010, 2011; Ininbergs et al. 2011; Ackermann et al. 2012; Gundale et al. 2012; Bay et al. 2013; Gundale et al. 2013; Leppänen et al. 2013; Kardol et al. 2016; Warshan et al. 2016; Warshan et al. 2017 (a, b, c); Whiteley-Gonzalez 2016; Jean et al. 2018; Holland-Moritz et al. 2018; Calabria et al. 2020; Arróniz-Crespo et al. 2022; DeLuca et al. 2022; Renaudin et al.

Bryophyte species	**Number of positive ARA results ≥ 1 in beach-ridge complex**	**Sample size (*n*)**	**N fixation previously reported**	**Cyanobacterial symbionts previously reported**
			DeLuca et al. 2022; Renaudin et al. 2022; Wang et al. 2022.	2022; Rodgriguez-Rodriguez et al. 2022; Wang et al. 2022.
Radula complanata (L.) Dumort.	No	2	No	No
Scapania nemorea (L.) Grolle	No	2	No	No
Scorpidium revolvens (Sw.) Rubers	Yes	6	Yes: Egorov 2007.	Yes: Egorov 2007.
Sphagnum L.	No	12	Yes: numerous references; see review by Kostka, J.E., Weston, D.J., Glass, J.B., Lilleskov, E.A., Shaw, A.J. and Turetsky, M.R., 2016.	Yes: numerous references; see review by Kostka, J.E., Weston, D.J., Glass, J.B., Lilleskov, E.A., Shaw, A.J. and Turetsky, M.R., 2016.
Syntrichia ruralis (Hedw.) F. Weber & D. Mohr	No	12	No: Snyder, J.M., & Wullstein 1973.	Yes: Synder & Wullstein 1973.
Thuidium delicatulum (Hedw.) Schimp.	Yes	28	No	Yes: Deane-Coe & Sparks, 2016.
Tortella tortuosa (Schrad. ex Hedw.) Limpr.	No	14	No	Yes: Pentecost & Whitton, 2012

Table 10. **Species of bryophytes associated with positive ARA rates in the beach-ridge complex in relation to published literature.** Each row represents a distinct taxonomic entity (most are species). Green rows represent bryophytes with at least one report of cyanobacterial symbionts or N fixation at the beach-ridge complex or in published literature reviewed by the author. **Bryophyte names in bold** represent species that were not previously associated with N fixation in the literature (i.e., potentially novel results). White rows represent bryophytes with no evidence of N fixation or cyanobacterial symbionts based on literature review and the results of this study.

* Gavazov et al. 2010 reports N fixation and cyanobacteria in association with *Drepanocladus cossonii* (Schimp.) Loeske; this taxon is of uncertain status at the genus level based on Tropicos (2023).

The rows in white represent species that are not known to associate with cyanobacteria or other N-fixing symbionts based on the study results and literature review. At this site, about one-third of the bryophyte flora (13 of 41 species) fall into this category. But most of the bryophytes present in the beach-ridge complex appear to be capable of N fixation or cyanobacterial symbiosis somewhere in the world (on site or elsewhere).

DISCUSSION

The ARA evidence from the beach-ridge ecosystem in Baileys Harbor, Wisconsin provides strong evidence that bryophytes in these systems host microbes involved in N fixation, and have the potential to be a net source of fixed N for vascular plants. N fixation activity is most robust in the wetland areas of the beach-ridge complex, as one might expect given the role of H_2O in enabling nitrogenase activity. In discussion I will contextualize these results focusing on (1) the beach ridge and swale sites at Moonlight Bay and the Baileys Harbor Town Beach, and (2) the upland ridge and swale sites within The Ridges Sanctuary. I will also discuss the implications of the N fixation data with respect to our understanding of symbioses and ecosystem function.

Bryophyte-associated N fixation in the beach ridge-and-swale systems along Lake Michigan. In the sites with the youngest sediments along the

lakeshore, the bryophyte flora of the low-lying wetlands, routinely subject to Lake Michigan wave action, are quite different from that of the first sandy ridge. The beach wetlands contain bryophyte families with high frequencies of symbiotic N fixation; the beach ridges are dominated by families with low frequencies of symbiotic N fixation, or no evidence of fixation.

Beach wetlands. The families most likely to be associated with N fixation are Amblystegiaceae, Bartramiaceae, Bryaceae, and Hypnaceae. Two of these (Bryaceae and Bartramiaceae) are limited to the beach wetlands. Hypnaceae is found primarily but not exclusively in the beach wetlands. By contrast, Amblystegiaceae is a dominant taxa in the beach wetlands, but has a substantial presence inland as well.

In Amblystegiaceae, the species associated with symbiotic N fixation at Moonlight Bay and the Baileys Harbor Bay include *Campylium chrysophyllum* (Brid.) Lange, *Campylium protensum* (Brid.) Kindb., *Campylium stellatum* (Hedw.) Lange & C.E.O. Jensen, *Drepanocladus polygamus* (Schimp.) Hedenäs, *Hamatocaulis vernicosus* (Mitt.) Hedenäs, *Scorpidium revolvens* (Sw.) Rubers, and *Drepanocladus revolvens* var *polycarpus*[11] (Crum 2004). These taxa are known as brown mosses, and are found in intermediate base-cation-rich environments. Symbiotic N fixation is a well-known feature of the family, first documented in *Drepanocladus uncinata* on Sidney Island in Antarctica (Fogg &

[11] There is no legitimate name associated with this particular variety of *Drepanocladus revolvens* in Tropicos (2023).

Stewart, 1968); the legitimate name for this taxon is now *Sanionia uncinata* (Hedw.) Loeske (Tropicos 2023). Since that initial report, over two dozen studies have verified cyanobacterial symbiosis and/or N fixation in mosses affiliated with Amblystegiaceae. These studies include habitats such as minerotrophic mires (e.g., Granhall & Selander, 1973), tundra (e.g., Schell & Alexander, 1973), sedge meadows (e.g., Stewart et al., 2013), coastal marshes (Garbary et al. 2008), glacial outwash (Solheim et al. 2002), freshwater spring systems associated with tufa (e.g., Pentecost 1981) or travertine (e.g., Pentecost 1998), permanent streams (e.g., Golubic 1957 in Golubic 2010), and deep freshwater lakes (e.g., McIntire et al., 1994), which may contain aquatic mosses. I am aware of only a single publication documenting a lack of N fixation activity in Amblystegiaceae (Line 1992), from a dry sub-Antarctic habitat; this may reflect a publication bias towards positive results.

While light microscopy evidence and most of the literature reviewed focuses on bryophyte-cyanobacterial symbioses, it is important to note that bryophytes are capable of hosting other N-fixing bacteria. Recent work on the brown mosses in Amblystegiaceae underscores the diversity of epiphytic microbes found in this family. A microbial ecology study of epiphytes associated with three mosses in Amblystegiaceae found the dominant bacterial taxa included Acidomicrobiales, Comamonamodaceae, Hyphomicrobiaceae, Pseudoanabaenaceae, and Sphingomonadaceae (Tveit et al. 2020). Pseudoanabaenaceae is a cyanobacterial group with N-fixing taxa. Nostocaceae was also present, as well

as N-fixing heterotrophic bacteria are also present among the dominant bacterial taxa in the study by Tveit et al. (2020). A separate study of bacterial diversity also detected non-cyanobacterial diazotrophic bacteria on two different Amblystegiaceaen mosses (Wang et al., 2018).

At my site, light microscopy indicates that heterocystous cyanobacteria are typically abundant on wetland Amblystegiaceaen species and most likely play a role in the positive ARA results, but that does not necessarily rule out a role for non-cyanobacterial diazotrophs in Amblystegiaceae. However, acetylene is known to distort and sometimes suppress N fixation in some heterotrophic microbes (e.g., DeBont & Mulder 1976, Fulweiler et al. 2015). The nature of the distortion (overestimation or underestimation) depends on the microbial taxon (Fulweiler et al. 2015). In some bryophyte-dominated ecosystems, e.g., *Sphagnum* peatlands (Saiz et al. 2019), the acetylene reduction assay may notably underestimate N fixation, most likely due to suppression of activity by heterotrophic bacteria. How acetylene impacts microbes associated with brown mosses in Amblystegiaceae, and hence the potential for bias in acetylene reduction estimates of N fixation rates, is not clear at this time.

Mosses in Bryaceae are also widely known to host N-fixing cyanobacteria. At my site, none of these mosses were determined to the species level given sterile condition, as well as a lack of nomenclatural clarity; all were assigned to Bryum *Hedw.* Like Amblystegiaceae, the family Bryaceae has been intensely studied

with respect to cyanobacterial symbionts, particularly in Antarctic habitats. At least two dozen studies affirm that mosses in Bryaceae, generally in *Bryum* and occasionally in *Pohlia* Hedw., host cyanobacteria. A number of these studies (13 of 24) also involved ARA; the majority (11/13) yielded positive results. The published research available to me provides robust evidence of symbiotic N fixation in Antarctic ecosystems (e.g., Fogg & Stewart 1968, Davey et al. 1982, Nakatsubo & Ino, 1987), but also provides tantalizing hints of potential roles in nitrogen cycling elsewhere. Reddy & Giddens (1981) documented N fixation in *Bryum argenteum* Hedw. in a fescue-dominated grassland in Georgia. Other positive ARA results for the genus *Bryum* were documented in moist depressions in *Salix* shrub ecosystems in the Niwot Ridge of the Rocky Mountains in Colorado (Wojciechowski & Heimbrook 1984), in eucalypt rainforest (Brasell et al. 1984), in freshwater tufa systems (Emeis et al. 1987), in primary successional lava habitats on the island of Surtsey in Iceland (Henriksson et al. 1987), and in various Arctic ecosystems (Solheim et al. 1996, Solheim et al. 2004, Egorov 2007). The relatively rare reports indicating mosses in Bryaceae fail to host cyanobacteria include Kanda et al. 2004 and Line 1992 (from the Antarctic and sub-Antarctic, respectively); Line also documented an absence of N fixation in *Bryum* utilizing ARA.

Within the family Hypnaceae, the moss *Calliergonella lindbergii* (Mitt.) Hedenäs was associated with positive ARA results in the beach-ridge complex. This taxon, like the mosses in Amblystegiaceae, has an affinity for calcareous habitats.

Hypnum pratense W.D.J. Koch ex Spruce was rare but present; the ARA results were negative. Published reports of symbiotic N fixation for the family Hypnaceae are modest in number. Early evidence of N fixation involved *Hypnum chrysogaster* Müll. Hal., a moss found in a clear-cut, recently burned eucalypt rainforest in Southern Tasmania, Australia (Brasell et al., 1984). More recently J.M. Stuart (2021) detected modest rates of N fixation using stable isotope methods in boreal forest mosses, including mosses in Hypnaceae: *Hypnum lindbergii* Mitt. and *Ptilium crista-castrensis* (Hedw.) DeNot. A few other studies have detected cyanobacteria in the family Hypnaceae, including *Hypnum* Hedw. (Sant'Anna, 1984), which was found on sun-exposed rocks associated with calcareous incrustations on a cliff in Brazil; *Ptilium crista-castrensis* (Hedw.) De Not., found in a boreal forest in Quebec, Canada (Houle et al. 2006); and *Vesicularia inflectens* (Brid.) Müll. Hal., found on a cliff in Ko'olau Mountain in Oahu, Hawaii (Ress 2012). The sole negative report for the family Hypnaceae involves *Hypnum* sp. in a study of dry plateau habitat in the sub-Antarctic Macquarrie Islands; neither cyanobacteria nor N-fixing activity were detected using microscopy and ARA (Line 1992). More recently, a microbial ecology study (Wang et al. 2018) detected diazotrophic bacteria including *Hyphomicrobium, Leptothrix*, and *Rhizobium* on a moss in Hypnaceae (*Pylasiella polyantha* Hedw. Grout). Overall this family is not as well-characterized as Amblystegiaceae, but there is sparse evidence of measurable symbiotic N fixation in boreal forests (Stuart 2020) and eucalypt rainforests (Brasell et al. 1984).

In Bartramiaceae, *Philonotis marchica* (Hedw.) Brid. was also typically associated with N fixation in the beach-ridge complex, but the high frequency may be an artifact of a small sample size (n = 4). This species, like others found in the low-lying swales, is characteristically found in mineral-rich wetland habitats. As far as I can tell, neither cyanobacterial symbionts nor N fixation have been previously reported in association with this species. But other mosses in the same genus are known to host cyanobacteria. *Philonotis fontana* (Hedw.) Brid. is known to host cyanobacteria in Arctic habitats (Solheim et al. 1996, Egorov 2007), although N fixation was not detected at either site. *Philonotis turneriana* (Schwägr.) Mitt. hosted cyanobacteria on a mountain cliff face in Hawai'i (Ress 2012); N fixation was not assayed. A study of another moss in Bartramiaceae identified to genus, *Breutelia* (Bruch & Schimp.) Schimp., yielded no evidence of N fixation or cyanobacteria in a dry sub-Antarctic habitat (Line 1992).

This study appears to be the first report of the potential importance of Amblystegiacean, Bartramiaceaen, Bryaceaen, and Hypnaceaen mosses in the nitrogen cycle of beach-ridge wetlands of the Great Lakes; as far as I know, bryophyte-associated N fixation has not been documented in these systems before. The evidence available suggests that these moss families may facilitate the enrichment of sandy soils in the beach-ridge ecosystems, making colonization by vascular plants more likely. These mosses are largely restricted in distribution to wetland areas, with N fixation most frequent and intense in younger sediments (< 100 years) along the lakeshore. These families

(Amblystegiaceae, Bartramiaceae, Bryaceae, Hypnaceae) are the most important biotic drivers for the significant difference in mean N fixation between wetland and upland areas in the mixed-effects models.

Sandy beach ridges. The dominant families of the sandy beach ridges are Ditrichaceae, Thuidiaceae, and Pottiaceae. Bryaceae is also present at the Town Beach site, but generated no evidence of N fixation in the sandy beach ridge.

The family Thuidiaceae, represented by the moss *Thuidium delicatulum* (Hedw.) Schimp., is known to host cyanobacteria in the forests of northeastern North America (Deane-Coe & Sparks, 2016). On the beach, the moss appears to tolerate the wind-blown sand, although it is often at the edge of sandy areas where vascular plants provide some protection. *Thuidium delicatulum* is widely distributed and common in eastern North America, all of Canada, and parts of the southwestern U.S., primarily in forest ecosystems. The moss is also found in other parts of the world; N fixation activity has been reported in a cloud forest in Costa Rica (Markham et al., 2021). Given its abundance in groundcover and broad distribution, better characterization of the biogeochemical impacts of *Thuidium delicatulum* may be of interest to forest ecologists.

Mosses in the family *Ditrichaceae* including *Ceratodon purpureus* (Hedw.) Brid. and *Ditrichum flexicaule* (Schwägr.) Hampe were also commonly found on the sandy beach, but rarely associated with N fixation. This family is widely known to

host cyanobacteria. Most of the published reports of cyanobacterial symbionts involve *Ceratodon purpureus*; about half of these are from Antarctic ecosystems (Horikawa & Ando, H. 1967; Matsuda, 1968; Kanda & Inoue, 1994; Kanda et al. 2004). Other reports for *Ceratodon purpureus* involve Canadian grasslands (Cullimore & McCann, 1972; Vlassak et al., 1973), post-fire tropical rainforest in Australia (Brasell et al. 1986), new lava on Surtsey Island in Iceland (Henriksson et al. 1987), and Mt. Fuji in Japan (Nakatsubo & Ohtani 1991). Only a single negative report (no cyanobacterial symbionts, no N fixation) has been published for *Ceratodon purpureus*; but other samples of the same moss at this Antarctic site were hosts associated with N fixation (Kanda et al. 2004). Other species in Ditrichaceae are also cyanobacterial hosts with positive ARA results, including *Ditrichum strictum* (Hook. f. & Wilson) Hampe in a mesic meadow in northern Canada (Jordan et al. 1978) and *Ditrichum strictum* (Hook. f. & Wilson) Hampe in a wet herbfield in sub-Antarctic McQuarrie Island (Line 1992). Given the widespread evidence of symbiotic N fixation for Ditrichaceae, especially for *Ceratodon purpureus,* it is somewhat surprising that only four of the ARA results were positive—two for each moss species. The positive ARA result from *Ditrichum flexicaule* in the beach-ridge complex appears to be the first report of N fixation for this taxon, which is of particular interest given its widespread distribution in early-successional dunes of the Great Lakes (Maun 2009).

In the context of the literature available on Ditrichaceae, the minimal N fixation associated with *Cerotodon purpureus* is mysterious. One would typically expect

to find a diverse cyanobacterial community on the phyllids and stems of this moss (e.g., Maraist 2018). The rarity of N fixation in *Ditrichum flexicaule* is harder to interpret; there are not enough studies on this genus to establish an expected condition with respect to cyanobacterial symbiosis. It is impossible to know whether the rarity of N fixation at this site represents a global anomaly or a globally typical outcome for *Ditrichum*.

Although there are only a few specimens of *Ditrichum flexicaule* from the Great Lakes region in the University of Wisconsin-Madison herbarium, at least one provided evidence that this moss has hosted cyanobacteria in the past. Cyanobacterial biofilms were visible to the naked eye on a specimen collected by Sally Freckman (#934) on August 8, 1973 and determined by Howard Crum. Filaments from Oscillatoriales as well as *Nostoc* colonies were apparent under light microscopy in 2013. Hence we have very tentative evidence (two points in time, two points in space) that mosses in the genus *Ditrichum* may host N-fixing cyanobacteria. Whether *Ditrichum flexicaule* routinely hosted N-fixing cyanobacteria in the past, or routinely does so today at other coastal dune sites in the Great Lakes region, is unclear.

Mosses in the family Pottiaceae, including the xeric-adapted *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr and *Tortella tortuosa* (Schrad. ex Hedw.) Limpr., are also a dominant element of the sandy beach ridges. *Syntrichia ruralis* is considered desiccation tolerant (Brown & Buck, 1979), and *Tortella tortuosa* is

well-adapted to mobile sand, with the ability to emerge rapidly from sand burial (Martínez & Maun, 1999). None of the samples affiliated with Pottiaceae yielded positive ARA results. In the literature, I was able to locate only two studies of symbiotic potential for *Syntrichia ruralis*. Snyder & Wullstein (1973), working in a desert ecosystem in Utah, detected cyanobacteria but no N fixation. Opelt & Berg (2004), studying moss from sand dunes along the Baltic Sea in Germany, failed to detect cyanobacteria in DGGE profiles of DNA sequence data; N fixation was not studied. Cyanobacteria have also been detected in a closely related species, *Syntrichia princeps* (De Not.) Mitt. in coastal Antarctica (Alfinito et al., 1998). I was able to locate a single published report of cyanobacteria in association with *Tortella tortuosa* (Pentecost & Whitton, 2012), but the habitat was not specified. The genus *Tortella* (Lindb.) Limpr. hosted cyanobacteria in moist depressions in a willow shrub tundra in the Rocky Mountains of Colorado (Wojciechowski & Heimbrook, 1984). Overall the evidence from the sandy, unstable beach ridge sites is in synch with the sparse evidence available from dry, sandy ecosystems elsewhere: there is no measurable N fixation associated with *Syntrichia ruralis* and *Tortella tortuosa* in these xeric habitats. However, with only one similar study of N fixation (Snyder & Wullstein, 1973), it is possible that the published results do not reflect the full range of environmental conditions. Symbiotic N fixation is quite common in Pottiaceae, making further investigation of N fixation potential intriguing.

Bryophyte-associated N fixation on inland sites of the beach-ridge system (The Ridges Sanctuary). The alternating ridge-swale topography is dominated by families with low frequencies of symbiotic N fixation, or no evidence of fixation. The primary question of interest is why do so many of these mosses, which are associated with symbiotic N fixation elsewhere, yield little or no evidence of N fixation in Baileys Harbor? Common upland families include Dicranaceae and Hylocomiaceae. Common wetland families are largely similar to those at the beach sites, and will not be discussed in further detail.

Mosses in Dicranaceae at the beach-ridge ecosystem were typically associated with negative ARA results. Positive ARA results were associated with three samples of *Dicranum polysetum* Sw., all from upland ridge sites. Two were marginally positive (just over the Method Detection Limit), one more strongly positive. Two of these three positive results were associated with the water treatment (saturation of field samples). Negative ARA results were associated with *Leucobryum glaucum* (Hedw.) Ångstr., known as the pin-cushion moss, as well as *Dicranum montanum* Hedw., *Dicranum scoparium* Hedw., and some samples of *Dicranum polysetum.* Overall the beach-ridge complex evidence suggests symbiotic incapacity or symbiotic inactivity with respect to most Dicranaceae samples.

In the literature, evidence on N fixation in Dicranaceae is mixed. About half of the studies report positive ARA or stable isotope results (Lambert & Reiners 1979,

Brasell et al. 1986, Christie 1987, Nakatsubo & Ohtani 1991, Stuart 2021). However, negative ARA studies are also associated with Dicranaceae (Fogg & Stewart 1968, Granhall & Hofsten 1976, Gundale 2011, Bay et al. 2013, Arróniz-Crespo et al. 2014), primarily with the genus *Dicranum* Hedw. A more recent study involving 8 species of *Dicranum* in Alaska (Stuart et al. 2021) provided a nuanced profile of N fixation activity. Most species (7 of 8) had detectable N fixation activity, but mean fixation rates by species were relatively low compared to mosses in other families. Also, the 95% confidence interval for the mean rate by *Dicranum* species included zero for 6 of 8 taxa (Table 2.3, Stuart 2021), suggesting that the mean rate by species may not necessarily differ from zero. Only *Dicranum elongatum* Schwgr. (1.46 ± 0.44 μg N g moss^{-1} day^{-1}) and *Dicranum scoparium* Hedw. (1.93 ± 0.63 μg N g moss^{-1} day^{-1}) had mean rates marginally greater than zero based on the confidence interval. It is unclear whether the positive N fixation results for *Dicranum* are associated with cyanobacteria or with other diazotrophic taxa in Stuart (2021). Salemaa et al. (2019) also reported rates above the detection limit for N fixation in *Dicranum* species of the boreal forest in Finland, but relatively close to zero (0.002-0.003 mg N kg^{-1} day^{-1}) or equal to zero. Rzepczynska et al. (2022) reported similar mean fixation rates (≤ 5 μmol N m^{-2} hr^{-1}) for *Dicranum scoparium* in Sweden, with a 95% confidence interval including zero.

Overall the evidence available in the literature does not provide a clear picture of symbiotic capacity for N fixation in Dicranaceae. Some taxa (outside the genus

Dicranum) are clearly capable of hosting N-fixing cyanobacteria. In many boreal ecosystems mosses in *Dicranum* appear to be incapable of symbiotic N fixation (e.g., Gundale 2011), and Bay et al. (2013) found evidence of symbiont incapacity in a colonization study involving *Dicranum* and *Nostoc*. But there is evidence of occasional low-positive N fixation within the genus. Whether this can be attributed to cyanobacteria, to other diazotrophic epiphytes, or both is unclear.

Inorganic N deposition and dissolved organic N inputs from the canopy have been implicated in the suppression of biological nitrogen fixation for *Dicranum* (Salemaa et al., 2018), with a deposition threshold of of 3-4 kg ha^{-1} $year^{-1}$. A map (Hamlin et al., 2020) based on the National Atmospheric Deposition Program Total Deposition Science Committee product for total nitrogen deposition indicates that rates are relatively low in Door County (< 7 kg ha^{-1} yr^{-1}) compared to most of the Great Lakes region (7-11 kg ha^{-1} yr^{-1} or more). Without direct measurement, it is not clear whether the beach-ridge ecosystem in Baileys Harbor typically exceeds the 3-4 kg ha^{-1} yr^{-1} of N deposition required for suppression of biological N fixation in *Dicranum*, but that is certainly a possibility. Experimental work demonstrating the suppression of symbiotic N fixation in other mosses in Dicranaceae (i.e., non-*Dicranum* species such as the pincushion moss *Leucobryum glaucum*) has not been conducted yet. Future work in this area might focus on developing a reasonable explanation for the apparent divergence in symbiotic capacity in Dicranaceae (negligible to low in *Dicranum*, but notable in some non-*Dicranum* mosses).

Hylocomiaceae is another abundant family found on upland dune ridges (away from the beach sites) and occasionally in wetland swales near the edge of the water. The most abundant species within the family at this site is *Pleurozium schreberi* (Willd. ex Brid.) Mitt., followed by *Hylocomium splendens* (Hedw.) Schimp. and *Hylocomiadelphus triquetrus* (Hedw.) Ochyra & Stebe; the latter is relatively rare. *Pleurozium schreberi* and *Hylocomium splendens* are dominant feather-mosses in boreal forests, contributing substantially to biological N fixation on a global scale (Gundale et al. 2012); researchers have suggested that *Pleurozium schreberi* may contribute more to biotic N fixation than any other species on Earth, considering taxa that are neither cultivated nor distributed by humans (Zackrisson et al. 2011). The potential for N fixation in feather mosses has been well understood for over 40 years; early studies on Canadian black spruce forest found rates ranging from 1 kg ha^{-1} yr^{-1} (Alexander & Billington, 1986) to 12.1 kg ha^{-1} yr^{-1} (Weber & Van Cleve, 1981). Dozens of field studies (Table 10) have been conducted involving *Pleurozium schreberi* and *Hylocomium splendens* in boreal forest ecosystems, with most in Canadian or Fennoscandian forests. More recent studies confirm relatively substantial rates of biotic fixation for feather mosses in these areas: up to 4 kg ha^{-1} yr^{-1} (e.g., DeLuca et al., 2002; Lagerström et al., 2007; DeLuca et al., 2008; Gundale et al., 2010, 2011; Stuart 2021).

Evidence of epiphytic N fixation in feather mosses in other regions is limited. In the continental United States, *Pleurozium schreberi* and *Hylocomium splendens* are widely distributed and common, found in 39 and 34 states respectively (Tesky 1992). But in the U.S., studies of N fixation potential have rarely been conducted outside of Alaska. In a sub-alpine forest in the White Mountains of New Hampshire, *Pleurozium schreberi* was associated with positive ARA results (Lambert & Reiners, 1979). In a temperate New York forest with poorly-drained soils, no cyanobacteria were detected on *Pleurozium schreberi* screened by epifluorescence microscopy (Deane-Coe & Sparks, 2016). And in a temperate North American grassland (Calabria et al. 2020), cyanobacteria and N fixation were detected on *Pleurozium schreberi* (Brid.) Mitt. and [12]*Rhytidiadelphus triquetrus* (Hedw.) Warnst., as well as in a related moss, *Racomitrium elongatum* Frisvoll; all three mosses belong to Hylocomiaceae. I could not locate any studies pertaining to symbiotic N fixation in *Hylocomium splendens* in the contiguous states of the U.S. The data from the beach-ridge complex in Baileys Harbor may be the first attempt to characterize N fixation in *Hylocomium splendens* at lower latitudes in North America. The ARA results were uniformly negative.

As atmospheric N deposition increases, N fixation per unit moss mass and per unit area decrease notably for feather mosses (Gundale et al. 2011); latitudinal gradients in N fixation are associated with N deposition (e.g., Zackrisson et al.

[12] *Rhytidelphus triquetrus* (Hedw.) Warnst. is a synonym of *Hylocomiadelphus triquetrus* (Hedw.) Ochyra & Stebe per Tropicos (2023): Tropicos.org. Missouri Botanical Garden. 25 May 2023 <https://tropicos.org/name/35217912>

2004, DeLuca et al. 2008, Zackrisson et al. 2009, Leppänen et al. 2013). But this is not necessarily true for all ecosystems; a recent study in Quebec, Canada demonstrated that N deposition rates across a latitudinal gradient were not important controls on bryophyte-associated N fixation in black spruce forest (Renaudin et al. 2022). Likewise, Scott et al. (2018) failed to find evidence of a response to a latitudinal gradient in N deposition along roadways in northern Sweden. In Fennoscandia, very low rates of N fixation (0.1 kg N ha^{-1} yr^{-1}) are typical for feather mosses at low latitudes (< 64° N), where atmospheric N deposition is higher (Zackrisson et al., 2009). *Pleurozium schreberi* appears to be more sensitive than *Hylocomium splendens*, as even low rates of atmospheric N deposition (3-4.25 kg ha^{-1} yr^{-1}) may inhibit symbiotic fixation in *Pleurozium* (Zackrisson et al. 2004, Gundale 2013). By contrast, even relatively high rates of atmospheric deposition (10 kg ha^{-1} yr^{-1}) may result in decreased fixation rates rather than inhibition in *Hylcomium splendens* (Zackrisson et al. 2009). Inhibition has been documented at 12.5 kg ha^{-1} yr^{-1} in *Hylocomium* (Gundale et al., 2013).

The near-total absence of N fixation in feather mosses at the Ridges is not readily explained by atmospheric N deposition based on Hamlin and colleagues' spatial mapping of these inputs (2020). These researchers estimate N deposition in Door County, Wisconsin is < 7 kg ha^{-1} yr^{-1}. One might expect to see inhibition in *Pleurozium schreberi* under these conditions, but not necessarily in *Hylocomium splendens.* One potential explanation might relate to local variability in N deposition; local hotspots may exceed the typical values, particularly along

the lakeshore, which receives atmospheric input from heavily industrial areas in the southern Great Lakes. But if the local rate of atmospheric deposition is relatively low (in accord with Hamlin et al. 2020), other control factors may be involved in the suppression of cyanobacterial N fixation.

Overarching control factors for bryophyte-associated N fixation include temperature and precipitation; the importance of temperature and precipitation, discussed in early Antarctic studies such as Fogg & Stewart 1968, has been verified in numerous research projects over the past several decades. In boreal ecosystems, higher moisture levels and higher temperatures are generally favorable to N fixation, although excessively high or low temperatures may have negative impacts, including inhibition. Temperature and precipitation may interact with one another (e.g., Zielke et al. 2005, Rousk et al. 2017). Work by Whiteley & Gonzalez (2016) and Gundale et al. (2012b) highlights the role of drought in inhibition of N fixation for feather-moss groundcover, with results suggesting moisture limitations may lead to inhibition more quickly than increases in temperature (Whiteley & Gonzalez 2016). Evidence from our mixed-effects model indicates that short-term moisture limitations suppress N fixation with respect to the entire bryophyte flora, but because there was almost no response to the water saturation treatment in *Pleurozium schreberi* and *Hylocomium splendens*, we can rule out hydration of the bryophyte sample as a general constraint on N fixation in feather mosses at the time of sampling. The treatment does not illuminate the potential impacts of precipitation over longer time periods,

however; one cannot rule out suboptimal inputs from snow or rain at a seasonal or annual scale.

Response to temperature for a given feather moss may vary by site. Rousk et al. 2017 found that N fixation generally increases as temperature increases for *Pleurozium schreberi* from temperate, sub-arctic, and arctic ecosystems, with an optimal rate between 20° C and 30° C. In Abisko, Sweden, Rzepczynska et al. (2022) found optimal rates occurred at 13° C, with a sharp decline in fixation (to 20% of maximum rate) at 9° C and 18° C. In addition to having a higher optimum, *Pleurozium schreberi* became acclimated to higher temperatures in the Rousk et al. (2017) experiment; this was not evident in the later Swedish study (Rzepczynska et al. 2022). The temperature-response curve for *Hylocomium splendens* resembles that for *Pleurozium schreberi* in northern Sweden (Rzepczynska et al. 2022). Given the potential site-specificity of the temperature-response curves for *Pleurozium schreberi* and *Hylocomium splendens*, it is difficult to know whether high temperatures during the summer season might limit or inhibit symbiotic N fixation at the Wisconsin beach-ridge ecosystem. Temperature is thus a potentially important control factor for future investigations of feather mosses at the beach-ridge system in Baileys Harbor.

Other potential limits on symbiotic N fixation in feather mosses might include the availability of molybedenum (Rousk et al. 2017), vanadium (Renaudin et al. 2022), and phosphorus (Basilier 1979, Rousk et al. 2017, Renaudin et al. 2022).

Molybdenum and vanadium are potential co-factors for different forms of the nitrogenase enzyme; phosphorus provides energy in the form of ATP for the fixation reaction. Increased availability of molybdenum and vanadium appears to facilitate symbiotic N fixation. However, recent work suggests that increased phosphorus does not always stimulate N fixation, as one might expect given the need for ATP. A meta-analysis by Zheng et al. (2019) indicates that P additions typically stimulate symbiotic N fixation, but inhibit free-living microbial fixation. Overall the impacts of P are complex, and may be site-specific. A recent random forests analysis indicated that moss tissue P concentrations are not important in determining rates of symbiotic N fixation in two boreal forest mosses, one of which is *Pleurozium schreberi*; but P concentrations do influence cyanobacterial biomass (Renaudin et al, 2022). Phosphorus appears to have complex interactions with other elements, including potential interactions with nitrogenase co-factors such as molybdenum (Scott et al., 2018). Its overall role in regulation of symbiotic N fixation is still a topic of debate.

Inputs from automobile emissions (Ackermann et al. 2012, Scott 2018) or nutrient-rich litter (Jean et al., 2020, Alvarenga & Rousk, 2021) may also decrease rates of fixation. Throughfall N, which may integrate nutrient inputs from multiple sources, is likewise implicated in limitations on symbiotic N fixation for moss (DeLuca et al. 2008, Ackermann et al. 2012, Rousk & Michelson 2017, Salemaa et al. 2019, Jean et al. 2020, Alvarenga & Rousk, 2021). Moss tissue stoichiometry, specifically C:N ratios, play a role in regulating symbiotic N fixation

in some boreal ecosystems (Leppänen et al. 2013, Darnajoux et al. 2018); in *Pleurozium schreberi*, for example, yellow-colored shoots have higher C:N ratios and consistently higher rates of N fixation compared to green shoots with lower C:N ratios (Darnajoux et al. 2018). Known exceptions include mosses on tropical forest trees Puerto Rico, where C:N ratios did not predict N fixation rates (Cusack et al., 2009). Other treatments that typically influence moss-associated N fixation (e.g., N fertilizer, moisture levels) also had no impact at this tropical site. Such interesting results can be found in a range of ecosystems, boreal, tropical, and otherwise, where local conditions and the composition of the bryophyte flora may interact with potential control factors, generating unexpected results by mechanisms not well characterized.

To summarize: there is a long list of potentially interacting influences that might explain the absence of cyanobacteria (documented with microscopy) and N fixation in *Pleurozium schreberi* and *Hylocomium splendens* in upland areas of the beach-ridge complex. Atmospheric N deposition likely plays a role in the absence of N fixation for *Pleurozium*, but its impact on *Hylocomium splendens* is less clear. Direct measurement of N deposition rates and throughfall on site might help to clarify. Temperature and precipitation are also fundamentally important, and with rising temperatures due to climate change and the potential for more-frequent desiccation at elevated temperatures, investigation of these factors would be useful. Among the other variables that might account for the lack of N fixation in feather mosses, auto exhaust stands out as potentially

informative. Door County is a tourism juggernaut within the state of Wisconsin; in 2022, post-pandemic tourism revenues for the county were $466 million dollars (Green Bay Press Gazette, 2023). Baileys Harbor has long been a center for ecotourism, but most travel involves automobiles, and the beach-ridge complex is bisected by a popular road along the lakeshore. There is a parking lot at the beach site (Town Beach) in the ridge complex, which is heavily used during summer. Automobile exhaust, with the potential for heavy metal contamination of plants and soil, probably affects sites close to Ridges Road along the lakeshore, including the first inland ridge (South Sandy Ridge). Automobile exhaust seems less likely to be influential at the older inland sites, which are more than 100 m from the road and from Route 57; Scott et al. (2018) documented a sphere of influence within 100 m for roads in northern Sweden. Likewise, the Moonlight Bay beach site near Stone Mill Lane is unlikely to receive a lot of exhaust; this dirt road is used only by a dozen or so homeowners. However, Highway 57 is parallel to Stone Mill Road (further inland) and has heavier traffic. Emissions from traffic along this road could impact symbiotic N fixation, if the zone of influence extends further than 100 m (to a half-kilometer or so). I see no reason to either prioritize or rule out any of the other potential control factors, including molybdenum, vanadium, phosphorus, nutrient-rich litter, and C:N ratios; all appear equally likely/unlikely. C:N ratios are an integrative signal and would necessarily be investigated in conjunction with atmopheric N deposition, throughfall, and litter quality.

A final consideration relates to the quality of the data collected. ARA can be conducted *in-situ* or in the laboratory. Each method has its own set of limitations. In this case, the process of transferring gases from the headspace to a new vial, and then transporting the vials to Madison for gas chromatography after several days of fieldwork resulted in some loss of signal. Quality control trials with acetylene indicated that losses were highly variable but could amount to up to 45% of the acetylene initially introduced into the vial. These data thus have a potentially strong bias toward negative results.

However, the combined evidence from light microscopy studies and an additional season of fieldwork, in which I brought living bryophyte samples to the laboratory to conduct ARA, suggests that the results described here may be reasonably accurate. The pattern of intense N fixation for taxa in Amblystegiaceae, Brachytheciaceae, and Bryaceae, combined with minimal or no N fixation in the feather-mosses of Hylocomiaceae and the mosses of Dicranaceae, is supported by data from laboratory studies not shown here.

Perspectives on symbiosis. Diverse perspectives exist with respect to the ubiquity of cyanobacterial associations in land plants, in particular among bryophytes. There is a large body of research (e.g., Meeks 1985, Adams and Duggan 2008, Ekman et al. 2013) focused on model taxa with well-defined morphological adaptations and relationships to cyanobacterial symbionts in the genus *Nostoc*. Examples include the liverworts *Blasia pusilla* L. and *Cavicularia*

densa Steph., which host *Nostoc* cyanobacteria in slime papillae. If one applies a narrow definition of symbiosis involving nutrient exchange and morphological adaptation in the host, cyanobacterial symbioses appear to be relatively rare among bryophytes as well as vascular plants. Rikinnen (2017) summarizes this widely-held view: “However, only a restricted and highly paraphyletic assemblage of land plants establish well-defined symbioses with cyanobacteria. These include two genera of thalloid liverworts (Marchantiophyta), all hornworts (Anthocerophyta), one genus of ferns (*Azolla*, Salviniales), all cycads (Cycadophyta), and one isolated genus of angiosperms (*Gunnera*, Gunnerales).” These plants are typically found with *Nostoc* symbionts.

By contrast, other researchers gravitate towards a broader definition of symbiosis, closer to that of Anton de Bary, who described “the living together of unlike organisms” (Bary 1978 in Egerton 2015). With a broader definition of symbiosis, the on-again, off-again relationships of diverse bryophytes and epiphytic cyanobacteria may be considered symbioses; Rousk (2022) summarizes this perspective: “All moss species harbor cyanobacteria, albeit to a varying degree.” This viewpoint is consistent with emerging genomic evidence, which supports the early development of microbial symbioses in algae and the early land plants. Knack et al. (2015) found evidence of an ability to host nitrogen-fixing bacteria including cyanobacteria and rhizobialeans in the charophyceaen algae, the closest relatives of the land plants. Evidence that charophyte algae share biochemical pathways and other symbiotic attributes with

basal land plants continues to accrue (e.g., Delaux & Schornack 2021, Picou 2022, Satjarak 2022); these symbioses involve both cyanobacteria and fungi (Delaux et al., 2015, Delaux & Schornack 2021).

The diverse bryophyte species involved in N fixation at my site, as well as the proportion of novel diazotrophic symbioses (approximately 15%), support the idea that bryophyte-cyanobacterial symbioses, defined broadly to include both intracellular and epiphytic association, may be relatively common. 26 of the 32 bryophyte genera studied are associated with symbiotic N fixation in study results or in published literature; this is in accord with the idea that cyanobacterial symbiosis may be a basal feature of the early land plants or a recurring phenomenon resulting from convergent evolution (e.g., Knack et al. 2015, DeLaux & Schornack 2021). There are a number of species (13 of 41) that did not support N fixation or cyanobacteria at my site or in the published literature, but many of these species were not studied in the publications I was able to review. The genus *Brachythecium,* for example, is a well-known host of cyanobacteria, but the species at my site that were not associated with N fixation (*B. acuminatum, B. rutabulum,* and *B. velutinum*) were not characterized in the available literature. However, some of these species hosted cyanobacteria at the Cana Island Road site (Ederer thesis, Part I, 2023), and may be capable of symbiotic N fixation under different conditions. Likewise, *Calliergon giganteum* is not associated with N fixation at the beach-ridge complex, but N-fixing microbes were documented in *Calliergon richardsonii* at other sites globally (Schell &

Alexander 1973, Solheim et al. 1996, Liegen & Olson 1997, Solheim et al. 2004). *Dicranum montanum* samples yielded negative ARA results, but (as discussed previously), mosses within the *Dicranum* genus are occasionally associated with low-positive ARA results. *Plagiomnium affine* did not provide evidence of N fixation on site, but *Plagiomnium cuspidatum* did, and there are numerous reports of N fixation and/or cyanobacterial symbionts at the genus level: *Plagiomnium cuspidatum* (Lambert & Reiners 1979, Zhang et al. 2014), as well as *Plagiomnium ellipticum* (Solheim et al. 1996) and various taxa originally identified to the genus *Mnium* Hedw.[13] (Schell & Alexander, 1973; Wojciechowski & Heimbrook, 1984). *Tortella tortuosa* is associated with negative ARA results at the beach-ridge complex, but known to host cyanobacteria (Pentecost & Whitton 2012). Likewise the genus *Tortella* (Lindb.) Limpr. can host cyanobacteria and is associated with symbiotic N fixation (Wojciechowski & Heimbrook, 1984). Moreover, almost all of the species associated with negative results have small sample sizes ($n \leq 4$). Only *Leucobryum glaucum* in Dicranaceae ($n = 10$), and *Tortella tortuosa* (n = 14) provide more convincing evidence of a lack of symbiotic N fixation. The latter result is surprising, given the abundance of cyanobacterial symbionts found on *Tortella tortuosa* at the boreal forest on Cana Island Road.

If we consider the question of whether the *genus* is associated with symbiotic N fixation (by cyanobacteria and/or other N-fixing microbes), about 80% of the taxa

[13] Some taxa formerly assigned to *Mnium* now belong to *Plagiomnium*; some belong to other genera. An older reference to *Mnium* sp. cannot be assigned with certainty to genus without reviewing herbarium material.

(26 of 32 bryophyte genera) are capable of associating with N-fixing bacteria. The published evidence supporting non-diazotrophic status at the genus level is summarized in Table 11. Note that the supporting evidence is either sparse or unavailable, presumably due to a lack of relevant publications, although limitations in digital access or search inefficiencies cannot be ruled out. Based on this information, it appears that most of the boreal bryophyte flora in the beach-ridge complex is capable of symbiosis with diazotrophic bacteria given appropriate environmental conditions.

Species not associated with N fixation at beach-ridge complex	**Genus**	**Published evidence of lack of symbiotic N fixation in genus**	**Nature of evidence**
Climacium dendroides (Hedw.) F. Weber & D. Mohr	*Climacium* F. Weber & D. Mohr	Neutral: no relevant publications discovered	None available to author
Conocephalum conicum (L.) Dumort.	*Conocephalum* Hill	Knack et al. 2015	No cyanobacterial sequences found in *Conocephalum conicum* metagenome
Leucobryum glaucum (Hedw.) Ångstr.	*Leucobryum* Hampe	Neutral: no relevant publications discovered	None available to author
Lophocolea heterophylla (Schrad.) Dumort.	*Lophocolea* (Dumort.) Dumort.	Neutral: no relevant publications discovered	None available to author
Plagiothecium laetum Schimp.	*Plagiothecium* Schimp.	Neutral: no relevant publications discovered	None available to author
Radula complanata (L.) Dumort.	*Radula* Dumort.	Neutral: no relevant publications discovered	None available to author

Table 11. Moss genera that did not generate detectable N fixation at the Baileys Harbor beach-ridge sites, as well as any relevant publications on symbiotic N-fixing capabilities.

Moreover, there is specific evidence of cyanobacterial symbiosis for about 75% of all taxa (24 of 32 bryophyte genera) in either the literature or my observational

data. The only genera that lack evidence of cyanobacterial symbionts in either source are *Brotherella, Calliergonella, Climacium, Conocephalum, Leucobryum, Plagiothecium, Platydictya,* and *Scapania.* Even among these taxa, symbiotic N fixation has been documented in some genera, although the nature of the diazotroph is unknown. *Brotherella* is associated with N fixation in single-species samples (Lambert & Reiners 1979) and more tentatively, in mixed-species samples (Han et al. 2010). *Calliergonella* and *Platydictya* are also associated with N fixation at the beach-ridge site. By contrast there is no evidence of diazotrophy for *Lophocolea,* but cyanobacteria were associated with this liverwort at the Cana Island Road site (Ederer thesis, Part III, 2023). There may be a variety of mechanisms responsible for the apparent lack of cyanobacterial symbionts in these taxa. For example, *Conocephalum conicum* has well-developed rhizoids and is known to harbor intercellular fungal symbionts (e.g., Ligrone & Lopes 1989, Turnau et al. 1999), suggesting that fungi, rather than cyanobacteria, may provide supplemental nitrogen.

Rates of N fixation and impact of water limitation. How biologically important is the increase in N fixation associated with the saturation treatment? I do not have enough data to build a nuanced model given the range of dates, sites, and seasons involved; the purpose of this experiment was to explore a potential control mechanism, not to robustly characterize variability in time and space. But I can estimate a range of plausible values for the potential loss of N fixation activity due to short-term water limitations, if I make some simplifying

assumptions. These include: (1) the impacts of short-term water limitations are uniform across all 210 days of the growing season, and negligible during the remainder of the year; (2) water limitation will suppress N fixation activity in about 12% of all bryophyte cover based on the frequency from our experiment, and (3) the impacts are similar across wetland and upland sites. Per Hardy et al. (1968), a standard calibration ratio (3 moles ethylene: 1 mole N_2 fixed) was utilized to compute the estimates of N fixation found in Table 12. The estimates of N fixation per hectare vary depending on the percent bryophyte cover; I have presented estimates for a range of plausible values based on the mean ethylene peak, given that bryophyte cover varies across the beach-ridge complex. Given the strong simplifying assumptions involved, the results in Table 12 (and inferences related to Table 12) are best regarded as something akin to a quantitative pilot study, rather than a model strongly grounded in substantial evidence.

Percent bryophyte cover	**Estimated N fixation per hectare suppressed by short-term water limitations (kg ha^{-1} yr^{-1})**
10	0.2
20	0.4
30	0.6
40	0.8
50	1.0
60	1.2
70	1.4
80	1.6
90	1.8
100	2.0

Table 12. Estimates of potential loss of fixed N per hectare due to water limitation.

Estimates of bryophyte percent cover at The Ridges range from 13-27% (upland sites) to 50-95% (wetland sites). This suggests that a plausible range for loss due to water limitation is 0.2-0.6 kg ha^{-1} yr^{-1} for the upland ridges, and 1.0-1.6 kg ha^{-1} yr^{-1} for the wetland swales. These are potentially nontrivial losses, both within the context of observed N fixation rates at The Ridges and within a global context. For example, the estimates of bryophyte-associated N fixation for the two lakeside ridges (based on detailed data from 2009) were 1.1 and 1.3 kg ha^{-1} yr^{-1}. Percent bryophyte cover for both upland sites is about 20%. The estimated reduction due to water limitation is 0.4 kg ha^{-1} yr^{-1} per site, about 30-35% of the observed total for the each of the upland beach sites. The estimated reduction for the lakeside wetland areas, about 1.6 kg ha^{-1} yr^{-1}, is larger in absolute value but may have a more modest impact: it constitutes a 6-8% reduction of the observed annual rates from 2010 (18.9 and 26.7 kg ha^{-1} yr^{-1}). I do not know of any comparable studies on bryophyte-cyanobacterial N fixation from the Great Lakes area; the data collected at The Ridges Sanctuary is the sole regional reference.

A global-scale model of free-living and symbiotic nitrogen fixation based on 253 high-quality field studies (Davies-Barnard & Friedlingstein 2020) provides additional context. The land cover categories utilized by these researchers do not map exactly onto the vegetation at The Ridges, but it may be reasonable to compare the wetland swales to reference values for Permanent Wetlands, and the upland ridges, to reference values for Mixed Forest. Table 13 provides

estimates of symbiotic biological N fixation from these reference ecosystems (Davies-Barnard & Friedlingstein 2020).

Land cover type classification	Median estimate of symbiotic biological N fixation (kg ha^{-1} yr^{-1})	Number of field studies providing basis for estimate
Permanent wetland	6.92	11
Mixed forest	7.47	1

Table 13. Global-scale reference values from Davies-Barnard & Friedlingstein (2020) for wetland and upland land cover classifications similar to those in the beach-ridge complex.

The estimated reduction in N fixation capacity for wetlands at the beach-ridge complex (1.0-1.6 kg ha^{-1} yr^{-1}) is 14-23% of the median global-scale reference value for wetland land cover. For the upland ridges, the estimated reduction is 5-8% of the median global-scale reference value. The reference value for this land cover classification type is based on a single study (Davies-Barnard & Friedlingstein 2020), so some caution is appropriate with respect to interpretation.

Based on the global-scale N fixation estimates in Table 13, such moisture limitations may have a notable impact on the wetland swales, and a minimal impact on the upland ridges. By contrast, the local estimates indicate that short-term water limitation may be of little importance in wetland areas (due to the relatively high rate of symbiotic N fixation overall), but more influential in the upland ridges.

Summary

This study provides strong evidence that wetland status and short-term moisture limitations influence the likelihood of bryophyte-associated N fixation in a beach-ridge complex on Lake Michigan in northern Wisconsin. Wetland sites, both inland and directly on the beach, are more likely to host N-fixing microbes (including but not limited to cyanobacteria) than the upland ridges. The likelihood and intensity of N fixation in the wetland swales decreases as distance from the lakeshore and sediment age increase. Experimental addition of water activated symbiotic N fixation in a small but statistically significant proportion of bryophyte samples (about 12%), indicating that dry conditions during the growing season constrain N fixation. The results with respect to the incidence of N fixation may be biased towards negative results, given the logistical difficulties of *in-situ* assessment of N fixation. However we can regard the positive results with high confidence, and the negative results are supported by laboratory assays from a subsequent field season.

The moss families Amblystegiaceae, Bryaceae, and Hypnaceae are strong drivers of the high rates of symbiotic N fixation found in the beach wetlands along Lake Michigan, potentially contributing fixed N to soils, vascular plants, and lake water, which flows through and washes over the beach wetlands. Real estate development typically involves disruption or elimination of the bryophyte ground cover along the shoreline, implying that a substantial loss in fixed N may be

associated with building along the coastline. The data from this study suggest that increased drought frequency, proposed as one possible effect of climate change, may notably reduce symbiotic N fixation even in relatively intact bryophyte-dominated wetlands in the Great Lakes region.

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