THE ROLE OF HOST IDENTITY IN HIGH LATITUDE MOSS-ASSOCIATED NITROGEN FIXATION

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ABSTRACT

THE ROLE OF HOST IDENTITY IN HIGH LATITUDE

MOSS-ASSOCIATED NITROGEN FIXATION

JULIA M. STUART

Mosses make up a significant portion of primary plant productivity in Arctic and boreal ecosystems and are important regulators of biogeochemical cycling. In addition to producing recalcitrant litter and insulating soils, mosses often host epiphytic microbes capable of fixing nitrogen (N) from the air at rates which make it the largest source of a limiting nutrient in these environments. Since the availability of N is linked to carbon (C) fixation and decomposition, the current and future rates of N_2 fixation are important topics of research in an area which stores large amounts of C belowground. Past evidence indicates that host moss identity and environmental conditions can alter rates of moss-associated N_2 fixation. However, past studies often focus on a limited number of species and use indirect methods to measure N_2 fixation. This dissertation employs $^{15}N_2$ incubations to measure rates of moss-associated fixation at sites ranging from 60° to 68° N in Alaska in both natural surveys and manipulative experiments in the field. We found that N_2 fixation is almost ubiquitous among mosses and that moss identity is consistently an important predictor of associated N_2 fixation rates. In subsequent analyses related to C stable isotopes and a reciprocal transplant, we also found a significant interaction between host identity and environment. The strength of the interaction term was typically host specific. As temperature and other abiotic conditions change along with climate and cause changes in moss biomass and diversity, it is critical to incorporate the interaction term into predictions of future N inputs.

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PREFACE

This dissertation follows the journal format for dissertations as described by the Graduate College of Northern Arizona University. Chapter I is an overall introduction including literature review on the topic of high latitude moss-associated nitrogen fixation. Chapter II is titled "Host identity as a driver of moss-associated N_2 fixation rates in Alaska" and was published in the journal Ecosystems on August 6th, 2020. Chapter III is called "The relationship of C and N stable isotopes to high latitude moss-associated N_2 fixation" and is currently in review with the journal Oecologia. The final data chapter will be submitted for peer review in the immediate future under the title "Tundra moss transplants reveal host species-specific response of associated N_2 fixation rates of environmental change". The final chapter is an overall discussion of results and conclusions. The materials and methods are discussed at length in each data chapter (Chapter II-IV) with sufficient detail to allow replication. The references are not separated by chapter, but collected at the end of the dissertation. Some redundancy arises from the inclusion of multiple articles on a similar topic and when combining these manuscripts with university formatting requirements.

CHAPTER I

Nitrogen (N) is a life-essential nutrient that is often limiting to plant growth in terrestrial ecosystems (Lebauer and Treseder 2008). Biological N_2 fixation is often the largest source of new N to ecosystems, particularly in pristine environments (Cleveland et al. 1999). In high latitude ecosystems such a boreal forest and Arctic tundra, the largest source of new N is moss-associated N_2 fixation (DeLuca et al. 2002; Gavazov et al. 2010a; Vile et al. 2014a; Kox et al. 2016). Estimates of N_2 fixation on the landscape vary from undetectable to over 20 kg N ha^{-1} yr^{-1}, through most estimates are closer to 1-2 kg N ha^{-1} yr^{-1} (DeLuca et al. 2002; Vile et al. 2014a; Rousk and Michelsen 2017). Low N_2 fixation rates associated with individual mosses are compensated for by the ubiquity and high biomass of mosses (Hobbie et al. 2005; Turetsky et al. 2010). Vascular plants with actinorhizal N_2 fixer associations, such as *Alnus spp.*, fix N_2 at locally high rates but are not common across the landscape (Mitchell and Ruess 2009). Similarly, cyanolichens fix more N_2 per unit biomass compared to mosses but are less abundant (Weiss et al. 2005) . While *Sphagnum spp.* and feather mosses such as *Hylocomium splendens* and *Pleurozium schreberi* have well-documented associative N_2 fixer communities, research presented in this dissertation also reveals that N_2 fixation activity is nearly ubiquitous among high latitude mosses (Zackrisson et al. 2009; Vile et al. 2014a; Calabria et al. 2020; Stuart et al. 2020).

Many measurements of moss-associated N_2 fixation have been made using the indirect method of acetylene reduction assays (ARA), where acetylene derived from CaC_2 and water is introduced into the headspace of a closed contained with the host mosses. Nitrogenase acts to break the triple-bonded acetylene molecule into ethylene. By measuring the amount of ethylene produced during an incubation period, the activity of the nitrogenase enzyme can be quantified.

A mole conversion ratio is then used to estimate the amount of N_2 fixed based on the rate of acetylene reduction (Rice and Paul 1971). Some studies will also use $^{15}N_2$ uptake paired with ARA measurements to calibrate the mole conversion ratio (DeLuca et al. 2002; Arróniz-Crespo et al. 2014; Vile et al. 2014a). However, the mole conversion ratio may not be consistent across time, host species, or between sites/treatments (Rousk et al. 2017a; Saiz et al. 2019a). Direct measurements of N_2 fixation using ^{15}N are preferable, particularly in environments where acetylene could inhibit diazotrophy (Warren et al. 2017).

Rates of moss-associated N_2 fixation are directly affected by a variety of abiotic factors, though the direction and magnitude of those effects are not always consistent (Lindo et al. 2013; Rousk et al. 2013). Rates of N_2 fixation generally increase with temperature until passing a threshold after which higher temperatures can inhibit fixation activity (Gentili et al. 2005; Gundale et al. 2012b, a; Jean et al. 2012). Moisture can also be a driver of rate variation. Zielke et al. (2002) found a low moisture threshold that suppresed N_2 fixation. However, rates of N_2 fixation recover quickly after water is made available and the frequency of precipitation may be more important than the amount of precipitation (Gundale et al. 2012b; Jean et al. 2012; Kardol et al. 2016). High availability of N or high N deposition rates downregulates N_2 fixation associated with mosses (DeLuca et al. 2007; Ackermann et al. 2012; Gundale et al. 2013b). The composition of the vascular plant community and the amount of light can also directly or indirectly affect rates of N_2 fixation (Gundale et al. 2012a; Jonsson et al. 2014). It is important to note that most experiments that manipulate the environment to understand the attendant effects on moss-associated N_2 fixation test their hypotheses with only one or two host moss species (Rousk et al. 2013).

There is strong evidence that the microbial communities associated with mosses are host species specific (Ininbergs et al. 2011; Bragina et al. 2012; Jean et al. 2020; Holland-Moritz et al. 2021). Mosses can chemo-attract cyanobacteria to induce hormogonia formation, which may be a source of this specificity (Bay et al. 2013a). Additionally, there is some evidence for material exchange between the host moss and the N_2 fixers (Warshan et al. 2017a). In addition to the microbial community compositional differences, different moss traits may also influence rates of N_2 fixation via the creation or maintenance of conditions within the moss carpet. Mosses have differential abilities to transport water and nutrients, maintain temperatures, or slow the rate of water loss based on anatomical or community features (Sonesson et al. 1992; van Breemen 1995; Elumeeva et al. 2011; Fukuta et al. 2012; Sokołowska et al. 2017; Brodribb et al. 2020). Moss identity may capture a combination of these many traits that can impact biogeochemical process rates (Eviner 2004).

Both moss biomass and diversity in high latitude ecosystems are expected to change with climate (Lang et al. 2012; Turetsky et al. 2012). Tundra bryophytes typically thrive in cold environments (He et al. 2016). Overall, passive warming experiments decrease moss biomass and diversity (Elmendorf et al. 2012), though that response is not universal or equally observed between moss species (Hudson and Henry 2010; Prather et al. 2019). Rates of moss-associated N_2 fixation could change directly with climate, indirectly through moss species composition, and/or as an interaction between environment and host species identity. Mosses are often treated as a single plant functional type or several coarsely differentiated groups within models or experimental manipulation experiments (Turetsky et al. 2012). Understanding the relative influence of environment, host identity, and the interaction between the two can help make better predictions for future N inputs to Arctic and boreal ecosystems.

Moss communities and their associated N_2 fixers have a disproportionate effect on the ecosystem relative to their size. In addition to being the largest source of new N, mosses control biogeochemical process rates through the production recalcitrant litter, promotion of high cation exchange capacity, insulation of soils, and competition with vascular plants (Malmer et al. 2003; Cornelissen et al. 2007a). Mosses directly benefit from N_2 fixed by epiphytic microbes, meaning that rate variation could impact moss growth over long time scales (Bay et al. 2013a; Berg et al. 2013). However, N_2 fixation may also benefit other ecosystem over short times scales via root uptake or drying events or over long time scales via decomposition, particularly through mycorrhizal associations (Rousk et al. 2016a). By providing N in a nutrient-limited environment (Shaver and Jonasson 1999; Lebauer and Treseder 2008), mosses may play a key role in plant growth and microbial respiration. Through all of these mechanisms, mosses impact carbon (C) cycling in a rapidly changing region that hosts vulnerable belowground C stores (Lindo et al. 2013; Hugelius et al. 2014).

In this dissertation, I examine moss-associated N_2 fixation and the drivers of process rate variation through a large natural survey, the natural abundance of stable isotopes, and a reciprocal transplant experiment in tundra. Each chapter considers moss host identity, environmental conditions, and the interaction between the two as drivers of process rates. Chapter II focuses on the broad scale of moss identity: broad both in the range of environments sampled and the scale of geographic variation. I used $^{15}N_2$ incubations to measure the fixation rates associated with more than 30 potential host moss species across 24 sites in three regions of Alaska. In Chapter III, I utilize the same dataset to specifically look at connections between N_2 fixation and natural abundance stable isotopes of C and N. The final data chapter, Chapter IV, makes species-specific comparisons of N_2 fixation rates and plant community structure in a

reciprocal transplant experiment between tundra sites with a mean annual temperature difference of more than 6°C. Overall, I found moss identity to be an important predictor of associated N_2 fixation rates across Alaska. I also found significant interactions between environment and host identity for some host mosses. This dissertation highlights the importance of including moss identity when predicting rates of N_2 fixation to avoid over- or under-estimating ecosystem N inputs as well as considering moss identity in conjunction with environmental change when considering the future of Arctic nutrient cycling.

CHAPTER II

Host identity as a driver of moss-associated N_2 fixation rates in Alaska

ABSTRACT

Moss-associated N_2 fixation provides a substantial but heterogeneous input of new N to nutrient limited ecosystems at high latitudes. In spite of the broad diversity of mosses found in boreal and Arctic ecosystems, the extent to which host moss identity drives variation in N_2 fixation rates remains largely undetermined. We used $^{15}N_2$ incubations to quantify the fixation rates associated with 34 moss species from 24 sites ranging from 60 to 68 degrees N in Alaska, USA. Remarkably, all sampled moss genera fixed N_2, including well-studied feather and peat mosses and genera such as *Tomentypnum, Dicranum*, and *Polytrichum*. The total moss-associated N_2 fixation rates ranged from almost zero to 3.2 mg N m^{-2} d^{-1}, with an average of 0.8 mg N m^{-2} d^{-1}, based on abundance-weighted averages of all mosses summed for each site. Random forest models indicated that moss taxonomic family was a better predictor of rate variation across Alaska than any of the measured environmental factors, including site, pH, tree density, and mean annual precipitation and temperature. Consistent with this finding, mixed models showed that trends in N_2 fixation rates among moss genera were consistent across biomes. We also found "hotspots" of high fixation rates in one fourth of sampled sites. Our results demonstrated the importance of moss identity in influencing N_2 fixation rates. This in turn indicates the potential utility of moss identity when making ecosystem N input predictions and exploring other sources of process rate variation.

INTRODUCTION

High latitude ecosystems, such as Arctic tundra and boreal forest, are globally important carbon (C) reservoirs that are often nitrogen (N) limited for vascular plants (Shaver and Jonasson

1999; Lebauer and Treseder 2008; Tarnocai et al. 2009; Hugelius et al. 2014). The largest source of new N in these ecosystems comes from microbial N_2 fixers that live as epiphytes on boreal and Arctic mosses (Alexander and Schell 1973; Basilier 1979; DeLuca et al. 2002; Lindo et al. 2013; Vile et al. 2014b). The N_2-fixing microbes that associate with mosses are generally considered to be autotrophic cyanobacteria, although some evidence does exist for a material exchange between host moss and symbiont as well as for the presence of heterotrophic N_2 fixers (Vile et al. 2014b; Warshan et al. 2017b). Rates of moss-associated N_2 fixation are connected to ecosystem nutrition, disturbance response, and C budget (Cornelissen et al. 2007a). Current evidence indicates that moss community structure and N_2 fixation rates will be affected directly or indirectly by warming temperatures (Gundale et al. 2012a; Turetsky et al. 2012; Deane-Coe et al. 2015; Carrell et al. 2019). Given this, making accurate predictions to changes in N inputs and its downstream effects on plant communities and C cycling must also rely on knowledge of interspecific variation in N_2 fixation (Hobbie 1995; Chapin 2003). Mosses are often undifferentiated from each other (or very coarsely differentiated) in vegetation models, but N_2 fixation rates could be an important classification trait, particularly since microbial symbionts can be considered an extension of plant phenotype (Turetsky et al. 2012; Wullschleger et al. 2015; St. Martin and Mallik 2017). The importance of moss-associated N_2 fixation rates in regulating C balance is clear (Lindo et al. 2013), and exploring the role of host identity in N_2 fixation can complement and improve biogeochemical predictions as climate changes.

While N_2 fixation associated with mosses is presumably as important in boreal and Arctic Alaska as it is in other high-latitude ecosystems, very few studies have been published on moss-associated N_2 fixation rates in Alaska (Alexander and Schell 1973; Holland-Moritz et al. 2018; Jean et al. 2018). Angiosperms with symbiotic N_2 fixers in Alaska, such as *Alnus* spp., fix N_2 at

locally high rates, but mosses are ubiquitous in the understory of the boreal forest and tundra ecosystems (Hobbie et al. 2005; Mitchell and Ruess 2009; Turetsky et al. 2010). Cyanolichens such as *Peltigera* spp. also fix N_2 at high rates per unit biomass, but are less abundant on the landscape (Weiss et al. 2005). Alaska is relatively pristine, largely underlain by permafrost, and expected to respond differently to climate change than similar ecosystems in Europe (Van Wijk et al. 2004; Holland et al. 2005; Pastick et al. 2015; Gisnås et al. 2017). The majority of reported N_2 fixation rates associated with mosses focus on northern Europe and common mosses such as *Sphagnum* spp., *Hylocomium splendens,* and *Pleurozium schreberi*. Feather mosses are often abundant in upland forest areas, where *Hylocomium splendens* and *Pleurozium schreberi* are co-dominant, but other mosses (*Aulacomnium turgidum, Aulacomnium palustre, Tomentypnum nitens*, etc.) can have patchy but high local abundances throughout Alaska (Vanderpuye et al. 2002; Walker et al. 2003; Turetsky et al. 2010). In Siberia, *P. schreberi* is less abundant than *H. splendens, T. nitens,* and *Aulacomnium turgidum*, which often co-dominate (Suzuki et al. 2007; Minke et al. 2009; Boike et al. 2013). Functional traits of mosses have been identified as influential on rates of N_2 fixation, and there is a large diversity in growth form and habitat preferences among the mosses listed above (Darell and Cronberg 2011; Elumeeva et al. 2011; Jonsson et al. 2014). Further, most studies have utilized $^{15}N_2$ calibrated or uncalibrated acetylene reduction assays to measure N_2 fixation, though recent evidence suggests that conversion factors for this method may be inconsistent temporally, spatially, or across moss species (Saiz et al. 2019b). Expanding the scope of N_2 fixation measurements to include more mosses and different geographic areas while utilizing $^{15}N_2$ uptake can improve the current state of knowledge about this process.

Numerous biotic and abiotic variables have been shown to affect rates of moss-associated N_2 fixation, but often experiments that focus on sources of environmental variation (such as temperature, moisture, N deposition, or phosphorus (P) availability) will test their hypotheses with only one or two species of host mosses (Rousk et al. 2013). Several studies report that the study location (and its associated biotic and abiotic factors) appear to be less important to microbial community composition and *nifH* gene expression than the host species in question, indicating a specificity between N_2 fixer communities and host mosses (Ininbergs et al. 2011; Bragina et al. 2012; Holland-Moritz et al. 2018; Jean et al. 2020). Bay and others (2013b) have shown that mosses likely chemo-attract cyanobacteria and induce hormogonia formation, which may be a source of specificity in host-microbe association. Mosses also have individual and community traits that may influence rates of N_2 fixation, such as community water retention or shade tolerance, and occupy specific micro-niches suitable to their growth (Mills and Macdonald 2004; Elumeeva et al. 2011; Jonsson et al. 2014). Differing microbial assemblages will react inconsistently to the same abiotic conditions (Gundale et al. 2012a; Leppänen et al. 2015), as, for example, cyanobacteria have different temperature optima than other N_2-fixing bacteria (Gentili et al. 2005). There may also be seasonal variation in N_2 fixation rates both within and between species, making it potentially difficult to disentangle these microbial community composition effects (Bay et al. 2013b; Lett and Michelsen 2014; Rousk and Michelsen 2017). Other studies indicate that site and abiotic factors are just as important, or more important, than host species identity in their effects on N_2 fixation (Gavazov et al. 2010a; Arróniz-Crespo et al. 2014). Nitrogen availability has consistently been shown to drive N_2 fixation rates (DeLuca et al. 2007; Ackermann et al. 2012; Bay et al. 2013b; Gundale et al. 2013b). Moisture, light availability, and micronutrient availability have also been shown to positively affect rates of N_2 fixation in

general (Gundale et al. 2012a; Rousk et al. 2013, 2017a). Vascular plant assemblage can have indirect effects on N_2 fixation rates (e.g. through canopy light penetration), which itself is affected by moisture and permafrost thaw depth (Yang et al. 2013; Jonsson et al. 2014). While environmental factors clearly influence rates of N_2 fixation, their impacts can be complex and likely interact with host identity.

In addition to the challenge of identifying the primary drivers of N_2 fixation rate variation, the presence of biogeochemical "hotspots" can further increase the difficulty of scaling N inputs to plant communities or ecosystems (Reed et al. 2011). After Reed and others (2010), a hotspot is defined as a rate of N_2 fixation that exceeds the median rate by more than three standard errors (SE). For other aspects of the N cycle, identifying where and why hotspots and hot moments occur was identified as critical for improving models (Groffman et al. 2009). Determining the geographic or temporal abundance of hotspots, as well as gaining insight into the causes of hotspots, can facilitate their inclusion in models (Reed et al. 2011). To our knowledge, hotspots have not been explicitly explored in moss-associated N_2 fixation, although past research indicates that increasing microbial diversity is tied to higher N_2 fixation rates and the occurrence of hotspots in tropical free-living N_2 fixers (Reed et al. 2010).

Our objective in this study was to evaluate the relative importance of host moss identity in driving landscape-level variation in associated N_2 fixation rates and, more specifically, to test for significant differences in N_2 fixation rates among mosses. We used $^{15}N_2$ incubation assays to determine the fixation rates associated with a total of 34 moss species across three broad geographic regions in Alaska. We used an exploratory random forest approach to determine variable importance in predicting N_2 fixation rates. We hypothesized that host moss genus would be a significant source of variation in N_2 fixation rates across a geographic region. To test for

differences in N_2 fixation rates between mosses, we used mixed models with moss genus as a fixed effect. We also assessed the occurrence of hotspots of N_2 fixation and what may contribute to their presence. In this context, evaluating the role of moss identity in predicting trends in associated N_2 fixation across a latitudinal gradient can provide valuable insights into the sources of process variation and the occurrence of hotspots. Through these goals, we highlighted possible tools and challenges for producing more accurate regional estimates of N_2 fixation rates. The diverse array of host mosses included in our research, along with a corresponding suite of environmental data from a range of ecosystems, allowed us to uniquely identify the importance of host moss identity in contributing to variation in N_2 fixation rates.

MATERIALS & METHODS

Sites

During late June 2016, 10 sites were sampled near Fairbanks, AK and 4 sites sampled near Toolik Field Station, AK, in boreal forest or alpine tundra and Arctic tundra, respectively. The following year, in June 2017, an additional 10 sites were sampled in the Anchorage, AK area, including *Tsuga* spp. dominated stands on the Kenai peninsula, *Picea* spp. areas near the University of Alaska Anchorage, and alpine tundra (Table 2.1, Table 2.2). Sites were selected based on the absence of obvious disturbance, their accessibility, and the presence of moss. At each site, a 30m transect was established, with replicate measurements of variables of interest along the transect at 5m intervals (n=6 per transect) referred to hereafter as subplots.

Site-level data collection

A 0.5×0.5 m frame was placed at every 5m increment subplot along the 30m transect to visually assess percent cover. Percent cover of vascular plants and bryophytes was agreed upon by two investigators. Thaw depth, the depth from the surface of the green moss to permafrost,

was measured by inserting a metal probe into the ground thrice at each subplot. A note was made if permafrost was either deeper than 1m or unmeasurable due to rocky soils. Organic layer depth was recorded after digging a small pit and having two researchers agree on the depth from the surface to the top of the mineral soil layer. Soil pH was measured at each subplot along the transect with a Milwaukee Instruments Professional Portable pH probe and a 2:1 water:soil slurry. Gravimetric water content was assessed by removing a 5×5×5 cm plug of moss at each subplot along the transect, placing it in an airtight plastic bag, transporting to the laboratory and immediately recording a field wet and, after 48 h in a 60° drying oven, dry weight. Water content was calculated as (field wet weight-dry weight)/dry weight. To assess exchangeable ammonium and nitrate, an index of N availability, plugs of moss were collected at each subplot along the transect and then extracted with 50mL 1M KCl under vacuum power through Büchner funnels and pre-leached Whatman 1 filter papers following 1 hour of manual agitation of the sample/KCl slurry. Extracts were frozen and transported to Northern Arizona University, where ammonium (NH_4^+-N) and nitrate (NO_3^--N) concentrations were analyzed colorimetrically on a SmartChem 200 Discrete Analyzer (Unity Scientific, Milford, MA USA) following the salicylate method and the cadmium coil reduction method, respectively. Inorganic N is expressed as mg N g dry moss^{-1} based on volume of extract and dry weight of extracted sample. Tree density was measured by taking the diameter at breast height (DBH) or basal diameter (BD) if a tree was shorter than breast height for every living tree within 1m on either side of the transect.

N_2 fixation measurements

At each site, all moss samples were identified to the genus or species level. Common mosses (appearing in six or more patches) were collected six times per site (one per subplot), while rare mosses (appearing in fewer than six patches) were sampled in all distinct subplots in

which the species was present. Bulk density of common moss species was measured by recording the dry weight of three 5×5×5 cm plugs of monospecific moss material per site. For each N_2 fixation measurement sample, roughly 40 moss ramets were collected. After collection, moss samples were returned to the lab and several moss ramets were removed as a voucher sample for identification at the University of Florida. Subsequently, the sample was divided into two subsamples, each containing ten ramets of moss, each of approximately 5 cm of length including green and senesced tissue. One subsample was immediately placed in a drying oven for 48h at 60° C, then shipped to Northern Arizona University to be measured for the natural abundance (NA) of ^{15}N. The second subsample was wetted with distilled water and placed in an airtight 60ml polypropylene syringe. The syringe was filled with 10ml of ambient air before 10ml of 98at% enriched $^{15}N_2$ gas was added for a final airspace volume of 20ml and a 50% enriched headspace (Sigma-Aldrich Inc., lot no. MBBB3807V and MBBB9003V). Samples were incubated for 24h in a common garden centrally located within each sampling area (Fairbanks, Anchorage, and Toolik). Previous studies have shown no significant difference in measured fixation rate from incubations *in situ* or incubations that occur in a similar but distinct environment (DeLuca et al. 2007). Three syringes of the same volume containing a Thermochron iButton (Model DS1921G-F5#, Embedded Data Systems, USA) were deployed simultaneously to record temperature every 10 minutes throughout the duration of the incubation. A temperature mean, minimum, and maximum was calculated for each incubation period based on iButton measurements. Following incubation, moss samples were removed from the syringes, bagged, dried as described above, and sent to Northern Arizona University for analysis.

Laboratory analysis and rate calculations

Both NA and incubated moss samples were finely ground. Six mg of each sample was rolled into tin capsules and run on a Costech ECS4010 elemental analyzer coupled to a Thermo Scientific Delta V Advantage Isotope ratio mass spectrometer to obtain $\delta^{15}N$ values. Fixation rates were calculated using the atom percent enrichment (APE) of each sample compared with its paired NA sample, and then scaling isotopic uptake by the sample weight and air:tracer ratio to calculate total ($^{15}N + {}^{14}N$) N_2 fixation (Jean et al. 2018). Rates are expressed on a per-mass basis as μg N g moss^{-1} day^{-1}. To scale rates to mg N m^{-2} d^{-1}, each genus was given an average bulk density based on measurements made at the study sites (values, in g moss cm^{-2} , were 0.146 ± 0.008, 0.067 ± 0.013, 0.066 ± 0.005, 0.037 ± 0.003, 0.043 ± 0.004, 0.027 ± 0.004, 0.046 ± 0.001, and 0.028 ± 0.002 for *Polytrichum, Dicranum, Aulacomnium, Pleurozium, Hylocomium* and *Rhytidiadelphus, Ptilium, Tomentypnum,* and *Sphagnum,* respectively) as described above or, for *Polytrichum spp.* only, from the literature (Fenton 1980). A site-level average percent cover was calculated for each genus, and that number was multiplied by the area-based N_2 fixation rate derived from the bulk density and the mass-based fixation rate. For each site, the average areal fixation rates of all mosses were summed together to estimate total mg N m^{-2} d^{-1} for each site, based on measured N_2 fixation rates and percent cover of mosses present at the site.

Sample distribution

Across all locations, N_2 fixation rates were measured for 580 samples. If samples did not have a paired NA sample, as was the case for ~60 samples, an average based on that host species within the site was used as reference. The average NA and enriched $\delta^{15}N$ was -3.07 ± 0.07‰ (mean ± SE) and 62.86 ± 4.24‰, respectively. The range of NA values was -7.64-5.31‰, and enriched samples ranged from -6.12 - 675.40‰. Samples with less than 2‰ difference between

NA and enriched samples were assumed to have a fixation rate of 0 based on the sensitivity of the isotope ratio mass spectrometer.

Statistical analyses

All analyses were conducted in R 3.4.1 (R Core Development Team) using the packages lme4 version 1.1-14 (Bates et al. 2015), emmeans version 1.3.0 (Lenth 2016), randomForest version 4.6-14 (Liaw and Wiener 2002), VSURF version 1.0.4 (Genuer et al. 2015), vegan version 2.5-5 (Oksanen et al. 2019), and car version 3.0-0 (Fox and Weisberg 2011).

To explore the importance of the environmental and taxonomic variables in explaining variation in fixation rates, we applied a random forest algorithm to each of the three geographic sampling areas: Anchorage, Fairbanks, and Toolik Field Station. Random forests are a flexible and unbiased approach that can create an informative and parsimonious model through a variable selection process while incorporating both continuous and categorical variables (Cutler et al. 2007). We opted to use random forest models over other approaches, such as variance partitioning or structural equation models, to more effectively deal with non-normally distributed data and for the ease of inclusion of categorical variables. For the random forest, collected mosses were divided into families based on the classification by Goffinet and Buck (2019) (see Table 2.3). Family was selected as the unit for analysis to capture taxonomic diversity and some trait cohesion while not overfitting the model by including a variable with many categories (i.e. moss genus), thus risking the inflation of variable importance or R^2 values. For each location, we used the VSURF variable selection package to identify variables that were most important at the threshold, interpretation, and prediction step. All variables from the "threshold" step were included in the random forest model. Each VSURF model started with the following variables to predict N_2 fixation rates ($\mu g\ N\ g\ moss^{-1}\ day^{-1}$) : tree density (trees m^{-2}, not included in Toolik

model as no trees were present at that location, as a relative index of light penetration), temperature minimum, maximum, and average during incubation (°C), gravimetric water content, altitude (m), pH, NH_4^+-N, NO_3^--N, and total extractable inorganic N (μg N g dry moss^{-1}), permafrost depth category (Shallow: 0-25 cm from surface, Moderate: 26-49 cm from surface, Deep: 50-100 cm from surface, or no permafrost above 1m), organic layer depth category (Shallow: 0-25 cm from soil surface, Moderate: 26-45cm from soil surface, Deep: below 46cm from surface, or under permafrost), mean annual temperature (MAT in °C, four-year average from 2014-2017 extracted from Climate NA), mean annual precipitation (MAP in mm, four-year average from 2014-2017 extracted from Climate NA), site of collection, and moss family (Table 2.3)(Wang et al. 2016). We then executed six averaged runs of a random forest with set randomization for all interpretation variables in each location, with mtree set at 10,000. We also ran one random forest model in the same fashion including all data and with broad geographic location (Toolik, Fairbanks, or Anchorage) as a predictor.

To test for genus-level differences in N_2 fixation rate on a per-unit mass basis, we used a linear mixed effects model with host moss genus as a fixed effect and subplot nested in site as a random effect for each of the three geographic areas sampled (Toolik, Fairbanks, and Anchorage, with site being one of the 24 locations summarized in Table 2.1 and 2.2). Genus was selected as a fixed effect due to the cohesion of traits such as growth form, anatomy, cell wall thickness, and rate of water loss within a genus (Elumeeva et al. 2011). Site was planned as a random effect in order to be able to collect target mosses across the spectrum of natural variation in habitats in which these genera occur. Due to the natural survey style of collection, only six genera had a large enough sample size to include in the model. Those genera (*Aulacomnium, Dicranum, Pleurozium, Hylocomium, Polytrichum,* and *Sphagnum*) represent a large spectrum of anatomical

diversity found in mosses of Alaska. The log+1 mass-basis N_2 fixation rate ($\mu g\ N\ g\ moss^{-1}\ day^{-1}$) was used as the response variable. Post-hoc analyses for all models were performed using the estimated marginal means to assign significance based on $\alpha = 0.01$. Excepting one random forest model for exploratory purposes, the geographic areas were analyzed separately due to the difference in sampling times, both in terms of year and progress of the growing season, to avoid confounding seasonal differences with variation from environment or identity. Site as a random effect can act as a proxy for some environmental variation (e.g. inter-site variation in altitude, MAT, and MAP) but cannot fully account for other sources of environmental variation that can change on a very fine scale (between 5m subplot intervals), such as tree density, organic layer depth, and moisture.

To test the effect of moss diversity on N_2 fixation rates, the number of moss genera present at each site (richness, *S*) was calculated. To account not just for presence, but for moss cover and dominance, the Simpson's diversity index D_1 (Simpson 1949) was also calculated for each site. These diversity indices were then regressed against the site-level summed N_2 fixation rates described above, and, if appropriate, analyzed using a linear model. Other site-level characteristics, such as MAT, MAP, N availability, gravimetric water content, and pH were also regressed against the site-level summed fixation rates to explore the occurrence of hotspots.

RESULTS

Across all three locations (Anchorage, Fairbanks, and Toolik), moss taxonomic identity consistently was the top ranked predictor of measured N_2 fixation rates (Figure 2.1). In other words, the identity of the moss was a better predictor of N_2 fixation rates than any of the measured environmental variables or the site of collection. For all three geographic areas, site of collection was the second-ranked variable and was consistently included at the "interpretation"

step of the random forest variable selection tool. In both Fairbanks and Anchorage, tree density was ranked in the top five predictors, though there was no clear linear trend relating N_2 fixation rates and tree density in post-hoc testing. Additionally, the depth of the organic layer was an important predictor in both Fairbanks and Anchorage, where post-hoc analysis revealed that shallow organic layers were associated with lower N_2 fixation rates. Toolik was the only geographic area for which pH was an important predictor of N_2 fixation rate (Figure 2.1), and all three locations included either MAP, MAT, or both as important predictors. In the random forest model that included all data, family was still the top-ranked predictor, followed by site. The geographic area (Toolik, Fairbanks, or Anchorage) was not a highly ranked predictor (Table 2.4).

When accounting for subplot nested in site as a random factor, host moss genus had a significant effect on N_2 fixation rate at all three geographic sampling regions (for Anchorage, Fairbanks, and Toolik, $p < 0.001$, Figure 2.2). Further, means comparisons indicated relatively consistent differences among host moss genera across the three geographic sampling locations. *Polytrichum* spp. and *Dicranum* spp. had fixation rates that were, at all locations, significantly lower than those of *Sphagnum spp.* or *Hylocomium* (Figure 2.2). *Aulacomnium* spp. was significantly lower than *Hylocomium* only at Toolik, whereas *Pleurozium* was never significantly higher than *Dicranum* spp. (Figure 2.2). Toolik typically had higher fixation rates, both overall and within moss genera, than the other two latitudinal sampling locations (Figure 2.2). Site as a random effect accounted for 6.7, 24, and 17% of variance explained in the model for Toolik, Fairbanks, and Anchorage, respectively. For the same models, subplot nested in site as a random effect explained very little variation: less than 10% at Toolik, and less than 3% in Anchorage and Fairbanks.

For each sampled site (n=24), the average areal N_2 fixation rate by host moss genus was summed within each site by adding the averages of all present families (Figure 2.3). The abundance-weighted N_2 fixation rates for individual sites ranged from less than 0.01 to 3.16 mg N m^{-2} d^{-1}, with a mean of 1.04 ± 0.19 mg N m^{-2} d^{-1}. The mosses with the largest contributions to N_2 fixation across all sites were *Sphagnum* spp. and *H. splendens*, but *T. nitens* and *Aulacomnium* spp. were locally important at some sites (e.g. Sites 6, 7, 13, and 14, Figure 2.3). Six of the 24 sampled sites were hotspots of N_2 fixation [three SE's over the median rate (Reed et al. 2010)]. Three of those sites occurred near Anchorage, two occurred near Toolik, and one near Fairbanks (Sites 7, 12, 14, 22, 23, and 24; median N fixation rate=0.86 mg N m^{-2} d^{-1}, SE=0.19 mg N m^{-2} d^{-1}). As this definition classified a full quarter of our sites as hotspots, we have added an additional tier of sites that exceeded the median N_2 fixation rate by more than 10 SE's, highlighting sites 7 and 14 as particularly active hotspots. We found no significant relationships between site-level N_2 fixation rate and moss richness (Figure 2.5), Simpson's Diversity Index (Figure 2.5), or any environmental variable (MAT, MAP, etc.) at the site level, but did see significant correlation between percent cover and N_2 fixation rate in *T. nitens* (Figure 4). There were non-significant positive trends between N_2 fixation rate and pH and fixation rate and gravimetric water content.

DISCUSSION

From the earliest attempts to quantify moss-associated N_2 fixation in Alaska, a large range of N_2 fixation rates have been observed in bryophyte-associated microbial communities while the main drivers of that variation have remained largely undetermined (Alexander and Schell 1973). Host species identity has been an intriguing avenue for explaining the high amounts of variation seen in this process and may be a valuable tool as moss communities and

associated N_2 fixation rates shift with climate (Gavazov et al. 2010a; Turetsky et al. 2012; Bay et al. 2013b; Warshan et al. 2017b; Holland-Moritz et al. 2018; Carrell et al. 2019). Here, we found host moss family to be the most important predictor of N_2 fixation rate across a broad geographic range (Figure 2.1, Table 2.4). We also found consistent and significant differences in N_2 fixation rate among moss genera (Figure 2.2). Site of collection was an important source of variation, but our analyses consistently found moss identity differences to be significant despite that variation. These findings indicate that host moss identity can play an important role in both predicting landscape-scale N inputs from moss-associated N_2 fixation and in further exploration of drivers of process rate variation.

Moss-associated N_2 fixation was nearly ubiquitous among collected mosses. Almost all potential host moss species (34 out of 35) collected in this survey had measurable rates of N_2 fixation (Table 2.3). By direct comparison of an incubated sample to a paired natural abundance sample, we were able to quantify even very low rates of N_2 fixation. Our results are in agreement with a previous study which used an isotopic approach to measuring moss-associated N_2 fixation (Gavazov et al. 2010a), indicating that the use of ^{15}N may be particularly valuable for measuring low rates of N_2 fixation that may be missed when using acetylene reduction assays. When making larger-scale calculations of N inputs, the ubiquity of measurable rates of N_2 fixation associated with mosses underscores the importance of including a diversity of host mosses when measuring or predicting N_2 fixation rates.

Diversity in free-living N_2 fixer communities in other ecosystems, such as tropical forests, has been shown to be associated with higher total rates of N_2 fixation (Reed et al. 2010, 2011). While past studies have focused directly on the positive relationship between microbial diversity and fixation rates, host moss diversity could reflect microbial diversity based on the

specificity between host identity and microbial community and thus also be positively correlated with N_2 fixation rates. Though we saw no relationship between diversity and function, it is notable that the two largest N_2 fixation hotspots had high moss diversity, indicating that diversity alone does not appear to cause hotspots but is a potential feature of hotspots (see Figure 2.5, Figure 2.3). Both active hotspots included *T. nitens*, which fixed N_2 at rates disproportionate to its cover (Figure 2.3). Additionally, we found a strong positive relationship between percent cover and N_2 fixation rate in *T. nitens*, a relationship that was absent in other high-fixing species such as *H. splendens* and *S. russowii* (Figure 2.4). Percent cover of all mosses was not associated with hotspots in a straightforward manner; all hotspots other than Site 14 had >50% moss cover, but other sites had high moss cover without a correspondingly high total N_2 fixation rate (Figure 2.3).

The data presented here are based on snapshot measurements from only one point in the growing season at each sampled location. Fixation rates are known to vary over the course of the growing season, perhaps in relation to N demand during reproduction or in response to environmental changes (Lett and Michelsen 2014; Warshan et al. 2016; Rousk and Michelsen 2017). Our sampling sites cover a wide range of naturally occurring differences in environmental conditions (see Table 2.2), but we cannot account for seasonal variation arising from phenology. Therefore, it is possible that our observed hotspots could also represent hot moments for *T. nitens*. Past measurements of *T. nitens* show a peak in N_2 fixation rates in mid-June to early July (Rousk and Michelsen 2017). Subplot nested in site was not a large source of variation within our models, but even subplot cannot fully capture diversity in microclimate conditions on a sub-5m scale. While some of these microclimate conditions can arise due to traits of the mosses and

their community structure and function, moss identity can only capture the combination of these traits as opposed to a single driver (Eviner 2004; Rixen and Mulder 2005; Gornall et al. 2007).

There are other possible explanations for the high observed N_2 fixation rates within *T. nitens*, none of which were experimentally addressed here. Non-acidic tundra surfaces which often contain *T. nitens* communities, such as in Site 14, have higher P availability when compared to other tundra types (Hobbie and Gough 2002). However, previous research indicates that high latitude moss-associated N_2 fixation is rarely limited by P (Zackrisson et al. 2004; Rousk et al. 2017a). Fixation rates were not disproportionately higher in all mosses at Site 14, despite the commingling growth of species in this location. Relative to its colony density, *T. nitens* retains moisture more effectively than similarly structured species (Elumeeva et al. 2011). This indicates perhaps a dual advantage for *T. nitens*, as it maximizes moisture while still allowing for light and air penetration into the colony structure. Other mosses, such as *P. commune* or *Sphagnum* spp., could also have anatomical features that affect their respective conditions for promoting or decreasing rates of N_2 fixation. *P. commune* contains transport cells that may allow it to obtain more water and nutrients from its substrate (Brodribb et al. 2020), thus decreasing the demand for N obtained via fixation. In our study, *Polytrichum* spp. tended to have higher tissue N. *Sphagnum* mosses are known to exert control over their environment through specialized hyaline cells for holding water (van Breemen 1995) which can create a moist microenvironment that is suited for optimization of rates of N_2 fixation. For *T. nitens*, as for other mosses, traits such as growth habitat and morphological features require further exploration to parse their role in driving rates of N_2 fixation, particularly since a combination of traits that constitute identity rather than a single trait may be important drivers of biogeochemical processes (Eviner 2004).

Random forest models also indicated the importance of certain environmental factors. The ranked predictors of N_2 fixation rate varied between the three latitudinal sampling sites (Toolik, Fairbanks, and Anchorage), where Arctic tundra was distinct from the other two locations. Site emerged consistently as a top predictor, perhaps as a distillation of a matrix of environmental variables that can influence process rates. Tree density was a large driver in Fairbanks and Anchorage ecosystems. Given the importance of light for phototrophic N fixers, such as *Nostoc*, canopy structure and its attendant light penetration is a logical driver of process rates (Gentili et al. 2005; Gundale et al. 2012a). Additionally, litter inputs from the canopy could be affecting moss community structure and/or N_2 fixation rates (Rousk and Michelsen 2017; Jean et al. 2020). Organic layer depths, another important predictor for N_2 fixation rates in Anchorage and Fairbanks, can affect soil temperature and moisture, which may in turn affect N_2 fixation rates indirectly through, for example, the surrounding vascular plant assemblage (Kasischke and Johnstone 2005; Gundale et al. 2012a; Jonsson et al. 2014). At Toolik Field Station, the importance of pH may be related to the relatively higher pH communities that contain *T. nitens* and its associated high N_2 fixation rates (Hobbie et al. 2005). Past studies have produced strong evidence for N availability downregulating N_2 fixation (Rousk et al. 2013). Extractable inorganic N was not a good predictor of N_2 fixation rates in our study. Nitrogen depositions rates are generally low across Alaska, but mosses may also be utilizing soil N or resorption N from senescent materials. Taken together, this means that extractable inorganic N from the mosses may not be the best index for N availability (Aldous 2002; Hember 2018; Liu et al. 2019). It is also important to note that inter-site differences in canopy structure and organic layer depth tended to be greater than those found in TIN or, within a geographic area, MAT.

Gravimetric water content and incubation temperature were generally not important predictors of N_2 fixation rates in our study, but MAT and MAP were often ranked highly in the random forest. Past studies have shown a positive effect of increased moisture on N_2 fixation rates (Rousk et al. 2013). Despite this, the lack of a direct effect of water content may be because only one sampled site (Site 8) was below the threshold identified by Zielke and others (2005) of 60% water content and N_2 fixation rates at this site were quite low. We did observe a non-significant positive trend between site-level N_2 fixation and gravimetric water content. In our study, N_2 fixation seemed more affected by long-term precipitation averages instead of the conditions on the day of sampling. The range of average temperatures in our incubations was 13.7-20.7°C, far below the threshold of where we would expect to see warm temperature-related inhibition of N_2 fixation (Gundale et al. 2012a). While there may have been some temporary suppression of N_2 fixation in association with high temperature maximums inside of syringes during incubation, temperature maximum was still not a strong predictor of rates. Again, the long-term temperature trend was more important for N_2 fixation variation. It is notable that temperature and moisture conditions may also be important in determining the distribution of host mosses, which in the longer term could alter landscape level N_2 fixation patterns (Deane-Coe et al. 2015).

The rates we obtained fall within the previous scope of rates of moss-associated N_2 fixation both in Europe and North America. We observed higher N_2 fixation rates for *S. fuscum, T. nitens, A. palustre, P. schreberi,* and *H. splendens* than Gavazov and others (2010a) despite the use of isotopic measurement, though there was some agreement of trends between mosses. The consistently lower rates associated with *P. schreberi* were surprising given the abundance of higher rates in the literature, but some papers do show a similar result particularly in comparison

with *H. splendens* (Gentili et al. 2005; Bay et al. 2013b; Gundale et al. 2013a; Leppänen et al. 2013; Jean et al. 2020). Rousk and Michelsen (2017) saw a similar mean rate of N_2 fixation for *T. nitens* as reported here. Looking at rates of N_2 fixation associated with cyanolichens in these ecosystems provides further contextualization for mosses. In some high-latitude ecotypes, cyanolichens account for the majority of fixed N_2 (Rousk et al. 2015). By a per-mass basis, cyanolichens from Toolik fixed an order of magnitude more N_2; however, their percent cover at Site 14 was also less than 4%, leading to a probable lower overall N source (Weiss et al. 2005).

Given the strength of moss identity as a predictor of N_2 fixation rates, the consistency of patterns between moss genera across broad geographic and environmental variation, and the importance of certain species in determining the presence of hotspots, we conclude that moss identity could be a valuable tool to increase the precision of regional-scale predictions of landscape N_2 fixation rates. Existing moss abundance datasets or advanced remote sensing techniques could be leveraged to make these predictions. Such landscape-level models could be further augmented by exploring the occurrence of hotspots on both spatial and temporal scales and incorporating that knowledge with information on moss community composition. Elucidating the relationships between N_2 fixation and host identity, as well as exploring the mechanisms underlying that specificity, can better inform how N dynamics in these valuable and vulnerable ecosystems will be affected by ongoing climate change.

TABLE 2.1

Site No.	Region	Location	Site Description
1	Fairbanks	N 64º 76.759’ W 148º 29.651’	*P. mariana* upland
2	Fairbanks	N 64º 77.113’ W 148º 27.303’	*P. mariana* upland
3	Fairbanks	N 64º 76.823’ W 148º 29.586’	*P. mariana* and *P. glauca* upland
4	Fairbanks	N 64º 70.662’ W 148º 30.995’	Mixed deciduous/conifer upland
5	Fairbanks	N 64º 70.377’ W 148º 29.731’	*P. mariana* wetland
6	Fairbanks	N 64º 70.213’ W 148º 29.165’	Open canopy *Sphagnum* wetland
7	Fairbanks	N 64º 86.718’ W 147º 85.897’	*P. mariana* tussock
8	Fairbanks	N 64º 95.692’ W 148º 36.926’	Alpine heath tundra
9	Fairbanks	N 64º 88.142’ W 148º 39.093’	*B. neoalaskana* upland
10	Fairbanks	N 64º 88.324’ W 148º 39.555’	*P. mariana* upland
11	Toolik	N 68º 64.132’ W 149º 58.541’	Heath tundra
12	Toolik	N 68º 63.869’ W 149º 56.812’	*B. nana* shrub tundra
13	Toolik	N 68º 63.902’ W 149º 56.761’	Moist acidic tussock tundra
14	Toolik	N 68º 63.404’ W 149º 63.964’	Moist non-acidic tussock tundra
15	Anchorage	N 61º 11.720’ W 149º 48.396’	*P. mariana* wetland
16	Anchorage	N 61º 11.853’ W 149º 48.584’	*P. mariana* upland
17	Anchorage	N 61º 09.397’ W 149º 47.879’	Mixed deciduous/conifer upland
18	Anchorage	N 60º 59.802’ W 149º 05.236’	*T. mertensiana* forest
19	Anchorage	N 60º 57.968’ W 149º 06.812’	Mixed conifer upland
20	Anchorage	N 60º 57.954’ W 149º 06.798’	Open canopy *Sphagnum* wetland
21	Anchorage	N 61º 08.384’ W 149º 46.458’	*P. mariana* upland
22	Anchorage	N 61º 09.996’ W 149º 47.046’	*P. mariana* upland
23	Anchorage	N 61º 11.772’ W 149º 48.710’	*P. mariana* upland
24	Anchorage	N 61º 13.410’ W 149º 25.498’	*B. nana* open canopy alpine

TABLE 2.2 Mean site characteristics for all sampled sites, including mean annual temperature (MAT) in °C (four year average), mean annual precipitation (MAP) in mm (four year average), altitude in m, depth of the organic layer, from the surface to the boundary of the mineral horizon (in cm, BPF=below permafrost, or organic layer extended to the active layer boundary), pH, tree density (trees m^{-2}), active layer depth (in cm, where NA means no permafrost was found in the top meter of soil), total extractable inorganic N (TIN, μg N g $moss^{-1}$, ± SE), and sampling date (dd/mm/yy). Shading in Site column corresponds to geographic area: lightest gray is Fairbanks, medium gray is Toolik, and darkest gray is Anchorage.

Site No.	MAT	MAP	Altitude	Org. layer depth	pH	Tree density	Active layer depth	TIN	Sampling Date
1	-4.3	298	425	26	5.02	47.0	41	16.2±3.6	06/21/16
2	-4.3	297	405	36	4.80	31.3	56	2.5±0.3	06/22/16
3	-4.3	298	425	28	5.09	51.3	39	4.8±0.6	06/22/16
4	-3.1	304	125	14	5.65	50.8	57	2.1±0.4	06/23/16
5	-3.1	304	119	BPF	4.55	11.6	29	9.6±1.1	06/23/16
6	-3.1	304	119	BPF	5.77	NA	41	10.1±1.0	06/23/16
7	-2.8	302	163	BPF	5.16	18.1	44	12.4±3.8	06/23/16
8	-6.1	296	790	14	4.90	NA	35	17.2±3.3	06/24/16
9	-3.9	297	240	9	5.58	80.6	60	7.3±0.8	06/24/16
10	-4.1	294	305	22	5.07	24.7	31	7.4±0.5	06/24/16
11	-11.4	224	735	10	4.95	NA	24	4.3±0.6	06/27/16
12	-11.5	227	765	16	5.76	NA	29	12.1±1.0	06/28/16
13	-11.5	227	765	BPF	5.31	NA	20	6.9±1.2	06/28/16
14	-11.4	225	728	14	6.20	NA	24	10.1±1.3	06/28/16
15	1.9	438	60	BPF	4.61	9.7	29	11.3±1.5	06/27/17
16	1.9	435	60	33	4.93	62.4	61	9.7±0.4	06/27/17
17	1.9	456	84	15	5.19	59.0	NA	14.2±1.0	06/28/17
18	1.7	1005	206	20	4.88	81.3	NA	29.8±3.2	06/28/17
19	2.2	1128	87	18	5.02	68.7	42	17.5±3.6	06/29/17
20	2.2	1128	87	>100	5.05	NA	NA	52.6±3.3	06/29/17
21	1.7	476	145	7	4.91	43.0	NA	20.2±1.9	06/30/17
22	1.9	457	86	33	5.41	54.6	71	15.5±1.4	06/30/17
23	1.9	435	60	22	5.18	77.5	40	10.1±0.4	06/30/17
24	-0.7	613	763	27	4.78	NA	NA	14.6±4.2	07/01/17

TABLE 2.3 *Based on Vanderpoorten and others (2001). Means of rate measurements across all geographic areas.

Family	*Species measured*	*Mean fixation rate ($\mu g\ N\ g\ moss^{-1}\ day^{-1}$, ±SE)*	*n*
Amblystegiaceae	*Tomentypnum nitens** (Hedw.) Loeske	14.58 ± 2.97	8
	Sanionia uncinata (Hedw.) Loeske	2.23 ± 1.82	5
Aulacomniaceae	*Aulacomnium turgidum* (Wahlenb.) Schwgr.	2.11 ± 0.73	18
	Aulacomnium palustre (Hedw.) Schwgr.	2.30 ± 0.42	48
	Aulacomnium acuminatum (Lindb. & Arnell) Kindb.	3.88 ± 0.35	3
Dicranaceae	*Dicranum acutifolium* (Lindb. & Arnell) C. Gens. ex Weinm	0.99 ± 0.40	14
	Dicranum elongatum Schwgr.	1.46 ± 0.44	10
	Dicranum fragifolium Lindb.	0.08 ± 0.05	5
	Dicranum fuscescens Turner	0.36 ± 0.19	4
	Dicranum montanum Hedw.	No measured fixation	2
	Dicranum polysetum Swartz	0.22 ± 0.14	21
	Dicranum scoparium Hedw.	1.93 ± 0.63	9
	Dicranum undulatum Brid.	0.34 ± 0.25	7
Grimmiaceae	*Racomitrium lanuginosum* (Hedw.) Brid.	11.69 ± 3.83	6
	Niphotrichum canescens (Hedw.) Brid.	2.62 ± 1.28	3
Hylocomiaceae	*Pleurozium schreberi* (Brid.) Mitt.	0.79 ± 0.18	90
	Hylocomium splendens (Hedw.) Schimp.	3.60 ± 0.43	99
	Rhytidiadelphus triquetrus Hedw.	0.09 ± 0.04	4
Hypnaceae	*Hypnum lindbergii* Mitt.	4.24 ± 1.60	5
	Ptilium crista-castrensis (Hedw.) DeNot	2.28 ± 0.56	37
Polytrichaceae	*Polytrichum juniperum* Hedw.	0.27 ± 0.05	2

	Polytrichum strictum Brid.	0.82 ± 0.28	25
	Polytrichum commune Hedw.	0.03 ± 0.02	33
Sphagnaceae	*Sphagnum alaskense* Andrus & Janssens	7.17	1
	Sphagnum angustifolium (Ehrh.) Hedw.	6.33 ± 0.98	15
	Sphagnum arcticum Flatberg & Frisvoll	1.85 ± 0.66	2
	Sphagnum capillifolium (Ehrh.) Hedw.	2.91	1
	Sphagnum fimbriatum Wilson	10.80 ± 5.00	2
	Sphagnum fuscum (Schimp.) H. Klinggr	5.35 ± 2.41	7
	Sphagnum girgensohnii Russow	5.20 ± 1.04	21
	Sphagnum magellanicum Brid.	2.51 ± 0.78	9
	Sphagnum russowii Warnst.	5.91 ± 0.95	28
	Sphagnum squarrosum Crome	6.28 ± 2.55	5

TABLE 2.4 Random forest results from a model that included all data from across Alaska. Geographic area was the 11th ranked variable. Model R^2=0.34, model RMSE=0.58.

Ranking	Predictor	Average Variable Importance Score
1	Family	183.0
2	Site	101.6
3	MAP	45.3
4	MAT	35.0
5	Organic layer depth	33.5

FIGURE 2.1 Variable importance scores of the top five predictors averaged across six random forest iterations for each sampling location (Anchorage, Fairbanks, and Toolik). The calculated R^2 for all models is based on a model run which contained variables identified at the "Threshold" step as identified through the VSURF variable selection tool, which eliminates all irrelevant variables. Initial models included MAT (°C, average from 2014-2017), MAP (mm, average from 2014-2017), permafrost category (see Methods), Organic layer depth category (see Methods), moss taxonomic family (see Table 2), pH, altitude (m), gravimetric water content, temperature minimum, maximum, and average during 24h incubation (°C), tree density (trees m^{-2}), extractable NH_4^+-N, NO_3^--N, and total inorganic nitrogen (μg N g dry $moss^{-1}$), and site of collection. The response variable was N_2 fixation rate (μg N g $moss^{-1}$ day^{-1}). Model root mean square error (RMSE) is 2.89, 0.63, and 5.0, for Anchorage, Fairbanks, and Toolik respectively. Please note scale changes in x-axis.

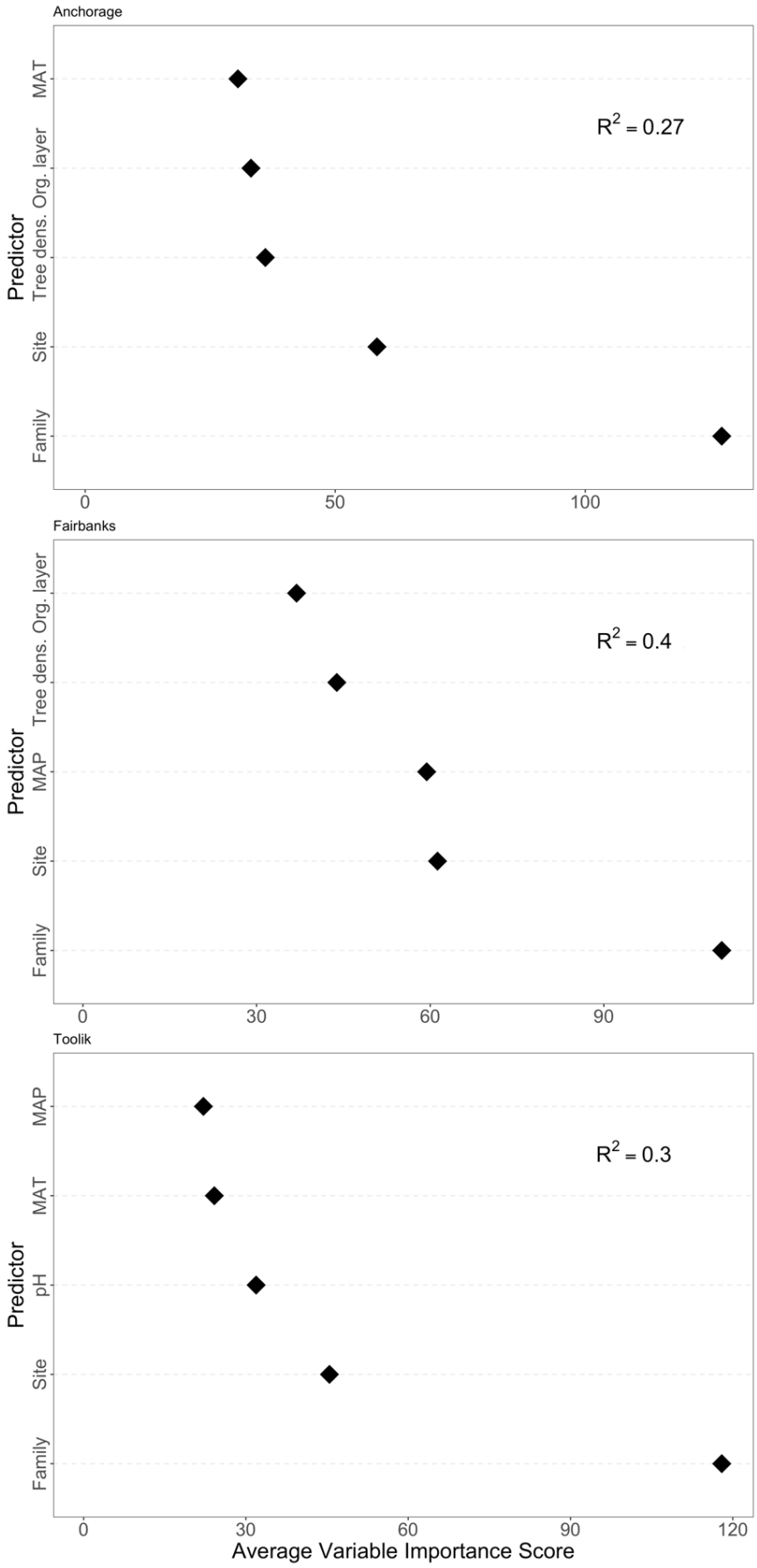

Anchorage
MAT
Org. layer
Tree dens.
Site
Family
Predictor
R^2 = 0.27
0
50
100
Fairbanks
Org. layer
Tree dens.
MAP
Site
Family
Predictor
R^2 = 0.4
0
30
60
90
Toolik
MAP
MAT
pH
Site
Family
Predictor
R^2 = 0.3
0
30
60
90
120
Average Variable Importance Score

FIGURE 2.2 Model results for N_2 fixation rates for each moss genus with subplot nested in site as a random factor in each region (Anchorage, Fairbanks, and Toolik in separate panels). Significant differences are represented by letters above each bar, which were based on post-hoc estimated marginal means pairwise comparisons at $\alpha=0.01$wherein data were log-transformed to meet model assumptions. Boxes are group means, bold bars are the 95% confidence interval, and grey points are raw data points.

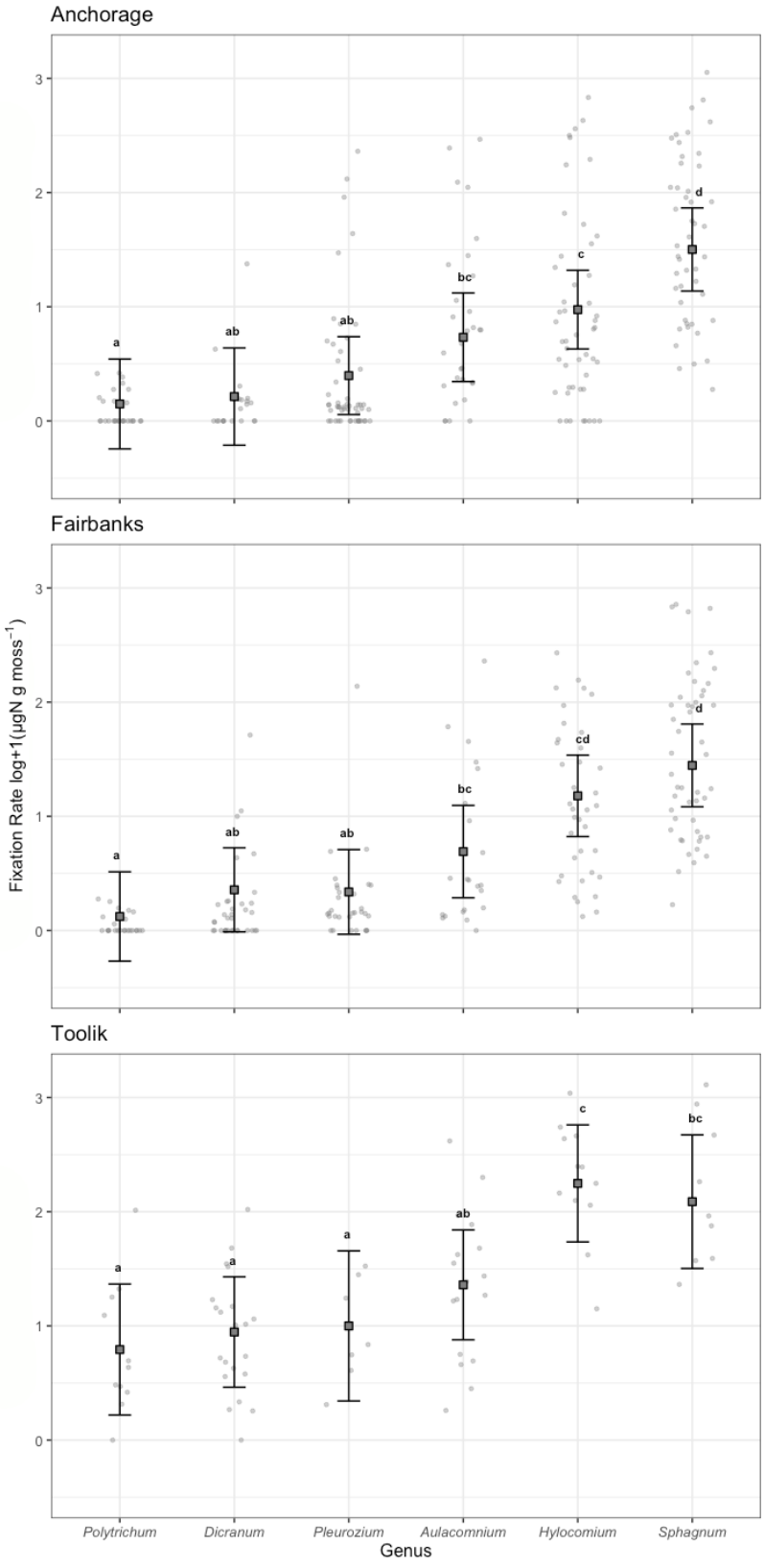

Anchorage
Fairbanks
Toolik
Fixation Rate log+1(μgN g moss^{-1})
a
ab
ab
bc
c
d
a
ab
ab
bc
cd
d
a
a
a
ab
c
bc
Polytrichum
Dicranum
Pleurozium
Aulacomnium
Hylocomium
Sphagnum
Genus

FIGURE 2.3 The total N_2 fixation rates (top) and percent cover (bottom) where each bar represents one site. Sites in Anchorage are on the left (Sites 15-24), Fairbanks sites in the middle (Sites 1-10), and Toolik sites (Sites 11-14) furthest to the right. All genera not in legend are represented by "Other"; see Table 2.3 for full list. Sites that are hotspots of N_2 fixation have a box around the site number in top panel.

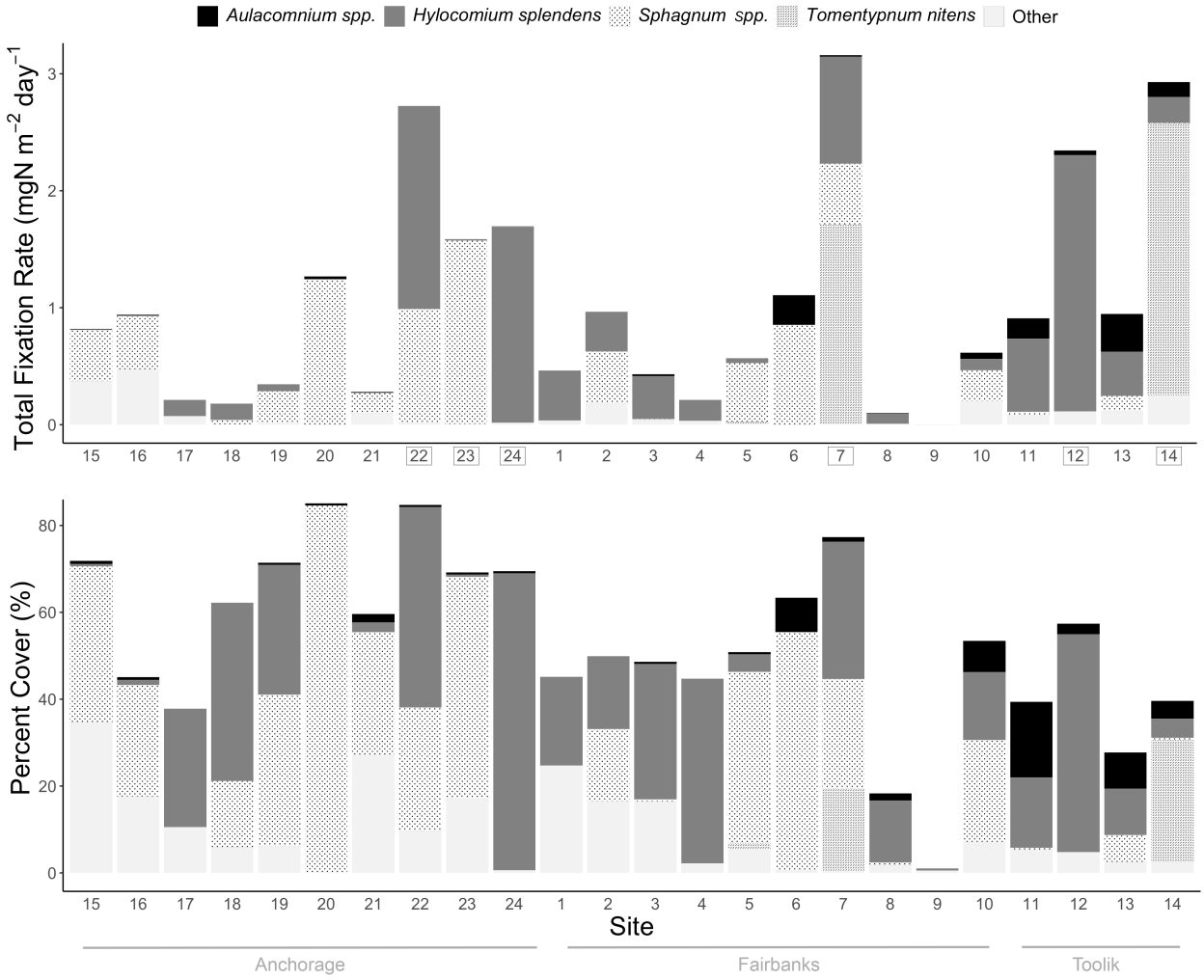


FIGURE 2.4 Linear model results for three host moss genera (*T. nitens, H. splendens, and S. russowii*) where the independent variable is percent cover and the dependent variable is N_2 fixation rate (in µg N g moss^{-1} day^{-1}). Only *T. nitens* had a significant positive relationship, where p=0.004 and R^2=0.73.

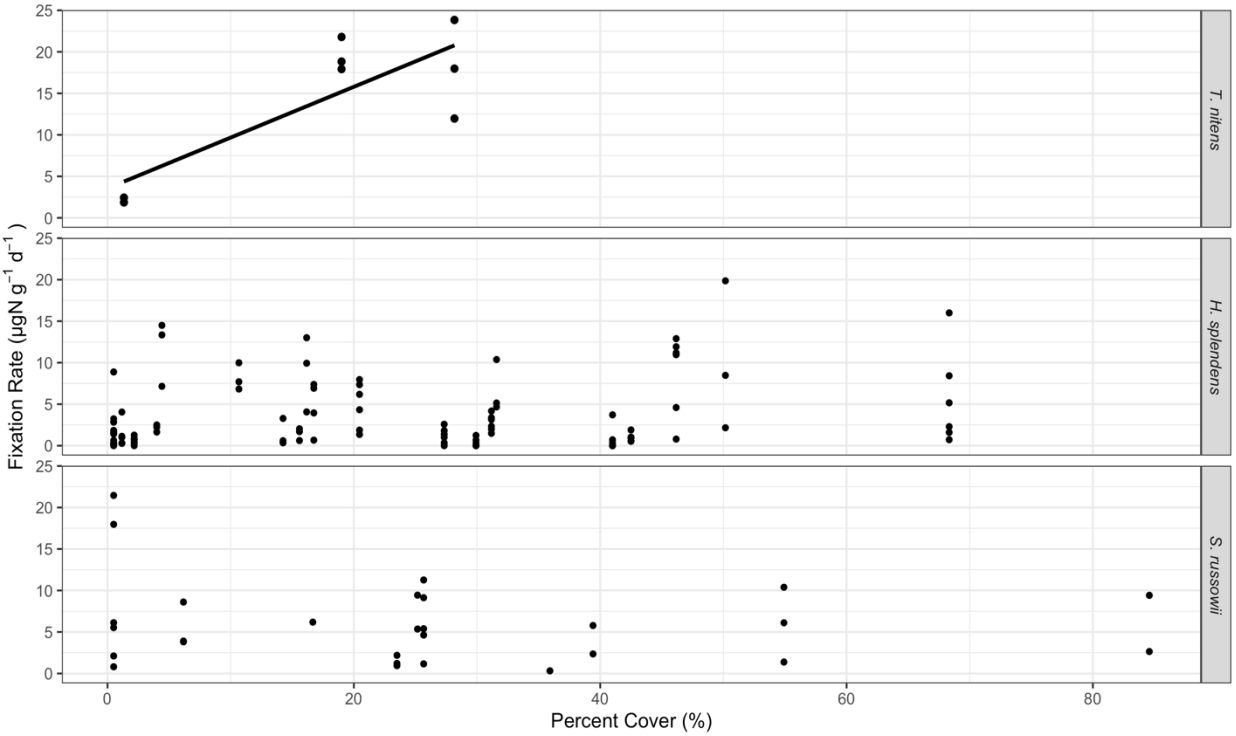
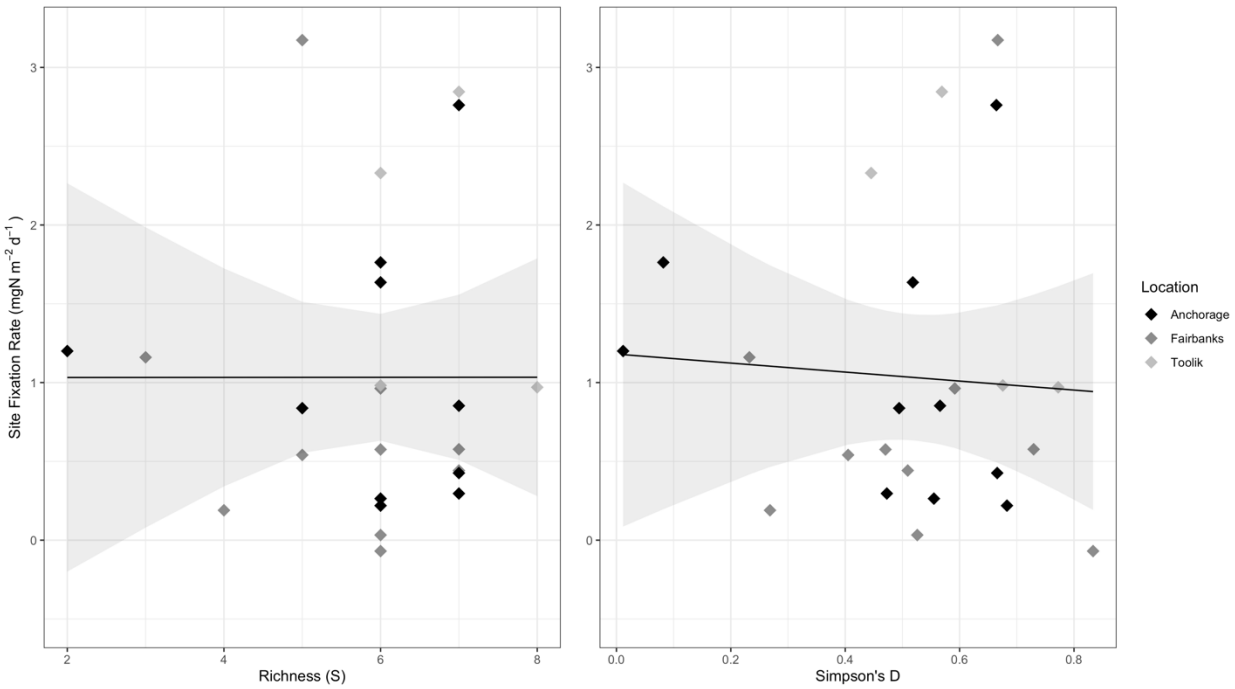


FIGURE 2.5 The left panel is Richness (S, number of moss genera present) regressed with total fixation rate (as a sum for each site). The right panel is Simpson's diversity index regressed with total fixation rate as a sum for each site. The line represents a linear model fit and the 95% confidence region is shaded in grey. For left panel, p=0.250 and R^2 is -0.04. For right panel, p=0.771 and R^2= -0.04.



CHAPTER III

The relationship of C and N stable isotopes to high latitude moss-associated N_2 fixation

ABSTRACT

Moss-associated N_2 fixation by epiphytic microbes is a key biogeochemical process in nutrient limited high-latitude ecosystems. Abiotic drivers, such as temperature and moisture, and the identity of host mosses are critical sources of variation in N_2 fixation rates. An understanding of the potential interaction between these factors is essential for predicting N inputs as moss communities change with the climate. To further understand the drivers and results of N_2 fixation rate variation, we obtained natural abundance values of C and N isotopes and an associated rate of N_2 fixation with $^{15}N_2$ gas incubations in 34 moss species collected in three regions across Alaska, USA. We hypothesized that $\delta^{15}N$ values would increase toward 0‰ with higher N_2 fixation to reflect the increasing contribution of fixed N_2 in moss biomass. Second, we hypothesized that $\delta^{13}C$ and N_2 fixation would be positively related, as enriched $\delta^{13}C$ signatures reflect abiotic conditions favorable to N_2 fixation. We expected that the magnitude of these relationships would vary among types of host mosses, reflecting differences in anatomy and habitat. We found little support for our first hypothesis, with only a modest positive relationship between N_2 fixation rates and $\delta^{15}N$ in a structural equation model. We found a significant positive relationship between $\delta^{13}C$ and N_2 fixation only in Hypnales, where the probability of N_2 fixation activity reached 95% when $\delta^{13}C$ values exceeded -30.4‰. We conclude that moisture and temperature interact strongly with host moss identity in determining the extent to which abiotic conditions impact associated N_2 fixation rates.

INTRODUCTION

Moss-associated nitrogen (N_2) fixation is the largest source of N inputs to boreal and Arctic ecosystems (Alexander and Schell 1973; Basilier 1979; DeLuca et al. 2002; Lindo et al. 2013; Vile et al. 2014a). High northern latitude ecosystems, which are currently experiencing rapid changes associated with climate warming, harbor large reservoirs of carbon (C) and are often N limited for vascular plants (Shaver and Jonasson 1999; Lebauer and Treseder 2008; Tarnocai et al. 2009; Hugelius et al. 2014). While the N_2 fixation rates associated with the autotrophic microbes in the bryosphere are relatively low compared to symbiotic N_2 fixers associated with angiosperms such as *Alnus spp.* or cyanolichens such as *Peltigera spp.*, the ubiquity of mosses in these ecosystems compensates for lower process rates (Hobbie et al. 2005; Weiss et al. 2005; Mitchell and Ruess 2009; Turetsky et al. 2010). Many biotic and abiotic variables have been proven to affect rates of moss-associated N_2 fixation, including temperature, moisture, and N deposition (Ackermann et al. 2012; Gundale et al. 2012a, 2013b; Lindo et al. 2013; Rousk et al. 2013). Other research shows that host moss identity can drive microbial community composition, *nifH* gene expression, and associated rates of N_2 fixation (Ininbergs et al. 2011; Bragina et al. 2012; Holland-Moritz et al. 2018; Jean et al. 2020; Stuart et al. 2020). As abiotic conditions as well as moss biomass and community composition are expected to change with climate, understanding the interaction between environment and host moss identity is critical for predicting future N inputs (Gundale et al. 2012a; Turetsky et al. 2012; Deane-Coe et al. 2015; Carrell et al. 2019).

The natural abundance of ^{15}N in plant material can provide clues regarding the source of plant N (Högberg 1997). For example, nitrogenase, the enzyme responsible for biological N_2 fixation, has a low discrimination against ^{15}N, leading to a $\delta^{15}N$ near -1‰ or 0‰ for plants that are utilizing fixed N (Vitousek et al. 1989; Högberg 1997). In contrast, other plant N sources

from soils or atmospheric N deposition are relatively enriched or depleted, respectively (Nadelhoffer et al. 1996; Bragazza et al. 2005). In mosses, natural abundances of $\delta^{15}N$ vary across ecological gradients, experimental treatments, and moss species (Bragazza et al. 2005; Gavazov et al. 2010b; Deane-Coe et al. 2015). Critically, mosses obtain N fixed by microbial epiphytes(Bay et al. 2013b; Berg et al. 2013; Rousk et al. 2016b). Previous studies have used moss $\delta^{15}N$ as a potential reflection of differences in associated N_2 fixation rates across space or potential hosts by assuming signatures increasing toward 0‰ from more depleted values ($\delta^{15}N <$ -3‰) had a higher N contribution via fixation, usually in combination with moss N concentrations, atmospheric N deposition signatures, and/or observed cyanobacteria colonization (Boddey et al. 2000; Deane-Coe and Sparks 2016; Novak et al. 2016; Živković et al. 2017).

Few studies have explicitly linked quantifications of moss-associated N_2 fixation to $\delta^{15}N$ signatures of the host mosses. In *Sphagnum spp.*, more depleted (0 to -3‰) $\delta^{15}N$ measurements were associated with higher rates of N_2 fixation (Leppänen et al. 2015), but in *Pleurozium schreberi* no relationship between these measurements was found (Hyodo et al. 2013). This discrepancy may be because the key measurements were made asynchronously or use only one natural abundance value linked with multiple N_2 fixation measurements. Mosses are also morphologically and ecologically hetereogeneous, and may vary in features that lead to greater soil N uptake or the translocation of N within the moss which can obscure recent inputs from N_2 fixation or other sources (Eckstein and Karlsson 1999; Aldous 2002; Bragazza et al. 2005; Ayres et al. 2006; Krab et al. 2008). Additionally, alternative forms of nitrogenase, such as vanadium (V)- and iron (Fe)-only nitrogenase, differ from molybdenum (Mo) nitrogenase in their levels of fractionation and thus their natural abundance signature, where V- and Fe-nitrogenase produce $\delta^{15}N$ signatures of -6 to -7‰ (Zhang et al. 2014). Sampled lichen thalli from Alaska fell mostly,

though not exclusively, above the Mo concentration threshold which would indicate an increased reliance on V-nitrogenase activity (Darnajoux et al. 2019). Experimental Mo additions have shown a brief positive effect on N_2 fixation in subarctic feather mosses, but overall Mo concentration in control plots also fell above the threshold suggested by cyanolichen research (Rousk and Rousk 2020). By directly pairing N_2 fixation and natural abundance measurements across a broad diversity of host mosses and environmental conditions, we can more effectively explore the link between process rates and $\delta^{15}N$.

Given the key role that climate has in affecting N_2 fixation, another tool for exploring N_2 fixation rate variation is $\delta^{13}C$. In mosses, $\delta^{13}C$ reflects temperature, moisture conditions, and plant productivity, both in natural and experimentally induced conditions (Williams and Flanagan 1996; Skrzypek et al. 2007; Deane-Coe et al. 2015; Royles et al. 2016; Granath et al. 2018). Unlike vascular plants, where fractionation against ^{13}C isotope occurs both in diffusion through stomata and enzymatic discrimination, mosses in moist environments often have a film of water on the surface which limits diffusion (Farquhar et al. 1989; Williams and Flanagan 1996; McCarroll and Loader 2004). Thus, while $\delta^{13}C$ in vascular plants becomes relatively depleted with increased temperature/decreased precipitation, mosses are relatively more enriched in ^{13}C in moist conditions, as limited diffusion decreases enzymatic discrimination (Stuiver and Braziunas 1987; Williams and Flanagan 1996; Rice 2000; Diefendorf et al. 2010). Therefore $\delta^{13}C$, which is often obtained concurrently with $\delta^{15}N$ analysis, is linked to climatic drivers of N_2 fixation.

The objective of our study was to evaluate the relationships between $\delta^{13}C$, $\delta^{15}N$, and N_2 fixation in high latitude mosses to identify links between stable isotope composition and process rates as well as to explore the interaction of host moss identity with these relationships. We used

$^{15}N_2$ incubations to measure the fixation rates associated with 34 different moss species across a latitudinal transect in Alaska from 60 to 68 degrees N, each with a paired natural abundance measurement from the same sample pre-incubation. We hypothesized that moss $\delta^{15}N$ would increase toward 0‰ with increasing N_2 fixation (**H1**). Past studies have shown a positive relationship between cyanobacteria colonization and $\delta^{15}N$ and that mosses utilize N fixed by microbial epiphytes (Berg et al. 2013; Deane-Coe and Sparks 2016). We also hypothesized that more enriched $\delta^{13}C$ would have a positive relationship with rates of N_2 fixation (**H2**), as higher $\delta^{13}C$ can indicate consistent moist conditions that facilitate microbial N_2 fixation. Finally, we expected that these relationships would vary in magnitude among mosses because of known differences in the associated rates of N_2 fixation as well as anatomy and habitat niche differentiation related to nutrient acquisition and water retention strategies. To test these hypotheses, we used mixed models and a multigroup structural equation model after first using exploratory random forest models to inform tested relationships. We also use a binomial generalized mixed effects model to establish a broad threshold of for N_2 fixation activity related to $\delta^{13}C$ signatures.

MATERIALS & METHODS

Sites

In June 2016, we selected 10 sites near Fairbanks, AK (N 64° W 148°) and 4 sites near Toolik Field Station, AK (N 68° W 149°). The following June, we selected an additional 10 sites near Anchorage, AK (N 60-61°, W 149°). These sites encompassed a diversity of ecotypes found in the regions, including *Picea spp.* dominated upland and peatland boreal forest, alpine tundra, *Tsuga* spp. coastal forests, and Arctic tundra. Sites were selected based on accessibility, low human disturbance, and the presence of moss. Each site was based on a 30m transect where

environmental measurements were made at 5m intervals, hereafter called subplots. For more details on the location and characteristics of sites, please see Stuart *et al.* (2020).

Environmental data collection

Gravimetric water content of the moss was measured via a 5×5×5 cm plug of moss from each subplot. These plugs were placed in an airtight plastic bag to be transported to the laboratory and immediately weighed for a field wet weight before being transferred to a 60° drying oven for 48h before reweighing for a dry weight. Moisture, or gravimetric water content, was calculated by dividing the difference between the wet and dry weights by the dry weight. Coordinates and elevation were recorded for each site using a Garmin GPSMAP 64x handheld GPS. Coordinate information was then used to extract the mean annual temperature (MAT, in °C) and precipitation (MAP, in mm) for each site from 2014-2017 from Climate NA (Wang et al. 2016).

Relative abundance of Nostocaceae

To compare our results to papers that looked only at cyanobacteria colonization, we compared Nostocaceae relative abundance with N_2 fixation rates and $\delta^{15}N$ in simple linear models (see Supplementary Figure 1). For full details of microbial methods, please see Holland-Moritz et al. (2021). Briefly, we used amplicon-based sequencing of a 253-bp region of the 16S rRNA bacterial and archaeal marker gene. We extracted DNA from a paired sample of moss tissue, homogenized with liquid N_2 and PCR-amplified the V4-V5 region of the 16S rRNA gene. Amplicons were sequenced on the Illumina MiSeq platform at the University of Colorado Next Generation Sequencing Facility using 2x150 bp paired-end chemistry. After sequencing we identified microbial phylotypes using the UNOISE pipeline (Edgar 2018) which denoises the reads and distinguishes between phylotypes using a unique sequence variant approach (i.e. all

sequences belonging to a single phylotype have 100% identical sequences). We assigned taxonomy to these phylotypes with the RDP Naive Bayesian Classifier (Wang et al. 2007) and GreenGenes database (McDonald et al. 2012). After filtering out phylotypes assigned as mitochondrial or chloroplast, we created a phylotype-by-sample table and controlled for differences in sequencing depth across samples by randomly selecting 3000 reads per sample. Finally, we converted our read-counts to relative abundances and calculated the percent relative abundance of Nostocaceae in each sample.

N_2 fixation and natural abundance measurements

All mosses at each site were identified and, if they appeared in at least two distinct patches, collected in each subplot where present. Common mosses, appearing in at least six distinct patches, were collected a maximum of six times (once per subplot). For each sample, circa 40 moss ramets were collected. From this original sample, several stems were removed for a voucher specimen to be identified at the University of Florida, ten stems were immediately placed in a drying oven for natural abundance values, and ten stems were placed in syringes to be incubated with $^{15}N_2$ gas. Each moss ramet included was of approximately 5cm of length, including both green and senescent moss tissue, and was cleaned of debris or other plant material. Natural abundance samples were dried at 60° C for 48 h before being shipped to Northern Arizona University. The incubation subsample was lightly sprayed with distilled water in an airtight 60 ml polypropylene syringe. Each syringe was plunged to 10 ml of ambient air before 10ml of 98at% enriched $^{15}N_2$ gas was added, making the final volume 20 ml (Sigma-Aldrich Inc., Lot # MBBB3807V and MBBB9003V). Incubations took place in a common garden (one in each geographic area of Toolik, Fairbanks, and Anchorage) for 24h, as past research showed no significant difference between *in situ* and common garden incubations

(DeLuca et al. 2007). Three additional syringes, each containing a Thermochron iButton (Model DS1921G-F5#, Embedded Data Systems USA), recorded field temperature every 10 minutes throughout the duration of the incubation. After incubation, moss material was removed from the syringes, dried as described above, and sent to Northern Arizona University.

Natural abundance and incubated samples were finely ground using either a clean coffee grinder or hand-chopped with scissors. Six mg of each sample was rolled into a tin capsule and run with a Costech ECS4010 elemental analyzer coupled to a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer to obtain %N, %C, $\delta^{15}N$, and $\delta^{13}C$. The atom percent enrichment (APE) of each sample was calculated by subtracting the natural abundance subsample atom percent from its paired incubated subsample atom percent. Isotopic uptake was then scaled by the sample weight and the air:tracer ratio to calculate total ($^{15}N+^{14}N$) N_2 fixation (Jean et al. 2018). Rates are expressed as μg N g $moss^{-1}$ day^{-1}.

We measured both natural abundance values and N_2 fixation rates associated with 505 samples. The average natural abundance and enriched $\delta^{15}N$ was -3.09± 0.08‰ (mean±standard error) and 54.67± 4.05‰, respectively. Natural abundance of $\delta^{13}C$ averaged -30.81±0.10 and percent N and percent C were 0.81±0.01 and 46.45±0.11 respectively. Based on the sensitivity of the isotope ratio mass spectrometer, samples with less than 2‰ $\delta^{15}N$ difference between the paired samples were assumed to have a N_2 fixation rate of zero.

Statistical analyses

All statistical analyses were performed in R Studio 1.2.1335 using R version 3.6.1 (R Core Development Team). To test the hypotheses of this study, we used piecewise structural equation models (SEMs) through the package piecewiseSEM 2.1.0 (Lefcheck 2016) and linear mixed-effects models in lme4 1.1-21 (Bates et al. 2015) paired with Satterwhwaite's degrees of

freedom method in lmerTest (Kuznetsova et al. 2017). Prior to implementing SEMs, we used random forests in the package randomForest 4.6-14 to explore both linear and non-linear relationships for each of the three response variables we were interested in modelling as endogenous variables within the SEM framework: $\delta^{13}C$, $\delta^{15}N$, and N_2 fixation rate (Liaw and Wiener 2002). Random forests are a flexible and informative approach in variable selection as they can incorporate both continuous and categorial variables and do not rely on assumptions of frequentist statistics (Cutler et al. 2007). As previous results demonstrated the importance of host moss identity in determining rates of N_2 fixation, we divided mosses into genera or orders for analyses: Hypnales (containing the genera *Hylocomium*, *Ptilium*, *Pleurozium*, *Hypnum*, *Tomentypnum*, *Rhytidiadelphus*, and *Sanionia*), Sphagnales (including 11 different species of *Sphagnum*), Rhizogoniales (three *Aulacomnium* spp.), Dicranales (eight *Dicranum* spp.), and Polytrichales (three *Polytrichum* spp.). Therefore, the only order than contains multiple genera is Hypnales. We compared the R^2 values between two different models for each endogenous variable, where one model contained moss genus as a predictor and the other substituted order for genus, to determine whether it was appropriate to test our hypotheses at a broader taxonomic scale. Each model also contained MAP, MAT, altitude, incubation temperature average, gravimetric water content, site, geographic area (Toolik, Fairbanks, or Anchorage), and the other endogenous variables as predictors. Each model averaged six executed runs with mtree set at 10,000.

After confirming the limited reduction of explained variance when using host moss order instead of genus, we implemented two mixed models. To account for spatial autocorrelation, we included the geographic area (Anchorage, Toolik, or Fairbanks), site nested in geographic area (24 sites, described above) and subplot nested in site and geographic area (six subplots per site)

as random effects. Interactive fixed effects of $\delta^{13}C$ and order were regressed with log+ 1 N_2 fixation rate (μg N g $moss^{-1}$ day^{-1}), while interactive fixed effects of N_2 fixation rate and order were used with $\delta^{15}N$ as the response variable. $\delta^{13}C$ was treated as the independent variable because it is a reflection of climate conditions, which in turn is hypothesized to relate to rates of N_2 fixation. Conversely, N_2 fixation rate was the independent variable when regressed with $\delta^{15}N$ because the natural abundance of N isotopes may reflect rates. For the Hypnales, we also ran a generalized linear mixed effects model on a binomial dataset, where N_2 fixation rates were allocated to zero if no fixation was observed and one if fixation did occur during the incubation. We were unable to test the effect of geographic area because all samples from Toolik fixed N_2 during our incubation (see Supplementary Figure 2). We regressed the presence/absence of N_2 fixation activity against the $\delta^{13}C$ natural abundance value in a binomial model with a bobyqa optimizer with random effects of site and subplot nested in site.

We used SEMs to further test our initial hypotheses for Hypnales and Sphagnales. The *a priori* model included MAT and MAP as exogenous predictors of $\delta^{13}C$ and log+ 1 N_2 fixation rate and %N as an exogenous predictor of $\delta^{15}N$ and log+ 1 N_2 fixation rate (see Figure 2). We opted for MAT and MAP indicators over snapshot measurements of temperature and moisture made at the time of sampling as they may correspond better with $\delta^{13}C$ values that reflect longer-term trends. Additionally, we previously found that MAT and MAP were generally better predictors of N_2 fixation rates than the snapshot measurements (Stuart et al. 2020). Measures that combine temperature and precipitation, such as climate moisture deficit, were considered but ultimately rejected to enable looking at each driver separately, as moisture has a consistently positive effect on N_2 fixation while temperature optima can differ among N_2 fixers (Gentili et al. 2005; Rousk et al. 2013). While %N could be a result of, rather than a cause of, N_2 fixation rate

variation, we worked under the assumption that increased N deposition would act more directly on %N and thus downregulate fixation activity (Solga and Fram 2006; Gundale et al. 2013b). This explanation seems plausible in our data, given the weakly negative relationship between %N and N_2 fixation in our data. In mixed models and the SEM, N_2 fixation rates were log-transformed to meet model assumptions. A multigroup SEM was employed to directly compare paths between Hypnales and Sphagnales within the same model. A multigroup piecewise SEM allows a direct comparison between two groups within the same model by allowing paths to vary if there is a significant interaction between the term and the grouping factor. The package automatically tests which paths to constrain, with automatic selections made for the best output. The piecewise SEM also allows for the inclusion of the hierarchical sampling design, as each relationship includes the random effect structure described for mixed effect models above.

RESULTS

Across all three variables of interest ($\delta^{13}C$, $\delta^{15}N$, and N_2 fixation), the substitution of moss order for moss genus as a predictor resulted in small or no change to the amount of variance explained by the model (Table 1). The largest diminution of explained variance between two models for the same variable of interest was for N_2 fixation rate, where the model including genus explained 9% more variation. Across all models, moss identity (genus or order) was the top-ranked predictor. Similarly, all models showed some influence of site of collection and relatively little influence from the geographic area (Toolik, Fairbanks, or Anchorage).

Mixed model analysis showed a significant interaction between $\delta^{13}C$ and moss order ($p < 0.001$, Figure 1, Table 2). Orders Hypnales and Sphagnales were significantly different from the intercept (CI=0.07-0.27, P=0.006 and CI=-0.28- -0.03, P= 0.013, respectively). Post-hoc graphing of model results showed that the direction of the relationship between $\delta^{13}C$ and log+1

N_2 fixation rate was different between these orders; while Hypnales displayed the expected positive relationship, Sphagnales had a negative relationship (Figure 1). Other moss orders did not have a significant relationship between $\delta^{13}C$ and log+1 N_2 fixation rates, though the direction of those relationships was positive (Figure 1). Random effects testing confirmed that geographic area and site nested in geographic area accounted for 32% and 14% of variation explained respectively, while subplot was not significant and accounted for less than 3% of variation. The interaction between log+1 N_2 fixation rate and order was significant when regressed with $\delta^{15}N$, but only significant for Polytrichales (Table 2, Figure 1). The $\delta^{15}N$ values of Polytrichales and Rhizogoniales were significantly higher than other moss orders. Only site nested in geographic area was significant as a random effect and accounted for 33% of variance explained.

Based on mixed model results, ecological prevalence, and higher associated N_2 fixation rates within Hypnales and Sphagnales, these two orders were compared in a multi-group SEM. All but four relationships were constrained to the global model (Figure 2). Therefore, there was a significant interaction between moss order and $\delta^{13}C$ in relation to log +1 N_2 fixation, percent N in relation to $\delta^{15}N$, and MAT in relation to $\delta^{13}C$ and log +1 N_2 fixation. For Sphagnales, MAT was negatively related to $\delta^{13}C$ and, unlike the mixed model results, log +1 N_2 fixation rate had a significant relationship $\delta^{15}N$ (P=0.0156). Other relationships, such as MAP and $\delta^{13}C$ or percent N and log +1 N_2 fixation rate, were not significant in the model (Figure 2).

The presence of N_2 fixation activity was modeled as a function of $\delta^{13}C$ values, which was found to be a significant relationship (CI=1.12-2.12, P=0.008, Figure 3). Geographic area could not be included as a model term, because all moss samples from Toolik fixed N_2 during our incubations (see Supplementary Figure 2). In the most depleted samples (-36‰), mosses were slightly more likely to be fixing than not fixing N_2, though the confidence region crosses below the 50%

threshold. However, at -30.4‰, samples crossed the threshold of a 95% probability prediction. All samples in our study displayed N_2 fixation activity at $\delta^{13}C$ values more enriched than -30‰. This trend did not appear to be driven by one moss species in particular, although *Tomentypnum nitens* had consistently high N_2 fixation rates and relatively depleted $\delta^{13}C$ (Figure 3).

DISCUSSION

Our observations of a large paired dataset that compares moss-associated N_2 fixation rates with natural abundance stable isotope values revealed a surprising disconnect between $\delta^{15}N$ and rates of N_2 fixation. We found little evidence to support our hypothesis that $\delta^{15}N$ values would increase toward 0‰ with N_2 fixation activity, even after accounting for host moss identity. We found a significant interaction of $\delta^{15}N$ and log+1 N_2 fixation rate in the Polytrichales (Table 2, Figure 1). Polytrichales had a generally higher $\delta^{15}N$ signature despite having consistently low N_2 fixation rates, resulting in a relationship of the opposite direction than we predicted in H1. Members of the Polytrichales have more developed internal transport systems, which perhaps facilitates greater soil uptake than other studied moss groups (Brodribb et al. 2020). The modest positive relationship between $\delta^{15}N$ and N_2 fixation in the SEM framework was significant for Hypnales and Sphgnales together. However, the actual $\delta^{15}N$ values were not close to -1 or 0‰, instead remaining largely below -2.25‰ (based on value of 3rd quantile when rate of fixation was greater than 5μg N g moss^{-1} day^{-1}). This is in contrast to a previous study, which saw a positive connection between the percentage of leaves with cyanobacteria colonization and elevated $\delta^{15}N$ in temperate forest mosses (Deane-Coe and Sparks 2016). While relative abundance does not necessarily equate to absolute abundance, we did see a significant positive relationship between Nostocaeae relative abundance and log-transformed N_2 fixation rates but no relationship between Nostocaeae relative abundance and $\delta^{15}N$ (see

Supplementary Figure 1). There is some evidence that N_2 fixation rates associated with bryophytes in temperate forests may be up to an order of magnitude greater than boreal or Arctic rates, thus increasing the footprint of fixation in the $\delta^{15}N$ signature (Menge and Hedin 2009). Lower rates of N_2 fixation relative to high latitudes have been measured in temperate grasslands in North America, and higher rates of N deposition in temperate ecosystems would be expected to lead to downregulation of the process (Gundale et al. 2013b; Hember 2018; Calabria et al. 2020).

One explanation for the absence of our hypothesize relationship is the increased reliance on alternative nitrogenases at higher latitudes, which would be expected to produce a much different natural abundance signature (Zhang et al. 2014; Darnajoux et al. 2019). The use of $\delta^{15}N$ as a straightforward proxy is also complicated by other N acquisition strategies in mosses which may dilute the signature of fixed N, including atmospheric deposition, uptake from soil, and translocation of N from senescent to live tissue (Aldous 2002; Turetsky 2003; Ayres et al. 2006; Krab et al. 2008). Based on experiments on *Sphagnum* spp. and *P. schreberi*, it seems likely that mosses obtain at least a portion of fixed N and that fixed N is retained in the green moss layer for at least one year, indicating the rapid loss of fixed N is an unlikely explanation for the weakness of the observed trend (Basilier 1980; Berg et al. 2013; Rousk et al. 2014, 2016a). Based on our results, we cannot recommend using $\delta^{15}N$ as an indicator of N_2 fixation in high latitude mosses without further elucidation of potential N sources and active nitrogenase forms.

Contrary to our initial prediction that host identity would impact only the magnitude of the hypothesized relationship between $\delta^{13}C$ and rates of N_2 fixation, we found instead that moss order was a key determinant of the presence and direction of significant relationships. A positive relationship between $\delta^{13}C$ and N_2 fixation was only significant in the feather moss order

Hypnales. Previous research has confirmed the importance of moss identity in the composition of microbial communities and the associated rates of N_2 fixation (Ininbergs et al. 2011; Leppänen et al. 2015; Jean et al. 2020; Stuart et al. 2020; Holland-Moritz et al. 2021). Our results add evidence to the premise that the distinct microbial communities harbored by mosses will respond differently to key process drivers of moisture and temperature, and thus that the response of N_2 fixation to changes in climate will not be universal among mosses.

In agreement with previous studies, we found negative relationships between MAT and $\delta^{13}C$ for Hypnales (Skrzypek et al. 2007; Deane-Coe et al. 2015). However, the direction of this relationship was reversed in Sphagnales within our SEM (Figure 2). The absence of the same relationship in Sphagnales may reflect the unique anatomy of these mosses which enables a high water-holding capacity (van Breemen 1995; Elumeeva et al. 2011). The range of $\delta^{13}C$ values for Sphagnales was only -34.0 to -26.8‰, compared to -37.4- to -24.3‰ for all other measured mosses. Unlike all other moss orders, every collected *Sphagnum* moss had detectable N_2 fixation activity across its more constrained $\delta^{13}C$ range. The high moisture retention of Sphagnales may increase latent heat loss (Fukuta et al. 2012). Previous research has shown that *Sphagnum palustre* (in the order Sphagnales) can buffer air temperatures, particularly when air temperatures exceed 20° C, while *Hylocomium splendens* (Hypnales) does not provide as much temperature insulation at the moss carpet (Sonesson et al. 1992; Fukuta et al. 2012). It follows that the trend between MAT and $\delta^{13}C$ would be more important in the Hypnales than the Sphagnales, which is borne out in our model. MAP was not significantly related to $\delta^{13}C$, but this may be due to sites which were in relatively hygric to mesic landscape positions. Only one site fell below the moisture threshold described by Zielke *et al.* (2005) as inhibiting N_2 fixation. For Hypnales and Sphagnales, $\delta^{13}C$ was a more significant predictor of N_2 fixation activity than either of the

climate variables in our model, which may better reflect the synthesis of conditions within the moss carpet than temperature or precipitation averages.

Using a binomial distribution of N_2 fixation activity associated with Hypnales, we identified a threshold of $\delta^{13}C$ values at which our model predicted a 95% probability of N_2 fixation. Across the spectrum of $\delta^{13}C$, feather mosses were fixing N_2 during our incubations. However, in samples more enriched than -30‰, all collected samples were fixing N_2 (Figure 3). Due to the uniform presence of N_2 fixation activity at sites near Toolik, we were unable to account for the effect of geographic area in the binomial model. However, Toolik has a lower mean annual temperature than the other two sampling areas, and we observed generally more enriched $\delta^{13}C$ and higher N_2 fixation rates (Supplementary Figure 2). Higher temperatures and/or lower moisture did not universally inhibit fixation activity; even at the most depleted $\delta^{13}C$ values, our model predicted a 62% probability of N_2 fixation activity. From our data, we infer that while relatively lower temperatures and higher moisture promote N_2 fixing activity, the opposite cannot be assumed.

One caveat to the research presented here is that each site was measured only once, providing a snapshot of N_2 fixation values. It is possible that the measured N_2 fixation rate at any given site was anomalous for the location or the host species in question, or that the present conditions that facilitated or diminished N_2 fixation activity did not correspond with the historical trend that would be encapsulated by the stable isotope values we measured. By sampling from many sites across a broad scale of geographic locations, however, we present a large dataset which can partially account for natural variation. Our nested random effects structure explained a notable proportion of model variance. Comparing the marginal and conditional R^2 in the SEM also shows that the random effects were a considerable source of

variation, particularly in the stable isotope signatures (Figure 2). This suggests that the stable isotope signature had a stronger geographic bias than N_2 fixation rates, possibly due to the more integrative stable isotope measurement of weather conditions or N sources over time.

Though we assumed that the strength of hypothesized relationships of N_2 fixation and stable isotope signature would vary between different groups of host mosses, the absence of significant relationships and/or different directions of relationships among orders indicates the degree to which the anatomy, life history, or micro-environment of these mosses could influence our attempts to understand the drivers of N_2 fixation rate variation. The most striking example of this is the difference between the relationship of $\delta^{13}C$ to fixation activity in Sphagnales and Hypnales. Though moisture and temperature are generally considered to be primary drivers of N_2 fixation rate variation, only in Hypnales did the expected positive relationship between a metric of moisture/temperature status and activity occur. While no significant trends were seen among the other moss orders included in this study, Dicranales, Polytrichales, and Rhizogoniales also had generally lower rates of N_2 fixation, making the trend harder to observe.

These observations underline the importance of thinking of moss identity as an interaction term along with environmental variables and, where possible, testing hypotheses with a diversity of host mosses. Both rates of N_2 fixation and moss community composition are expected to change with the climate (Turetsky et al. 2010; Gundale et al. 2012a; Deane-Coe et al. 2015; Carrell et al. 2019). Moss-associated N_2 fixation, along with other biogeochemically relevant moss traits, plays an important role in high latitude C balance (Cornelissen et al. 2007b; Lindo et al. 2013). Interspecific knowledge and trait-based approaches to exploring moss-associated N_2 fixation can complement and improve biogeochemical predictions as climate changes and are especially important to consider when employing proxies of the process.

TABLE 3.1 Comparison of random forest R^2 for modeling variables of interest with different moss taxonomic levels as a predictor with the same suite of environmental predictors (including MAT, MAP, altitude, incubation temperature average, gravimetric moss water content, site, and geographic region).

Predicted Variable	**Model R^2**	
	Moss Genus	*Moss Order*
$\delta^{13}C$ (‰)	0.651	0.650
N_2 fixation rate (μg N g $moss^{-1}day^{-1}$)	0.422	0.333
$\delta^{15}N$ (‰)	0.551	0.561

TABLE 3.2 ANOVA tables (Type III with Satterthwaite method) for log+1 N_2 fixation rate (μg N g $moss^{-1}day^{-1}$) as a function of the interaction of moss order and $\delta^{13}C$ (M1) and $\delta^{15}N$ as a function of the interaction of moss order and log+1 N_2 fixation rate (μg N g $moss^{-1}day^{-1}$, M2). Model marginal R^2 was 0.320 in M1 and 0.172 in M2.

M1: log +1 N_2 fixation model

Model term	*df*	*F*	*p*
$\delta^{13}C$	1	0.36	0.551
Order	4	10.48	**>0.001**
$\delta^{13}C$:Order	4	10.55	**>0.001**

M2: $\delta^{15}N$ model

Model term	*df*	*F*	*p*
Log+1 N_2-fix	1	0.001	0.973
Order	4	42.80	**>0.001**
Log+1 N_2-fix:Order	4	6.98	**>0.001**

FIGURE 3.1 Top panel shows estimated marginal means predictions of mixed model results regressing the interaction between moss order and $\delta^{13}C$ with log-transformed N_2 fixation rate (μg N g $moss^{-1}day^{-1}$). Lower panel shows estimated marginal means predictions of mixed model results regressing the interaction between moss order and log-transformed N_2 fixation rate (μg N g $moss^{-1}day^{-1}$) with $\delta^{15}N$. Lines represent predicted relationships, and ribbons are the 95% confidence interval of the predicted relationships. Both models included geographic region, site nested in geographic region, and subplot nested in site and geographic region as random effects.

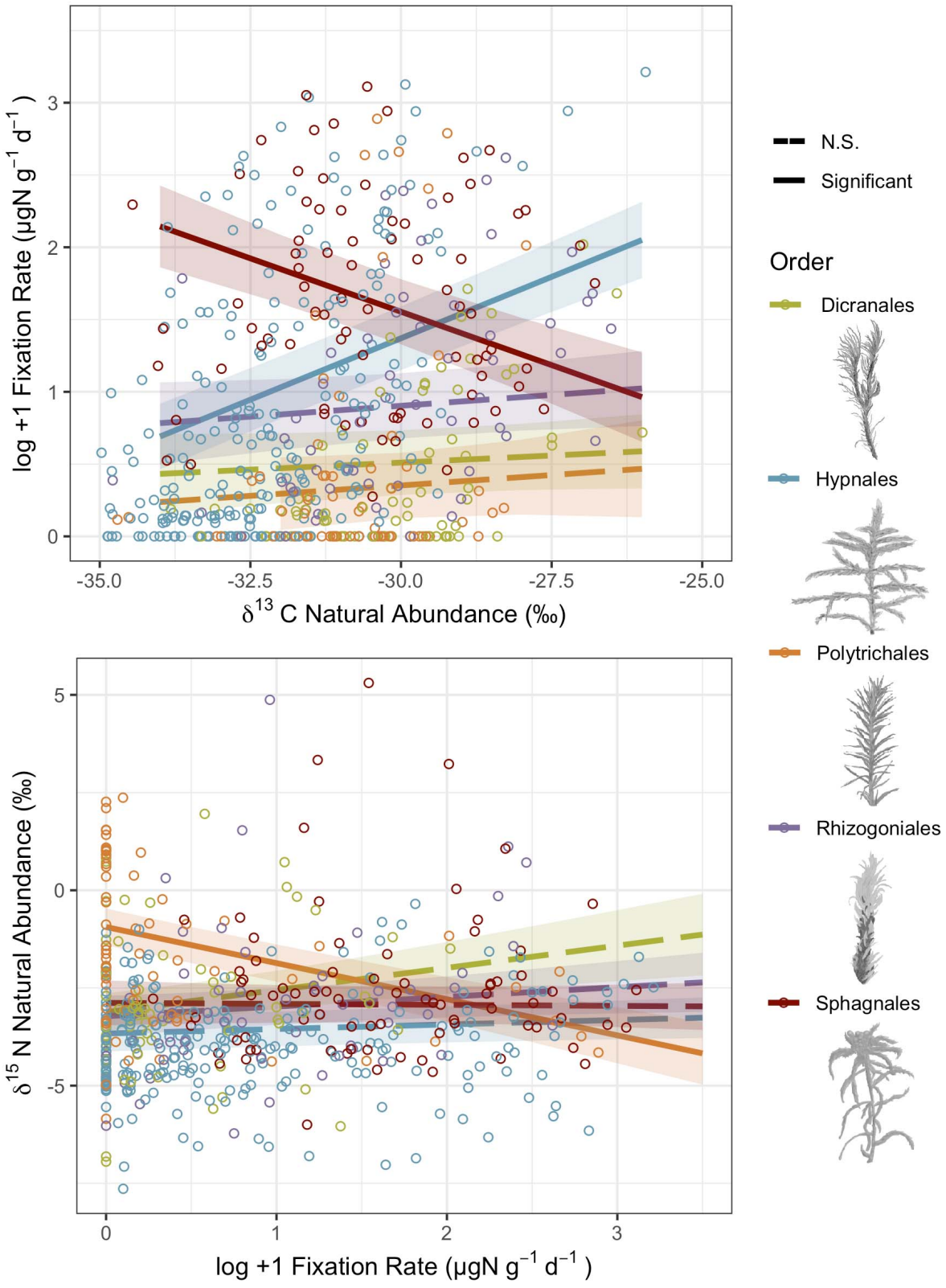

log +1 Fixation Rate (µgN g^{-1} d^{-1})
3
2
1
0
-35.0
-32.5
-30.0
-27.5
-25.0
δ^{13} C Natural Abundance (‰)
N.S.
Significant
Order
Dicranales
Hypnales
Polytrichales
Rhizogoniales
Sphagnales
δ^{15} N Natural Abundance (‰)
5
0
-5
0
1
2
3
log +1 Fixation Rate (µgN g^{-1} d^{-1})

FIGURE 3.2 Panel **a** is the *a priori* model of proposed relationships within the SEM, where the hypothesis-testing relationships are highlighted. Panel **b** shows the multi-group model results, including the marginal and conditional R^2 values of the endogenous variables. The numbers on each path are the path coefficients, with significant p values denoted with *($p<0.05$) and ***($p<0.001$). Model AIC was 56.3 with a Fisher's C of 12.32 ($p = 0.138$, df=8). MAT was measured in °C, MAP in mm, N_2 fixation rates in μg N g moss^{-1}day^{-1}, and $\delta^{13}C$ and $\delta^{15}N$ in ‰.

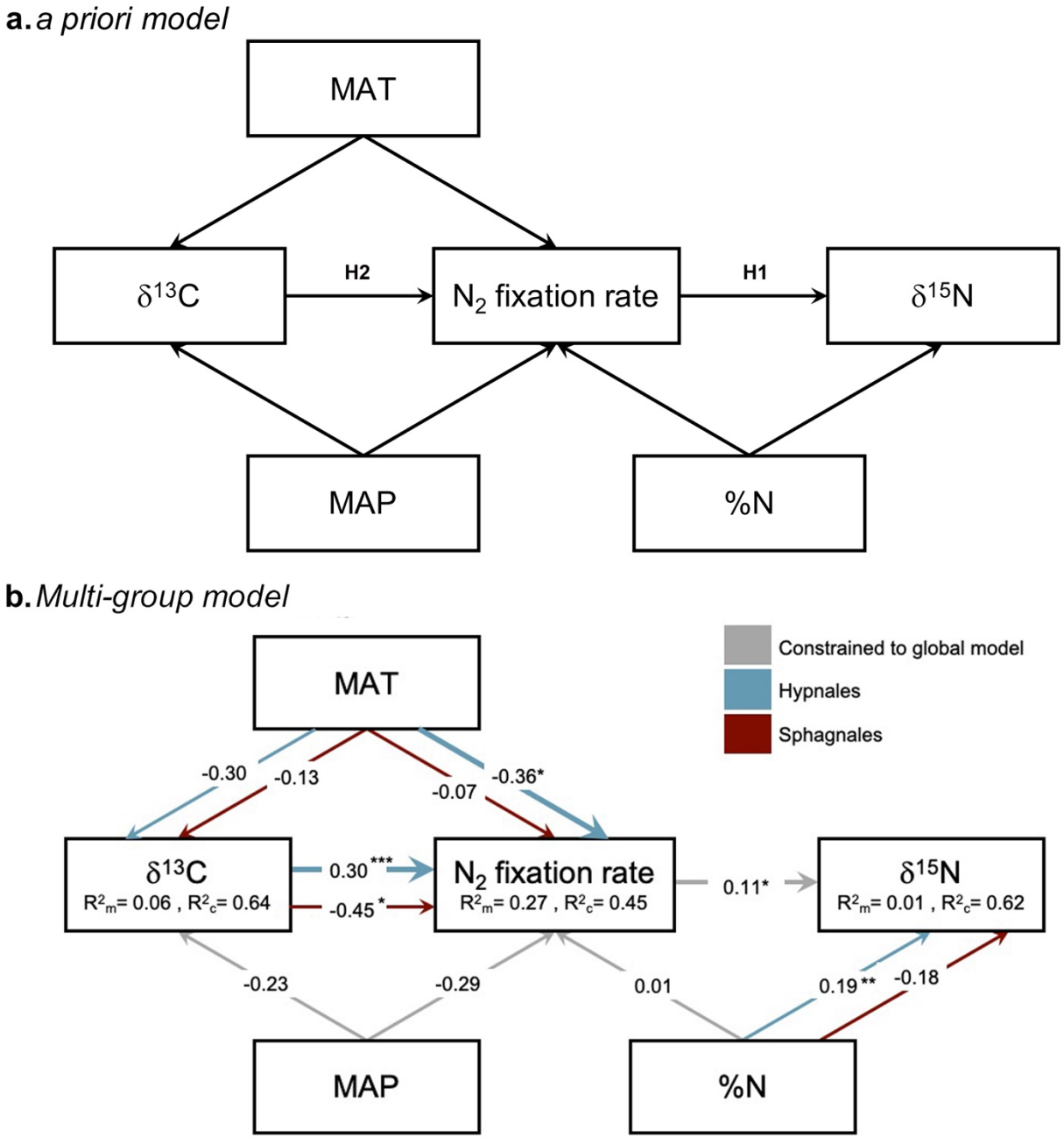


FIGURE 3.3 N_2 fixation activity was modeled as a function of $\delta^{13}C$ with random effects of site and subplot nested in site, where rates of >0 µg N g $moss^{-1}day^{-1}$ were assigned a value of 1. Shading represents the 95% prediction intervals. Points are staggered for visual differentiation and colored based on moss species. Vertical dotted line represents the point on the x-axis at which the predicted probability reached 95%. Graph and model contained data from all three geographic areas included in the study (Anchorage, Fairbanks, and Toolik).

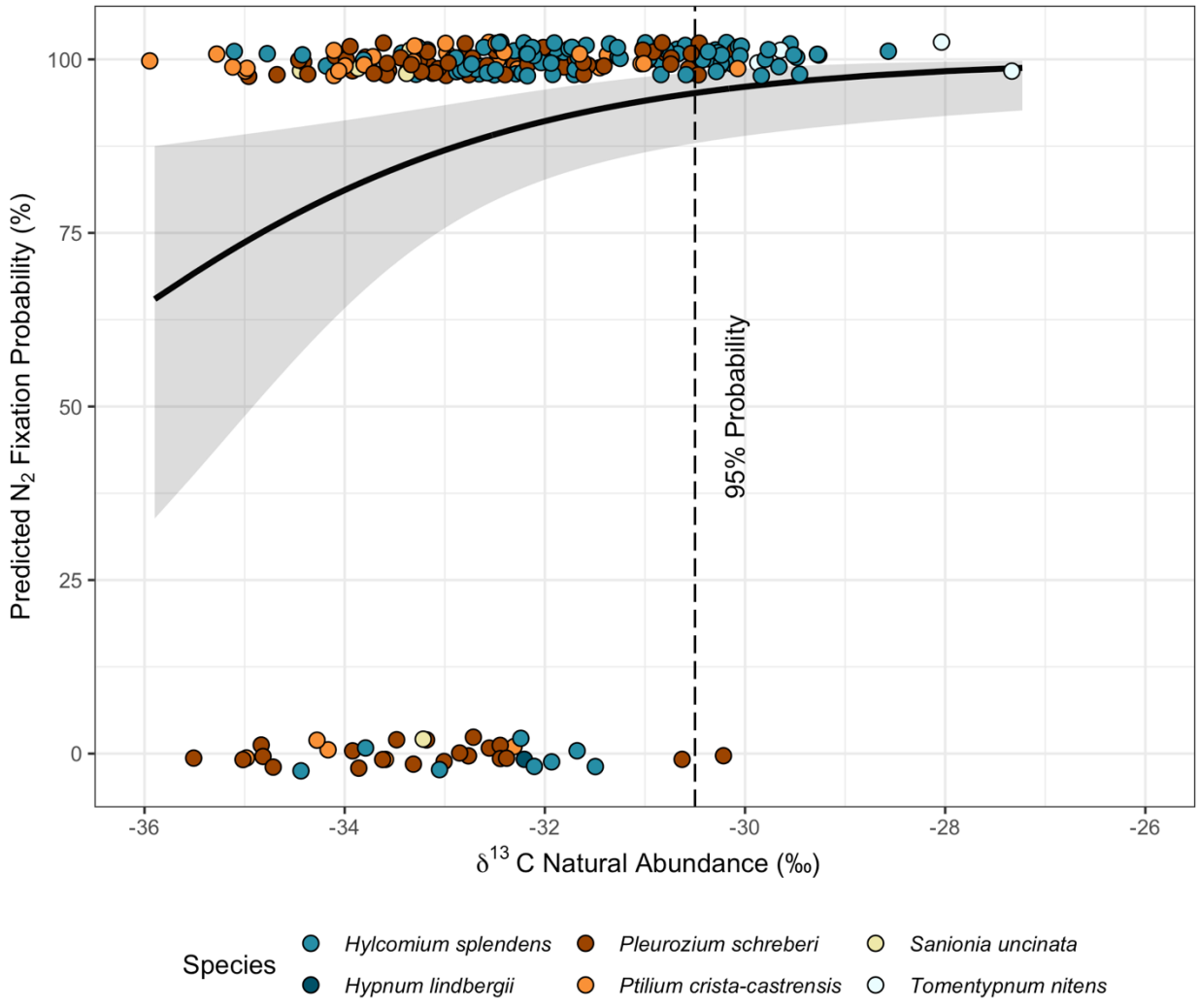


FIGURE 3.4 Linear model results comparing the relative abundance of Nostocaceae in each sample with the corresponding log-transformed N_2 fixation rate or $\delta^{15}N$, respectively. Each point represents one sample. Solid lines represent a significant relationship ($p<0.001$, $R^2 = 0.36$), and dotted line represents non-significant relationship ($p=0.947$). Shaded area is 95% confidence interval.

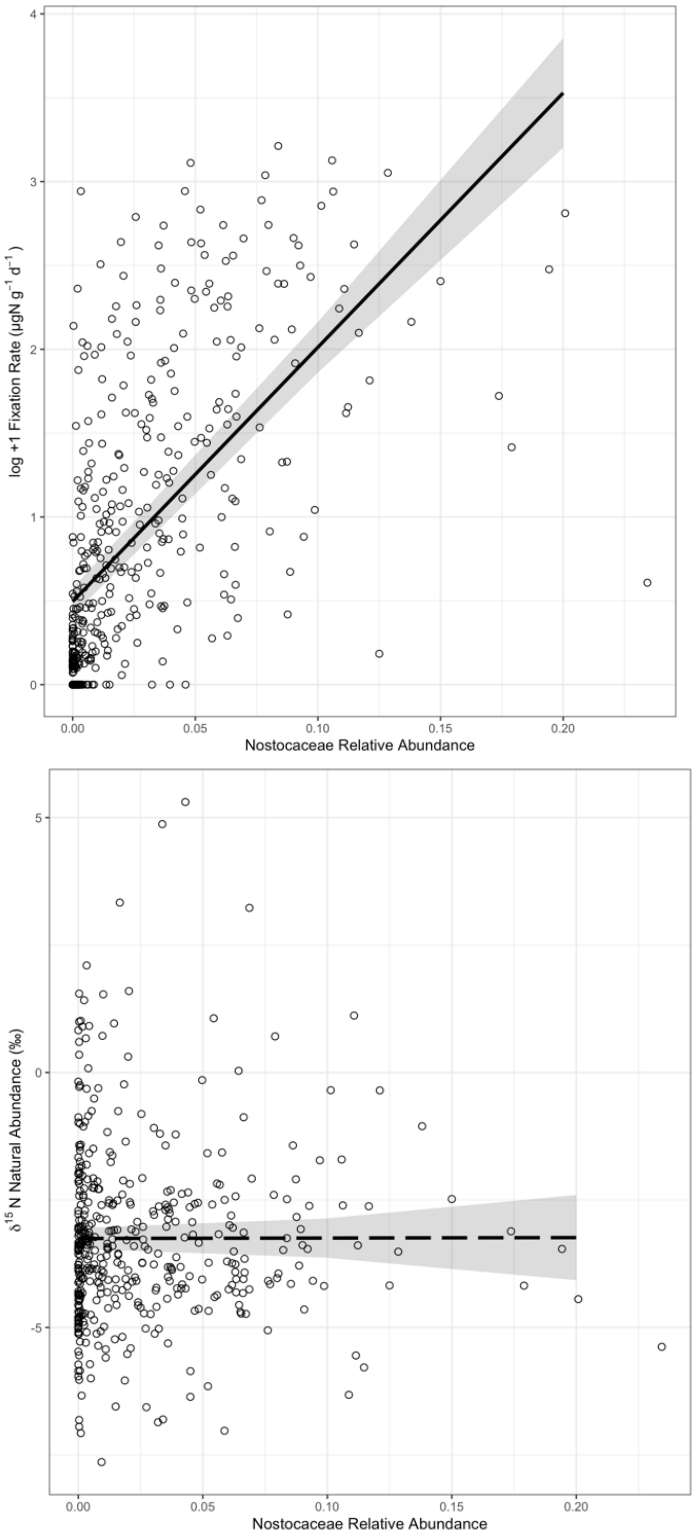
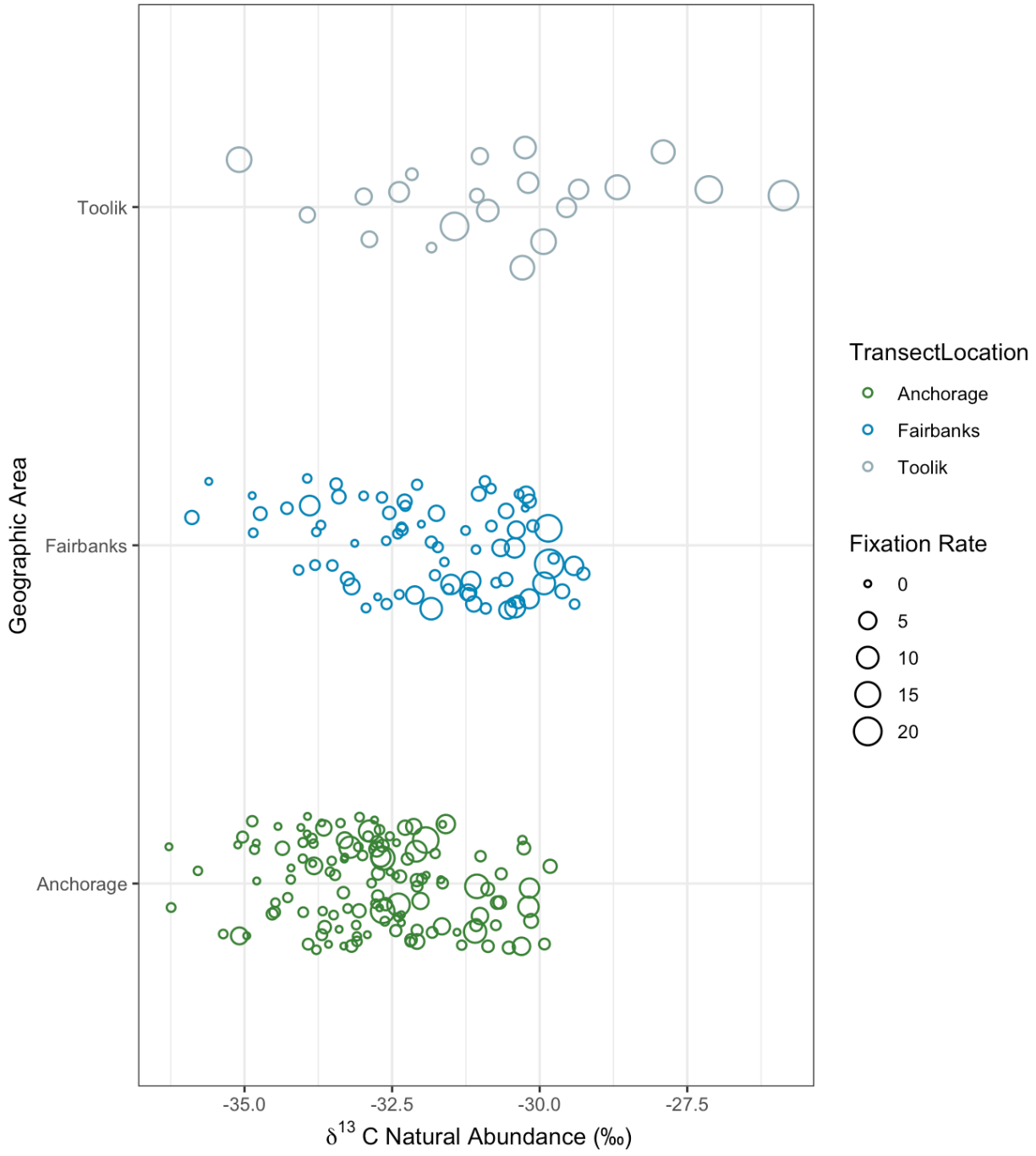


FIGURE 3.5 Dotplot of $\delta^{13}C$ values for samples at each geographic area. N_2 fixation rate values (in µg N g $moss^{-1}day^{-1}$) are sorted into the bins described in the figure legend.



CHAPTER IV

Tundra moss transplants reveal host species-specific response of associated N_2 fixation rates to environmental change

ABSTRACT

Rapid warming in Arctic and boreal ecosystems is expected to alter moss biomass and diversity. Process rates of moss associated N_2 fixation, the largest source of novel N to a nutrient-limited environment, are also expected to change directly with the environment and indirectly through changes in host moss assemblages. Understanding the interaction between moss identity and environmental change in determining rates of N_2 fixation can improve future N input predictions in vulnerable regions which store vast amounts of C. We measured vascular and non-vascular community composition and moss species-specific rates of N_2 fixation in a reciprocal tundra transplant experiment between Healy (63°N, 149°W) and Toolik Lake (68°N,149°W), Alaska, sites which have similar moist acidic tussock plant communities but differ by over 5°C in mean annual temperature. We hypothesized that transplanted mosses would not differ in N_2 fixation rates from their home environment within a moss species one year after transplantation due to the key role of host identity in determining associated N_2 fixation rates. We found that the magnitude of response to transplantation varied greatly among mosses. While *Hylocomium splendens* showed no change associated with transplantation, *A. turgidum* varied moderately by location and *P. schreberi* had a strong increase in N_2 fixation rates associated with movement to a colder environment. Overall, N_2 fixation rates were lower in our warmer site both for native and transplanted mosses. We saw no change in vascular or non-vascular species composition with transplantation, though both communities were distinct between home locations. We conclude that moss-associated N_2 fixation responses to climate changes are host-dependent.

Previous experiments have frequently seen a decline in moss biomass and moss diversity over time in response to warming. The host-specific responses of N_2 fixation to changing temperature could precede or contribute to these observed declines.

INTRODUCTION

Mosses are a key constituent of high latitude plant communities. In boreal and Arctic ecosystems, mosses account for 20-60% of net primary productivity on average (Chapin et al. 1995; Bisbee et al. 2001; Turetsky et al. 2010; Deane-Coe et al. 2015). The presence of mosses affects carbon (C) cycling directly and indirectly through traits such as the production of recalcitrant litter and the insulation of soils (Cornelissen et al. 2007a; Turetsky et al. 2012). Mosses exert control over vascular plant growth through peat accumulation, soil temperature, and allelopathy (Turetsky et al. 2012). High latitude ecosystems are also often nitrogen (N) limited for vascular plants (Shaver and Jonasson 1999; Lebauer and Treseder 2008). The largest source of new N is from associated N_2 fixers living as epiphytes on many moss species (Alexander and Schell 1973; DeLuca et al. 2002; Vile et al. 2014b; Stuart et al. 2020). Mosses are able to access this N resource as well as limit ecosystem N uptake via litter recalcitrance and high cation exchange capacity (Malmer et al. 2003; Cornelissen et al. 2007a; Berg et al. 2013; Rousk et al. 2016a). Through these mechanisms, mosses have a disproportionate effect on C cycling dynamics in a region of the world which hosts vast and vulnerable C stores (Lindo et al. 2013; Hugelius et al. 2014).

Changes in both moss biomass and community composition have been observed in manipulative experiments in boreal and tundra ecosystems (Lang et al. 2012; Turetsky et al. 2012; Deane-Coe et al. 2015; Alatalo et al. 2020). The optimum temperature range for tundra bryophytes is between 5-15° C, though many can photosynthesize even at temperatures below

freezing (He et al. 2016). Overall, passive warming experiments in high latitude ecosystems have shown a decrease in moss biomass and diversity (Wahren et al. 2005; Elmendorf et al. 2012; Deane-Coe et al. 2015; Alatalo et al. 2020). However, there are exceptions where no change or an increase in diversity and/or biomass of mosses were observed with experimental warming (Hudson and Henry 2010; Prather et al. 2019). Frequently, the direction or magnitude of the warming effect was moss species specific. Declining moss diversity could have cascading effects on the ecosystem, as high tundra moss diversity is linked to higher productivity and moisture retention (Rixen and Mulder 2005). Moss identity also impacts the magnitude of functional trait on the landscape as, for example, *Sphagnum* mosses can more effectively insulate soil than a feather moss like *Hylocomium splendens* (Sonesson et al. 1992; Fukuta et al. 2012).

The mechanism for moss biomass or diversity loss is often attributed to an increase in vascular plant growth, typically graminoids or deciduous shrubs, due to an increase in nutrient availability or changes in hydrology (Bisbee et al. 2001; Graglia et al. 2001; Shaver et al. 2001; Wahren et al. 2005; Bret-Harte et al. 2008; Deane-Coe et al. 2015). While the decline of N_2-fixer associated moss species could depress N inputs, loss of mosses also facilitates higher soil temperatures, permafrost thaw, and altered litter decomposition than can contribute to higher N availability and increased vascular plant growth (Wahren et al. 2005; Lang et al. 2012).

Rates of moss-associated N_2 fixation are affected directly by the environment and indirectly by alterations in moss biomass and species composition over time. Temperature, moisture, and light are important sources of process rate variation (Gundale et al. 2012a; Rousk and Michelsen 2017; Rousk et al. 2017b), but not all host moss species respond identically to changes in abiotic conditions over short and long time scales (Sorensen et al. 2012). Moss traits that differ between species, such as shade tolerance, microhabitat preference, or community

water retention, could influence the relative impact of changing edaphic or abiotic conditions (Mills and Macdonald 2004; Elumeeva et al. 2011; Jonsson et al. 2014). There is strong evidence that host species identity influences the structure of the N_2 fixing microbial community (Ininbergs et al. 2011; Bragina et al. 2012; Jean et al. 2020; Holland-Moritz et al. 2021). Mosses can chemo-attract cyanobacteria, which may play a role in the diverging structures of microbial assemblages associated with differen moss species (Bay et al. 2013b). There is some evidence that this specificity also leads to differences in N_2 fixation rates between species that can propagate across large environmental differences (Leppänen et al. 2015; Stuart et al. 2020). Previous work in Alaska demonstrated the relative importance of host moss genera in determining associated N_2 fixation rates compared with abiotic conditions such as temperature and moisture (Stuart et al. 2020). Assessing the degree of interaction between environmental conditions and host identity is critical in predicting future changes in ecosystem N inputs as both abiotic conditions and biotic communities change with climate.

The objective of our study was to compare the associated N_2 fixation rates of three common tundra moss species (*Aulacomnium turgidum*, *Hylcomium. splendens*, and *Pleurozium schreberi*) in a reciprocal transplant between warm and cool moist acidic tundra in Healy and Toolik Lake, Alaska. These sites represent areas of similar elevation, species pools, and mean annual precipitation (MAP) amounts but which vary in mean annual temperature (MAT) by over 5°C. We used $^{15}N_2$ incubations and percent cover assessments to measure N_2 fixation rates and community composition, respectively. We hypothesized that the N_2 fixation rates associated with transplanted mosses would not differ relative to their home locations due to the importance of host species identity over environment in structuring the microbial communities responsible for N_2 fixation. To test our main hypothesis, we used linear mixed effect models and linear models

to compare the effect of home location, transplantation status, and moss species. We did not expect to see significant changes in community composition over a single year. We used perMANOVA tests and nonmetric multidimensional scaling to compare the vascular and non-vascular plant community compositions between treatments. Finally, we utilized site weather data from long-term experiments to ensure that conditions were similar to long-term means during our measuring period.

MATERIALS & METHODS

Moss transplant experiment

In July 2018, two moist acidic tundra sites were selected in Healy near Eight Mile Lake (65°52'51" N,149°14'12"W) and Toolik Field Station (68°37'27"N,149°36'18"W), Alaska, USA. Sites were similar in vascular and non-vascular plant species pools, MAP, and elevation. The MAT at Toolik is -6.4°C and -1.0°C at Healy. Sites were near, but not within, long-term experimental plots in both locations. For a site description of sites at Eight Mile Lake, see Schuur et al. (2009).

At both sites, twelve circular cores (30cm diameter, approximately 15cm in depth) were carefully excavated. Patches were selected randomly from inter-tussock spaces, with at least one meter in between core edges. Six of the twelve cores were randomly assigned to the "home" treatment. Home cores were immediately re-transplanted into the locations from which the "away" cores had been excavated. Away cores were placed in buckets which contained multiple cold packs beneath a shelf within the bucket designed to hold the core intact. In both locations, away cores were kept in refrigerated rooms overnight before being transported to novel locations. During transplantation, all efforts possible were made to keep cores intact and cool. All transplantations were completed within 48h.

Following transplantation into either original or novel environments, the plant community of each core was assessed by two researchers. Ocular assessments of non-vascular percent cover were made for each core. Mosses were identified to genus or species. Liverworts and lichens were typically only identified to genus if cover exceeded 5%. The vascular community was characterized by counting the number of individuals of each species within the core. Each core was assessed again one year later in August 2019 prior to N_2 fixation measurements in the same fashion.

N_2 fixation measurements

Approximately one year after transplantation, in August of 2019, N_2 fixation rates were measured in all cores. Three target moss species were measured in this experiment: *Hylocomium splendens* (Hedw.) Brid.*, Pleurozium schreberi* (Brid.) Mitt.*,* and *Aulacomnium turgidum* (Wahlenb.) Schwgr. Each species was sampled in every core in which it was present. For each sample, 10 moss ramets of a single species were removed from the plot and placed in an airtight 60ml polypropylene syringe. Each moss ramet included was of approximately 5cm of length, including both green and senescent moss tissue, and was cleaned of debris or other plant material. The included moss was lightly sprayed with distilled water. Each syringe was plunged to 10 ml of ambient air and moss before 10ml of 98at% enriched $^{15}N_2$ gas was added, making the final volume 20 ml (Sigma-Aldrich Inc., Lot # MBBB9003V). Following a 24h incubation, samples were dried at 60° C for 48 h before being shipped to Northern Arizona University.

Dried moss samples were finely hand-chopped with scissors. Six mg of each sample was rolled into a tin capsule and run on a Costech ECS4010 elemental analyzer coupled to a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer to obtain %N, %C, $\delta^{15}N$, and $\delta^{13}C$. The atom percent enrichment (APE) of each sample was calculated by subtracting the natural

abundance atom percent (calculated using the average species $\delta^{15}N$ value: *A. turgidum*: -3.39‰; *P. schreberi*: -3.59‰; *H. splendens*: -3.34‰ from previously published data, see Stuart et al. 2020) from the incubated sample atom percent. Isotopic uptake was then scaled by the sample weight and the air:tracer ratio to calculate total ($^{15}N+^{14}N$) N_2 fixation (Jean et al. 2018). Rates are expressed as μg N g $moss^{-1}$ day^{-1}. The average enriched $\delta^{15}N$ value (± standard error) averaged across all samples was 2573.3± 148.4.

Statistical analyses

All statistical analyses were performed in R Studio 1.2.1335 using R version 3.6.1 (R Core Development Team). To test the hypothesis of this study, we used linear models and linear mixed-effects models in lme4 1.1-21 (Bates et al. 2015) paired with Satterthwaite's degrees of freedom method in lmerTest (Kuznetsova et al. 2017). Additional analyses used the vegan 2.5-7 package for nonmetric multidimensional scaling, running permutational multivariate analysis of variance (perMANOVA) tests on community composition data, and creating treatment group ellipses (Oksanen et al. 2019).

To test the effect of transplantation on moss associated N_2 fixation rates, we used a linear mixed effects model with rate of N_2 fixation (in μg N g $moss^{-1}$ day^{-1}) as the response variable and fixed effects of an interaction between home location (Toolik or Healy) and transplant status (transplanted or untransplanted). The distribution of the target species was not equal between the two tundra sites. While all mosses were present in each location, Toolik was dominated by *H. splendens* while *P. schreberi* was dominant in Healy. Under the assumption that moss species will affect the results but could not be directly tested in this model due to unequal distribution between treatment groups, we included it as a random effect. We also included sampling core as

a random effect to account for autocorrelation within cores but removed it from the final model as the between-group variability was not sufficient to warrant its incorporation.

In order to draw some conclusions about the role of moss identity in determining response to transplantation given the constraints of sample size, we analyzed each measured species in a separate linear model. Core was not included as a random effect in these models as only one sample of each species was measured in each core. As *A. turgidum* was well represented in all treatments, N_2 fixation rates associated with those samples was regressed against an interaction between home location and transplant status. For *H. splendens* and *P. schreberi*, only samples originating in Toolik or Healy, respectively, were included in the models. For each species model, N_2 fixation rates were regressed with transplant status to compare mosses that remained in their home environment to ones that were transported to a novel environment.

We generated nonmetric multidimensional scaling (NMDS) ordinations using the Bray-Curtis dissimilarity index separately for the vascular and non-vascular community composition of each core. Each ordination used 2 dimensions after confirming a low (<0.05) reduction in stress from adding additional axes. After checking for homogeneity of group variances, we ran a perMANOVA for each community to test for the effect of the interaction between home location and transplant status using the *adonis2* function with the Bray-Curtis dissimilarity index and 999 permutations. Depending on perMANOVA results, 95% confidence interval ellipses were constructed in ordination space for each group and then extracted for plotting. Using the function *envfit* we also fit species vectors within each community.

Weather datasets for each site were checked for anomalies in the time leading up to N_2 fixation rate measurements. Data for weather at Toolik Field Station were provided by the Toolik

Field Station Environmental Data Center (Environmental Data Center Team 2021) and Healy weather data were provided by the Schuur laboratory through the Bonanza Creek LTER (Schaedel et al. 2021).

Some additional linear models were explored based on other outputs from the isotope ration mass spectrometer, such as $\delta^{13}C$ and %C. Each output was coded as response variable to the interaction between home location and transplant status with an additional effect of moss species. No strong effects were observed apart from the effect of moss species on percent carbon. Results are presented in the supplementary material.

RESULTS

N_2 fixation rates within the transplant experiment were strongly affected by the interaction between home location and transplant status (Figure 4.1, $F=13.18$, $P < 0.001$). Mosses that we re-transplanted into their home environment in Healy fixed N_2 at lower rates than all other treatment groups, while mosses transplanted from Healy to Toolik fixed as much N_2 as native Toolik mosses. In this mixed model, moss species identity was treated as a random effect due to unequal distribution of species within treatments. The random effect of host species accounted for 64% of variance explained within the model. The marginal and conditional R^2 of model was 0.11 and 0.68, respectively.

Individual host moss species' N_2 fixation rates had divergent responses to transplantation. *H. splendens* originating in Toolik did not respond to transplantation (Figure 4.2, $F=0.28$, $P=0.611$). *Aulacomnium turgidum* was found in almost all treatment cores and its associated N_2 fixation rates were modeled as an interaction between home location and transplant status. Home location had the strongest impact on N_2 fixation rates, where mosses originating from Toolik had higher associated rates than those from Healy ($F=4.79$, $P=0.04$). However, the interaction

between home location and transplantation status appeared to have a moderate effect compared to transplantation status (F=3.71, P=0.072 for interaction term, F=0.84, P=0.374 for transplantation status). *Pleurozium schreberi* from Healy responded strongly to transplantation (Figure 4.2, F=42.52, P < 0.001).

Neither the vascular nor the non-vascular plant community responded to transplantation or the interaction between home location and transplant status (Vascular community: F=1.02, P=0.412 for transplantation status; F=1.37, P=0.240 for interaction term; Non-vascular community: F=0.46, P=0.680 for transplantation status; F=0.66, P=0.482 for interaction term). Conversely, for both plant community types, home location was significant (Figure 4.3; Vascular community: F=6.29, P<0.001; Non-vascular community: F=22.95, P<0.001). Within the non-vascular community species vectors, our three target moss species had a P value smaller than 0.01 (see Figure 4.5). Several vascular species, including *Rubus chamaemorus, Rhododendron tomentosum,* and *Cassiope tetragona* had low P values and appeared to be important in structuring differences between communities (see Figure 4.6).

In the days leading up to the N_2 incubations, average daily PAR at Toolik was higher compared to Healy (Figure 4.4). Healy had more precipitation in the summer of 2019, with 297 mm total falling during the two-month period prior to incubation. Out of those 60 days, 34 included some precipitation falling. In Toolik, the same time increment before incubation included 208mm of rain and 33 rainy days. In the fortnight preceding N_2 fixation measurements, Toolik received 73mm of precipitation to Healy's 158mm, with 10 and 11 days with rain events, respectively. As expected, daily mean and maximum temperatures were higher in Healy compared to Toolik. Relative humidity was higher at Healy (>75% in all days leading up to incubation) than Toolik (50-80%, see Figure 4.8).

DISCUSSION

The object of our experiment was to test the effect on moss-associated N_2 fixation rates when tundra mesocosms were transplanted reciprocally over 500km and a 5° C MAT shift. Contrary to our original hypothesis that the N_2 fixation rates of transplanted mosses would not differ relative to their home locations, we found a full spectrum of responses depending upon the host moss species. Our hypothesis was only supported for *H. splendens*, while *A. turgidum* N_2 fixation rates differed between home locations and *P. schreberi* responded strongly to transplantation. Given the scale of differences in response to experimental conditions, it is unsurprising that treating moss identity as a random effect explained a great deal of variation within the experiment. *A. turgidum* N_2 fixation rates were generally higher than those of the feather mosses, in contrast to previously observed trends in Alaska (Stuart et al. 2020). Overall, the N_2 fixation rates presented here are relatively high but within the range of previously published rates associated with mosses both in Alaska and Northern Europe (Bay et al. 2013b; Vile et al. 2014b; Jean et al. 2018).

As expected, no detectable changes occurred in plant community composition one year after transplantation as previously observed changes in community composition or diversity were only seen after an extended monitoring period (Wahren et al. 2005; Alatalo et al. 2020). Despite sharing a similar pool of plant species, experimental mesocosms were structured by home location (Figure 4.3). Three species vectors with strong effects on the vascular community appear to be *Rubus chamaemorus, Rhododendron tomentosum,* and *Cassiope tetragona*, with the former two in Healy plant communities and the latter in Toolik. Both Healy species are deciduous, with *R. chamaemorus* in particular having large leaves. *C. tetragona*, one of those most common Toolik vascular plant species, is relatively small and evergreen. Both light penetration and litterfall can impact rates of N_2 fixation (Zielke et al. 2002; Sorensen and

Michelsen 2011; Gundale et al. 2012a; Jean et al. 2020), though the direction and magnitude of the response is not equal across temperature gradients or host species.

The non-vascular community of each home location was significantly structured by the dominance of either *H. splendens* or *P. schreberi*, closely related feather mosses which are often co-dominant (Zackrisson et al. 2009; Huttunen et al. 2012). In the two tundra sites selected for our experiment, one feather moss or the other was dominant. Healy mesocosms had, on average, 4% *H. splendens* cover and 62% *P. schreberi* cover, while Toolik mesocosms held 73% *H. splendens* and 10% *P. schreberi*. The unequal species distribution prevented the inclusion of moss species as a fixed effect in the full reciprocal transplant model and places some limits on the conclusions that can be drawn within species.

Another caveat to the research presented is the short-term nature of the experiment. Since measurement were made one year after transplantation, it is difficult to disentangle the speed of responses from the strength of the response. What appears to be a moderate response to transplantation in N_2 fixation rates associated with *A. turgidum* could also be a slower response to temperature shifts relative to *P. schreberi*. The relatively dramatic shift in temperature regime may partially compensate for the short duration of the experiment. Most passive warming experiments that saw shifts in moss biomass elevated the growing season air temperature by only 1.5°C relative to the controls (Wahren et al. 2005). Further, the large temperature manipulation experiments by Gundale et al. (2012a) of 5.7°C induced changes in N_2 fixation rates associated with *P. schreberi* and *H. splendens* in just two weeks. A previous natural survey in Alaska also found MAT to be a relatively important predictor of associated N_2 fixation rates across many host moss species (Stuart et al. 2020). Incubation temperature was not found to be an important predictor, but on the days of incubation Healy had higher maximum (22.9°C) and average

(13.9°C) temperatures than Toolik on the day of incubation (9.0 and 6.7°C). Neither incubation period had temperatures that would be expected to inhibit N_2 fixation (Zielke et al. 2002; Gundale et al. 2012a; Jean et al. 2012).

Weather conditions leading up to the time of incubation were not anomalous and most likely had small effects on rates of N_2 fixation. While precipitation amounts were higher in Healy than in Toolik, precipitation has either been uncorrelated with N_2 fixation or more closely tied to the frequency of rain events than the total amount (Markham 2009; Gundale et al. 2012b). Light has long been known to affect N_2 fixation given the prevalence of phototrophic N_2 fixers (Basilier 1980; Gentili et al. 2005; Gundale et al. 2012a). The higher average daily PAR at Toolik may reflect the longer daylight hours at 68°N and could contribute to higher N_2 fixation activity at Toolik. Relative humidity was generally higher at Healy than at Toolik (see Supplementary Figure 4.4). Higher moisture is associated with an increase in N_2 fixation and may be just as or more important than temperature (Rousk et al. 2013, 2018). *P. schreberi* may be at an advantage in drier environments relative to *H. splendens* due to internal apoplasmic transportation of water along a central strand of hyoid cells (Sokołowska et al. 2017). As humidity decreases, *P. schreberi* is capable of increasing long-distance internal water transportation within the moss body (Sokołowska et al. 2017). Internal water transport capabilities may also limit water availability to epiphytic N_2 fixers, meaning drier conditions could favor moss survival while restricting available moisture for N_2 fixation.

Moss species often respond uniquely to changes in climate (Wahren et al. 2005; Hudson and Henry 2010; Prather et al. 2019; Alatalo et al. 2020). Previous experimental temperature increases have observed either no change or a decrease of N_2 fixation rates in association with *H. splendens* (Sorensen and Michelsen 2011; Gundale et al. 2012a; Sorensen et al. 2012). Similarly,

studies that quantified biomass or percent cover also saw steady or decreasing *H. splendens* with warming (Wahren et al. 2005; Alatalo et al. 2020). In contrast, *P. schreberi* often responds rapidly to warming both in biomass and rates of associated N_2 fixation (Gundale et al. 2012a; Deane-Coe et al. 2015; Rousk et al. 2017b). Transcriptome analysis of *A. turgidum* revealed more genes related to oxidative stress from heat than cold stress despite their acclimation to cold ecosystems (Liu et al. 2010). Biomass of *A. turgidum* has responded inconsistently to increases in air temperature (Wahren et al. 2005; Hudson and Henry 2010; Sorensen and Michelsen 2011), but N_2 fixation rates have decreased relative to the control after 20 years of experimental warming (Sorensen et al. 2012). In short, responses to temperature changes interact both with moss species and likely with other environmental conditions given the divergence of both biomass and N_2 fixation rates in response to temperature alterations. The mechanism behind species specificity, similarly, may have multiple and interacting sources. Arctic and boreal mosses have unique microbiome assemblages (Ininbergs et al. 2011; Jean et al. 2020; Holland-Moritz et al. 2021) which may be more or less resilient to changing conditions over time (Gentili et al. 2005). Differences in moss anatomy, microhabitat, and community structure can also directly affect temperature and moisture within the bryosphere (Elumeeva et al. 2011).

Within moss species that were responsive to experimental manipulation, we saw a striking trend of increases in N_2 fixation with movement to a colder environment. There was also some evidence that mosses transplanted from cold tundra to warmer tundra had lower rates of N_2 fixation relative to untransplanted mosses. Given the strong evidence for moss acquisition of fixed N_2 resources (Bay et al. 2013b; Berg et al. 2013; Rousk et al. 2016a), changes in process rates may influence biomass loss over time. As temperatures warm quickly at northern latitudes, some moss species could demonstrate a relatively fast negative response, a potential mechanism

for the decrease in moss diversity observed in response to warming (Lang et al. 2012; Alatalo et al. 2020). Decreases in moss cover or biomass can affect the resilience of high latitude ecosystems through a decrease in new N inputs, faster decomposition rates, and changes in understory heat fluxes (Blok et al. 2011; Turetsky et al. 2012). Further insights into the interaction between host moss identity and environmental change could elucidate the mechanisms behind seemingly stochastic responses of bryophytes to a changing climate.

FIGURE 4.1 Mixed model results of an interaction between home location and transplant status on associated N_2 fixation rates. Moss species measured was a random effect in the model. The interaction term had an F value of 13.18. Squares represent estimated marginal means predictions with bars indicating the 95% confidence interval. Letters represent pairwise comparison *P* value differences where α=0.05 using the Kenward-Roger degrees-of-freedom method with Tukey's adjustment. Points are the raw data included in the model.

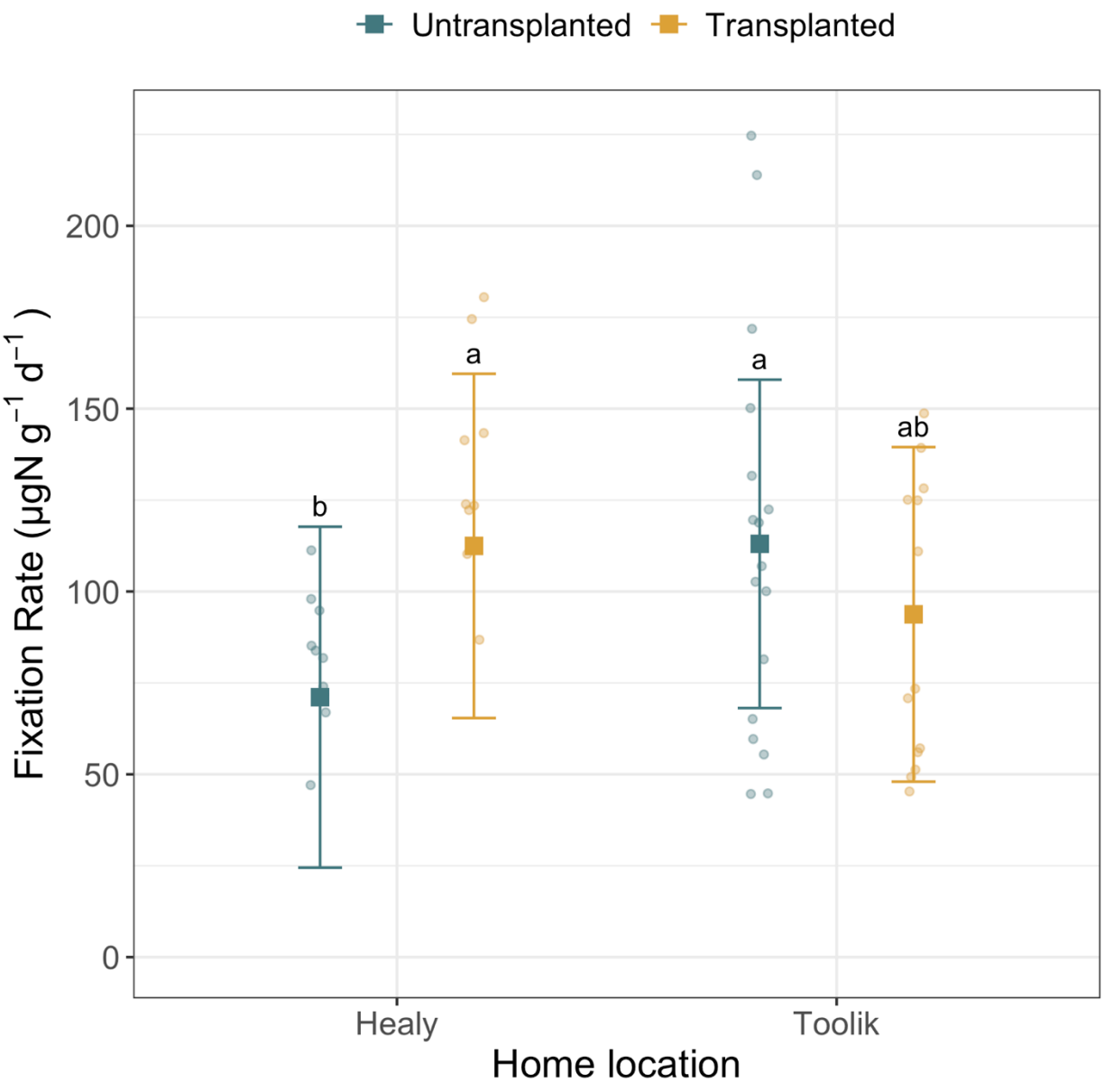


FIGURE 4.2 Individual linear models were run for each tested moss species. The *H. splendens* model included only samples from Toolik and *P. schreberi* only samples from Healy due to incomplete replication within treatment groups. Red boxes were placed over data excluded from the models and within those areas squares represent group mean and bars are standard error. Otherwise, box points represent estimated marginal means and bars are 95% confidence intervals. *H. splendens* did not respond to transplantation, while *P. schreberi* responded strongly. *A. turgidum* N_2 fixation rates were most affected by home location but there was a moderate interaction between home location and transplantation status (P=0.07).

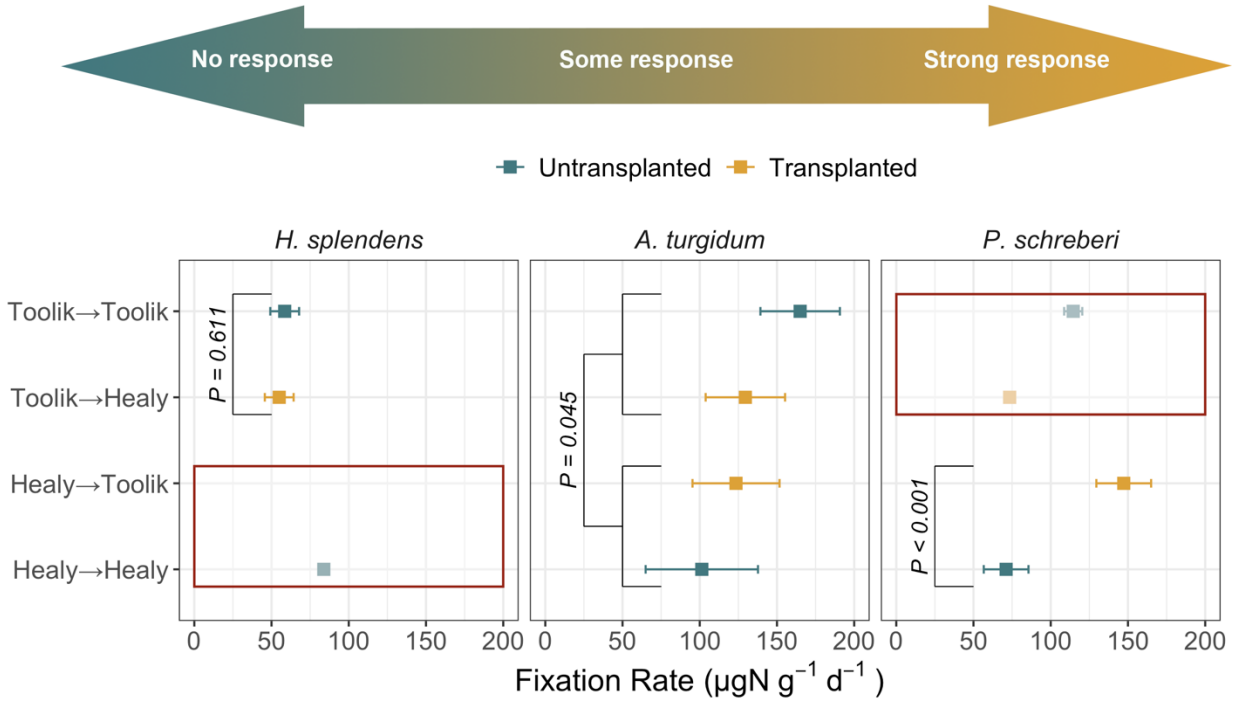
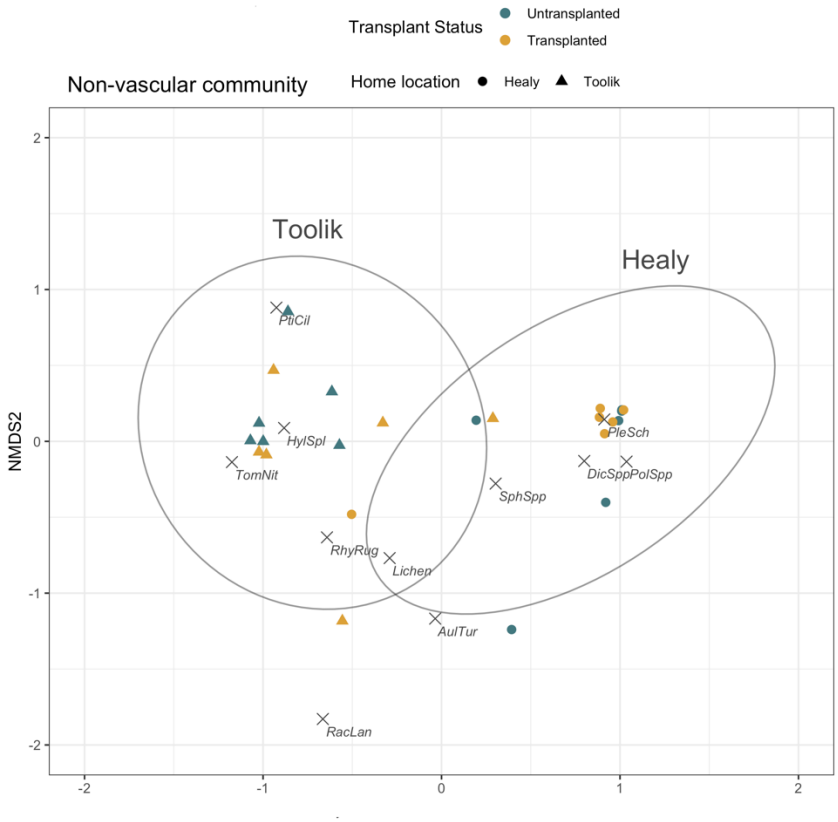


FIGURE 4.3 NMDS ordination of observed community structure. Ellipses representing the 95% group confidence interval using the standard deviation of points for home locations in the non-vascular (top) and vascular (bottom) plant communities. perMANOVA results showed no effect of transplant status. Species ordination scores are represented by X's. Species codes are the first three letters of the genus followed by the first three letters of the species. If species were not differentiated within a genus, *Spp* appears in place of the species code.


Transplant Status
Untransplanted
Transplanted
Non-vascular community
Home location
Healy
Toolik
Toolik
Healy
PtiCil
HylSpl
TomNit
PleSch
DicSppPolSpp
SphSpp
RhyRug
Lichen
AulTur
RacLan
NMDS2
2
1
0
-1
-2
-2
-1
0
1
2

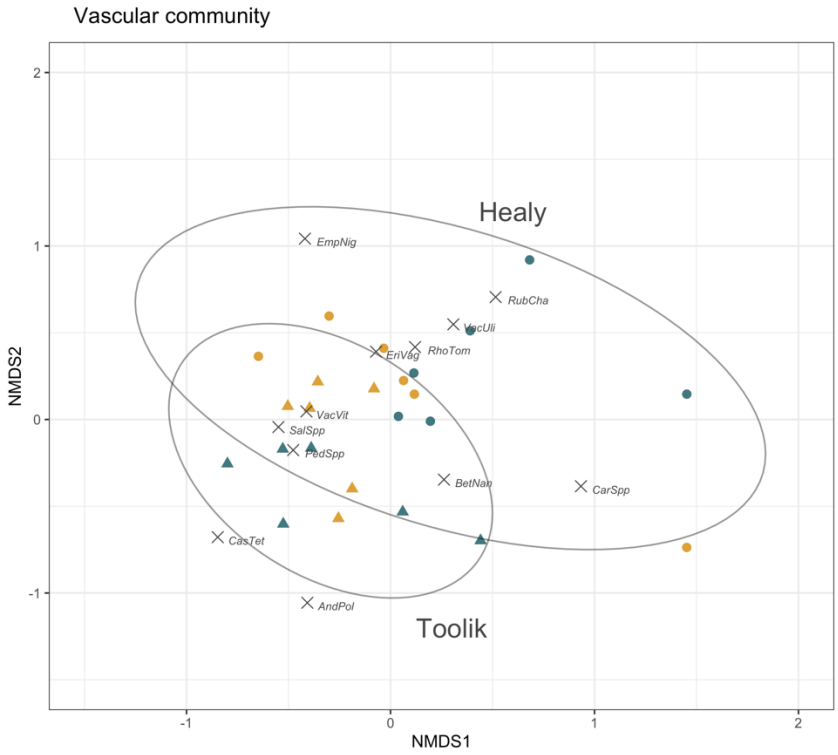

Vascular community
Healy
EmpNig
RubCha
VacUli
EriVag
RhoTom
VacVit
SalSpp
PedSpp
BetNan
CarSpp
CasTet
AndPol
Toolik
NMDS2
NMDS1
2
1
0
-1
-1
0
1
2

FIGURE 4.4 Average daily PAR (top) and precipitation (bottom) in the growing season leading up to N_2 fixation measurements. Dates of incubation are indicated by a red bar on each graph. The *x*-axis is the Julian day of year.

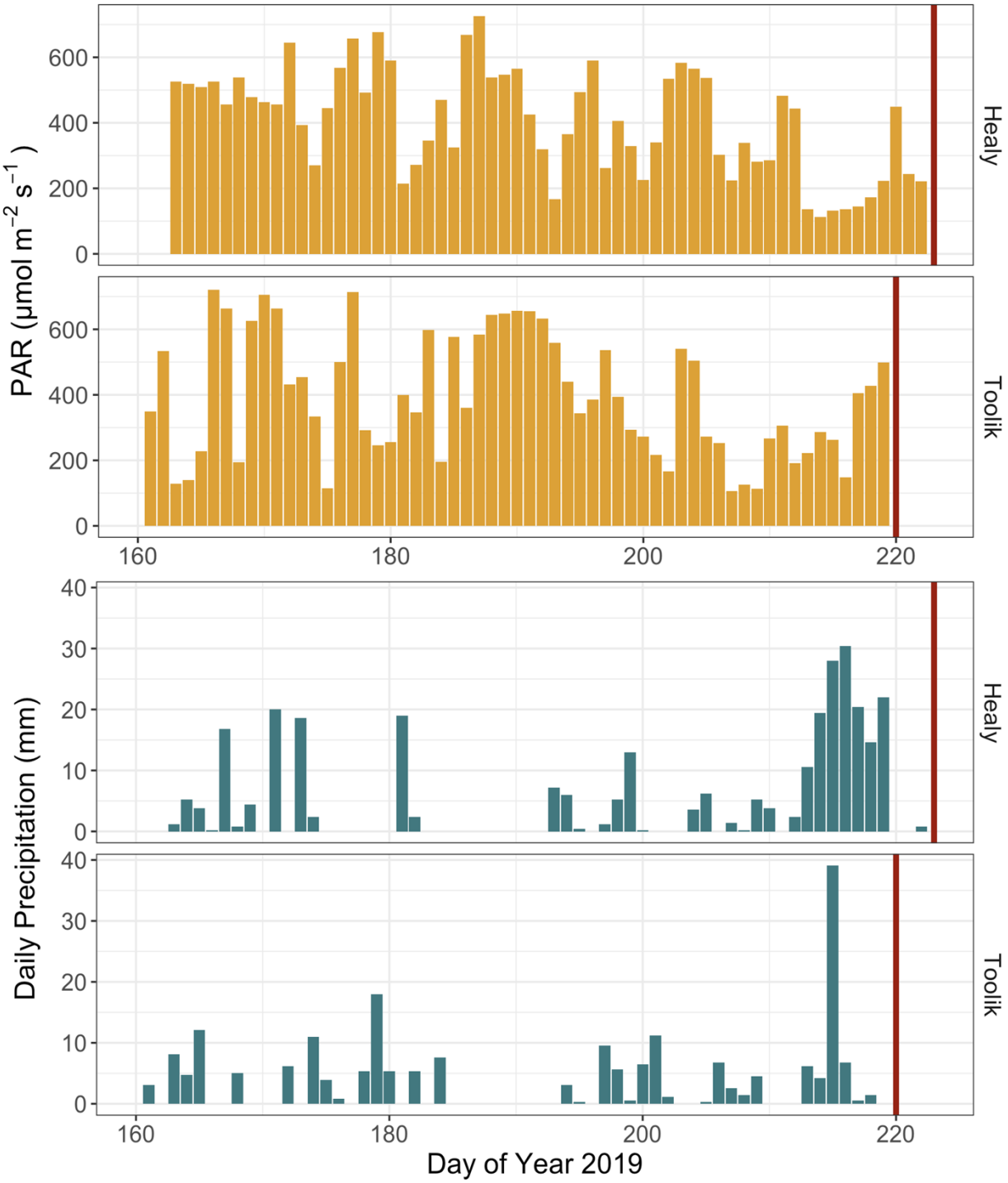


FIGURE 4.5 Vectors of species fit on non-vascular plant community composition from both experimental sites. Vectors are pictured if the P value was less than 0.05, and the P values are included next to the species code in the graph. Species codes are the first three letters of the genus followed by the first three letters of the species. If species were not differentiated within a genus, *Spp* appears in place of the species code.

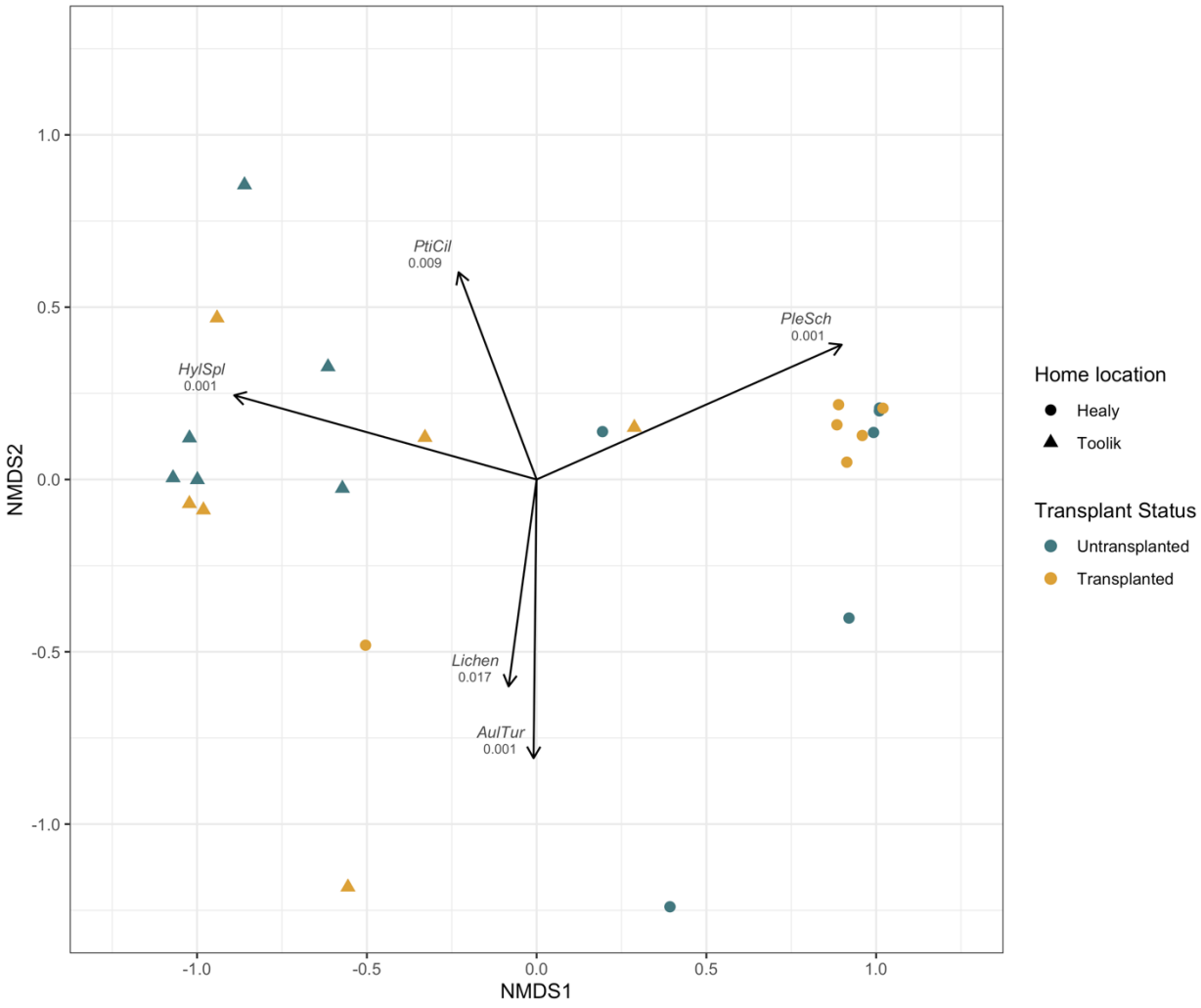


FIGURE 4.6 Vectors of species fit on vascular plant community composition from both experimental sites. Vectors are pictured if the P value was less than 0.05, and the P values are included next to the species code in the graph. Species codes are the first three letters of the genus followed by the first three letters of the species. If species were not differentiated within a genus, *Spp* appears in place of the species code.

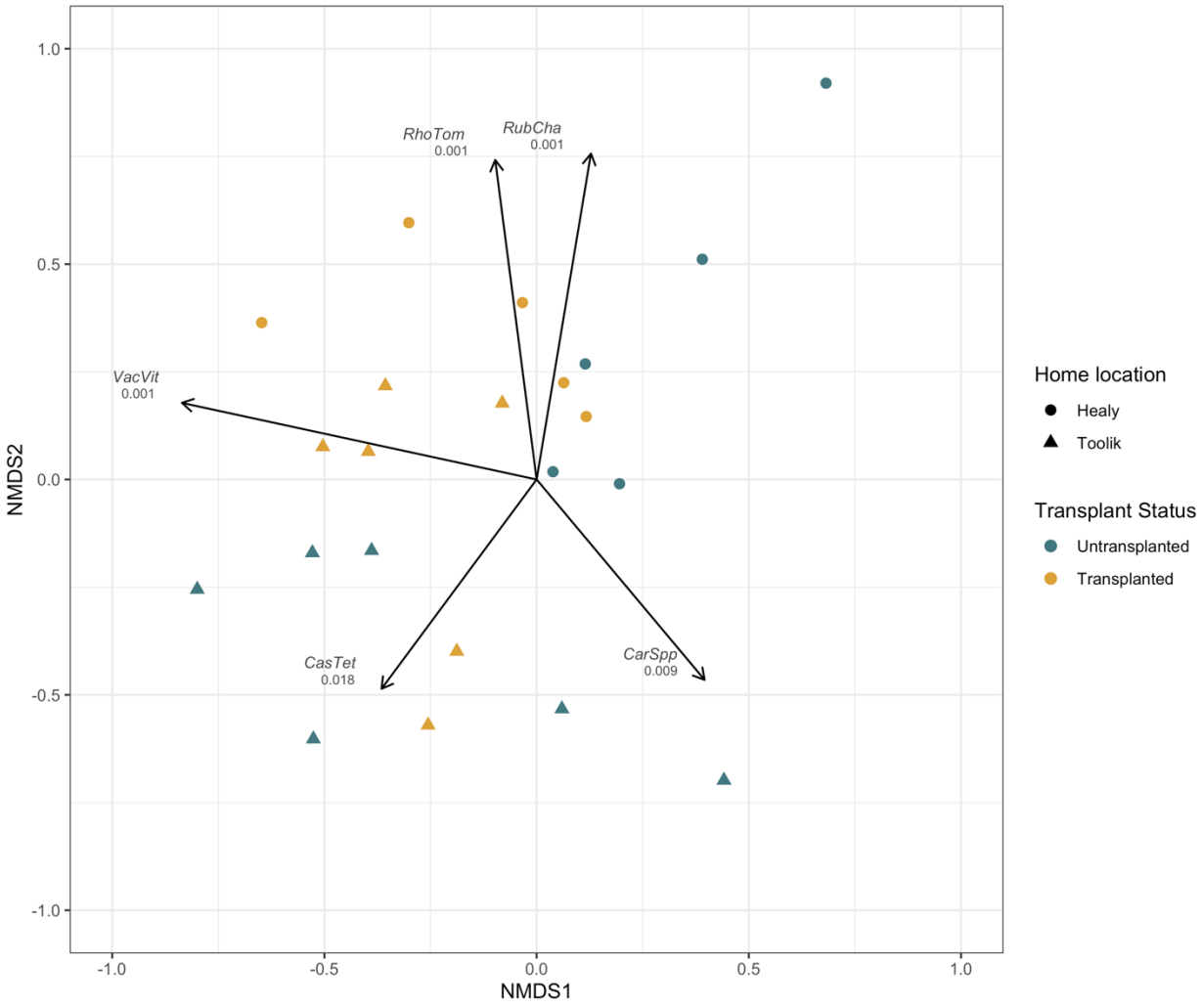


FIGURE 4.7 Linear model results for percent carbon of each sample modeled as an interaction between home location and transplant status and an additive effect of moss species. Only moss species had a strong effect. Boxes and bars represent estimated marginal means and 95% confidence intervals from the model, while grey points are the underlying raw data.

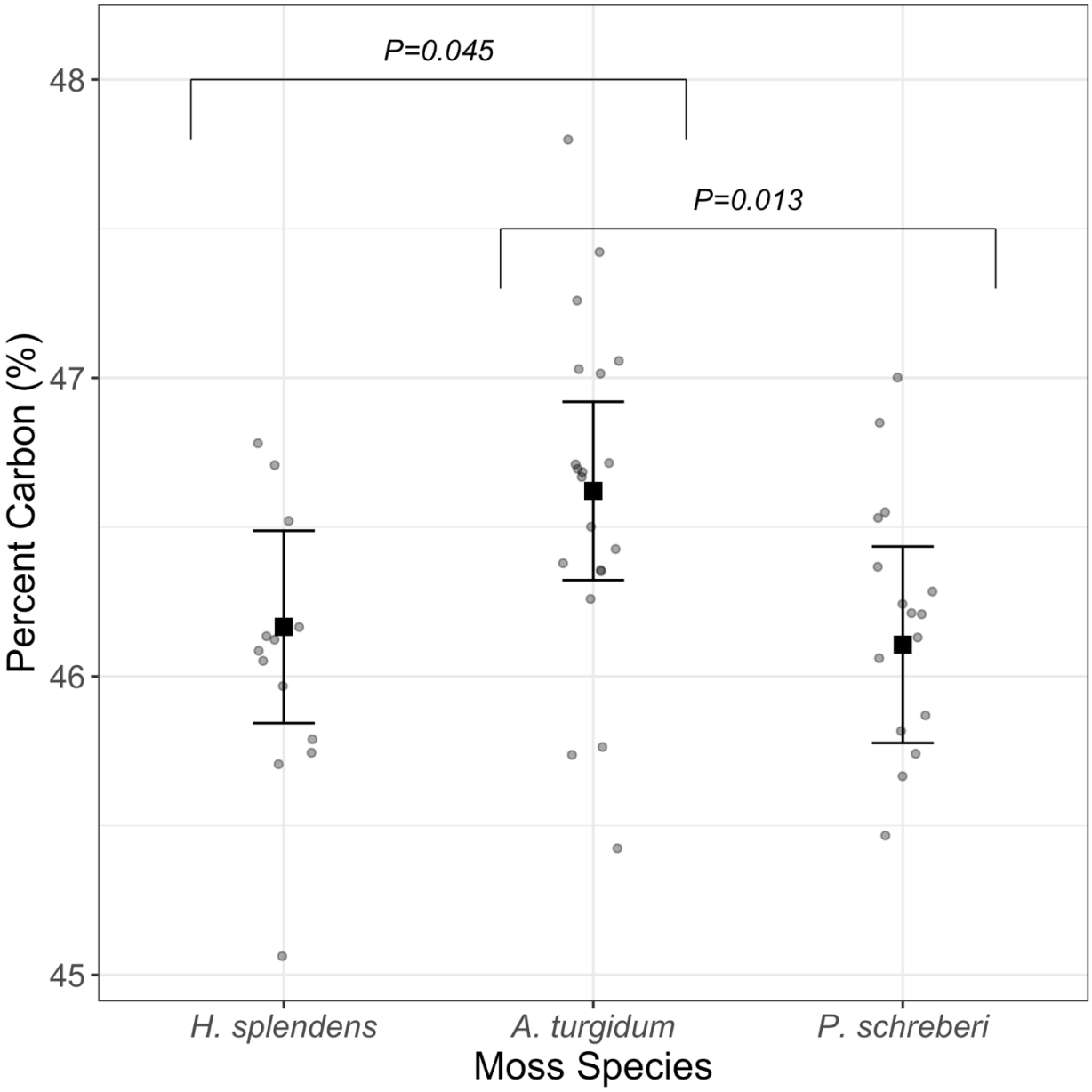
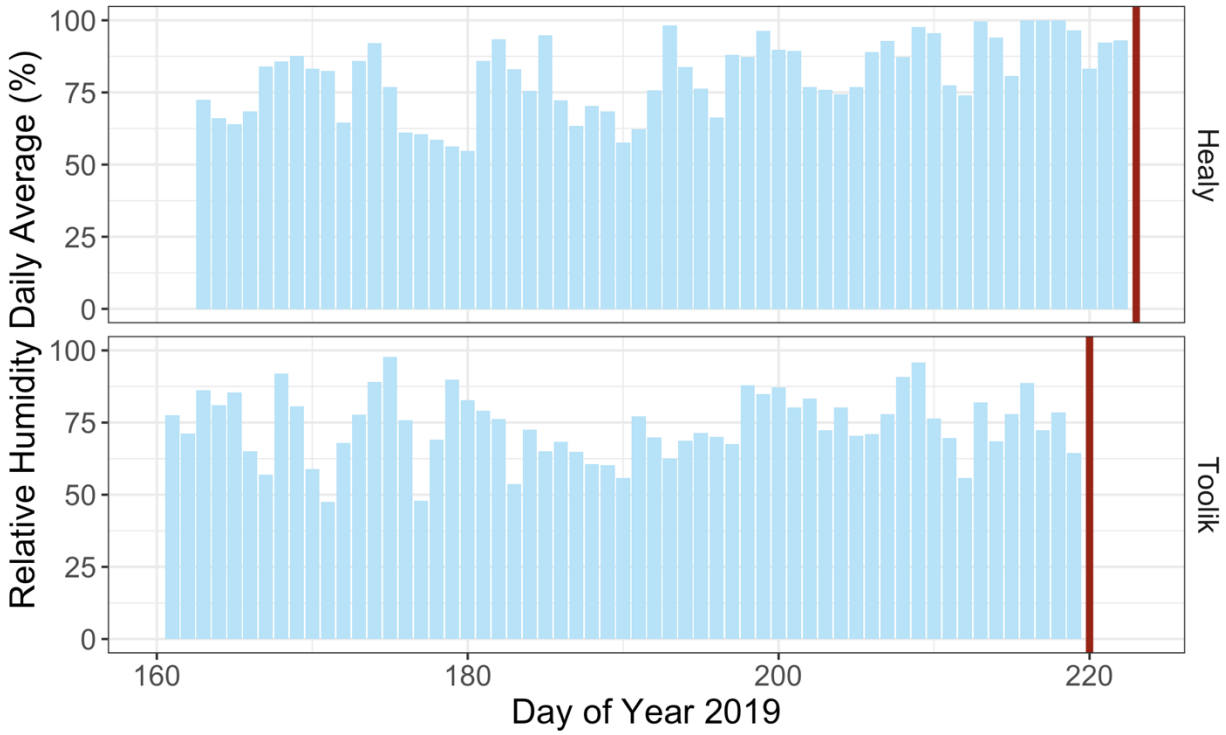


FIGURE 4.8 Average daily relative humidity in the growing season leading up to N_2 fixation measurements. Dates of incubation are indicated by a red bar on each graph. The *x*-axis is the Julian day of year.



CHAPTER V

Research highlights:

- N_2 fixation is nearly ubiquitous among a diversity of high latitude mosses
- Host moss identity is a key driver of associated N_2 fixation rates
- Significant, consistent N_2 fixation rate variation was observed between moss genera
- Measuring all present mosses revealed hotspots of N_2 fixation
- We found only a modest correlation between $\delta^{15}N$ and N_2 fixation rates, potentially indicating the presence of alternative nitrogenases
- An interaction between $\delta^{13}C$ and host moss order showed that host moss identity impacts how strongly N_2 fixation rates respond to abiotic conditions
- When $\delta^{13}C$ values exceed -30.4‰, the probability of N_2 fixation was over 95% in feather mosses
- The magnitude of response in associated N_2 fixation rate to transplantation varied greatly between host mosses, which may serve as a mechanism for decreased biomass and diversity observed in other warming experiments
- Vascular and non-vascular community structure did not change one year after transplantation

Surveying a broad diversity of potential host moss species revealed definitively that associations with microbial N_2 fixers are widespread, geographically and taxonomically, in Alaska. While the rates per unit mass that were found in this dissertation are within the same range of values found in previous studies, the inclusion of all species revealed hotspots that may have been missed if only one or two target species were measured. Species-specific incubations also allowed for the testing of the hypothesis that host moss identity would be a

significant source of variation even when accounting for differences between sampling sites. As these sampling sites encompassed a diversity of conditions, including differences in elevation and plant community, we conclude that sampling site conditions were less important than moss identity in determining N_2 fixation rates. While environment likely serves as an interaction term with host moss identity, the strength of identity as a predictor underlines the importance of its inclusion in future studies.

Past studies have used $\delta^{15}N$ as a proxy for N_2 fixation activity. While a relationship was expected to be observed in this dissertation, little evidence was found to support that hypothesis. Since previous research has shown that mosses do utilize and retain fixed N, it is likely that the prevalence of alternative nitrogenases at high latitudes influenced the natural abundance of ^{15}N. Thus, the use of $\delta^{15}N$ is likely not useful for inferring N_2 fixation rates without further characterization of potential N sources.

Moss $\delta^{13}C$, an indicator of long-term trends in temperature and moisture, had a strong relationship to N_2 fixation within feather mosses. While this relationship was not observed broadly across all mosses, it is evident that past conditions within the moss carpet may connect to current N_2 fixation rates in feather mosses. Generalizing the magnitude or direction of "moss-associated N_2 fixation" as a whole in response to environmental change can overgeneralize between host mosses that show substantive differences in the degree of resilience or reaction to abiotic shifts. Future research disentangling the role of species-specific microbiomes and individual/ community moss traits in mediating environmental conditions could provide further insight into the drivers of host moss specific responses observed in this study.

The transplantation of mosses from areas representing a large temperature gradient highlighted the interaction between host moss identity and environmental conditions in mediating rates of moss-associated N_2 fixation. The three target species had divergent reactions, with *Pleurozium schreberi* showing greatly increased N_2 fixation rates in a cooler environment while the closely related *Hylocomium splendens* did not decrease N_2 fixation in a warmer environment. Overall, N_2 fixation rates were lower in the warmer environment. No community composition changes were observed in either the vascular or non-vascular community in response to transplantation. Though the change in temperature between sites was dramatic, community changes are not typically observed after only one year. However, the strong effect of transplantation in only some host moss species may help explain long-term community changes. Species-specific decreases in N_2 fixation may feed back to decrease species diversity or overall moss biomass.

Each experiment detailed in this dissertation relates in some way to the effect of host moss identity on N_2 fixation. While previous studies have found species-level differences, particularly in microbial community composition, this is the first strong evidence for host moss differences in N_2 fixation across a broad array of potential host mosses. Some of the published N_2 fixation rates are the first presented in the literature for that species. Additionally, this dissertation expands the geographic range of published N_2 fixation rates, since the vast majority are from Europe. Finally, by using $^{15}N_2$ incubations, no potentially inaccurate conversion ratio is required to quantify the amount of N_2 fixed. By highlighting the interaction between host moss and environment, these conclusions add more nuance to future N input predictions and hopefully encourage other scientists to consider the differences among mosses and their importance for high latitude ecosystems.

Additional data collected during this dissertation may help answer the next pressing topics related to moss-associated N_2 fixation. These include tracing the fate of fixed N on longer timescales, assessing the impact of the vascular plant community on N_2 fixation rates in tundra mosses in an experimental manipulation of shrubs, and looking at interannual differences in N_2 fixation rates.

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