Bacterial communities of lichens and mosses and nitrogen fixation in a warming climate

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Dissertation submitted in partial fulfillment of a
Philosophiae Doctor degree in Biology

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Abstract

Climate warming in sub-Arctic regions leads to shifts in plant communities and retreating glaciers. Mosses and lichens contribute to important ecosystem processes in these environments, including nitrogen fixation via their microbiome. The first objective of this dissertation was to understand the extent to which long-term warming affects bacterial communities associated with the lichen *Cetraria islandica*. In the same context, the bacterial community and nitrogen fixation rates associated with the moss *Racomitrium lanuginosum* were investigated. These species are among the most common lichen and moss species in Iceland, respectively. Paper I shows that long-term warming affects the structure and composition of the bacterial community associated with *C. islandica* and that this change is partly mediated via changes in the plant community. The same is true for the bacterial communities associated with *R. lanuginosum*, although nitrogen fixation rates are apparently not affected by warming, potentially due to warming-induced shifts in nitrogen-fixing taxa (Paper II). The second objective was to evaluate the extent to which bacterial communities of two common *Racomitrium* species and the underlying soil as well as the moss-associated nitrogen fixation change during primary succession and whether these changes are related to changes in moss functional traits. The bacterial community composition associated with *Racomitrium* mosses was correlated with the successional stage in the Fláajökull forefield, and also with moss moisture content. The bacterial communities of the underlying soil also shifted with succession and were in addition related to the moss C:N ratio. Nitrogen fixation rates did not change with time since deglaciation, but were correlated with the bacterial community structure.

Útdráttur

Meðal afleiðinga hlýnunar loftslags á norðurslóðum má nefna hörfun jökla og ýmsar gróðurbreytingar. Mosar og fléttur leggja mikið af mörkum til vistkerfislegra ferla í hinu kalda umhverfi norðurslóða, þar á meðal bindingu köfnunarefnis með aðstoð þeirra örvera sem í þeim þrífast. Eitt af meginmarkmiðum þeirra rannsókna sem greint er frá í ritsmíð þessari er að leiða fram að hve miklu leyti loftslagshlýnun á áratuga skala hefur áhrif á örverusamfélög í fléttunni *Cetraria islandica* (fjallagrös) og í gamburmosanum *Racomitrium lanuginosum* (hraungambri). Þessar tegundir eru meðal algengustu fléttu- og mosategunda í mörgum íslenskum vistgerðum. Í Grein I er sýnt fram á að langtímahlýnun hefur áhrif á samsetningu örverulífríkisins í fjallagrösum og að þau áhrif eru að hluta tengd breytingum á aðlægum gróðri. Hið sama reyndist eiga við um örverusamfélög hraungambra, nema hvað, að hlýnunin reyndist ekki hafa marktæk áhrif á niturbindingu, og kom fram að sú niðurstaða gæti skýrst af breyttri tegundasamsetningu niturbindandi baktería (Grein II). Annað meginmarkmið rannsóknanna var að meta að hve miklu leyti örverusamfélög í tveimur gamburmosategundum og í jarðveginum sem þær vaxa á taka breytingum með gróðurframvindu og hvort þær breytingar tengjast ástandi mosans og starfsemi á borð við niturbindingu. Örverusamfélögin voru þannig kortlögð með tilliti til aldurs jökulgarða Fláajökuls, og einnig við rakainnihald mosans. Bakteríusamfélög í jarðveginum tóku skýrum breytingum með aldri jökulgarðanna, og stóðu einnig í samhengi við C:N hlutfall mosans sem á honum óx. Niturbinding stóð ekki í marktæku samhengi við aldur jökulgarðanna, en var hins vegar skýrt fylgin samsetningu örverulífríkisins.

Samenvatting

Klimaatverandering in het subarctische gebied leidt onder meer tot veranderingen in plantengemeenschappen en smeltende gletsjers. Mossen en korstmossen dragen bij aan belangrijke ecosysteemprocessen zoals stikstoffixatie in deze gebieden, deels via hun microbioom. Het doel van dit proefschrift was ten eerste om te begrijpen in hoeverre langdurige opwarming de bacteriële gemeenschappen van het korstmos *Cetraria islandica* (IJslands mos) en de bacteriële gemeenschappen en stikstoffixatie van het mos *Racomitrium lanuginosum* (Wollige bisschopsmuts) beïnvloedt. Paper I laat zien dat langdurige opwarming de structuur en compositie van de bacteriele gemeenschap van *C. islandica* beïnvloedt en dat dit ten dele komt door veranderingen in de plantengemeenschap. Dezelfde bevindingen vond ik voor de bacteriële gemeenschap van *R. lanuginosum*, hoewel stikstoffixatie niet beïnvloed leek door opwarming, wellicht veroorzaakt door een verschuiving in de stikstoffixerende bacteriële taxa. Het tweede doel van dit proefschrift was om te evalueren in hoeverre bacteriële gemeenschappen van *Racomitrium* soorten en de onderliggende bodem en stikstoffixatie veranderen gedurende primaire successie en of deze veranderingen gerelateerd zijn tot moskenmerken. De bacteriële gemeenschappen van *Racomitrium* mossoorten waren gerelateerd tot successiestadium, maar ook tot vochtgehalte van de mossen. De bacteriële gemeenschappen van de onderliggende bodem veranderden ook met successie, en werden tegelijkertijd beïnvloed door mos C:N ratio. Stikstoffixatie was niet gerelateerd aan tijd sinds deglaciatie, maar aan de structuur van de bacteriële gemeenschap.

To my parents.

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List of Original Papers

This thesis is based on the following three papers. Hereafter they will be referred to by their numbers as follows:

Paper I: Klarenberg, I.J., Keuschnig, C., Warshan, D., Jónsdóttir, I.S., Vilhelmsson, O. (2020) The total and active bacterial community of the chlorolichen *Cetraria islandica* and its response to long-term warming in sub-Arctic tundra. Frontiers in Microbiology, 11, 3299, https://doi.org/10.3389/fmicb.2020.540404

Paper II: Klarenberg, I.J., Keuschnig, C., Russi Colmenares, A.J., Warshan, D., Jungblut, A.D., Jónsdóttir, I.S., Vilhelmsson, O. Long-term warming effects on the microbiome and nitrogen fixation or common moss species in sub-Arctic tundra. https://doi.org/10.1101/838581

Paper III: Klarenberg, I.J., Keuschnig, C., Salazar, A., Benning, L.G., Vilhelmsson, O. Nitrogen fixation and bacterial communities of *Racomitrium* mosses and underlying substrate in an Icelandic glacier forefield.

Other papers published during this study:

Gowers, G.-O.F., Vince, O., Charles, J.-H., Klarenberg, I., Ellis, T., Edwards, A. (2019) Entirely off-grid and solar-powered DNA sequencing of microbial communities during an ice cap traverse expedition. Genes, 10(11), 902. https://doi.org/10.3390/genes10110902

Author contributions

The following authors have contributed to the papers and manuscripts for this dissertation: Ingeborg Klarenberg (IJK), Oddur Vilhelmsson (OV), Ingibjörg Svala Jónsdóttir (ISJ), Christoph Keuschnig (CK), Ana J. Russi Colmenares (AJRC), Denis Warshan (DW), Anne D. Jungblut (ADJ), Alejandro Salazar (AS) and Liane G. Benning (LGB). The contribution of authors to the papers part of this dissertation are as follows:

Paper I: IJK initiated the idea and methodology together with ISJ and OV. IJK collected the samples, performed the DNA and RNA extraction and the cDNA synthesis and performed the bioinformatics and statistical analyses. CK performed the qPCR. IJK drafted the paper with revisions from co-authors CK, DW, ISJ and OV.

Paper II: IJK initiated the idea and methodology together with ISJ and OV. IJK collected the samples, performed the DNA and RNA extraction and the cDNA synthesis and performed the bioinformatics and statistical analyses. CK performed the qPCR. AJRC collected samples and performed the ARAs. IJK drafted the paper with revisions from co-authors CK, ARJC, DW, ADJ, ISJ and OV.

Paper III: IJK defined the questions and the sampling design. IJK and OV collected the samples. IJK conducted the DNA extractions, bioinformatics and statistical analyses. IJK also performed the ARAs with input from AS and measured moss and soil characteristics. CK performed the qPCR. IJK drafted the paper with input from AS, CK, OV and LGB.

List of Published Nucleotide Sequences

The nucleotide sequences produced during the work within this dissertation were made publicly available at the European Nucleotide Archive under the following accession numbers:

PRJEB37116: partial 16S rRNA sequences from bacteria associated with the lichen *Cetraria islandica*, published in Paper I.

PRJEB40635: partial 16S rRNA sequences from bacteria associated with the moss *Racomitrium lanuginosum*, reported in Paper II.

Abbreviations

ARA – Acetylene Reduction Assay

ASV – Amplicon Sequence Variant

cDNA – complementary Deoxyribonucleic Acid

CSS – Cumulative Sum Scaling

DNA – Deoxyribonucleic Acid

GLMM – Generalized Linear Mixed Model

MCMC – Markov Chain Monte Carlo

N – Nitrogen

N_2 - Dinitrogen

OTC – Open Top Chamber

PCoA – Principal Coordinate Analysis

PCR – Polymerase Chain Reaction

PERMANOVA – Permutational Multivariate Analysis of Variance

qPCR – quantitative Polymerase Chain Reaction

RNA – Ribonucleic Acid

rRNA – Ribosomal Ribonucleic Acid

SEM – Structural Equation Modelling

TC – Total Carbon

TN – Total Nitrogen

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1 Introduction

The overall scope of this doctoral thesis was to investigate the extent to which the bacterial community composition associated with certain mosses and a lichen as well as moss-associated nitrogen fixation are affected by environmental change. Among the studied factors were long-term warming and time since deglaciation. The studies were conducted using a long-term warming experiment in an Icelandic dwarf-shrub heath as well as along a successional gradient in an Iceland glacier forefield.

1.1 Geographical setting and climate change

Iceland is located on the mid-Atlantic ridge, just south of the Arctic circle. Iceland is for the largest part located in the sub-Arctic (Figure 1). The sub-Arctic boundary was defined by Circumpolar Arctic Vegetation Map Team and is based on the boundaries between the tree line and the timberline, or the forest-tundra ecotone (Love 1970; Meltofte et al. 2013; CAVM Team 2003).

Figure 1 Map of the top of the northern hemisphere with the high and low Arctic subzones, as well as the sub-Arctic zone, according the Circumpolar Arctic Vegetation map. Source: CAVM Team 2003 and Meltofte et al. 2013.

The Icelandic climate is milder than its geographic position suggests. A branch of the Gulf Stream, the Irminger Current flows west of Iceland and the associated northward heat flux is key for the terrestrial Icelandic climate. As a result, the climate can be classified as maritime, with cool summer and mild winters with annual average temperatures ranging from 3 to 6 °C at the coasts and colder in the highlands based on the period 1981–2010 (Einarsson 1984; Icelandic Meteorological Office 2021). Annual accumulated precipitation ranges from less than 1,000 to 5,000 mm, with the highest amounts in the glaciated areas in the south of Iceland and the lowest amounts north of those glaciers (Icelandic Meteorological Office 2021).

We are living in a time of rapidly changing climates driven by human activities, the Anthropocene (Lewis and Maslin 2015). While the impact of global change on the climate system is complex, CO_2 is the main greenhouse gas contributing to global warming (Intergovernmental Panel on Climate Change 2014). At the time of writing (16. February 2021) the atmospheric CO_2 level measured at the Mauna Loa Observatory in Hawaii is 415 ppm. We have to go back at least 7 million years and possibly further to find equivalent atmospheric CO_2 levels (Cui, Schubert, and Jahren 2020).

Anthropogenic climate change will affect the entire Earth, but temperatures in the Arctic have increased more double compared to the global average over the last two decades (IPCC 2019). The causes for this Arctic amplification are, amongst others, changes in albedo and increases in cloud cover and water vapor (Serreze and Barry 2011). For Iceland, average temperatures are expected to rise between 1.8 °C and 3.1 °C in the 21st century, depending on the scenario (RCP4.5 or RCP8.5) (Gosseling 2017). Warming will be more pronounced in the northern part of Iceland and at higher elevations (Gosseling 2017). The projected trends in precipitation are not clear. Mostly an increase in total precipitation is predicted, but the spatial trends are uncertain (Gosseling 2017). In addition, the number of dry days is expected to increase, especially in the later part of the 21st century (Gosseling 2017).

1.2 Effects of climate change on microbial communities

The effects of climate change on Arctic terrestrial systems are, amongst others, changes in glacier mass balances, permafrost thaw and shrub expansion (Box et al. 2019), which can in turn affect albedo and carbon cycling and ultimately feed back to the climate system. Climate change can for instance enhance permafrost thaw and stimulate microbial breakdown of organic carbon stored in these frozen soils, thereby releasing CO_2 and methane (Schuur et al. 2015). Similarly, climate change may affect microbes associated with other living organisms such as plants, but our current understanding of the associated consequences for ecosystem processes and climate feedbacks is limited.

Microbial organisms are rarely discussed in the light of climate change, especially with regard to the effect of climate change on microorganisms, compared to animals and plants (Cavicchioli et al. 2019). Microbial organisms (such as bacteria, fungi and archaea) are at the base of biogeochemical cycles and can be found in nearly any environment on Earth. Climate change induced shifts in microbial diversity and activities are likely to alter those biogeochemical cycles. In addition, microbial organisms are part of the microbiome of living hosts and can influence evolutionary (Delaux and Schornack 2021) and ecological processes

(Fitzpatrick, Mustafa, and Viliunas 2019). Therefore, these microbial organisms may affect the ability of their hosts to respond to climate change.

Microbial organisms may be directly influenced by climate factors such as elevated CO_2, warming or drought, or interactions between these drivers (Bardgett, Freeman, and Ostle 2008). Reactions of microbial communities to climate change can take different forms. Microbial communities may be able to adapt to environmental change by genetic evolution (Romero-Olivares, Taylor, and Treseder 2015), physiological adaptation (Crowther and Bradford 2013) or shifts in community structure (Wallenstein and Hall 2012). All these mechanisms may be operating simultaneously (Bradford 2013).

Indirect effects of climate change on microbial organisms via plants may be stronger than the direct effects of climate on shaping microbial community structure and function (Classen et al. 2015; Ma et al. 2018). These indirect effects include changes in plant composition (Finks et al. 2021; Rinnan et al. 2007) and litter composition (Rinnan et al. 2007; Deslippe et al. 2012). Soil bacterial communities are indeed strongly controlled by changes in above-ground plant conditions such as net primary productivity. Soil environmental conditions also strongly control the response of soil bacterial composition, structure and functional activity to warming (Zhou et al. 2012). This also illustrates the difficulty of predicting the response of microbial communities to warming due to the many interacting factors influencing them and their diversity in physiology and functions, individual responses and interactions between them (Deslippe et al. 2012; Post et al. 2009; Bardgett et al. 2013).

1.3 Mosses and lichens

While mosses and lichens are organisms from different Kingdoms, they both represent important parts of the alpine and polar vegetation. Lichens and mosses contribute significantly to net primary production in Arctic ecosystems, estimated at 5-30% depending on the site (Shaver and Chapin 1991). Mosses and lichens are also important contributors to species richness in the Arctic (Bültmann 2008). Bryophytes (which include mosses) are abundant in most vegetation types in the Arctic and on small spatial scales bryophyte species diversity is higher than that of vascular plants (Väre et al. 2013). Mosses and lichens can be found in most Icelandic vegetation types and in some areas (post-glacial lava flows and in glacier forefields) mosses can develop into thick moss mats (Arnalds 2015).

Lichens are traditionally defined as a symbiosis between a fungus (mycobiont) and an algae and/or a cyanobacterium (photobiont). In recent years, the finding that bacteria (other than cyanobacteria) and additional fungi are associated with lichens, has questioned the traditional definition of a lichen. The lichen symbiosis is thus more complex than a partnership between a mycobiont and one or two photobionts and can be seen as a miniature ecosystem, which has been defined as ‘a self-sustaining ecosystem formed by the interaction of an exhabitant fungus and an extracellular arrangement of one or more photosynthetic partners and an indeterminate number of other microscopic organisms’ by Hawksworth and Grube (2020). Apart from bacteria and other fungi, lichens are also home to small invertebrates (Bokhorst et al. 2015). Lichens develop a wide variety of morphological shapes, for instance crustose (crustlike), foliose (leaflike) or fruticose (branching), which are mainly determined by the fungal partner. The photobiont provides photosynthetically derived sugars to the fungus (Hill 1972). In lichens with both an alga (the phycobiont) and a

cyanobacterium, the cyanobacterium (or cyanobiont) is mainly responsible for nitrogen fixation and provides the fungal partner with ammonia (Rowell, Rai, and Stewart 1985).

Cetraria islandica, the lichen host of the bacterial community studied here in relation to long-term warming, is common in the low- and highlands of Iceland (Thell and Moberg 2011) and can be found throughout the Arctic, sub-Arctic and alpine regions in the northern hemisphere as well as sub-Antarctic (Kärnefelt, Mattsson, and Thell 1993). It is a mat-forming chlorolichen with foliose thalli.

Figure 2 Photo of Cetraria islandica in a Betula nana heath in northwest Iceland.

Mosses are among the earliest land plants (Shaw, Szövényi, and Shaw 2011). They are positioned within the plant Kingdom in the phylum Bryophyta. Most of them lack specialized tissues such as tracheids or vessels such as vascular plants have and are therefore limited in active transport of water and nutrients. Their state of hydration is controlled by the environment (they are poikilohydric) (Raven 2002). Similarly to the idea of a lichen as minuscule ecosystem, mosses have been defined as the *bryosphere*, representing 'the combined complex of living and dead moss tissue and associated organisms' (Figure 3) (Lindo and Gonzalez 2010), although this term is not much used. This term acknowledges the existence of additional components such as a microbiome, micro- and mesofauna.

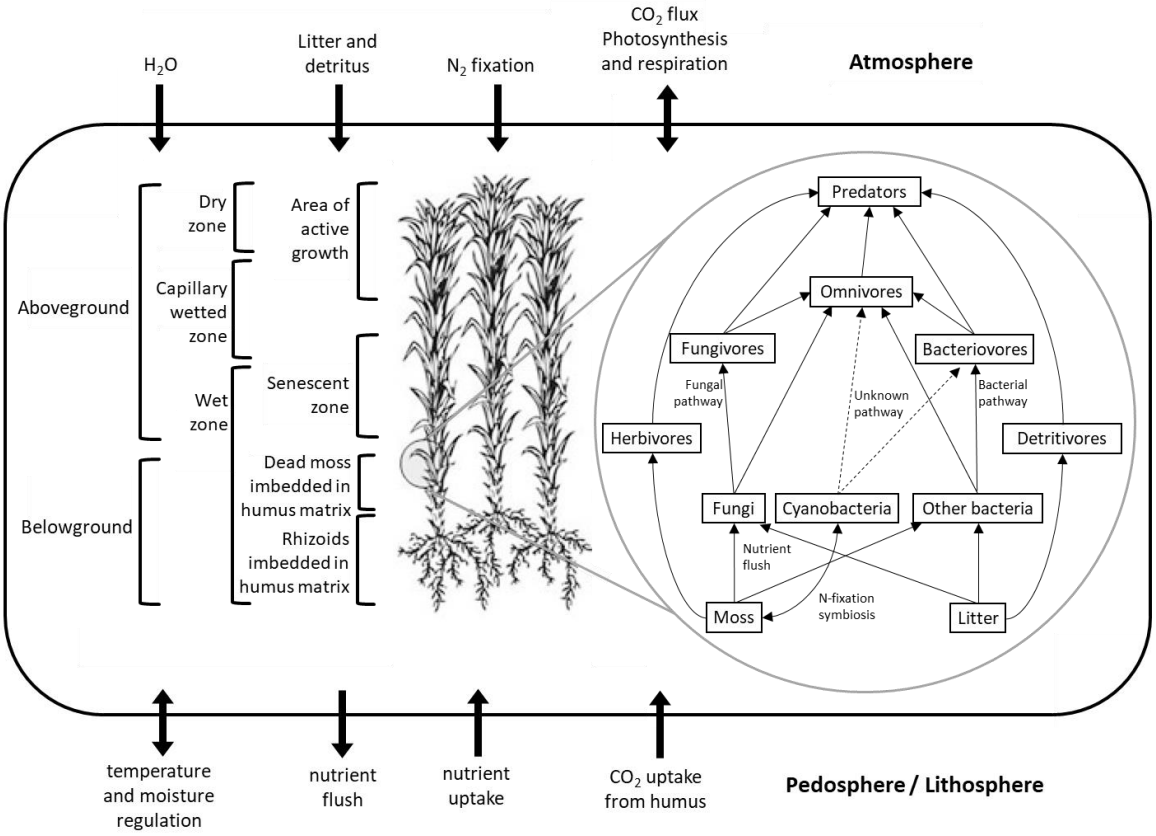

Figure 3 The bryosphere within its boundaries with the atmosphere and pedosphere/lithosphere. Shown also are ecological linkages with these boundaries as well as the different vertical zones of mosses. In addition, and arbitrarily placed, a detrital foodweb is shown. After: Lindo and Gonzalez 2010.

Peat mosses (*Sphagnum spp*) have received particular scientific attention especially because of the economic value of the peatlands they form and their role as carbon stocks (Nichols and Peteet 2019). In Iceland however, the most abundant mosses belong to the genus *Racomitrium* (Figure 4), which can develop into thick moss mats when conditions allow (Bjarnason 1991). The bacterial communities of these mosses were the subject of study in this dissertation with regard to long-term warming and primary succession.

Both lichens and mosses contribute to important ecosystem functions in high-latitude ecosystems. Among these ecosystem properties or functions are water retention, ecosystem carbon budget, ecosystem nutrition and soil cohesion (Figure 3) (Cornelissen et al. 2007). Lichens and especially mosses are generally recalcitrant, or resistant to decomposition. Therefore, their abundance can play an important role in ecosystem carbon budgets (Cornelissen et al. 2007). Nitrogen is often limited in the tundra environments where lichens and mosses are generally found. As mentioned before, lichens can have symbiotic relationships with Cyanobacteria that fix atmospheric N_2 (Henriksson and Simu 1971). But mosses also host Cyanobacteria (Smith and Russell 1982). Thus, both lichens and mosses can contribute to ecosystem nitrogen input. In glacier forefields for instance, the establishment of mosses can provide the local environment with nitrogen and thereby influence the soil microbial community (Miller and Lane 2019). Glaciers cover

approximately 11% of Iceland (Björnsson and Pálsson 2008), but are retreating. Vatnajökull for instance, lost 164 km^2 between 1890 and 2010 (H. Hannesdóttir et al. 2015). As mosses are among the first colonizing plants of glacier forefields, they may be important determinants of soil development during succession in these environments.

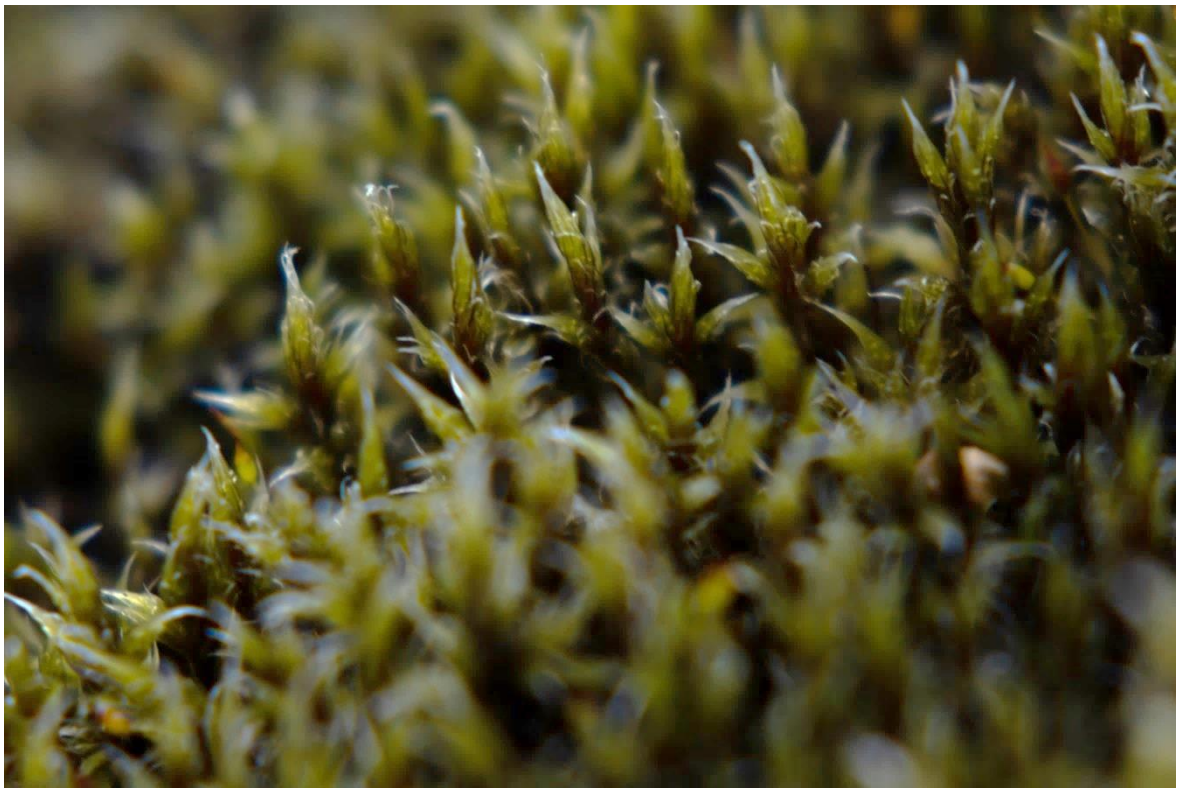

Figure 4 Photo of Racomitrium lanuginosum.

Both mosses and lichens are expected to suffer from climate change in regions where warming leads to shrub expansion (Myers-Smith et al. 2011; Cornelissen et al. 2001; Jonsdottir et al. 2005). These shrubs increase shading and litter accumulation and thereby outcompete slow-growing lichens (Cornelissen et al. 2001) and mosses (van Zuijlen et al. 2021).

1.4 Lichens and mosses as hosts for bacterial communities

Within the concept of lichens and mosses as small ecosystems, the host organisms and their microbiota are defined as the holobiont (Margulis 1983). These microbiota form complex communities that are shaped by active recruitment of the host, stochastic transfer of microorganisms, vertical transmission and abiotic and biotic environmental conditions (Vandenkoornhuyse et al. 2015). As the microbiota add a set a functional genes to the holobiont, plant microbiomes have been suggested to be the powerhouse of adjustments to local conditions (Vandenkoornhuyse et al. 2015).

1.4.1 Lichen-associated bacterial communities

The first discovery of non-cyanobacterial lichen-associated bacteria (a deltaproteobacterium now known as *Melittangium lichenicola*) was probably by Thaxter in 1892. Throughout the 20th century, diverse bacteria genera have been isolated from lichens. The development of culture-independent techniques in recent years has led to a surge in studies describing the composition of lichen-associated bacterial communities (Cardinale, Puglia, and Grube 2006). The abundance and location of bacteria in lichen thalli have been revealed by fluorescent *in-situ* hybridization (FISH) and confocal laser scanning microscopy. Cardinale et al. (2008) for instance, found a density of 6×10^7 bacteria.g^{-1} in the reindeer lichen *Cladonia arbuscula*. With the development of 'omics' technologies, research on lichen microbiomes shifted to more holistic approaches using metagenomics (all genomic information in a certain habitat), metatranscriptomics (all expressed genes in a given community) and metaproteomics (all synthesized proteins in a given community). Metabolomics (all metabolites in a given community) approaches have so far not been applied to lichens.

Most lichen-associated bacterial communities are dominated by Alphaproteobacteria (Cardinale et al. 2008; Bragina, Maier, et al. 2012; Printzen et al. 2012; Hodkinson et al. 2012; Bates et al. 2011; West et al. 2018). Within the Alphaproteobacteria, the order Rhizobiales is often the most abundant, together with Rhodospirillales (in chlorolichens) and Sphingomonadales (in cyanolichens) (Hodkinson et al. 2012). Acidobacteria are the main phylum in other lichens, such as *Ophioparma* (Hodkinson et al. 2012) and *Solorina crocea* (Grube et al. 2012). West et al. (2018) found that marine cyanolichens *Lichina pygmaea* and *L. confinis* are dominated by Bacteroidetes. Other phyla, such as Actinobacteria, Betaproteobacteria and Firmicutes are present in lower abundances (Cardinale et al. 2008; González et al. 2005).

While these bacterial communities may be less tightly integrated partners than the myco- and photobiont, they do contribute to the functioning of the holobiont. Their roles include supplying nutrients such as nitrogen, phosphorous and sulfur (Grube et al. 2015; Sigurbjörnsdóttir, Andrésson, and Vilhelmsson 2015), production of vitamins (Erlacher et al. 2015; Grube et al. 2015), pathogen defense (Grube et al. 2015), resistance against abiotic factors (Grube et al. 2015), detoxification of metabolites (Grube et al. 2015) and decomposition of older parts of the lichen thallus (Grube et al. 2015). There are also indications that chlorolichens, lacking cyanobacterial partners, host other bacteria taxa capable of nitrogen fixation (Liba et al. 2006) and a higher diversity of nitrogen fixing bacteria than cyanolichens (Almendras et al. 2018).

The drivers of the composition of lichen-associated bacterial communities have been explored to some extent. Lichens harbor species-specific bacterial communities (Grube et al. 2009; Sierra et al. 2020) that differ at least partly from the substrate they grow on (Leiva et al. 2021). Part of their bacterial community may be co-dispersed with lichen propagules, while other parts may be selected from the surrounding environment (Leiva et al. 2021). The photobiont may also affect the bacterial community composition (Hodkinson et al. 2012), in addition to the age (Cardinale et al. 2012) and location (Noh et al. 2020) of the thallus parts. But the bacterial community composition has also been shown to shift with abiotic factors such as large-scale geography (Hodkinson et al. 2012), substrate type and sun exposure (Cardinale et al. 2012), and elevation (Coleine et al. 2019). The shift with elevation and sun

exposure suggest changes in bacterial community structure with temperature (Coleine et al. 2019).

Considering the variety of roles that members of the bacterial communities are likely to play in the lichen holobiont, as outlined above, one can postulate that changes in lichen-associated bacterial community composition and, hence, function may support adaptation of the lichen holobiont to environmental change.

1.4.2 Moss-associated bacterial communities

Most of what we know about moss-associated bacterial communities originates from studies on peat mosses (*Sphagnum spp.*) and boreal forest mosses (*Hylocomium splendens* and *Pleurozium schreberi*). The emphasis on peat mosses is not surprising as peatlands constitute 30% of the world's soil carbon storage (Oke and Hager 2020). Similarly, boreal forest feather mosses have received much attention due to their contribution to nitrogen fixation in these ecosystems. Considering the importance of other mosses than peat mosses and boreal forest mosses, studies of their bacterial communities are highly relevant.

Due to the relevance of mosses for nitrogen fixation, we know most about moss-cyanobacterial interactions (Figure 5) as compared to other bacterial associates. A synergistic relationship between nitrogen fixing cyanobacteria and *Sphagnum* and *Drepanocladus* mosses was first suggested by Granhall and Selander (1973). Opelt and Berg (2004) later suggested that mosses host diverse bacterial communities. Using culture-dependent and -independent techniques, sequences closely related to *Pseudomonas*, *Acetobacter*, *Methylobacterium*, *Sphingomonas* and many other known plant-associated taxa were found. *Burkholderia* isolates have also been found (Opelt et al. 2007). On the phylum level, Proteobacteria are the most abundant, followed by Acidobacteria, Verrucomicrobia, Bacteroidetes, Cyanobacteria and Actinobacteria (Holland-Moritz et al. 2018; Ma et al. 2017). The families Acetobacteraceae (Alphaproteobacteria) and Acidobacteriaceae (Acidobacteria) are commonly found (Holland-Moritz et al. 2018; Tveit et al. 2020).

The functions of these bacterial associates, apart from nitrogen fixation, which is discussed in more detail in the next subchapter, range from pathogen defense (Shcherbakov et al. 2013), methanotrophy (Tveit et al. 2020), nutrient acquisition (Opelt et al. 2007) to production of plant hormones (Opelt et al. 2007). A draft genome of a highly abundant member of the candidate phylum WPS-2 in moss samples harbored genes for anoxygenic phototrophy (Holland-Moritz et al. 2018).

Similar to lichens, mosses are thought to host species-specific bacterial communities (Holland-Moritz et al. 2021; Warshan et al. 2016). These host-specific communities may be driven by differences in host traits, such as tissue nutrient content, moisture status or secondary metabolites, leading to variation in microbial communities as in foliar microbial communities of vascular plants (Bálint et al. 2013). The environmental drivers of the composition of moss-associated bacterial communities are largely unknown, but there are indications that hydrology (Tveit et al. 2020), light availability (Holland-Moritz et al. 2021), trophic interactions (Kardol et al. 2016), altered litter inputs (Jean et al. 2020a) as well as location along the moss shoot (Chen et al. 2019) can affect the bacterial community structure. Temperature can also influence moss-bacterial community structure (Tveit et al. 2020; Holland-Moritz et al. 2021; Carrell et al. 2019), but only two studies have specifically tested the effect of increased temperatures on bacterial communities of peat mosses. They

have shown that increased temperatures can lead to a decrease in the diversity of the overall and the nitrogen fixing bacterial communities after four weeks of warming (up to +30°C) and one and two years of warming (up to +9°C) (Carrell et al. 2019; Kolton et al. 2019). Whether this can be generalized to other moss species is unclear, and the long-term effects of climate warming on moss microbiomes have never been explored. Finally, the successional stage in glacier forefields can drive the composition of cyanobacterial genera on mosses (Arróniz-Crespo et al. 2014), and presumably non-cyanobacterial moss-associates may also shift with successional stage.

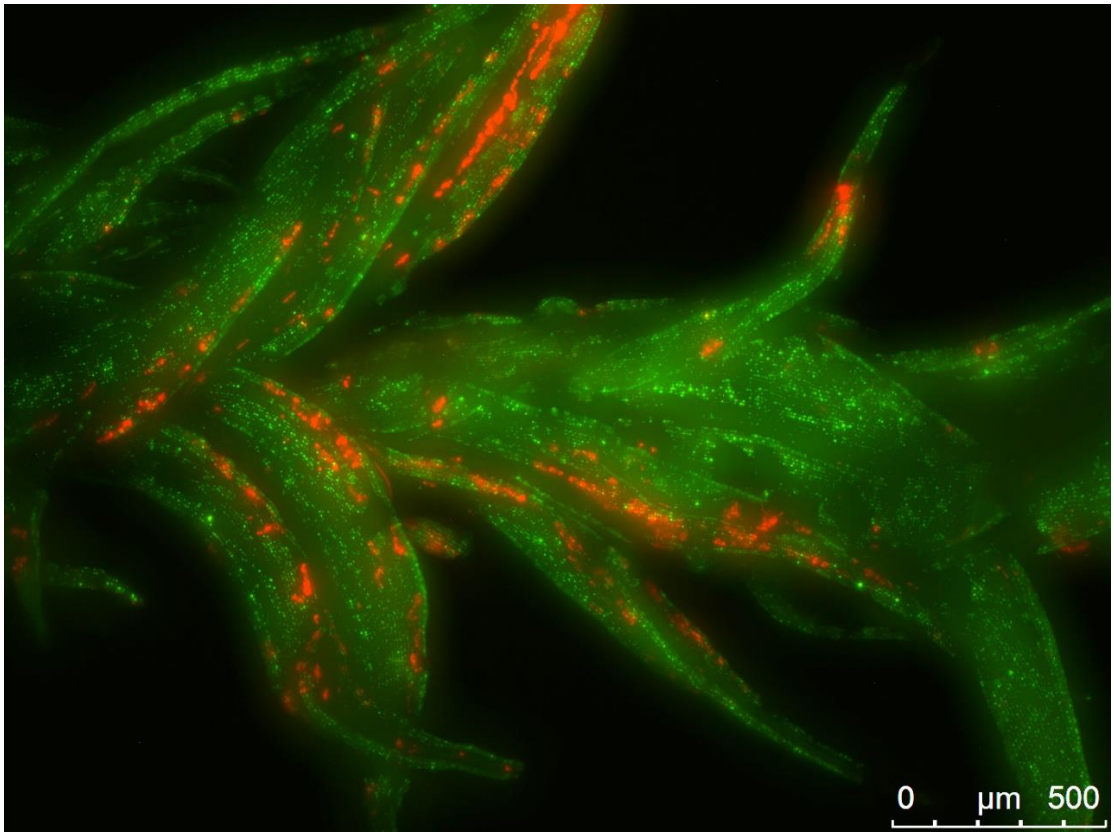

Figure 5 Image of a Racomitrium sp. gametophyte with cyanobacteria (red) taken with epifluorescence microscopy.

1.5 Moss-associated nitrogen fixation

1.5.1 Nitrogenases

Biological nitrogen fixation is the conversion of atmospheric dinitrogen (N_2) to ammonia (NH_3) by the enzyme complex nitrogenase. The core of the complex is formed by two proteins, nitrogenase (MoFe or molybdenum-iron protein) and nitrogenase reductase (or Fe protein). *NifH*, the gene encoding nitrogenase reductase, is highly conserved and its phylogeny is generally in agreement with phylogenies based on 16S rRNA gene sequences (Zehr et al. 2003). *NifH* homologs can be divided into four or five clusters (Zehr et al. 2003; J. Raymond et al. 2004).

The largest group of *nifH* sequences belongs to cluster I and contains sequences belonging to bacterial nitrogen fixers, such as the Cyanobacteria, *Frankia,* Proteobacteria as well as some Clostridia, Bacilli and Nitrospirae (Angel et al. 2018). Cluster I also contains some sequences of the alternative nitrogenase *vnfH*. Cluster II contains alternative nitrogenases that are paralogs of *nifH*: *vnfH* and *anfH*. These nitrogenases use alternative cofactors, either an iron-only (FeFe) or a vanadium-iron (VFe) (Bishop et al. 1986; Chisnell, Premakumar, and Bishop 1988). These alternative nitrogenases are also thought to contribute to nitrogen fixation in lichens and mosses in high-latitude ecosystems (Rousk, Degboe, et al. 2017; Darnajoux et al. 2016; Villarreal et al. 2021). As these complementary nitrogenases are less efficient at reducing acetylene, they could obscure the correlation between acetylene reduction assays and nitrogen fixation rates. Besides, the vanadium nitrogenase has been shown to be more efficient at lower temperatures than molybdenum nitrogenase (Bellenger et al. 2020). Cluster III consists of *nifH* sequences of Archaea and anaerobic bacteria such as methanogens, Clostridia, Spirochaetes and green sulfur bacteria. Cluster IV sequences are paralogs of the *nifH* gene that are generally uncharacterized or non-functional, although exceptions exist (Zheng et al. 2016). Sometimes a fifth cluster is recognized, which contains paralogs of the *nifH* gene involved in photopigment biosynthesis (J. Raymond et al. 2004). Many microorganisms possess multiple copies of nitrogenase genes (Zehr et al. 2003).

Nitrogen fixation is a highly energy intensive process, that uses at least 16 ATPs for reducing one mole of dinitrogen (Burris and Roberts 1993):

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$$

This process is the primary source of new nitrogen in natural ecosystems (Cleveland et al. 1999).

1.5.2 Nitrogen fixation

In polar ecosystems, which receive low amounts of biologically available nitrogen in atmospheric deposition, where decomposition and nitrogen mineralization are slow, nitrogen is the main limiting nutrient to vascular plant growth (Shaver and Chapin 1980). Cyanobacteria, either free-living or in association with mosses and lichens are thought to be the most important nitrogen fixers in these environments (Zielke et al. 2005; Stewart et al. 2011). Moss-associated nitrogen fixation is a major pathway for ´new´ nitrogen in Arctic ecosystems (Rousk, Sorensen, and Michelsen 2017). This is not only an important process in often nitrogen-limited Arctic tundra, but also during primary succession after glacier retreat (Arróniz-Crespo et al. 2014).

Again, most of what we know about moss-associated nitrogen fixation comes from studies on *Sphagnum* and boreal forest mosses, but a wide variety of moss species associates with diazotrophic bacteria, including *Racomitrium spp* (Solheim, Endal, and Vigstad 1996).

Cyanobacteria are thought to be the most important nitrogen fixing bacteria colonizing mosses. The most common cyanobacterial genera are *Nostoc*, *Cylindrospermum* and *Stigonema* (Ininbergs et al. 2011). Apart from Cyanobacteria, it has been suggested that other bacterial taxa could also play a role in moss-associated nitrogen fixation (Opelt et al. 2007; Holland-Moritz et al. 2018; Holland-Moritz et al. 2021), for instance members of the family Methylocystaceae and the genera *Bradyrhizobium*, *Methylibium* and *Acidisoma* (Holland-Moritz et al. 2021) and members of the family Beijerinckiaceae (Carrell et al. 2019).

Moss-associated nitrogen fixation rates are influenced by several biotic and abiotic variables. **Moss species** (Stuart et al. 2020) is among these factors, which may influence nitrogen fixation rates via host-specific microbial associations. **Species-specific traits** such as water retention (Elumeeva et al. 2011), or leaf area and tissue nutrient concentrations (Jonsson et al. 2015a) can also influence nitrogen fixation rates.

Nitrogen fixation rates show **seasonal variations** (Lett and Michelsen 2014; Warshan et al. 2016), which may be driven by changes in nitrogen demand or changes in environmental factors.

Moisture has been identified as the most important factor influencing nitrogen fixation, by directly stimulating the activity of nitrogen fixing bacteria (Rousk, Sorensen, and Michelsen 2018a).

Temperature generally has a positive effect on nitrogen fixation rates (Rousk, Pedersen, et al. 2017). The temperature optimum for nitrogen fixation lies between 19-35 °C (Vitousek et al. 2002) and climate warming in high-latitude ecosystems could thus in theory increase nitrogen fixation rates. Lett and Michelsen (2014) for instance found a strong increase in nitrogen fixation activity after 11 years of warming. However, as mosses and cyanobacteria dry out quickly at higher temperatures (Rousk, Jones, and DeLuca 2014b), higher temperatures can also decrease moisture and inhibit nitrogen fixation (Rousk et al. 2015). This may have led to the small negative and no effect of warming on nitrogen fixation rates associated with *Sphagnum warnstorfii* and *Hylocomium splendens* respectively, after 10 years of warming in a sub-Arctic dwarf shrub heat in northern Sweden (Sorensen and Michelsen 2011). Similarly, no changes and a negative response in nitrogen fixation rates were observed after 21 years of warming in *Hylocomium splendens* and *Aulacomnium turgidum* respectively in an experiment near the previous study (Sorensen, Lett, and Michelsen 2012). This study, however, explains the lack of response to a moderation of the effect of warming at the soil level due to lower light intensity at the moss level as a result of an increase in vegetation height.

As moss-associated nitrogen fixation is often performed by Cyanobacteria, **light** may be a limiting factor for nitrogen fixation. Indeed, at ´normal´ temperatures, light has been shown to have a positive effect on nitrogen fixation, but at high temperatures, nitrogen fixation rates can be negatively affected by increased light, at least in feather mosses (Gundale et al. 2012). Changes in the canopy cover above mosses, such as with shrubification in certain Arctic ecosystems, may thus negatively affect nitrogen fixation rates.

Nitrogen fixation is further limited by the availability of molybdenum, as well as phosphorous (Rousk, Degboe, et al. 2017). And nitrogen deposition can inhibit nitrogen fixation (Ackermann et al. 2012). As shrub expansion will also lead to changes in the quantity and quality of litter (Buckeridge et al. 2010; McLaren et al. 2017), this may change **nutrient** turnover and availability (DeMarco, Mack, and Bret-Harte 2014) and thereby affect nitrogen fixation rates. In the study by Lett and Michelsen (2014) long-term litter addition alone did not affect nitrogen fixation, but in combination with warming nitrogen fixation rates increased. This may be due to warming-induced phosphorous limitation mediated by increased litter abundance. On the other hand, litter addition can also inhibit nitrogen fixation when it increases nitrogen availability (Rousk and Michelsen 2017). The effect of increased shrub abundance on moss-associated nitrogen fixation will thus depend on the litter quality and potential interactions with other environmental factors. Apart from external nitrogen

input, nitrogen fixation is also directly related to moss N content and specific leaf area (Jonsson et al. 2015a).

The above-mentioned factors interfere with each other and the host, making it difficult to disentangle the direct and indirect effects of environmental change on nitrogen fixation rates. In addition to these drivers, moss-associated **microbial community composition** can affect nitrogen fixation rates. This has been suggested in many studies on the effect of environmental change and nitrogen fixation rates, but most of these studies do not assess shifts in the microbial community or specific microorganism responses simultaneously with nitrogen fixation rates. Only Carrell et al. (2019) showed that warming leads to a decrease in diazotroph diversity and that this is associated with a decrease in nitrogen fixation rates in *Sphagnum*. Moss-associated nitrogen fixation rates can be positively linked to cyanobacterial cell abundance (DeLuca et al. 2007), but not all cyanobacterial genera contribute equally to nitrogen fixation (Warshan et al. 2016).

With the state of knowledge presented in the previous sections, it is clear that understanding how bacterial communities associated with mosses and lichens respond to environmental change will improve our understanding of the role of mosses and lichens in ecosystem processes, especially nitrogen fixation. Among the many unknowns are to which extent bacterial communities of mosses and lichens respond to long-term warming in terms of composition, whether nitrogen fixation rates of *R. lanuginosum* are affected by long-term warming or bacterial community composition and how stable moss-associated bacterial communities are during primary succession in a glacier forefield. Iceland, with a vegetation rich in lichens and mosses and with its glaciers retreating due to global climate change, is a suitable location to conduct studies elucidating the extent to which long-term warming and time since deglaciation affect moss- and lichen-associated bacterial communities.

1.5 Research aims and hypotheses

The overall aim of this dissertation was to investigate the effect of climate warming and primary succession on the bacterial communities of common lichen and moss species, as well as moss-associated nitrogen fixation in sub-Arctic ecosystems in Iceland. In **Paper I**, the effect of warming on the bacterial community structure and response of individual bacterial phylotypes of the chlorolichen *Cetraria islandica* in a sub-Arctic heathland are described. The study presented in **Paper II** was conducted in the same warming experiment, but focused on nitrogen fixation and the bacterial communities associated with the moss *Racomitrium lanuginosum.* In **Paper III**, a chronosequence approach was used to determine the degree to which moss-associated and underlying soil bacterial communities, nitrogen fixation and moss traits are affected by time since deglaciation. Further, this study explores whether moss traits correlate with the composition of moss-associated and underlying soil bacterial communities, as well as nitrogen fixation rates. The hypotheses tested in each paper specifically are shown in Table 1.1.

Table 1.1 Hypotheses tested in this dissertation.

Paper I
Warming and the lichen bacterial community
• Long-term warming and the associated increase in tundra shrubs and litter will lead to an increase in heterotrophic, biopolymer-degrading bacterial taxa and a higher incidence of potentially lichenivorous or lichenopathogenic bacteria.
• The potentially metabolically active (16S rRNA based) community shows a larger change in richness, diversity and community structure to the warming treatment than the total bacterial community.
Paper II
Warming and the moss bacterial community
• Long-term warming leads directly and/or indirectly via the warming-induced increase in labile *B. nana* litter to a shift in bacterial community composition and a decrease in bacterial diversity.
• Long-term warming leads to a decrease in oligotrophic taxa and an increase in copiotrophic taxa.
• Changes in N_2-fixation rates will depend on the combination of the direct effect of warming and indirect effects of warming via shading and increased litter, and/or changes in the bacterial community.
Paper III
The moss bacterial community along a primary successional gradient
• Moss shoot traits will change with time since deglaciation with total nitrogen (TN) and total carbon (TC) increasing with time since deglaciation.
• Changes in moss functional traits and time since deglaciation will lead to shifts in moss-associated bacterial communities and the underlying soil bacterial community.
• Moss-associated N_2-fixation rates and *nifH* gene abundance will depend on time since deglaciation, moisture content, total carbon and/or bacterial community composition.

2 Methods

2.1 Experimental design

2.1.1 Open top chambers

To study the effect of long-term warming on lichen- and moss-associated bacterial communities and moss-associated nitrogen fixation **(Paper I** and **II**), we used open top chambers (OTCs) (Figure 6). OTCs are hexagonal plexiglass chambers with an open top (Molau and Mølgaard 1996; Marion et al. 1997) that increased the air temperature on average by 1.4 °C (Supplementary Material Paper I) in the sampling site.

OTCs are low maintenance, cost efficient and easy to replicate devices, which has made them a popular passive warming device in remote tundra locations without direct access to electricity. OTCs also come with potentially unwanted effects. Among these artifacts are reduced and altered incident radiation and changes in moisture (Marion et al. 1997). Relative humidity inside OTCs is often lower than outside due to the increased temperature (Marion et al. 1997). OTCs also trap snow, which may induce higher temperatures at the soil/vegetation level (Bokhorst et al. 2013). Nevertheless, the response of plant species to OTC warming is comparable to the response to natural temperature variations (Hollister and Webber 2000) and they have been used in a large number of studies as an analogue of climate warming.

The site where the OTCs are deployed since 1996/1997, lies above the treeline in the northwest Icelandic highlands and is called Auðkúluheiði (Figure 6). The conditions at this sub-Arctic alpine site resemble low Arctic conditions. The vegetation can be described as a species-rich dwarf shrub heath dominated by *Betula nana* with *Racomitrium lanuginosum* and *Cetraria islandica* as the most common moss and lichen species respectively (Jonsdottir et al. 2005).

Figure 6 Photo of an Open top chamber (OTC) in a dwarf-shrub heath in Auðkúluheiði.

2.1.2 Chronosequence

The concept of time as a driving factor behind succession can be studied by using a chronosequence. A chronosquence can be defined as a set of sites formed from the same parent material or substrate that differs in the time since they were formed (Lawrence R. Walker et al. 2010). Forefields of retreating glaciers are such places where time is substituted by space (Matthews 1992). We used this concept to study the effect of time since deglaciation on moss-associated and belowground bacterial communities as well as nitrogen fixation rates in the glacier forefield of Fláajökull in the southeast lowlands of Iceland (**Paper III**) (Figure 7 and 8).

The Fláajökull glacier is an outlet glacier of Vatnajökull and has been retreating since the end of the Little Ice Age with an average recession rate of ~25 m yr^{-1} between 1894 and 2016 (Wojcik et al. 2020). Mosses of the genus *Racomitrium* are among the dominant colonizing plants and plant cover increases from almost absent to a cover of 25-50% on the oldest moraine (Wojcik et al. 2020). We collected samples of two *Racomitrium* species in the glacier forefield: *R. ericoides* and *R. lanuginosum*.

One of the key assumptions regarding chronosequences is that all sampled sites have started with the same initial conditions and followed the same direction and magnitude of change. Glacier forefields however are often very heterogeneous in terms of topography and geomorphological disturbances. The largest disturbance factors in glacier forefields are glaciofluvial and hillslope activity (Matthews 1992), which can affect the development of ecosystems directly or indirectly by redistributing substrates and water (L. R. Walker 1999). To avoid sampling sites disturbed by glaciofluvial processes, we collected moss and underlying substrate from the top of recessional moraine ridges, following the chronosequence sampling sites described by Wojcik et al. (2020).

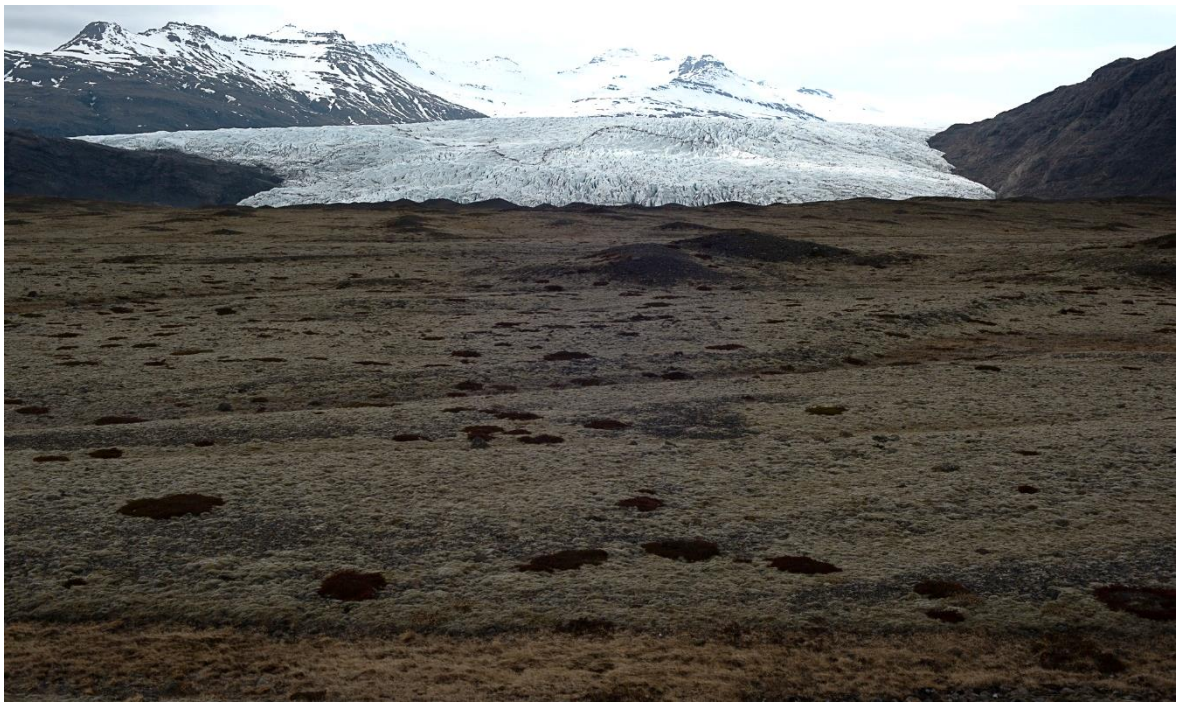

Figure 7 Photo of the glacier Fláajökull and its forefield with vegetation dominated by Racomitrium spp.

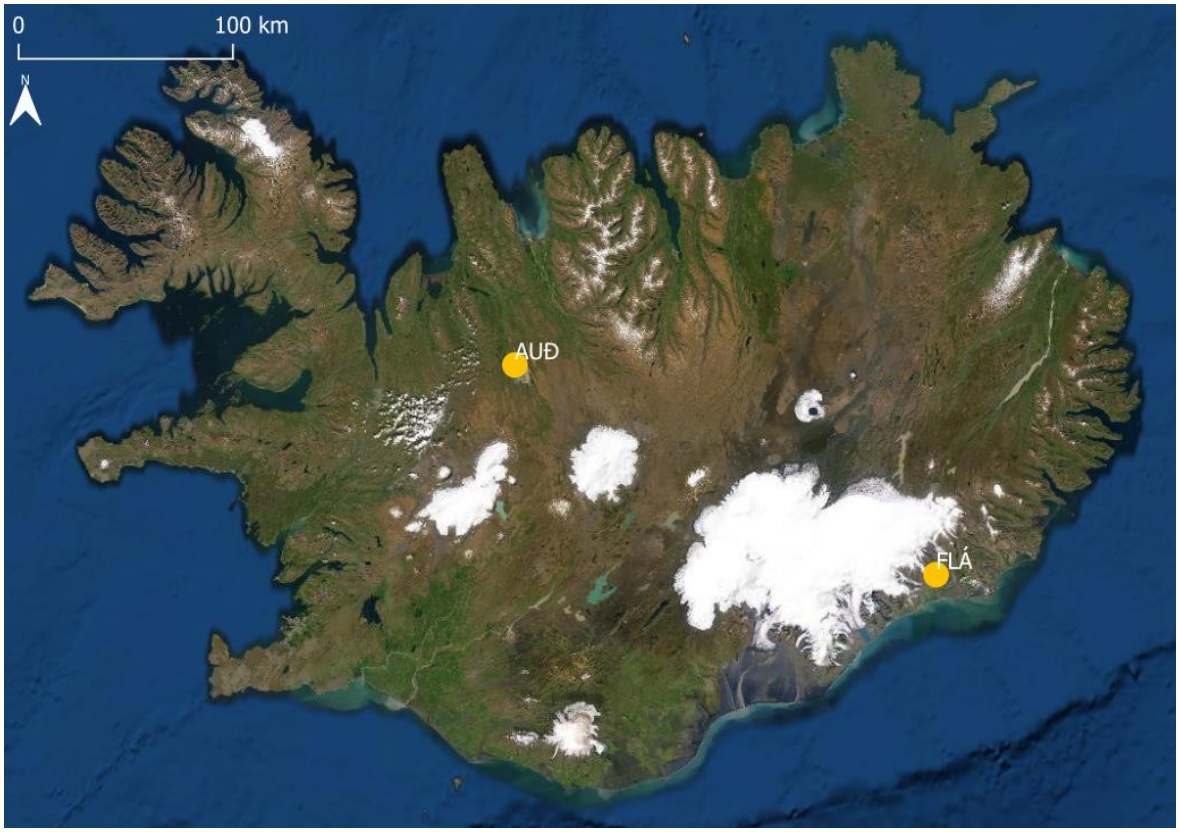

Figure 8 Satellite image of Iceland showing the two sampling locations with a yellow dot. Auðkúluheiði is indicated with AUÐ and Fláajökull with FLÁ. Map created with QGIS and ESRI World Imagery.

2.2 Amplicon sequencing and bioinformatics

While both climate change and bacterial community dynamics take place on scales and time for humans incomprehensible, we have developed methods to study these phenomena. We live in exciting times regarding the study of the microbial world. Until recently, we had to rely on culture-dependent techniques to get an idea of the taxonomic composition and functional potential of a microbial community. Next-generation sequencing techniques have changed this. While many 'omics' approaches are possible, this study used amplicon sequencing of the 16S rRNA gene to describe the bacterial communities of the lichen, the mosses and the soil.

There are biases associated with amplicon sequencing, such as primer biases and sequencing errors (Schöler et al. 2017). The taxonomic assignment of 16S rRNA sequences is dependent on the database coverage and some bacterial taxa have multiple 16S gene copies, making them disproportionally abundant. Nevertheless, amplicon sequencing facilitates the comparison of many samples and the relative changes in abundances of taxa between these samples.

We sequenced both total DNA and complementary DNA (cDNA) derived from extracted RNA. rRNA is often related to growth rate, but this may not be valid for all organisms and some dormant cells contain measurable amounts of rRNA (Setlow and Kornberg 1970). Therefore, we follow Blazewicz et al. (2013) and refer to the cDNA-based bacterial community as potentially metabolically active, or potentially protein synthesizing. Some studies have found that the potentially metabolically active bacterial communities are more sensitive to environmental changes, such as drought (Bastida et al. 2017).

The sequences in all three papers were processed using a pipeline called DADA2 (Callahan et al. 2016). This pipeline uses a machine learning algorithm to learn error rates of the different samples. Additionally, DADA2 infers exact amplicon sequence variants (ASVs) and therefore results in higher-resolution data than when sequences are clustered based on a similarity threshold (resulting in operational taxonomic units or OTUs). To correct for unequal sample sizes, we used cumulative sum scaling (Paulson et al. 2013).

Datasets containing high-throughput sequences come with unresolved problems, such as being compositional and sparse. Many statistical methods do not handle datasets with many zeros well and compositional data often do not meet the requirements for independence between samples. New statistical methods are being developed (Gloor et al. 2017), but it will take time until these have been verified and integrated in common microbial bioinformatics practices.

2.3 Abundance of potential nitrogen fixing bacteria

Quantitative polymerase chain reaction (qPCR) is a highly sensitive technique to quantify the abundance of sequences. We used qPCR to quantify the abundance of *nifH* genes (**Paper I, II and III**) and 16S rRNA genes (**Paper I and II**).

For *nifH* gene quantification, we used the PolF/PolR primer set, as this primer set has a high specificity (Gaby and Buckley 2017). However, as this primer set only covers 25% of *nifH* gene sequences known in public sequence databases (Gaby and Buckley 2012), we may have underestimated the number of *nifH* genes in our samples. To screen primer specificity, we cloned *nifH* amplicons and sequenced them. Amplicons from moss samples were all very similar to Cyanobacterial *nifH* sequences.

2.4 Acetylene reduction assay

We used the acetylene reduction assay (ARA) to estimate nitrogen fixation rates (in **Paper II and III**) (Hardy et al. 1968). The nitrogenase enzyme that breaks the triple bond between two nitrogen atoms in atmospheric nitrogen, also breaks the triple bond between the two carbon atoms in acetylene (C_2H_2) to produce ethylene (C_2H_4). Acetylene and ethylene can be quantified by gas chromatography. As nitrogenase has a different affinity for acetylene than for dinitrogen, a conversion factor is needed to estimate nitrogen fixation rates. This conversion factor is often assumed to lie around 3, but it can range from less than 1 to over 30 (Bellenger et al. 2014). As we did not measure nitrogen incorporation, we don't have a conversion factor for acetylene reduction to nitrogen fixation for *R. lanuginosum* or *R. ericoides* in our studied habitats. Therefore, we simply used acetylene reduction as a proxy for nitrogen fixation.

2.5 Statistical methods

A range of statistical methods were applied to test the significance of the effect of treatment (control versus warmed) and time since deglaciation, as well as other environmental variables on the bacterial community composition, on richness and diversity, on gene abundance and on nitrogen fixation rates.

PERMANOVAs (permutational MANOVAs) (Anderson 2001) were used to test the effect of treatment and environmental variables on the bacterial community compositions (**Paper I, II and III**).

DESeq2 (Love, Huber, and Anders 2014) is a method to identify differentially expressed genes, and we used it to identify differently abundant ASVs between the control and warmed treatment, as well as ASVs changing in relative abundance with soil age (**Paper III**). In addition, we used indicator species analysis (De Caceres and Legendre 2009) to identify ASVs indicative for the control or warmed treatment (**Paper I and II**). We combined these two methods (**Paper I and II**), as DESeq2 only works for ASVs found in both treatments, while indicator species analysis also identifies ASVs only found in one of the two treatments.

To compare the relative abundance of taxa on coarser taxonomic levels between control and warmed plots, Wilcoxon rank-sum tests were used (**Paper I & II**).

Linear models were used to test whether measured environmental variables and moss characteristics changes with time since deglaciation in the glacier forefield (**Paper III**).

To test the effect of the warming experiment on bacterial richness, diversity, 16S rRNA and *nifH* gene abundance and nitrogen fixation rates, we used generalized linear mixed-effect models (glmm) fitted in a Bayesian framework using Markov Chain Monte Carlo (MCMC) sampling (Hadfield 2010). We corrected for multiple sampling from the same OTC and control plots by incorporating a random factor in the models.

Structural Equation Modelling (SEM) (Rosseel 2012) was used to identify direct and indirect linkages between treatment, the bacterial community structure and nitrogen fixation rates in the warming experiment (**Paper II**). This method was also used to detect direct and indirect linkages between time since deglaciation, environmental factors, the bacterial community structure and nitrogen fixation rates in the glacier forefield (**Paper III**).

3 Results and discussion

3.1 *Cetraria islandica* bacterial communities in a warming Arctic

Paper I describes the total and potentially metabolically active bacterial community of the chlorolichen *C. islandica* and its response to 20 years of *in situ* warming in a dwarf-shrub heath in northwest Iceland.

3.1.1 Bacterial community composition

The total and potentially metabolically active bacterial community composition of the lichen *C. islandica* was dominated by the orders Acetobacterales (of the class Alphaproteobacteria) and the Acidobacterales (of the phylum Acidobacteria). The most abundant genera were the proteobacterial genera *Acidiphilium* and *Endobacter* and the acidobacterial genera *Bryocella* and *Granulicella*. The dominance of Alphaprotebacteria has been described as a general characteristic of lichen microbiota (Cardinale et al. 2008; Printzen et al. 2012), but the dominance of Acetobacteriaceae in *C. islandica* is remarkable as the dominant Alphaprotebacteria in lichens are often Rhizobiaceae (Hodkinson et al. 2012). Acidobacteria have also been described from lichens before (Pankratov 2012; Muggia et al. 2013). The presence of these acidophilic taxa may be associated with organic acid secondary metabolites produced by *C. islandica* (Xu et al. 2018).

We also found that 295 ASVs were only present in the potentially active bacterial community (cDNA), but not in the total community. These ASVs belonged to the most abundant genera, such as *Endobacter* and *Acidiphilium*, although some genera were exclusively detected in the potentially active bacterial community, for instance *Rhizobacter*, *Ktedonobacter*, *Telmatobacter* and *Acidiphilium*. Some studies call these rare taxa "phantom taxa" and they could be a result of cDNA synthesis errors. However, there are also indications that a rare fraction of the microbiome is disproportionally active and contributes more to ecosystem functioning than one would expected based on their abundance (Campbell et al. 2011; Baldrian et al. 2012; Klein et al. 2016; Jia et al. 2019).

3.1.2 Long-term warming induced changes in the bacterial community

Treatment (warming versus control) did not affect 16S rRNA gene copy numbers nor *nifH* gene copy numbers. However, *Betula nana* abundance positively influenced both *nifH* and 16S rRNA gene abundance, whereas litter abundance negatively affected *nifH* gene abundance. The presence of *nifH* gene copies suggests the presence of potential nitrogen fixing bacterial taxa and the sequencing data indeed confirms the presence of putative nitrogen fixing taxa such as *Curvibacter* and members of the Burkholderiaceae. Chlorolichens can show significant nitrogenase activity (Torres-Cruz et al. 2018), but the

nitrogen-fixing capacity of *C. islandica* is unknown and calls for more studies to elucidate the role of chlorolichens in nitrogen fixation in tundra ecosystems.

Treatment (warming versus control) explained 7% of the variation in both the total and the potentially active bacterial community. In addition, litter abundance affected the potentially active bacterial community structure, and a positive effect of *B. nana* abundance was found on the richness and diversity of the potentially active bacterial community. This indicates that long-term warming may indirectly influence lichen-associated bacterial communities via changes in the vegetation structure and litter quantity (and potentially quality), especially the potentially active bacterial community. Another indication that the potentially active community was more strongly affected by warming than the total bacterial community, was the higher number of potentially active indicator species. While to our knowledge, no other studies have investigated the potentially active bacterial community of lichens, potentially active soil bacterial communities seem also more sensitive to changes in soil properties (Herzog et al. 2015).

Shifts in the relative abundances of taxa occurred mainly at lower taxonomic levels (class, order and ASV). On ASV level in the total bacterial communities, phylotypes of the acidobacterial genera *Granulicella* and *Bryocella* and the alphaproteobacterial genus *Acidiphilium* were proportionally less abundant in the warmed treatment than in the control treatment. Both genera are chemoorganotrophic or chemolithotrophic and *Granulicella* includes acidophilic, cold-adapted species (Männistö et al. 2013) with hydrolytic properties (Pankratov and Dedysh 2010), which suggests that they are involved in the degradation of senescing lichen thalli. While some ASVs of the genus *Granulicella* decreased in relative abundance, the increased potential activity might result in increased degradation of dead lichen material. In contrast, the decreased relative abundance and potential activity of *Bryocella* and *Acidiphilium* might result in slower degradation of dead lichen material.

The alphaproteobacterial genera *Acidisphaera*, *Sphingomonas*, and *Endobacter* showed an increased relative abundance and potential activity with warming. *Endobacter* is a poorly described genus, of which only one species has been described (Ramirez-Bahena et al. 2013). *Acidisphaera* is a bacteriochlorophyll containing chemoorganotroph (A Hiraishi et al. 2000) and *Sphingomonas* is known to degrade plant biomass, utilize recalcitrant matter in oligotrophic environments as well as sulfonated compounds as sources of carbon and sulfur (Aylward et al. 2013). The increase in relative abundance and potential activity of these taxa may lead to increased carbon and nutrient availability in the lichen thalli.

3.2 *Racomitrium lanuginosum* bacterial communities and nitrogen fixation in a warming Arctic

Paper II focused on the effect of long-term *in situ* warming on the total and potentially active bacterial communities associated with the moss *R. lanuginosum*. This study was performed at the same site as in Paper I. Here, we also included *nifH* gene abundance and nitrogen fixation rate measurements and linked these to changes in the vegetation structure, litter abundance and the bacterial communities.

3.2.1 Shifts in the bacterial community structure with OTC treatment

A small part of the variation in both the total and the potentially active bacterial communities associated with *R. lanuginosum* was due to the treatment (warming versus control). However, we found that litter and *Betula nana* abundance also affected the bacterial community structure. In fact, the indirect effect of warming via *Betula nana* abundance was stronger than the direct effect of warming on the bacterial community structure. This suggests that the effect of long-term warming on moss-associated bacterial communities is mainly mediated through changes in the plant community. This is in agreement with the finding that changed leaf litter inputs alter moss-associated bacterial community composition (Jean et al. 2020a) and the idea that climate change effects on soil organisms are largely plant-mediated (Deslippe et al. 2012), even though we are looking at moss-associated bacterial communities here.

3.2.2 Genera shifting in relative abundance with OTC treatment

We found lower relative abundances in the warmed plots of taxa such as Acidobacteria (and more specific ASVs of the genera *Granulicella*, *Solibacter*, *Bryocella*, *Bryobacter* and *Acidipila*) and the Alphaproteobacterial genus *Acidiphilium*. These are bacterial taxa that are generally classified as oligotrophic (adapted to low substrate concentrations, but more efficient substrate users and relatively slow growers) (Dedysh and Sinninghe Damsté 2018; Fierer, Bradford, and Jackson 2007; Hiraishi and Imhoff 2015). On the other hand, we found higher relative abundance of potential copiotrophic taxa (taxa with higher growth rates, a broader range of substrate affinities and more responsive to carbon availability) in the warmed plots, such as members of the proteobacterial genera *Rhizobacter*, *Nitrobacter* and *Rhizobium*.

Acidobacteria often dominate tundra soils, in particular soils with high concentrations of phenolic compounds such as *Sphagnum* peat and *Empetrum* heath (Gallet, Nilsson, and Zackrisson 1999; Männistö et al. 2013). Proteobacteria dominate the soil bacterial community in shrub tundra dominated by *Betula nana* and *Salix* species (Wallenstein, McMahon, and Schimel 2007). The decrease in Acidobacteria and increase in copiotrophic Proteobacteria could thus be due to an increase in labile *Betula nana* litter which may increases carbon flux rates (Parker et al. 2018), potentially due to shifts towards more copiotrophic bacterial taxa, at least in the moss layer. These changes again suggest that long-term warming indirectly affects moss-associated bacterial community composition via changes in the plant community.

3.2.3 Effect of long-term warming on nitrogen fixation

We did not detect differences in nitrogen fixation rates, nor *nifH* gene abundance between the control and the warmed treatment. Nevertheless, we did find indications for shifts in nitrogen fixing taxa. Cyanobacteria for instance were proportionally less abundant in the warmed treatment. On the other hand, the majority of ASVs that increased in relative abundance belong to taxonomic groups capable of nitrogen fixation (*Sphingomonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Rhizobacter*). This shift in nitrogen fixing taxa might lead to some degree of functional redundancy and could explain why nitrogen fixation rates were not directly affected by the warming treatment. We did

however detect a negative effect of litter abundance on *nifH* gene abundance and a negative effect of *Betula nana* abundance on nitrogen fixation rates. These results are partly in agreement with Jean et al. (2020a), who found a negative effect of leaf litter addition and canopy cover on nitrogen fixation rates and Nostocaceae abundance of the moss *Pleurozium schreberi*. Thus changes in the vegetation structure may affect nitrogen-fixing bacterial abundance (possibly via nutrients leaching from litter or increased shading) and nitrogen fixation rates (potentially via shading).

3.3 *Racomitrium* moss bacterial communities and nitrogen fixation during primary succession

In paper III, the bacterial communities of the mosses *R. lanuginosum* and *R. ericoides* and the underlying substrate were studied along a chronosequence in the Fláajökull glacier forefield in the south-east lowlands. In addition, moss traits such as C and N content were measured as these could act as drivers for the moss-associated bacterial community composition. And lastly, this paper also describes the potential links between time since deglaciation, moss traits, bacterial community composition, *nifH* gene abundance and nitrogen fixation rates.

3.3.1 Moss traits

We found little statistical evidence for changes in moss traits with time since deglaciation. Only moss shoot length significantly increased with time since deglaciation. Nevertheless, shoot TC and C:N ratio also showed an increasing trend, at least in the younger soils. This is in agreement with the general increase in TC in soils in glacier forefields with succession (Wojcik et al. 2020; Schulz et al. 2013). Shoot TN however did not show any trends with time since deglaciation, similar to TN in *R. lanuginosum* on lava flows of different ages in Iceland (Cutler 2011). Other studies have shown that denitrification increases in soils as plant cover develops in glacier forefields (Brankatschk et al. 2011). This may also explain the lack of nitrogen accumulation in moss shoots (including the decomposing part), in addition to potential leaching of nitrogen.

3.3.2 Moss and soil bacterial community composition

The bacterial communities of both the mosses and the soil were affected by time since deglaciation. The effect of time since deglaciation was stronger on the moss-associated bacterial communities, potentially due to a shift in moss species along the chronosequence, as mosses appear to have species-specific bacterial communities (Holland-Moritz et al. 2021).

Moss moisture content also contributed significantly to variation in the moss-associated bacterial communities. As moisture is an important driver of microbial decomposition (Schimel et al. 1999), it may also impact bacterial community structure, especially in the decomposing parts of the moss shoots. Moisture content has also been identified as a driver for moss-associated fungal communities (Hirose et al. 2016). Additionally, microbial

organisms and plants are tightly coupled to water availability in glacier forefields (Zumsteg et al. 2013).

The soil bacterial community was, in addition to time since deglaciation, also affected by moss C:N ratio. Moss nutrients may leach from the moss shoots during drying-wetting cycles and thereby affect the underlying soil bacterial community and similarly, plant traits such as leaf N have been shown to influence soil bacterial community composition (Vries et al. 2012). This illustrates how moss establishment in glacier forefields can in turn influence the bacterial communities of the underlying soil by provision of resources.

On the phylum level, the moss-associated bacterial community exhibited more changes with time since deglaciation than the soil bacterial community. We found that Chloroflexi increase in proportion (both in the moss and in the soil) and that Proteobacteria, Cyanobacteria and Bacteroidetes decreased in proportion in the moss. The increase in Chloroflexi has been found before in the soil and rhizosphere of *Saxifraga oppositifolia* in a high-Arctic glacier forefield (Mapelli et al. 2018), while Proteobacteria, Cyanobacteria and Bacteroidetes are often becoming less abundant with succession in glacier forefield soils (Bajerski and Wagner 2013; Jiang et al. 2018; Fernández-Martínez et al. 2017; Bradley et al. 2016). Interestingly we found the same patterns in the moss, but not in the soil.

On the ASV level however, we found most changes with time since deglaciation in the soil. All ASVs changing in relative abundance, showed a proportional increase with time since deglaciation. Many of them were classified as genera known to be able to degrade plant-organic matter, such as Ca. *Solibacter* (Ward et al. 2009), *Nocardioides* (Guo et al. 2021), Chitinophagaceae (Yong Li et al. 2011) and Micropepsaceae (Harbison et al. 2016), indicating that increased moss abundance with succession also increases the potential for degradation of dead moss material in the soil. While Cyanobacteria decreased in relative abundance with time since deglaciation in the moss, heterotrophic nitrogen fixers became more abundant with time since deglaciation (for instance *Devosia* (Rivas et al. 2002), Rhizobiaceae (Dobbelaere, Vanderleyden, and Okon 2003), *Methylocapsa* (Dedysh et al. 2002) and Rhodoplanes (Buckley et al. 2007) in soil, and Acetobacteriaceae (Saravanan et al. 2008) in the mosses), probably because of increased substrate availability. An increase in potential denitrifiers (Ca. *Solibacter* (Ward et al. 2009) and *Rhodanobacter* (Kostka et al. 2012)) suggests an increase in nitrates and/or nitrites with succession and loss of fixed nitrogen via denitrification with succession, at least in the soil.

3.3.3 Nitrogen fixation

Similar to moss shoot TN, nitrogen fixation rates did not show any clear trend with time since deglaciation. As moss cover increases with time since deglaciation (Wojcik et al. 2020), nitrogen fixation per area may also increase. *nifH* gene abundance however, decreased with time since deglaciation, indicating a decreasing abundance of nitrogen fixing bacterial taxa with succession.

Moss-associated nitrogen-fixation rates were not affected by moss nitrogen content, time since deglaciation or moisture content, but rather by the abundance of diazotrophs and bacterial community composition, at least in *R. lanuginosum*. The abundance of diazotrophs was negatively linked to nitrogen fixation rates, which suggests that not all potential nitrogen fixing taxa are actively fixing nitrogen, or that nitrogen fixation depends on the diazotrophic community composition. We detected for example a decrease in the relative abundance of

Cyanobacteria in the mosses with soil age and an increase in an ASV of the Acetobacteraceae, which contain nitrogen fixing members (Saravanan et al. 2008). Additionally, shifts in *nifH* gene diversity with succession occur in soil in glacier forefields (Duc et al. 2009) and our study suggests that these shifts may also take place in mosses.

4 Conclusions

Moss and lichen-associated bacterial communities are diverse and species-specific, and this dissertation places the bacterial communities in the context of climate change by exploring their response to 20 years of warming (**Paper I and II**) and changes in bacterial community structure with time since deglaciation after glacier retreat (**Paper III**). In addition to community structural changes and shifts in individual taxa, this dissertation also focuses on moss-associated nitrogen fixation, an important trait mediated by their associated bacterial communities (**Paper II and III**).

Both the bacterial communities of the lichen *Cetraria islandica* and the moss *Racomitrium lanuginosum* were affected by 20 years of *in situ* warming with OTCs in a *Betula nana* dwarf-shrub heath (**Paper I** and **II**). Both of these bacterial communities were also indirectly affected by long-term warming via increases in *Betula nana* abundance and litter abundance. This indicates that lichen and moss-associated bacterial communities are not only directly affected by warming, but also via warming-induced changes in the vegetation structure.

The bacterial community of the lichen *C. islandica* was dominated by acidophilic taxa and harbored rare, but potentially active taxa (**Paper I**). Warming-induced changes in the microbiota were mainly detected at lower taxonomic levels, particularly in ASVs of genera potentially involved in the degradation of the lichen thallus where shifts in proportional abundance were observed, although the direction of change differed between ASVs.

Warming-induced changes in the moss bacteriota (**Paper II**) were detectable on the phylum, class, order and ASV level. Many of the taxa decreased in relative abundance with warming, for instance members of the Acidobacteria, while others such as members of the genus *Rhizobacter* increased in relative abundance.

In addition to the response of the bacterial communities of *R. lanuginosum* to long-term warming, **Paper II** discusses the effect of warming on moss-associated nitrogen fixation. Our results showed no direct response of nitrogen-fixation rates and *nifH* gene abundance to warming. Nevertheless, nitrogen-fixation rates and *nifH* gene abundance were affected negatively by *B. nana* and litter abundance respectively. Additionally, long-term warming led to changes in abundances of potential nitrogen fixing taxa. On the ASV level, these changes were characterized by a decrease in the relative abundance of Cyanobacteria and an increase in abundance and potential metabolic activity of non-cyanobacterial diazotrophs, which may explain the lack of response of nitrogen-fixation rates to warming.

Paper III describes the development of *Racomitrium* moss bacterial communities as well as those of the underlying substrate with reference to moss functional traits along a chronosequence in the glacier forefield of Fláajökull in southeast Iceland. While moss functional traits such as TN and moisture content did not show clear trends along the chronosequence, moss shoot length increased with time since deglaciation. Time since deglaciation as well as moss C:N ratio and moss moisture content were related to moss bacterial community structure, showing for the first time how moss functional traits are important drivers for moss-associated bacterial communities. The bacterial communities of

the underlying soil were also affected by time since deglaciation and by the moss C:N ratio, highlighting the influence of moss cover on soil development. Moss and underlying soil bacterial communities differed strongly from each other, suggesting that little transfer between them takes place.

Paper III also describes the potential drivers of moss-associated nitrogen-fixation rates in the glacier forefield. Nitrogen-fixation rates were not affected by time since deglaciation or moss TN, but nitrogen-fixation rates were linked to bacterial community structure and negatively linked to *nifH* gene abundance. This may indicate a shift in diazotrophic taxa with different N_2-fixing efficiencies along the chronosequence and our data indeed show a proportional decrease in Cyanobacteria and an increase in heterotrophic N_2-fixing taxa.

5 Future outlook

While the studies in this dissertation have provided indications that the bacterial communities of mosses and lichens are changing with long-term warming, partly through indirect effects of warming via changes in the vegetation and litter abundance, many questions remain unresolved. For instance, the exact mechanisms as to why certain taxa are shifting and the mechanisms behind these shifts are unclear. There are many more ways in which warming can indirectly affect the bacterial communities of mosses and lichens. One such way may be via warming-induced changes in moss and lichen secondary metabolites.

Another way in which warming could affect the bacterial communities of mosses and lichens are trophic cascades. Warmer temperatures may affect the abundance of microfauna and thereby affect bacterial abundance. This top-down control on the bacterial community and nitrogen fixation via changes in foodweb has been shown for Cyanobacteria on the boreal feathermoss *Pleurozium schreberi* (Kardol et al. 2016), and it might well play a role in *Racomitrium* mosses or lichens.

In Paper I and II, we detected indirect effects of long-term warming via changes in the plant community. To gain a greater insight of the direct effects of warming on moss-associated bacterial communities, another long-term warming experiment in Iceland in Þingvellir could be used. The OTCs are located in a moss heath dominated by *R. lanuginosum*. Here no clear changes in the vascular plant community have taken place (Jonsdottir et al. 2005). Therefore, this experiment would enable a study of the effect of warming on the nitrogen-fixation rates and the bacterial community composition (as well as moss functional traits) without the indirect effects of warming via changes in the plant community.

In Paper I and II we only measured the effect of long-term warming after 20 years. This leaves it unclear what the short-term effects of warming are on lichen- and moss-associated bacterial communities. In future warming experiments, multiple sampling points in time could reveal the temporal changes in the bacterial communities.

Additionally, in Paper II, we detected a shift towards a more copiotrophic bacterial community associated with *R. lanuginosum* after long-term warming. Copiotrophic bacterial communities could be less resistant but more resilient to climate extremes than oligotrophs (De Vries and Shade 2013). This offers the opportunity to study how long-term warming affects the resistance and resilience of moss-associated bacterial communities to for instance drought. Do they recover quickly (in terms of composition and activity) and what happens after multiple cycles of high temperatures and/or drought? This could then affect nitrogen fixation rates for instance. This could be tested by collecting mosses from long-term warmed and control plots and subjecting these to drought treatment(s) and analyzing the changes in bacterial communities before, during and after the treatment.

In Paper I, we detected the presence of *nifH* genes in *C. islandica*. Chlorolichens indeed have the potential to contribute to nitrogen fixation in, for instance, biocrusts (Torres-Cruz et al. 2018) and they should therefore not be ignored in nitrogen fixation estimates. Follow-up

studies could investigate the potential nitrogen fixers of *C. islandica* in more detail, as well as measure its capacity for nitrogen fixation by ^{15}N incorporation or ARAs.

The degree to which changes in abundances of diazotrophic bacterial taxa lead to changes in moss-associated nitrogen fixation rates would be worthwhile to investigate. Certain taxa may be "free-riders" and not contribute much to nitrogen fixation, while others do the job (Warshan et al. 2016). In addition, the contribution of non-cyanobacterial nitrogen fixers to nitrogen fixation has been suggested and in Paper II we have also detected potential non-cyanobacterial diazotrophs associated with *R. lanuginosum*. Other studies have also indicated links between nitrogen fixation rates and non-cyanobacterial diazotrophs (Holland-Moritz et al. 2021). Are they actually fixing nitrogen and if so, how does their activity compare to that of Cyanobacteria? Are they active in other parts of the moss shoot, or in other seasons, for instance under snow cover? Considering that moss-associated nitrogen fixation is important in many ecosystems, this warrants further research.

Moss-associated fungi and bacteria are specific to the photosynthetic, non-photosynthetic and decaying part of moss shoots (Chen et al. 2019). We do not know if the changes we found in Paper II are consistent along the senescence gradient of *R. lanuginosum* or whether long-term warming only affects bacterial communities in certain parts of the moss. In future studies, moss samples collected from long-term warming experiments could be divided into the photosynthetic, senescent and decomposing parts and analyzed separately.

In Paper I, II and III, we have focused on the taxonomic composition of the bacterial communities of mosses and lichens using 16S rRNA gene amplicon sequencing, giving an idea of 'who is there/active'. But ultimately, for ecosystem functioning, the question is whether the functions of the microbial communities are affected by warming. Whether that is related to community composition is the question. Metagenomics is more informative regarding the potential functional changes of these bacterial communities and may be applied in future experiments regarding the effect of environmental change on moss-associated bacterial (and fungal) communities. In addition, metatranscriptomics could be used to infer changes in actual functions of moss- and lichen-associated bacterial communities with environmental change.

In Paper III no evidence was found for nitrogen accumulation in mosses during succession. Further studies investigating the potential reasons for this could elucidate the fate of nitrogen in mosses in glacier forefields. Is nitrogen lost via leaching or denitrification? ^{15}N incubations to quantify nitrogen fixation rates as well as tracer studies could be used to study whether and how long fixed nitrogen is stored in the moss tissue or whether it leaches to other ecosystem pools.

Many unknowns regarding moss- and lichen-associated bacterial communities and the extent to which they are affected by environmental change exist, especially with regard to functional changes and links between community composition, activity and nitrogen fixation.

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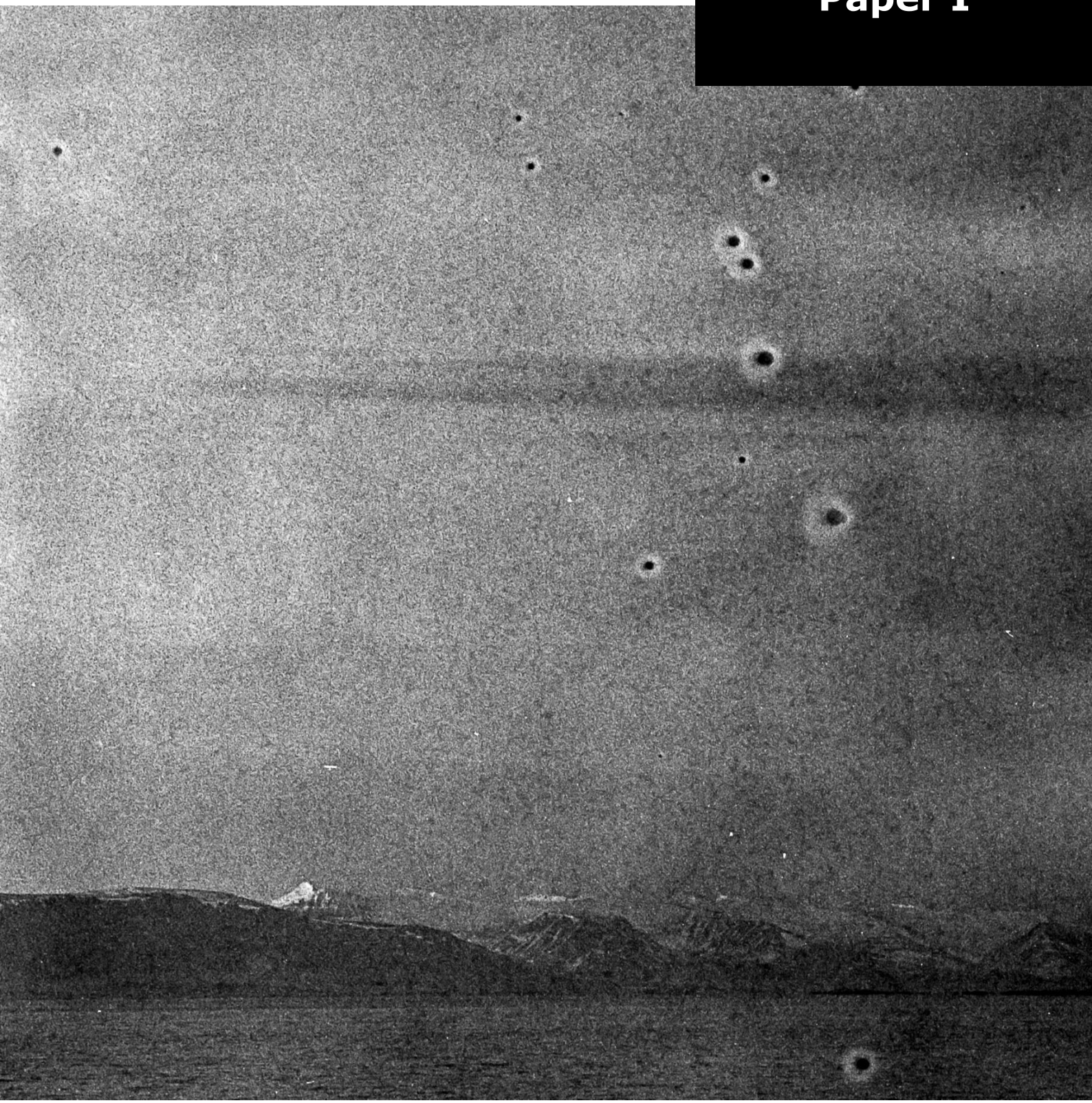
The photographs between the papers are work I made during my PhD. I took these photos in Iceland on black-and-white film and to develop them, I experimented with a developer based on washing soda, vitamin C, salt and the moss *Racomitrium lanuginosum*. This idea stems from others working with alternative developers, such as caffenol and seaweed developers. While hiking I always kept my eyes open for a nice patch of moss to use for my developer. One of the most interesting collection sites was by Fagradalshraun, just in front of a lava flow that would otherwise have destroyed the moss. Without the moss, the images would not have been visible. This for me represents another way of looking through the lens of the moss.

Paper I



frontiers in Microbiology

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The Total and Active Bacterial Community of the Chlorolichen *Cetraria islandica* and Its Response to Long-Term Warming in Sub-Arctic Tundra

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Lichens are traditionally defined as a symbiosis between a fungus and a green alga and or a cyanobacterium. This idea has been challenged by the discovery of bacterial communities inhabiting the lichen thalli. These bacteria are thought to contribute to the survival of lichens under extreme and changing environmental conditions. How these changing environmental conditions affect the lichen-associated bacterial community composition remains unclear. We describe the total (rDNA-based) and potentially metabolically active (rRNA-based) bacterial community of the lichen *Cetaria islandica* and its response to long-term warming using a 20-year warming experiment in an Icelandic sub-Arctic tundra. 16S rRNA and rDNA amplicon sequencing showed that the orders Acetobacterales (of the class Alphaproteobacteria) and Acidobacteriales (of the phylum Acidobacteria) dominated the bacterial community. Numerous amplicon sequence variants (ASVs) could only be detected in the potentially active community but not in the total community. Long-term warming led to increases in relative abundance of bacterial taxa on class, order and ASV level. Warming altered the relative abundance of ASVs of the most common bacterial genera, such as *Granulicella* and *Endobacter*. The potentially metabolically active bacterial community was also more responsive to warming than the total community. Our results suggest that the bacterial community of the lichen *C. islandica* is dominated by acidophilic taxa and harbors disproportionally active rare taxa. We also show for the first time that climate warming can lead to shifts in lichen-associated bacterial community composition.

Keywords: lichen, lichen microbiome, tundra, climate change, host–microbiome, lichen-associated bacteria, long-term warming

INTRODUCTION

The notion that lichens harbor diverse bacterial and fungal communities has challenged the traditional view of the lichen as a symbiosis between a fungus (mycobiont) and an alga and/or a cyanobacterium (photobiont) (González et al., 2005; Spribille et al., 2016). Nonetheless, the first lichen-associated bacteria were already discovered in the 1920s (Uphof, 1925). To date, bacterial communities of a wide-range of lichen species have been revealed by molecular approaches (Cardinale et al., 2006, 2008; Grube et al., 2009; Hodkinson and Lutzoni, 2009; Bjelland et al., 2011; Mushegian et al., 2011; Weiss et al., 2011; Sigurbjörnsdóttir et al., 2015; Park et al., 2016). Alphaproteobacteria usually dominate the lichen microbiome, but other taxa such as Actinobacteria, Firmicutes, Acidobacteria, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria are also found. These bacteria can form highly structured, biofilm-like assemblages on fungal surfaces and within the lichen thallus (Grube et al., 2009). The bacterial communities inhabiting the lichen thalli play important roles in the lichen holobiont (the lichen and its microbiome), by contributing to nutrient supply, resistance against biotic and abiotic stresses, production of vitamins and support of fungal and algal growth by the production of hormones, detoxification of metabolites and degradation of senescing parts of the lichen thallus (Grube et al., 2015; Sigurbjörnsdóttir et al., 2015). Thereby, the bacterial part of the lichen holobiont is suggested to contribute to the survival of lichens under extreme and changing environmental conditions.

The composition of associated bacterial communities of lichens may be shaped by intrinsic and extrinsic factors. Among intrinsic factors affecting the lichen microbiome are thallus age (Cardinale et al., 2012b), mycobiont species, and photobiont species (Grube et al., 2009; Weiss et al., 2011; Hodkinson et al., 2012; Wedin et al., 2016; Coleine et al., 2019). The composition of lichen bacterial communities can also be influenced by extrinsic factors such as sunlight exposure and substrate type (Cardinale et al., 2012b; Park et al., 2016), geography and local habitat (Cardinale et al., 2012a; Hodkinson et al., 2012; Printzen et al., 2012; West et al., 2018), altitude (Coleine et al., 2019), drought (Cernava et al., 2019), and arsenic contamination (Cernava et al., 2018). Some lichens can adapt to changing environmental factors by switching photobionts depending on the ecological niche of the photobiont (Domaschke et al., 2013; Rolshausen et al., 2018). Some lichens have also been shown to be able to acclimate to higher temperature by increasing their respiration (Lange and Green, 2005) or net photosynthesis (Colesie et al., 2018). However, not all lichens are able to adapt to changing environments in these ways. Lichens might also be able to acclimate through changes in their associated bacterial communities. This strategy has been demonstrated for several environmental factors, such as drought (Cernava et al., 2019) and arsenic contamination (Cernava et al., 2018). Substrate type is another extrinsic factor that can influence the composition of bacterial communities (Cardinale et al., 2012b). Therefore, changes in C (carbon) or N (nitrogen) availability in the environment, for instance as a result of changes in plant litter quality due to shrubification (McLaren et al., 2017), might be factors altering the structure of the lichen microbiome. However, little is known about the effect of long-term environmental changes on the bacterial communities associated with lichens.

High-latitudes are especially rich in lichen species and biomass (Cornelissen et al., 2007; Nash, 2008), where they make significant contributions to ecosystem functioning (Asplund and Wardle, 2017). Mat-forming lichens such as Cetraroid species contribute to primary production and nutrient cycling, control soil chemistry and water retention (Cornelissen et al., 2007). Currently, climate in high-latitudes warms twice as fast as elsewhere (IPCC, 2019) resulting in increased abundance of shrubs, particularly in the low and sub-Arctic (Elmendorf et al., 2012; Myers-Smith et al., 2019). Direct effects of warming on lichens include changes in C-based secondary compounds (Asplund et al., 2017) and increased biomass (Biasi et al., 2008). Warming also has indirect effects on lichens. In many low and sub-Arctic tundra ecosystems shrubification results in increased shading and greater amounts of litter, which can lead to decreased lichen photosynthesis rates causing a decline in lichen biomass (Nash and Olafsen, 1995; Cornelissen et al., 2001; Elmendorf et al., 2012; Fraser et al., 2014; Alatalo et al., 2017). Yet, the effect of long-term warming on the bacterial communities of lichens in high-latitudes needs to be investigated.

In this study we investigate the total and potentially metabolically active bacterial community of the lichen *Cetraria islandica* (L.) Ach. (English "Iceland moss") and its response to two decades of warming in open top chambers (OTCs) in an Icelandic sub-Arctic alpine dwarf-shrub heath. *C. islandica* is a mat-forming chlorolichen with foliose thalli and forms a major component of the vegetation in Arctic, sub-Arctic and alpine environments throughout the northern hemisphere (Kärnefelt et al., 1993). 16S rRNA and rDNA sequencing was used to characterize the potentially active and total bacterial community in control plots and OTCs. We also quantified 16S rRNA gene abundance by quantitative PCR to compare the absolute abundance of bacteria in the controls and OTCs. Finally, it was recently demonstrated that N_2-fixing bacteria could associate with chlorolichens (Almendras et al., 2018). Thus, we also quantified the number of *nifH* genes by quantitative PCR in order to test if the *C. islandica* microbiome could potentially perform N_2-fixation and how warming influences the abundance of associated N_2-fixers.

We predicted that long-term warming and the associated increase in tundra shrubs and litter will lead to an increase in heterotrophic, biopolymer-degrading bacterial taxa and a higher incidence of potentially lichenivorous or lichenopathogenic bacteria, such as shown for the plant phyllosphere (Aydogan et al., 2018). Thus, in terms of taxonomic composition, we expected an increase in detritivorous taxa, endosymbionts and pathogens of fungi such as chitinolytic bacteria (Kobayashi and Crouch, 2009), whereas the relative abundance of cold-adapted and facultatively lithotrophic bacteria may decrease.

We also hypothesized that the potentially metabolically active (16S rRNA based) community shows a larger change in richness, diversity and community structure to the warming treatment

than the total bacterial community, as has been shown for the effect of drought (Bastida et al., 2017).

MATERIALS AND METHODS

Study Site and Experimental Design

The study site is located in a *Betula nana* heath in the Icelandic central highlands at an altitude of 450 m. According to Köppen's climate definitions, the sampling site, called Auðkúluheiði (65°16′N, 20°15′W, 480 m above sea level) is situated in the lower Arctic. The vegetation is characterized as a relatively species-rich dwarf shrub heath, with *B. nana* being the most dominant vascular species and the moss *Racomitrium lanuginosum* and the lichen *C. islandica* as the dominating cryptogam species (Jonsdottir et al., 2005).

Ten OTCs were set up to simulate a warmer summer climate in August 1996 in a fenced area to exclude sheep grazing (Hollister and Webber, 2000; Jonsdottir et al., 2005). The OTCs raise the mean daily temperature by 1–2°C during summer and minimize secondary experimental effects such as differences in atmospheric gas concentration and reduction in ambient precipitation. Control plots were established adjacent to the OTCs, but without any treatment, thus exposing the environment to ambient temperatures. Air temperatures measured in the growing season from 1999 to 2002 at the surface of the cryptogam layer indicated an average increase of 0.7–1.0°C in the OTCs (Jonsdottir et al., 2005). Air temperature measured 10 cm above the moss layer in summer 2016 was on average 1.4°C higher in the OTC than in the control plot of one of the plot pairs ($t = -8.2$, $P < 0.001$) (**Supplementary Table 1**). Relative humidity measured in the same plot pair and period was 3% lower in the OTC than in the control plot ($t = 26.9$, $P < 0.001$) (**Supplementary Table 1**). Temperatures on the moss surface measured in all OTCs and control plots from mid-August 2018 to mid-June 2019 were on average 0.22°C higher in the OTCs compared to the control plots ($t = -16.4$, $P < 0.001$) (**Supplementary Table 1**).

The response of the vegetation was monitored by a detailed vegetation analysis after peak biomass at a few year intervals using the point intercept method following standard protocols of the International Tundra Experiment (ITEX: 100 points per plot, all hits (intercepts) per species recorded in each point through the canopy; relates to biomass) (Jonsdottir et al., 2005). In 2014, the abundance of *B. nana* was on average 2.5 times larger in the OTCs than in the control plots and litter was 2.7 times more abundant in the OTCs than in the control plots (Jónsdóttir, unpublished data). In this study we use these data on abundance (total number of hits per plot) of *B. nana* and litter to test the effect of vegetation change on the richness, diversity, community structure of the lichen associated bacterial community as well 16S rRNA and *nifH* gene abundance.

Per warmed (OTC) and control plot, the upper parts (2 × 2 cm) of five lichen thalli were randomly selected and collected with sterile tweezers. The samples were immediately soaked in RNAlater (Ambion) to prevent RNA degradation and kept cool until storage at −80°C. The lichen samples were collected in June 2017.

RNA and DNA Extraction and Sequencing

Prior to RNA and DNA extraction, the samples were washed with RNase free water to get rid of soil particles and RNAlater and ground for six minutes using a Mini-Beadbeater and two sterile steel beads. RNA and DNA were extracted simultaneously using the RNeasy PowerSoil Total RNA Kit (Qiagen) and the RNeasy PowerSoil DNA Elution Kit (Qiagen), following the manufacturer's instructions. DNA and RNA concentrations were measured with a Qubit Fluorometer (Life Technologies) and purity was assessed with a NanoDrop (NanoDrop Technologies) and integrity by Bioanalyzer (Agilent Technologies). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher) following the manufacturer's instructions and quantified on a Qubit Fluorometer (Life Technologies). From the 100 DNA and 100 cDNA samples, we selected 48 DNA samples (24 from each treatment) and 48 cDNA samples (24 from each treatment) for sequencing based on the RNA and DNA quantity and quality. Library preparation and sequencing of the V3–V4 region of the 16S rRNA gene on an Illumina MiSeq platform was performed by Macrogen (Seoul), using the standard Illumina protocol. The primer pair 337F/805R and the same PCR condition described in Klindworth et al. (2013) were used.

Sequence Processing

In order to obtain high-resolution data, we processed the raw sequences using the DADA2 pipeline (Callahan et al., 2016, 2017). Hereby, sequences are not clustered into operational taxonomic units (OTUs), but exact sequences or amplicon sequence variants (ASVs). Forward reads were truncated at 260 bp and reverse reads at 250 bp. Assembled ASVs were assigned taxonomy against the SILVA_132 database (Quast et al., 2013) using the Ribosomal Database Project (RDP) naïve Bayesian classifier (Wang et al., 2007) in DADA2. We discarded samples with less than 10,000 non-chimeric sequences and/or less than 50 ASVs. We removed ASVs assigned to chloroplasts and mitochondria, singletons, doubletons and ASVs occurring in only one sample. In total, for 82 samples, 1,954 ASVs remained. The data were normalized using cumulative-sum scaling (CSS) (Paulson et al., 2013) to account for uneven sequencing depths.

The 16S rDNA based community is hereafter sometimes referred to as the DNA based community and the 16S rRNA (cDNA) based community is hereafter referred to as the cDNA based community. We interpret the cDNA based community as the "potentially metabolically active bacterial community," acknowledging that 16S rRNA is not a direct indicator of activity but rather protein synthesis potential (Blazewicz et al., 2013).

Quantitative Real-Time PCR of nifH and 16S rRNA Genes

We used all 100 DNA extractions (50 replicates per treatment) for quantification of *nifH* and 16S rRNA genes, which was

performed by quantitative PCR (Corbett Rotor-Gene) using the primer set PolF/PolR and 341F/534R, respectively (Poly et al., 2001). The specificity of the *nifH* primers for our samples was confirmed by SANGER sequencing of 10 clone fragments. Standards for *nifH* reactions were obtained by amplifying one cloned *nifH* sequence with flanking regions of the plasmid vector (TOPO TA cloning Kit, Invitrogen). Standard curves were obtained by serial dilutions (E = 0.9–1.1, R^2 = > 0.99 for all reactions). Each reaction had a volume of 20 μL, containing 1× QuantiFast SYBR Green PCR Master Mix (Qiagen), 0.2 μL of each primer (10 μM), 0.8 μL BSA (5 μg/μL), 6.8 μL RNase free water, and 2 μL template. The cycling program was 5 min at 95°C, 30 cycles of 10 s at 95°C and 30 s at 60°C.

Statistical Analysis

Statistical analyses were conducted in R version 3.6.1. Richness (number of ASVs) and Shannon diversity were calculated with the R packages "vegan" (Oksanen et al., 2013) and "phyloseq" (McMurdie and Holmes, 2013).

Differences in 16S rRNA and *nifH* gene abundance, ASV richness and Shannon diversity between the treatments and the DNA and cDNA were assessed with Bayesian (Markov chain Monte Carlo) generalized linear models using the R package "MCMCglmm" (Hadfield, 2010) with treatment as a fixed and plot as a random factor to take into account the variation caused by pseudoreplication. We considered differences significant if the modeled 95% confidence intervals did not overlap. In addition to the effect of treatment, we included *B. nana* and litter abundance in these generalized linear models. The effect of *B. nana* and/or litter abundance was considered significant if 95% High Posterior Density Credible Interval (95% CrI) were not overlapping zero.

Distances between the community composition of the control and OTC samples were based on Bray-Curtis distances. The effect of the treatment, *B. nana* abundance and litter abundance on the bacterial community composition was tested by permutational-MANOVA (PERMANOVA) (Anderson, 2001) analysis of the Bray-Curtis distance matrices using the *adonis* function in the R package "vegan" with plot as strata. Principal coordinate analysis was used to ordinate the Bray-Curtis distance matrices and to visualize the relationships between samples from OTC and control plots.

For the comparisons of relative abundances of taxa on phylum, class and order level between the warmed and the control samples, pseudoreplicates were averaged by the OTC or control plot they originated from. These average relative abundances were then compared using Wilcoxon rank-sum tests.

We used two methods to determine taxa sensitive to warming. For both methods, we used the average abundances of ASVs in each plot. First, differential abundance of bacterial ASVs between warmed and control samples was assessed using DESeq2 (Love et al., 2014) on the non-CSS normalized datasets with the R package "DESeq2" (Love et al., 2014). The adjusted *P*-value cut-off was 0.1 (Love et al., 2014). Differential abundance analysis only uses ASVs present in both the OTC and control samples. The second method we used to find taxa sensitive to warming, was indicator species analysis. To find bacterial taxa indicative for the warming or the control treatment, correlation-based indicator species analysis was done with all possible site combinations using the function *multipatt* of the R package "indicSpecies" (De Caceres and Legendre, 2009) based on 10^3 permutations. The indicator species analysis takes into account ASVs present in both OTC and control samples, but also ASVs present in only one of the treatments. We combined results of the DESeq2 and indicator species analysis for a final list of ASVs sensitive to warming.

For visualizations of the data, we showed all samples when we could account for pseudoreplication (Bayesian generalized linear models and Permanovas) and we showed plot averages when we compared between the control and warmed treatment (Wilcoxon rank-sum tests).

RESULTS

Effect of OTC Treatment on ASV Richness, Diversity and Community Structure

The ASV richness and Shannon diversity of the bacterial communities associated with *C. islandica* were not significantly affected by the warming treatment (**Figure 1** and **Supplementary Figures 1–4**). However, we found that *B. nana* abundance tended to positively influence the cDNA-based bacterial richness and Shannon diversity (**Supplementary Figures 3, 4**). A significant difference was found for the richness, which was higher for the cDNA-based bacterial community than the DNA-based community in the warmed treatment (**Supplementary Figure 7**). The cDNA-based Shannon index tended to be higher than the DNA-based Shannon index (**Figure 1** and **Supplementary Figure 5**).

Some level of clustering between the control and warmed samples could be observed in the principal coordinate analysis (**Figure 2**). Based on the results of a PERMANOVA, the warmed lichen associated bacterial communities were significantly different from the communities in the control samples (DNA: R^2 = 0.07 and P = 0.001; RNA: R^2 = 0.07 and P = 0.005) (**Supplementary Tables 2, 3**). In addition, litter abundance was associated with variation in the cDNA-based bacterial community (Permanova: R^2 = 0.04 and P = 0.05).

Effect of OTC Treatment on the Taxonomic Composition and Abundance of the *C. islandica* Bacteriota

The bacterial community found associated with the lichen *C. islandica* is described at the phylum level (**Figure 3A**) and at the class and order level (**Figure 4**). No clear differences were found for the relative abundance at the phylum level between the control and warmed treatment for the total bacteria community (**Figure 3A**). Similarly, we did not detect differences between the control and warmed

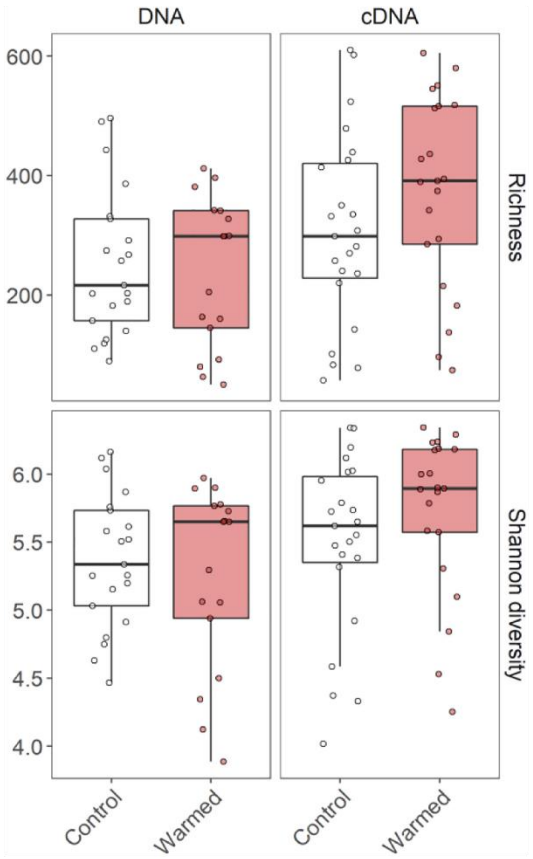

FIGURE 1 | ASV richness and Shannon diversity of the DNA and cDNA-based bacterial communities associated with the lichen *C. islandica* in control and warmed samples. $N = 82$, $n = 21$ for DNA control, $n = 17$ for DNA warmed, $n = 23$ for cDNA control and $n = 21$ for cDNA warmed.

treatment in the cDNA-based bacterial community at the phylum level (**Figure 3A**). The total bacterial community was dominated by Proteobacteria and Acidobacteria (DNA: 58 and 34% average relative abundance across all control and warmed samples, for Proteobacteria and Acidobacteria, respectively). Proteobacteria and Acidobacteria were also the main phyla in the cDNA-based bacterial community (cDNA: 63 and 29%, respectively) (**Figure 3A**). At lower taxonomic level, the orders Acetobacterales and Acidobacteriales were the dominant taxa (DNA: 44%; cDNA: 51%, DNA: 34%; cDNA 29%, **Figure 4A**). Within the acidobacterial family Acetobacteraceae, about 14% could not be assigned to a genus (**Supplementary Figure 7**). The most abundant genera in the cDNA and DNA-based bacterial communities were the proteobacterial genera *Acidiphilium* (DNA: 8%, cDNA: 11%) and *Endobacter* (DNA: 19%, cDNA: 20%) and the acidobacterial genera *Bryocella* (DNA: 10%, cDNA: 9%) and *Granulicella* (DNA: 15%, cDNA: 11%) (**Supplementary Figure 7**).

A total of 295 ASVs were only detected in the cDNA-based samples and not in the DNA-based samples. These taxa belonged to abundant genera such as *Endobacter* and *Acidiphilium* (**Supplementary Tables 4, 5**). Genera that were exclusively found in the cDNA-based community were *Lactococcus*, *Lachnospiraceae NK4A136* group, *Ktedonobacter*, *Methylovirgula*, *Frigoribacterium*, *Amnibacterium*, *Rhizobacter*, *Telmatobacter*, *Kineosporia*, and *Acidiphilium* (**Supplementary Tables 4, 5**).

The bacterial load was on average 671 and 1944 16S rRNA copies per ng DNA for control and warmed plots, respectively (**Figure 3B**). However, no differences could be found between the overall 16S rRNA gene copy numbers, or *nifH* gene copy numbers in the control and warmed plots (**Figure 3B** and **Supplementary Figures 8, 9**). Nevertheless, *B. nana* abundance positively affected both *nifH* and 16S rRNA gene copy numbers, whereas litter abundance negatively affected *nifH* gene copy numbers (**Supplementary Figures 8, 9**).

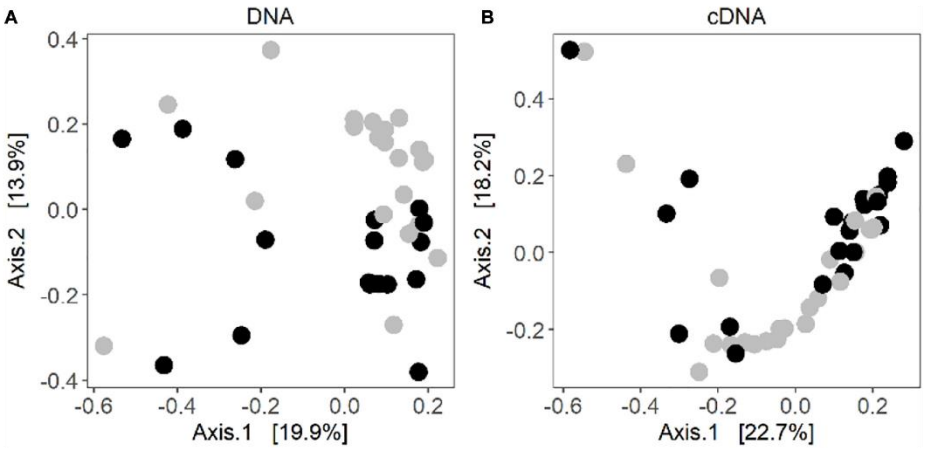

FIGURE 2 | Principal coordinate analysis of Bray-Curtis distances of the **(A)** DNA-based community composition and **(B)** cDNA-based community composition of the lichen *C. islandica*. Warmed samples are represented as black circles and control samples as gray circles. $N = 28$, $n = 21$ for DNA control, $n = 17$ for DNA warmed, $n = 23$ for cDNA control and $n = 21$ for cDNA warmed.

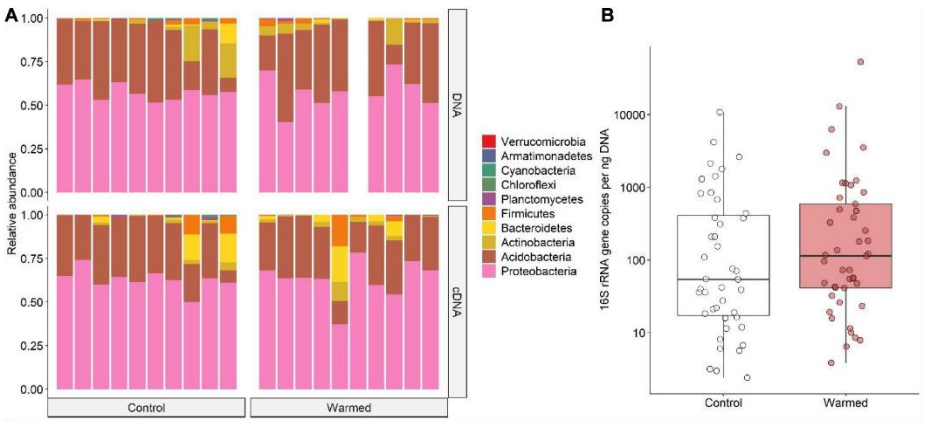

FIGURE 3 | (A) Barplots of the bacterial community composition associated with the lichen *C. islandica* in the control and warmed plots. Data are presented at the phylum level for DNA and cDNA. Bars represent pooled samples from each separate plot. **(B)** 16S rRNA gene abundance per ng extracted DNA in control (white) and warmed (red) samples. $N = 88$, $n_{warmed} = 45$, $n_{control} = 43$.

In the DNA-based samples, the only proteobacterial class found significantly affected by warming were the Gammaproteobacteria with an increase of 50% in the warmed samples (Wilcoxon rank-sum test, $P = 0.033$; **Figure 4A**). Most of this increase was due to an increase in relative abundance of the order Betaproteobacteriales ($P = 0.046$) (**Figure 4B**). The order Sphingomonadales (Alphaproteobacteria) increased by a factor of 2.5 in relative abundance ($P = 0.009$) (**Figure 4B**). In the cDNA-based bacterial community, we did not detect differences in relative abundance on Proteobacterial class level between the control and warmed treatment (**Figure 4A**). Changes were found on order level with the alphaproteobacterial order Caulobacterales being twice as abundant in the cDNA-based bacterial community in the warmed plots ($P = 0.038$) than in the control plots. Similarly, the order Sphingomonadales was 2.7 times more active in the warmed treatment ($P = 0.007$) (**Figure 4B**). The order Diplorickettsiales (Gammaproteobacteria) was four times as abundant in the cDNA-based bacterial community in the warmed plots ($P = 0.040$) (**Figure 4B**).

While the phylum Actinobacteria was not among the most common phyla (DNA: 4%, cDNA: 2%), its order

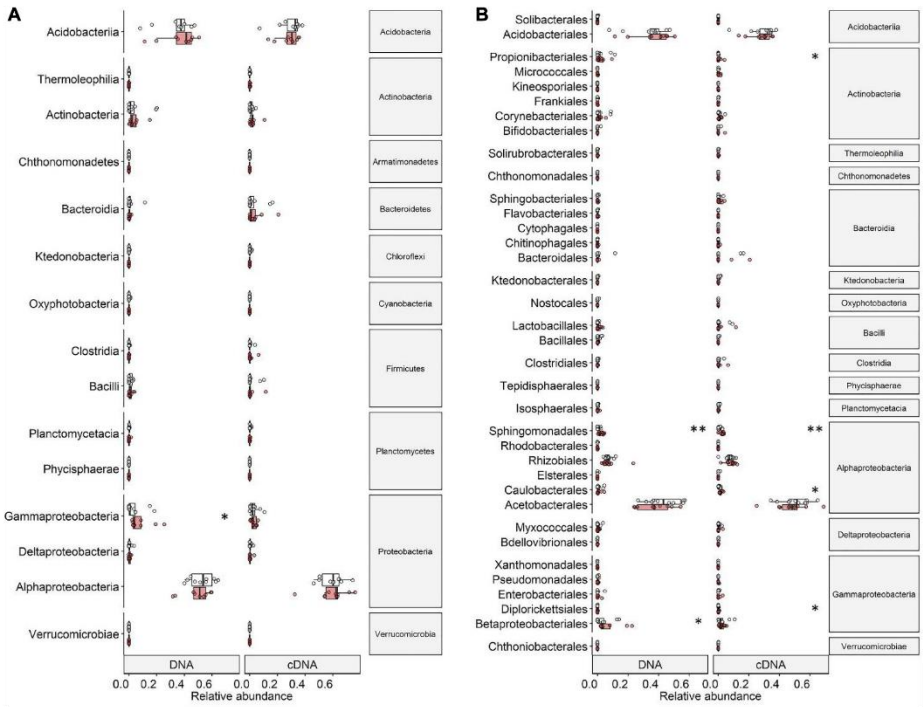

FIGURE 4 | Relative abundances of **(A)** classes and **(B)** orders of DNA- and cDNA-based bacterial communities associated with the lichen *C. islandica* in control (white) and warmed (red) samples. Points indicate average relative abundance values per control or warmed plot. Boxplots represent minimum values, first quartiles, medians, third quartiles, and maximum values. Significance levels ($*p < 0.05$ and $**p < 0.01$) are based on Wilcoxon rank-sum tests.

Propionibacteriales was 20 times as abundant in the cDNA-based bacterial community in the warmed plots compared to the control plots ($P = 0.040$) (**Figure 4B**).

Effect of OTC Treatment on the Relative Abundance of Bacterial ASVs

For the DNA-based communities, we detected 61 ASVs with a higher relative abundance in the warmed samples with a total relative abundance of 1% (**Supplementary Table 4**). We detected 96 ASVs with a lower relative abundance in the warmed samples compared to the control samples making up 1.7% of the total abundance (**Supplementary Table 4**). For the cDNA-based bacterial communities, we detected 190 ASVs with a higher relative abundance in the warmed samples (2.12%) and 77 ASVs with a lower relative abundance in the warmed samples compared to the control samples (0.9%) (**Supplementary Table 5**).

Of the ASVs only detected in cDNA-based bacterial community, 14 ASVs had a higher relative abundance in the warmed plots. All these rare ASVs belonged to the Proteobacteria, except one ASV that was classified as Bacteroidetes.

Amplicon sequence variants within the Proteobacteria showed mainly increased relative abundance in the warmed samples (**Figure 5** and **Supplementary Tables 4, 5**). Only ASVs classified under the genus *Acidiphilium* had more often a lower relative abundance in the warmed samples as well as a few ASVs of the genera *Acidisphaera* and *Endobacter*. In the cDNA-based samples, more proteobacterial ASVs with increased relative abundances under warming were detected than in the DNA-based samples.

Acidobacterial ASVs showed mixed differences between the control and warmed samples (**Figure 5** and **Supplementary Tables 4, 5**). ASVs of the genus *Bryocella* were less abundant under warming in both the DNA- and cDNA-based samples. ASVs of the genus *Granulicella* were equally more and less abundant in the warmed DNA-based samples, but had more often higher relative abundances in the warmed cDNA-based samples (**Figure 5** and **Supplementary Tables 4, 5**).

DISCUSSION

We assessed the effect of long-term (20 years) warming by OTCs on the bacterial community composition associated with the lichen *C. islandica* in an Icelandic sub-Arctic alpine dwarf-shrub heath. The community was dominated by Acidobacteria

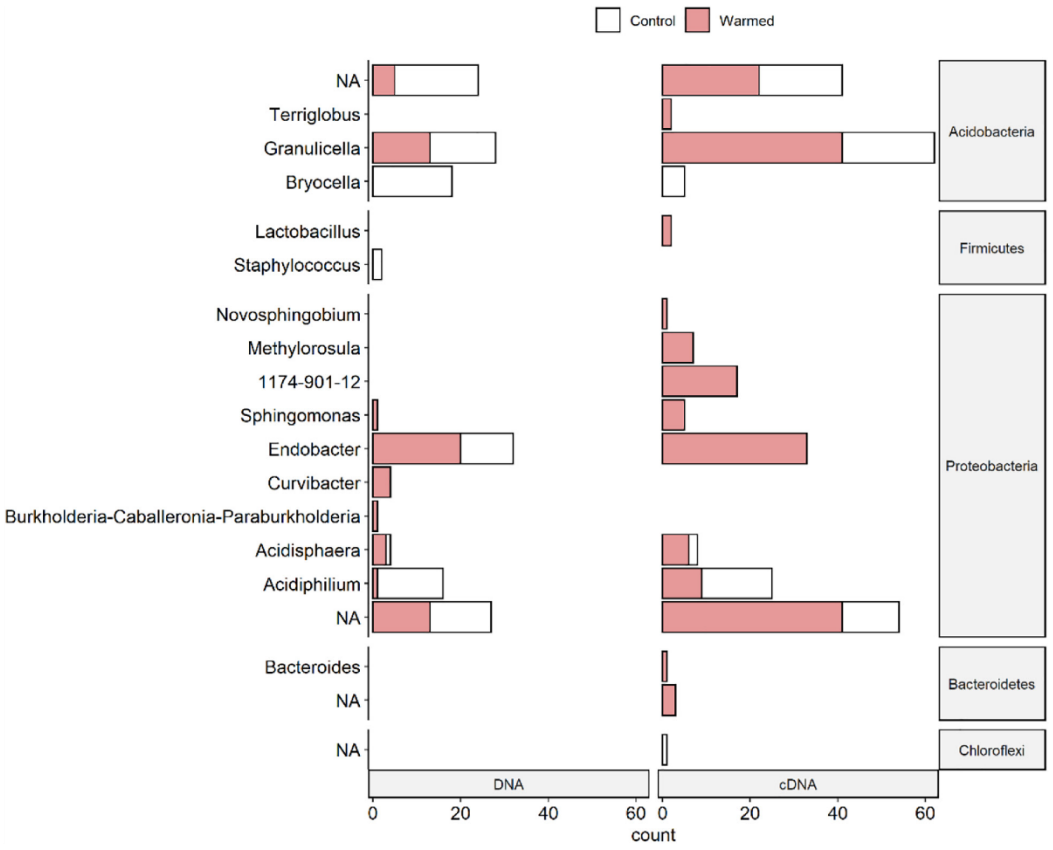

FIGURE 5 | Number of ASVs (amplicon sequence variants) per genus sensitive to warming for DNA and cDNA-based bacterial communities associated with the lichen *C. islandica*. Sensitivity to warming was determined by differential abundance analysis (DESeq2) and indicator species analysis. ASVs not assigned to genus level are called "NA." The number of ASVs indicative for the OTC (warmed) treatment is indicated in red and the number of ASVs indicative for the control treatment is indicated in white.

and Proteobacteria in both total and potentially active bacterial communities in both control and warmed plots. Warming did not induce compositional or structural changes at higher taxonomical levels. Nevertheless, we found indications of multiple warming-induced shifts in the community composition at the class, order and ASV levels. The most prominent increases in relative abundance were found in several genera belonging to the Proteobacteria. Our results illustrate that the long-term warming treatment affects the bacterial community composition of the lichen symbiosis at fine taxonomical levels.

The Bacterial Community of *Cetraria islandica*

While the dominance of the class Alphaproteobacteria has been described as a general characteristic of lichen bacterial communities (Cardinale et al., 2008; Weiss et al., 2011; Printzen et al., 2012), a striking feature of the *C. islandica* microbiome is the strong dominance of the family Acetobacteriaceae (Alphaproteobacteria). While the presence of Acetobacteriaceae in lichens has been observed before, notably in the reindeer lichen *Cladonia arbuscula* (Cardinale et al., 2008), this strong dominance does seem unusual. Indeed, the Rhizobiaceae, often held to be the dominant Alphaproteobacteria in lichens (Weiss et al., 2011; Hodkinson et al., 2012), only make up a minor part of the *C. islandica* bacteriome. The second dominant group in the *C. islandica* bacteriome are members of the family Acidobacteriaceae (Acidobacteria). Even at the genus level, the *C. islandica* bacteriome is surprisingly homogeneous, with approximately half of the reads being assigned to only four genera, the acetobacterial genera *Endobacter* and *Acidiphilium*, and the acidobacterial genera *Granulicella* and *Bryocella*. This pronounced dominance of presumptively acidophilic taxa is noteworthy. Acidobacteria were also reported earlier in

living parts of bog and tundra (Pankratov, 2012) and lichens in Alpine soil crusts (Muggia et al., 2013). The presence of acidophilic taxa may be explained by organic acid secondary metabolites produced by *C. islandica*, such as protolichesterinic and fumaroprotocetraric acids (Xu et al., 2018).

Another feature of the *C. islandica* microbiota is the difference between the potentially metabolically active and total community. The richness of the potentially active bacterial communities was higher compared the to total bacterial communities. One possible explanation for this is that the detection of taxa in 16S rRNA sequences, but not in 16S rDNA sequences, can occur when rare taxa have a high metabolic potential. The occurrence of these "phantom taxa" could be a result of cDNA synthesis errors that do not occur in the rDNA samples, but are introduced in the rRNA sequences. Another explanation could be variation in metabolic activity among taxa (Campbell et al., 2011; Baldrian et al., 2012; Klein et al., 2016; Jia et al., 2019). Rare taxa have been observed to be disproportionally active compared to abundant members (Jones and Lennon, 2010) and thereby might contribute more to ecosystem functioning than one would expect based on their abundance (Jousset et al., 2017). The rare bacterial taxa of *C. islandica* were mostly composed of not assigned genera and members of the genera *Endobacter*, *Acidiphilium*, *Lactococcus*, *Mucilaginibacter*, and *Bacteroides*. The fermenting bacteria from the genus *Lactococcus* have been described before in a bioreactor as being rare while having high potential activity levels (Lawson et al., 2015).

N_2-fixation is an important process in N-limited tundra ecosystems and previous work has shown that biocrust chlorolichens can show significant nitrogenase activity (Torres-Cruz et al., 2018). As *C. islandica* is a chlorolichen and does not have N_2-fixing Cyanobacteria as a photobiont, this raises the question if other taxa could be N_2-fixers. The N_2-fixing capability of *C. islandica* is unknown, but the presence of *nifH* genes indicates that potential N_2-fixers are present in the *C. islandica* bacterial community. Indeed, we detected putative N_2-fixers such as *Curvibacter* (Ding, 2004) and members of the Burkholderiaceae. On the other hand, lichens might also obtain nutrients dissolved in precipitation or through runoff from taller vegetation, or via a moisture gradient resulting in upward movement of soil moisture and dissolved nutrients (Longton, 1992). Nevertheless, our data indicate that the indirect effect of warming through changes in litter and *B. nana* abundance can influence the abundance of N_2-fixing bacteria. More studies on the N_2-fixation capabilities of chlorolichens in tundra ecosystems are necessary to elucidate their role as N_2-fixers.

The *C. islandica*-associated microbiota was found to be markedly different to that of the moss *Racomitrium lanuginosum* which was studied in the same warming experiment (Klarenberg et al., 2019), further supporting the host-specific selection of bacteria from the environment and symbiotic nature of both bryophyte and lichen holobionts proposed in the recent literature (Aschenbrenner et al., 2016; Holland-Moritz et al., 2018). Specifically, we found that *C. islandica* harbored a less rich and diverse bacterial community than *R. lanuginosum*, and the microbiota composition was profoundly different. Whereas the moss was dominated by the genera *Haliangium*, *Acidiphilium*, *Nostoc*, *Conexibacter*, *Granulicella*, *Solibacter*, and *Bryobacter*, the lichen was dominated by a few genera (*Bryocella*, *Granulicella*, *Acidiphilium*, and *Endobacter*) as reported herein. The same difference between the bacterial diversity of a lichen and a moss was shown by Aschenbrenner et al. (2017).

The Effect of OTC Warming on Bacterial Richness, Diversity and Community Structure

While we did not see any significant changes in richness or diversity of the bacterial community with warming, the warmed bacterial community structure significantly differed from the control community, both for the total as well as the potentially active communities. Overall, the potentially active community tended to be more affected by warming than the total bacterial community. For instance, more indicator taxa were found in the potentially active community and many more of these indicators were found in the warmed treatment. In addition, indirect effects of warming via shrubification and litter modification were found to affect the bacterial community. A positive effect of *B. nana* abundance was found on the richness and diversity of the potentially active community as well as on 16S rRNA and *nifH* gene abundance. Litter abundance was positively associated with *nifH* gene abundance and with the structure of the potentially active bacterial community.

At a coarse taxonomic level, the bacterial community structure was quite similar between the control and warmed treatment. One possible explanation for the similarity between the richness, diversity and composition of the warmed and control lichen bacterial communities could be that over long periods of warming bacterial communities acclimatize (Bradford et al., 2008; Crowther and Bradford, 2013; Romero-Olivares et al., 2017). Nonetheless, at lower taxonomic levels (class, order, and ASV) we detected differences in relative abundances. Shifts in individual taxa can affect microbe–microbe and microbe–host interactions and potentially change functionality or stability of the lichen-associated bacterial communities and thereby influence host health and ecosystem functioning, as proposed for plant–microbiomes (Agler et al., 2016; van der Heijden and Hartmann, 2016; Aydogan et al., 2018).

Long-term warming decreased the relative abundance of ASVs belonging to the Acidobacterial genera *Granulicella* and *Bryocella* and the alphaproteobacterial genus *Acidiphilium* in the total bacterial communities. *Acidiphilium* and *Granulicella* have been observed in other lichen microbiomes (Bates et al., 2011; Pankratov, 2012; Park et al., 2016; Aschenbrenner et al., 2017). These genera are chemoorganotrophic or chemolithotrophic and might thus survive on C sources present in the lichen thallus. *Granulicella* encompasses several acidophilic, cold-adapted species described from tundra soil isolates (Männistö et al., 2012). It has hydrolytic properties such as the ability to degrade chitin (Pankratov and Dedysh, 2010; Pankratov, 2012; Park et al., 2016; Belova et al., 2018), which suggest a role for these bacteria in the degradation of senescing lichen thalli. While some ASVs of the genus *Granulicella* decreased

in relative abundance, the increased potential activity might result in increased degradation of dead lichen material. In contrast, the decreased relative abundance and potential activity of *Bryocella* and *Acidiphilium* might result in slower degradation of dead lichen material. The genus *Acidiphilium* showed an increase in relative abundance in a moss microbiome in the same warming experiment (Klarenberg et al., 2019). This suggests that the responses of microbiome components to environmental change are at least in part dependent upon host vegetation identity rather than constituting a direct response of the bacteria themselves to extrinsic environmental factors. Thus leading to different outcomes for the various microbiomes within the same environment.

The alphaproteobacterial genera *Acidisphaera*, *Sphingomonas*, and *Endobacter* showed an increased relative abundance and potential activity with warming. *Sphingomonas* and *Acidisphaera* have been identified in other lichen bacterial communities (Cardinale et al., 2008). *Endobacter* is a poorly characterized genus of which only one species has been described (Ramirez-Bahena et al., 2013). *Acidisphaera* is chemoorganotrophic and contains bacteriochlorophyll (Hiraishi et al., 2000). *Sphingomonas* is known for its ability to degrade plant biomass, the utilization of recalcitrant matter in oligotrophic environments, and the use of sulfonated compounds as sources of C and sulfur (Aylward et al., 2013), which may be linked to the increase in litter abundance. The increase in relative abundance and potential activity of these genera in the warmed conditions might enhance C and nutrient availability in the lichen thalli.

Overall, all genera that dominated the bacterial community of *C. islandica* contained ASVs that were affected by the warming treatment in their relative abundances and potential metabolic activity. The genera that were affected in relative abundance are likely to play roles in nutrient recycling and supply in the lichen symbiosis. As most of these ASVs increased in relative abundance with warming, nutrient turnover in the lichen might be accelerated.

OTCs have been deployed to study warming effects in a wide range of ecosystems and plant responses correspond well to responses to natural climate warming (Hollister and Webber, 2000). We have shown that the OTC treatment leads to changes in the composition of the bacterial community associated with the lichen *C. islandica*, and that part of this change could be attributed to the increase in *B. nana* and litter abundance in the OTCs. This secondary effect of the OTC treatment may shield radiation from reaching the lichen layer or soil and thereby reduce the warming effect of the OTCs (Bokhorst et al., 2013), reducing the amount of PAR reaching the lichen and potentially affecting the bacterial communities. The increase in *B. nana* leaf litter may also increase C turnover as it is easily decomposable (McLaren et al., 2017) and thereby influence the lichen bacterial community. It should be noted that multiple caveats can be associated with the use of OTCs. Snow trapped in OTCs can increase temperatures at the soil surface, but at the same time decrease photosynthetically active radiation (Bokhorst et al., 2013). The walls of the OTCs may act as a barrier for new species to arrive (Richardson et al., 2000), even though it is unknown how important this side effect of the OTCs is on microbial communities. In addition, temperature-induced changes in lichen traits such as thallus nutrient content, as well as soil organic matter content, and soil moisture are environmental factors that could potentially influence the lichen bacterial community composition. Warming could also affect the secondary metabolites of the lichen (Asplund et al., 2017) and thereby alter the composition of the lichen microbiota.

In conclusion, we found that the bacterial community of *C. islandica* was dominated by acidophilic taxa and harbored rare, but potentially active taxa. Our results also showed that twenty years of warming and an increase dwarf-shrub and litter abundance can lead to changes in lichen bacterial communities at a fine taxonomic level as well as richness and diversity. The lichen microbiome plays an important role in the growth of lichens and climate-driven changes in the lichen microbiota, irrespective of whether they are due to direct or indirect effects of climate change, might affect decomposition of lichens and thereby nutrient cycling in sub-Arctic ecosystems.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in NCBI, under accession PRJEB37116.

AUTHOR CONTRIBUTIONS

IJ, IK, and OV designed the study. IK conducted the sampling, the laboratory work, the bioinformatics processing, and the statistical analysis. CK performed the qPCR measurements. IK wrote the manuscript with contributions from OV, IJ, CK, and DW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.540404/full#supplementary-material

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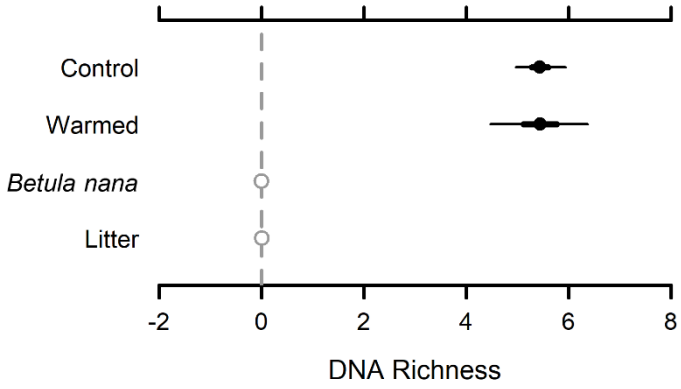
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

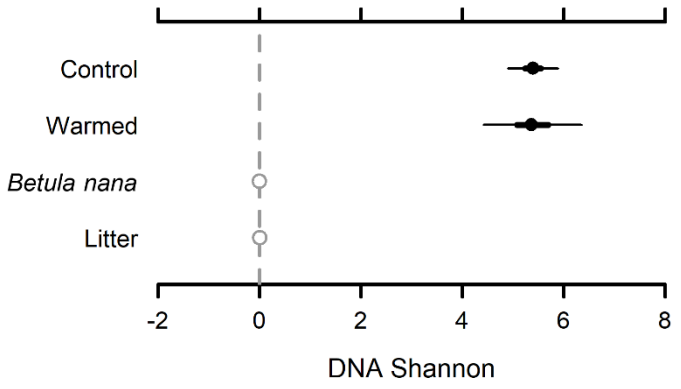
Supplementary Material Paper I

1.1. Supplementary Figures

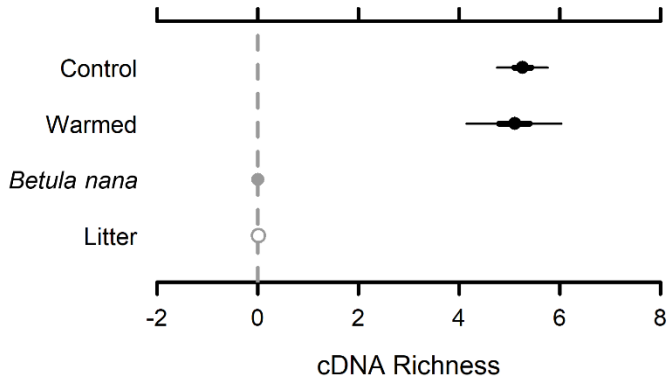
Supplementary Figure 1 Fixed effect structure of the linear mixed-effect model testing the effect of treatment, *Betula nana* abundance and litter abundance on the DNA-based richness. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



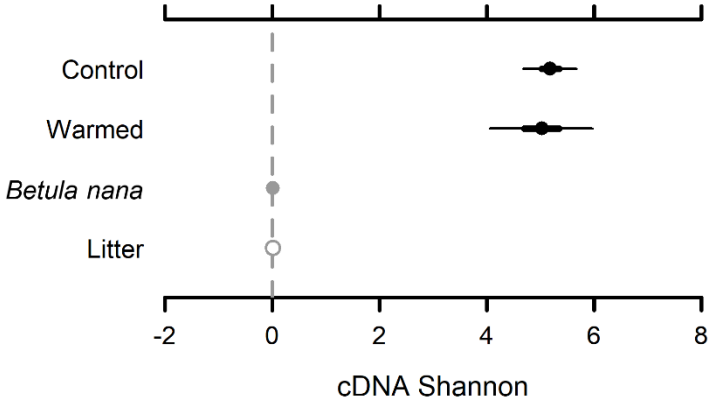
Supplementary Figure 2 Fixed effect structure of the linear mixed-effect model testing the effect of treatment, *Betula nana* abundance and litter abundance on the DNA-based Shannon diversity. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



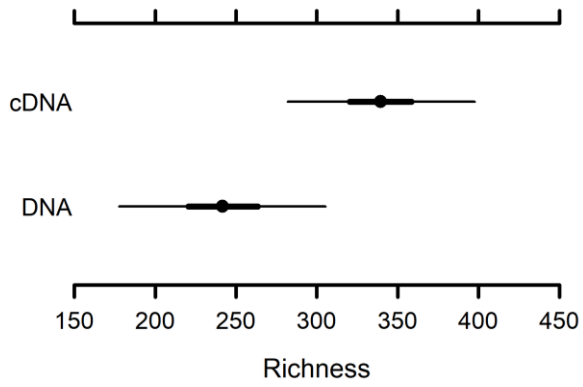
Supplementary Figure 3 Fixed effect structure of the linear mixed-effect model testing the effect of treatment, *Betula nana* abundance and litter abundance on the cDNA-based richness. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



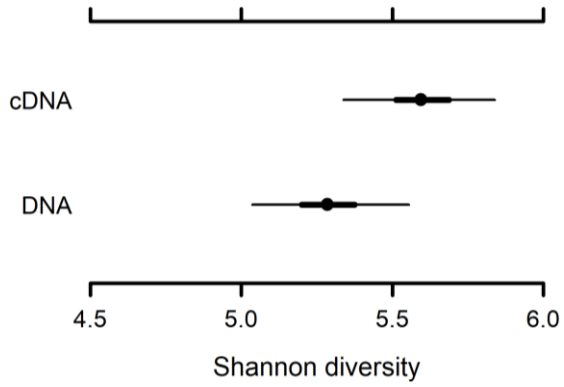
Supplementary Figure 4 Fixed effect structure of the linear mixed-effect model testing the effect of treatment, *Betula nana* abundance and litter abundance on the cDNA-based Shannon diversity. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



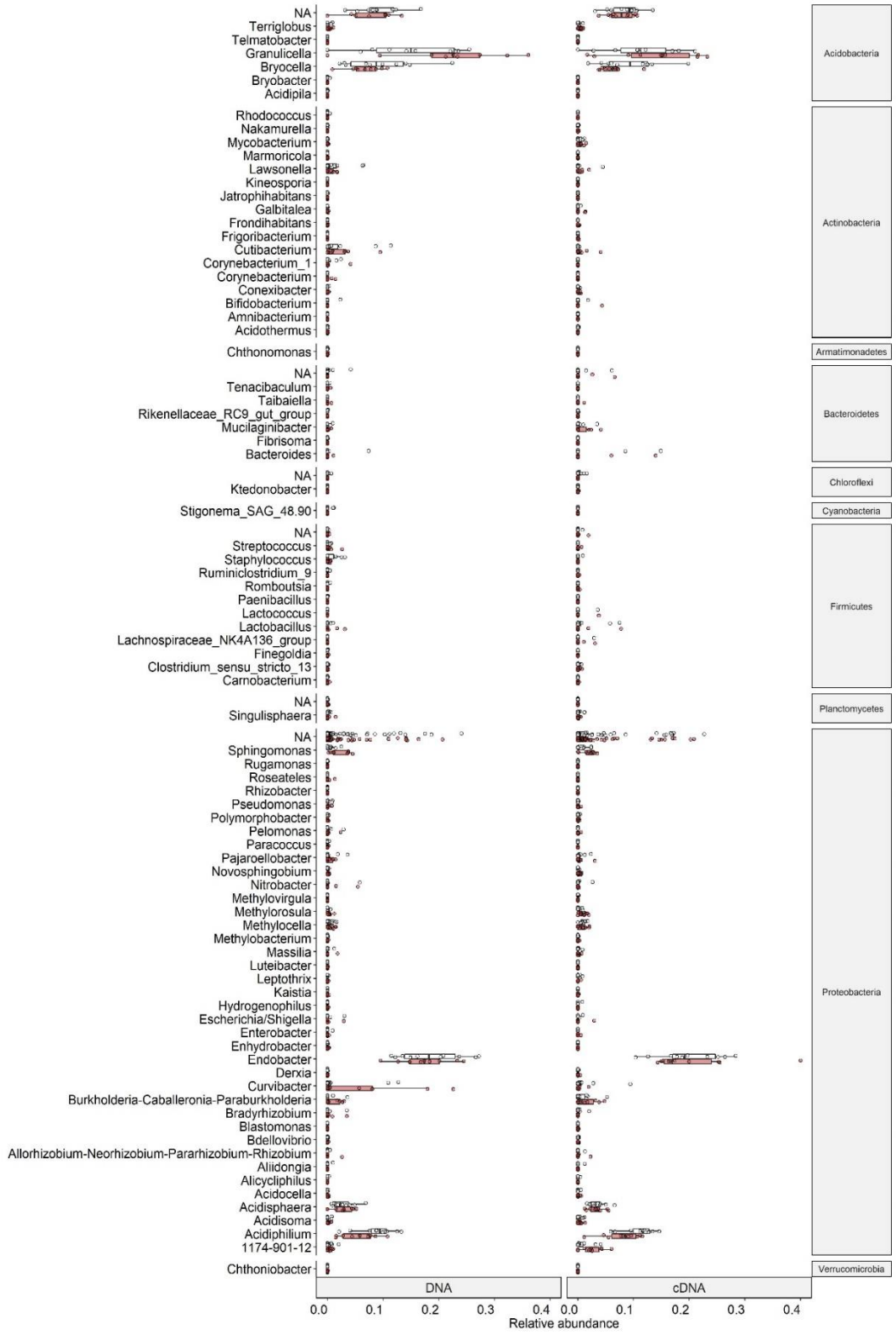
Supplementary Figure 5 Fixed effect structure of the linear mixed-effect model testing the effect material (DNA vs cDNA) on the ASV richness. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



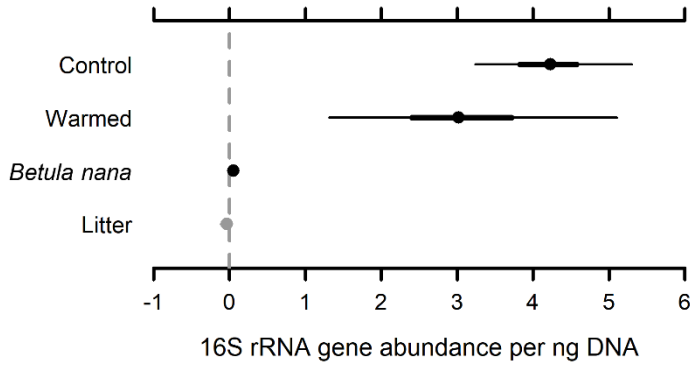
Supplementary Figure 6 Fixed effect structure of the linear mixed-effect model testing the effect material (DNA vs cDNA) on the Shannon diversity. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



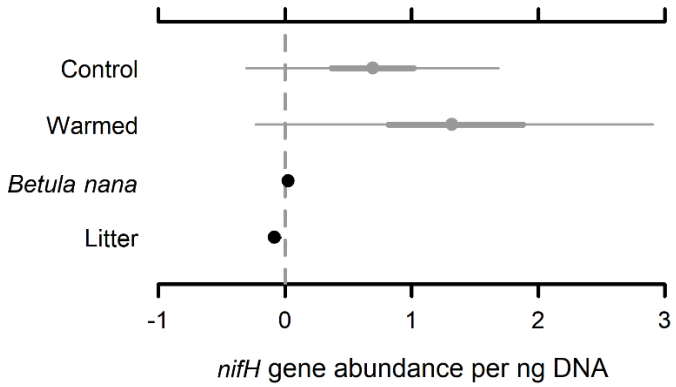
Supplementary Figure 7 Relative abundances of genera of DNA- and cDNA-based bacterial communities associated with the lichen *Cetraria islandica* in control (white) and warmed (red) samples. Points indicate average relative abundance values per control or warmed plot. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values.



Supplementary Figure 8 Fixed effect structure of the linear mixed-effect model testing the effect of treatment, *Betula nana* abundance and litter abundance on the 16S rRNA gene copy numbers. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



Supplementary Figure 9 Fixed effect structure of the linear mixed-effect model testing the effect of treatment, *Betula nana* abundance and litter abundance on the *nifH* gene copy numbers. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.



1.2. Supplementary Tables

Supplementary Table 1 Temperature and relative humidity for the OTC (warmed) and control plots measured in June-August 2016 (temperature and relative humidity 10 cm above the moss layer) and August 2018-June 2019 (temperature on the moss surface). Shown are mean ± standard error of the mean. Significant differences (t-test, $P < 0.05$) are indicated in bold.

Air temperature June – August 2016			Moss surface temperature August 2018 – June 2019			Relative humidity Air June – August 2016		
OTC	Control	Δ°C	OTC	Control	Δ°C	OTC	Control	Δ%
11.4 ± 0.1	10.0 ± 0.1	1.4	1.28 ± 0.01	1.06 ± 0.01	0.22	78.8 ± 0.36	81.8 ± 0.37	-3

Supplementary Table 2 Summary for the Permanova testing the effect treatment, *Betula nana* abundance and litter abundance on the DNA-based bacterial community variation of the lichen.

	DF	Sum of Squares	Mean Squares	F.Model	R^2	Pr(>F)
Treatment	1	0.6646	0.66461	2.7591	0.07019	<0.001
Betula nana	1	0.3654	0.36536	1.5168	0.03859	0.1646
Litter	1	0.2483	0.24830	1.0308	0.02622	0.6616
Residuals	34	8.1900	0.24088		0.86499	
Total	37	9.4683			1.00000	

Supplementary Table 3 Summary for the Permanova testing the effect treatment, *Betula nana* abundance and litter abundance on the cDNA-based bacterial community variation of the lichen.

	DF	Sum of Squares	Mean Squares	F.Model	R^2	Pr(>F)
Treatment	1	0.6183	0.61835	3.1828	0.06659	<0.001
Betula nana	1	0.5283	0.52833	2.7194	0.05690	0.06879
Litter	1	0.3674	0.36736	1.8909	0.03956	0.04850
Residuals	40	7.7712	0.19428		0.83694	
Total	43	9.2852			1.00000	

Supplementary Table 4 Indicator and differentially abundant ASVs (every row represents a single ASV) in the DNA-based bacterial communities associated with *Cetraria islandica*. NA indicates ASVs not assigned to genus level.

Phylum	Family	Genus	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Diffab	Indicator species	comb	A	B	Indstat	Indpvalue
Proteobacteria	Acetobacteraceae	Endobacter								c	c	0.90	0.38	0.59	0.02
Proteobacteria	Acetobacteraceae	Endobacter								c	c	0.91	0.33	0.55	0.03
Proteobacteria	Acetobacteraceae	Endobacter								c	c	0.74	0.52	0.62	0.03
Proteobacteria	Acetobacteraceae	Endobacter								c	c	0.73	0.52	0.62	0.04
Proteobacteria	Acetobacteraceae	Endobacter								c	c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	Endobacter								c	c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	NA								c	c				
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c				
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.83	0.48	0.63	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.79	0.48	0.61	0.04
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.29	0.54	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.24	0.49	0.03
Proteobacteria	Acetobacteraceae	Acidisphaera								w	w	0.88	0.81	0.84	0.00
Proteobacteria	Acetobacteraceae	Acidisphaera								w	w	0.89	0.76	0.82	0.00

Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.89	0.67	0.77	0.00
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.87	0.67	0.76	0.00
Proteobacteria	Acetobacteraceae	Endobacter	w	w				
Proteobacteria	Acetobacteraceae	Endobacter	w	w				
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.65	0.90	0.77	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.64	0.90	0.76	0.02
Proteobacteria	Beijerinckiaceae	NA	c	c	0.85	0.41	0.59	0.01
Proteobacteria	Beijerinckiaceae	NA	c	c	0.78	0.41	0.57	0.03
Proteobacteria	Beijerinckiaceae	NA	c	c	0.90	0.48	0.66	0.01
Proteobacteria	Beijerinckiaceae	NA	w	w	0.82	0.38	0.56	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.66	0.81	0.73	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.66	0.81	0.73	0.02
Acidobacteria	Acidobacteriaceae	Bryocella	c	c	0.64	0.81	0.72	0.04
Acidobacteria	Acidobacteriaceae	Bryocella	c	c	0.82	0.35	0.54	0.03
Acidobacteria	Acidobacteriaceae	Bryocella	c	c	0.77	0.53	0.64	0.02
Acidobacteria	Acidobacteriaceae	Bryocella	c	c	1.00	0.29	0.54	0.01
Acidobacteria	Acidobacteriaceae	Bryocella	c	c	0.76	0.59	0.67	0.01
Acidobacteria	Acidobacteriaceae	Bryocella	c	c	0.75	0.53	0.63	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.75	0.53	0.63	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	1.00	0.24	0.49	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.70	0.81	0.75	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.69	0.81	0.75	0.01

Acidobacteria	Acidobacteriaceae	Granulicella	19.91	-23.39	2.97	-7.88	0.00	0.00	c		c	0.66	0.76	0.71	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	18.43	-23.28	2.97	-7.84	0.00	0.00	c		c	0.92	0.35	0.57	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	0.90	0.29	0.51	0.04
Acidobacteria	Acidobacteriaceae	NA								c	c	0.86	0.35	0.55	0.01
Acidobacteria	Acidobacteriaceae	NA								w	w	1.00	0.24	0.49	0.03
Acidobacteria	Acidobacteriaceae	NA								w	w	0.89	0.48	0.65	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	0.80	0.52	0.65	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	0.88	0.43	0.62	0.02
Acidobacteria	Acidobacteriaceae	NA								c	c	0.88	0.43	0.61	0.02
Acidobacteria	Acidobacteriaceae	NA								c	c	0.86	0.38	0.57	0.04
Acidobacteria	Acidobacteriaceae	NA	58.92	-4.13	1.34	-3.08	0.00	0.07	c		c				
Acidobacteria	Acidobacteriaceae	NA	48.08	-4.64	1.35	-3.43	0.00	0.02	c		c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	Endobacter								c	c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	Endobacter								c	c				
Proteobacteria	Acetobacteraceae	Endobacter								w	w				
Proteobacteria	Sphingomonadaceae	Sphingomonas								w	w	0.77	0.48	0.61	0.04
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.83	0.43	0.60	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.24	0.49	0.04
Proteobacteria	Acetobacteraceae	NA								c	c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	Acidisphaera								c	c	1.00	0.24	0.49	0.04
Proteobacteria	Acetobacteraceae	NA								c	c	0.86	0.76	0.81	0.00
Proteobacteria	Acetobacteraceae	NA								w	w	0.85	0.76	0.80	0.00

Proteobacteria	Acetobacteraceae	Acidisphaera								w	w	0.89	0.67	0.77	0.00
Proteobacteria	Acetobacteraceae	NA								w	w	0.85	0.67	0.75	0.00
Proteobacteria	Acetobacteraceae	Endobacter								w	w				
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.85	0.35	0.55	0.02
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.88	0.29	0.51	0.05
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.82	0.47	0.62	0.01
Proteobacteria	Beijerinckiaceae	NA								c	c	1.00	0.24	0.49	0.02
Proteobacteria	Beijerinckiaceae	NA								c	c	1.00	0.24	0.49	0.02
Proteobacteria	Beijerinckiaceae	NA								c	c	0.80	0.41	0.57	0.03
Proteobacteria	Beijerinckiaceae	NA								w	w	0.80	0.41	0.57	0.04
Proteobacteria	Beijerinckiaceae	NA								w	w	0.90	0.29	0.52	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.62	0.90	0.75	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.61	0.90	0.74	0.05
Acidobacteria	Acidobacteriaceae	Bryocella								c	c				
Acidobacteria	Acidobacteriaceae	Bryocella								c	c				
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.67	0.76	0.72	0.02
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.92	0.48	0.66	0.00
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.84	0.43	0.60	0.02
Acidobacteria	Acidobacteriaceae	Bryocella	16.57	-23.13	2.97	-7.79	0.00	0.00	c		c	0.94	0.38	0.60	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.67	0.81	0.74	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.67	0.81	0.74	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	34.32	-24.13	2.97	-8.13	0.00	0.00	c		c	0.64	0.81	0.72	0.04

Acidobacteria	Acidobacteriaceae	Granulicella	32.63	-24.06	2.97	-8.10	0.00	0.00	c		c	0.67	0.71	0.69	0.03
Acidobacteria	Acidobacteriaceae	NA								c	c	1.00	0.24	0.49	0.04
Acidobacteria	Acidobacteriaceae	NA								c	c	0.74	0.59	0.66	0.01
Acidobacteria	Acidobacteriaceae	NA								w	w	0.73	0.53	0.62	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.73	0.53	0.62	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	1.00	0.29	0.54	0.02
Acidobacteria	Acidobacteriaceae	NA								c	c	1.00	0.24	0.49	0.04
Acidobacteria	Acidobacteriaceae	NA								c	c	0.66	0.76	0.71	0.05
Acidobacteria	Acidobacteriaceae	NA								c	c	0.66	0.76	0.71	0.05
Acidobacteria	Acidobacteriaceae	NA								c	c	0.95	0.35	0.58	0.01
Acidobacteria	Acidobacteriaceae	NA	75.98	-4.10	1.40	-2.94	0.00	0.10	c		c	0.92	0.35	0.57	0.01
Proteobacteria	Burkholderiaceae	Curvibacter								w	w	0.93	0.29	0.52	0.02
Proteobacteria	Burkholderiaceae	Burkholderia-Caballeronia-Paraburkholderia								w	w				
Proteobacteria	Acetobacteraceae	Endobacter								w	w	1.00	0.24	0.49	0.03
Proteobacteria	Beijerinckiaceae	NA								w	w	1.00	0.24	0.49	0.03
Proteobacteria	Beijerinckiaceae	NA								w	w	0.92	0.43	0.63	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.91	0.43	0.63	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.88	0.38	0.58	0.03
Proteobacteria	Burkholderiaceae	Curvibacter								w	w	0.87	0.38	0.58	0.04
Proteobacteria	Acetobacteraceae	Endobacter								c	c				

Proteobacteria	Acetobacteraceae	Endobacter								c	c				
Firmicutes	Staphylococcaceae	Staphylococcus	16.14	-23.10	2.97	-7.78	0.00	0.00	c		c	0.77	0.35	0.52	0.05
Firmicutes	Staphylococcaceae	Staphylococcus	13.21	-22.83	2.97	-7.69	0.00	0.00	c		c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.29	0.54	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c				
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c				
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.24	0.49	0.03
Proteobacteria	Acetobacteraceae	NA								c	c	1.00	0.24	0.49	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.24	0.49	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.86	0.81	0.83	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.86	0.76	0.81	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.86	0.67	0.76	0.00
Proteobacteria	Acetobacteraceae	Endobacter								w	w				
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.84	0.35	0.55	0.04
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.64	0.90	0.76	0.03
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.62	0.90	0.75	0.04
Proteobacteria	Acetobacteraceae	Endobacter								w	w	0.79	0.41	0.57	0.04
Proteobacteria	Beijerinckiaceae	NA								c	c	1.00	0.24	0.49	0.02
Proteobacteria	Beijerinckiaceae	NA								c	c	0.79	0.59	0.68	0.01
Proteobacteria	Beijerinckiaceae	NA								w	w	0.90	0.29	0.52	0.04
Proteobacteria	Beijerinckiaceae	NA								w	w	0.89	0.38	0.58	0.02
Proteobacteria	Beijerinckiaceae	NA								w	w	0.67	0.76	0.71	0.03

Proteobacteria	Beijerinckiaceae	NA	26.76	6.30	2.11	2.98	0.00	0.09	w		w	0.66	0.76	0.71	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.79	0.59	0.68	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.80	0.53	0.65	0.01
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	1.00	0.24	0.49	0.03
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.96	0.29	0.52	0.04
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.90	0.38	0.59	0.02
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.91	0.33	0.55	0.03
Acidobacteria	Acidobacteriaceae	Bryocella	16.74	-23.04	2.97	-7.76	0.00	0.00	c		c	0.74	0.52	0.62	0.03
Acidobacteria	Acidobacteriaceae	Bryocella	16.88	-23.16	2.97	-7.80	0.00	0.00	c		c	0.73	0.52	0.62	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	1.00	0.29	0.54	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.29	0.54	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								c	c				
Acidobacteria	Acidobacteriaceae	Granulicella	42.66	-24.40	2.97	-8.22	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	38.51	-24.08	2.97	-8.11	0.00	0.00	c		c	0.83	0.48	0.63	0.02
Acidobacteria	Acidobacteriaceae	NA								w	w	0.79	0.48	0.61	0.04
Acidobacteria	Acidobacteriaceae	NA								w	w	1.00	0.29	0.54	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	1.00	0.24	0.49	0.03
Acidobacteria	Acidobacteriaceae	NA								c	c	0.88	0.81	0.84	0.00
Acidobacteria	Acidobacteriaceae	NA								c	c	0.89	0.76	0.82	0.00
Acidobacteria	Acidobacteriaceae	NA								c	c	0.89	0.67	0.77	0.00
Acidobacteria	Acidobacteriaceae	NA	87.02	-3.76	1.27	-2.95	0.00	0.10	c		c	0.87	0.67	0.76	0.00
Proteobacteria	Burkholderiaceae	Curvibacter								w	w				

Proteobacteria	Acetobacteraceae	Endobacter	c	c				
Proteobacteria	Acetobacteraceae	Endobacter	c	c	0.65	0.90	0.77	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.64	0.90	0.76	0.02
Proteobacteria	Beijerinckiaceae	NA	w	w	0.85	0.41	0.59	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.78	0.41	0.57	0.03
Proteobacteria	Burkholderiaceae	Curvibacter	w	w	0.90	0.48	0.66	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	0.82	0.38	0.56	0.04
Proteobacteria	Acetobacteraceae	NA	c	c	0.66	0.81	0.73	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.66	0.81	0.73	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.64	0.81	0.72	0.04
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.82	0.35	0.54	0.03
Proteobacteria	Acetobacteraceae	NA	w	w	0.77	0.53	0.64	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	1.00	0.29	0.54	0.01

Supplementary Table 5 Indicator and differentially abundant ASVs (every row represents a single ASV) in the cDNA-based bacterial communities associated with *Cetraria islandica*. NA indicates ASVs not assigned to genus level.

Phylum	Family	Genus	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Diffab	Indicator species	comb	A	B	Indstat	Indpvalue
Acidobacteria	Acidobacteriaceae	Terriglobus								w	w	0.90	0.24	0.46	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.86	0.43	0.61	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.84	0.43	0.60	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.92	0.38	0.59	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.72	0.48	0.59	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.69	0.57	0.63	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.71	0.52	0.61	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.71	0.48	0.58	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.79	0.52	0.65	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.79	0.43	0.58	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.68	0.76	0.72	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.30	0.55	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.30	0.55	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.92	0.38	0.59	0.00

Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.33	0.58	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.29	0.54	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.75	0.43	0.57	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.82	0.33	0.52	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.69	0.67	0.68	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.82	0.48	0.62	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.91	0.38	0.59	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.72	0.48	0.59	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.70	0.61	0.65	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.74	0.57	0.65	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.71	0.48	0.58	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.78	0.48	0.61	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.65	0.71	0.68	0.05
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	1.00	0.26	0.51	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	1.00	0.26	0.51	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.29	0.54	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.84	0.29	0.49	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.84	0.29	0.49	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.83	0.48	0.63	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.77	0.38	0.54	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.70	0.57	0.63	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.19	0.44	0.05

Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.19	0.44	0.05
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.90	0.52	0.69	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.83	0.52	0.66	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.92	0.38	0.59	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.70	0.48	0.58	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.71	0.57	0.63	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.71	0.52	0.61	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	0.73	0.48	0.59	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.75	0.43	0.57	0.05
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.67	0.81	0.74	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.66	0.71	0.69	0.05
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	1.00	0.30	0.55	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	c	c	1.00	0.30	0.55	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.88	0.43	0.62	0.01
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.33	0.58	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	1.00	0.33	0.58	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.77	0.38	0.54	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.81	0.33	0.52	0.02
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.69	0.57	0.63	0.05
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.93	0.52	0.70	0.00
Acidobacteria	Acidobacteriaceae	Granulicella	w	w	0.92	0.33	0.55	0.01
Acidobacteria	Acidobacteriaceae	NA	w	w	0.93	0.57	0.73	0.00

Acidobacteria	Acidobacteriaceae	NA	w	w	1.00	0.52	0.72	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	0.92	0.52	0.69	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	1.00	0.29	0.54	0.01
Acidobacteria	Acidobacteriaceae	NA	c	c	0.76	0.70	0.73	0.00
Acidobacteria	Acidobacteriaceae	NA	c	c	0.78	0.65	0.71	0.00
Acidobacteria	Acidobacteriaceae	NA	c	c	0.80	0.57	0.67	0.01
Acidobacteria	Acidobacteriaceae	NA	w	w	0.80	0.62	0.70	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	0.74	0.62	0.68	0.01
Acidobacteria	Acidobacteriaceae	NA	w	w	0.78	0.52	0.64	0.01
Acidobacteria	Acidobacteriaceae	NA	c	c	0.71	0.52	0.61	0.04
Acidobacteria	Acidobacteriaceae	NA	w	w	1.00	0.48	0.69	0.00
Acidobacteria	Acidobacteriaceae	NA	c	c	0.87	0.74	0.80	0.00
Acidobacteria	Acidobacteriaceae	NA	c	c	0.80	0.70	0.74	0.00
Acidobacteria	Acidobacteriaceae	NA	c	c	0.82	0.65	0.73	0.00
Acidobacteria	Acidobacteriaceae	NA	c	c	0.90	0.57	0.71	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	0.80	0.71	0.76	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	0.69	0.62	0.66	0.01
Acidobacteria	Acidobacteriaceae	NA	w	w	0.74	0.52	0.62	0.02
Acidobacteria	Acidobacteriaceae	NA	c	c	0.70	0.57	0.63	0.04
Acidobacteria	Acidobacteriaceae	NA	w	w	0.93	0.62	0.76	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	1.00	0.57	0.76	0.00
Acidobacteria	Acidobacteriaceae	NA	w	w	1.00	0.57	0.76	0.00

Acidobacteria	Acidobacteriaceae	NA								c	c	0.85	0.74	0.79	0.00
Acidobacteria	Acidobacteriaceae	NA								c	c	0.80	0.70	0.75	0.00
Acidobacteria	Acidobacteriaceae	NA								c	c	0.78	0.70	0.74	0.00
Acidobacteria	Acidobacteriaceae	NA								w	w	0.71	0.62	0.66	0.01
Acidobacteria	Acidobacteriaceae	NA								w	w	0.76	0.57	0.66	0.01
Acidobacteria	Acidobacteriaceae	NA								w	w	0.75	0.52	0.63	0.01
Acidobacteria	Acidobacteriaceae	Terriglobus								w	w	0.93	0.33	0.56	0.01
Acidobacteria	Acidobacteriaceae	Bryocella	30.99	-7.60	2.29	-3.31	0.00	0.05	c		c				
Acidobacteria	Acidobacteriaceae	Bryocella	25.28	-6.96	1.92	-3.63	0.00	0.02	c		c				
Acidobacteria	Acidobacteriaceae	Bryocella	15.39	-6.59	2.01	-3.28	0.00	0.05	c		c				
Acidobacteria	Acidobacteriaceae	Bryocella	30.05	-7.21	2.32	-3.11	0.00	0.08	c		c				
Acidobacteria	Acidobacteriaceae	Bryocella	12.29	-7.18	2.36	-3.04	0.00	0.10	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	26.60	-24.29	2.96	-8.21	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	25.20	-24.22	2.96	-8.19	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	31.95	-24.54	2.96	-8.30	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	30.71	-24.50	2.96	-8.28	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	27.02	-24.32	2.96	-8.22	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	24.91	-23.52	2.95	-7.96	0.00	0.00	c		c				
Acidobacteria	Acidobacteriaceae	NA	80.77	-3.64	1.20	-3.04	0.00	0.10	c	c	c	0.83	0.74	0.79	0.00
Acidobacteria	Acidobacteriaceae	NA	9.16	6.22	1.79	3.47	0.00	0.03	w		w				
Acidobacteria	Acidobacteriaceae	NA	12.09	6.63	1.92	3.46	0.00	0.03	w		w				
Acidobacteria	Acidobacteriaceae	NA	13.86	6.82	1.88	3.62	0.00	0.02	w	w	w	1.00	0.52	0.72	0.00

Acidobacteria	Acidobacteriaceae	NA	72.11	-4.68	1.13	-4.14	0.00	0.00	c		c					
Acidobacteria	Acidobacteriaceae	NA	64.79	-3.86	1.03	-3.75	0.00	0.01	c		c					
Acidobacteria	Acidobacteriaceae	NA	39.40	-3.76	1.25	-3.02	0.00	0.10	c		c					
Acidobacteria	Acidobacteriaceae	NA	9.91	6.34	1.79	3.54	0.00	0.03	w		w					
Acidobacteria	Acidobacteriaceae	NA	14.56	6.89	1.69	4.07	0.00	0.00	w		w					
Acidobacteria	Acidobacteriaceae	NA	15.19	-23.53	2.96	-7.95	0.00	0.00	c		c					
Acidobacteria	Acidobacteriaceae	NA	82.32	-3.86	1.17	-3.29	0.00	0.05	c		c					
Acidobacteria	Acidobacteriaceae	NA	71.81	-3.50	1.04	-3.37	0.00	0.04	c		c					
Bacteroidetes	Bacteroidaceae	Bacteroides	4.36	20.40	2.96	6.89	0.00	0.00	w		w					
Bacteroidetes	Muribaculaceae	NA	4.14	20.36	2.96	6.88	0.00	0.00	w		w					
Bacteroidetes	Muribaculaceae	NA	4.41	20.44	2.96	6.91	0.00	0.00	w		w					
Bacteroidetes	Muribaculaceae	NA	5.94	20.66	2.96	6.98	0.00	0.00	w		w					
Chloroflexi	Ktedonobacteraceae	NA	17.67	-23.74	2.96	-8.03	0.00	0.00	c		c					
Firmicutes	Lactobacillaceae	Lactobacillus	7.47	21.18	2.96	7.16	0.00	0.00	w		w					
Firmicutes	Lactobacillaceae	Lactobacillus	9.03	21.44	2.96	7.25	0.00	0.00	w		w					
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.92	0.52	0.69	0.00	
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.76	0.52	0.63	0.02	
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.64	0.91	0.76	0.01	
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.67	0.87	0.76	0.01	
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.62	0.91	0.75	0.02	
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.88	0.52	0.68	0.00	
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	1.00	0.43	0.66	0.00	

Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.86	0.57	0.70	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.83	0.52	0.66	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.86	0.38	0.57	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.82	0.33	0.52	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	1.00	0.33	0.58	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.82	0.33	0.52	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.85	0.57	0.69	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.75	0.52	0.63	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.65	0.96	0.79	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.69	0.87	0.78	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.63	0.91	0.76	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.83	0.48	0.63	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.86	0.38	0.57	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	0.86	0.29	0.50	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.67	0.65	0.66	0.05
Proteobacteria	Acetobacteraceae	Acidiphilium	22.36	-5.45	1.45	-3.75	0.00	0.01	c	c	c	0.96	0.57	0.74	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium	21.96	-5.23	1.63	-3.22	0.00	0.06	c	c	c	0.95	0.52	0.70	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium	11.00	-5.42	1.73	-3.13	0.00	0.08	c	c	c	0.95	0.39	0.61	0.01
Proteobacteria	Acetobacteraceae	Acidisphaera								w	w	0.73	0.67	0.70	0.01
Proteobacteria	Acetobacteraceae	Acidisphaera								w	w	0.69	0.62	0.65	0.02
Proteobacteria	Acetobacteraceae	Acidisphaera								w	w	0.69	0.52	0.60	0.04
Proteobacteria	Acetobacteraceae	Acidisphaera								c	c	0.66	0.87	0.76	0.01

Proteobacteria	Acetobacteraceae	Acidisphaera	w	w	0.76	0.62	0.69	0.01
Proteobacteria	Acetobacteraceae	Acidisphaera	w	w	0.70	0.67	0.68	0.02
Proteobacteria	Acetobacteraceae	Acidisphaera	w	w	0.74	0.43	0.56	0.05
Proteobacteria	Acetobacteraceae	Acidisphaera	c	c	0.74	0.78	0.76	0.00
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.82	0.33	0.52	0.05
Proteobacteria	Acetobacteraceae	Endobacter	w	w	1.00	0.24	0.49	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.71	0.71	0.71	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.70	0.71	0.71	0.03
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.68	0.71	0.70	0.03
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.74	0.62	0.68	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.91	0.52	0.69	0.00
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.90	0.43	0.62	0.00
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.89	0.38	0.58	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	1.00	0.29	0.54	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.78	0.62	0.70	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.79	0.38	0.55	0.03
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.78	0.38	0.54	0.04
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.77	0.38	0.54	0.04
Proteobacteria	Acetobacteraceae	Endobacter	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.71	0.71	0.71	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.70	0.71	0.71	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.69	0.71	0.70	0.02

Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.90	0.43	0.62	0.00
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.83	0.43	0.60	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.77	0.57	0.67	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.80	0.38	0.55	0.03
Proteobacteria	Acetobacteraceae	Endobacter	w	w	1.00	0.29	0.54	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.72	0.71	0.72	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.70	0.71	0.71	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.70	0.71	0.71	0.02
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.90	0.43	0.62	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.81	0.33	0.52	0.04
Proteobacteria	Acetobacteraceae	Endobacter	w	w	1.00	0.19	0.44	0.04
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.76	0.57	0.66	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.76	0.57	0.66	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.73	0.71	0.72	0.01
Proteobacteria	Acetobacteraceae	Endobacter	w	w	0.72	0.71	0.72	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.24	0.49	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.65	0.87	0.75	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.64	0.87	0.75	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.71	0.48	0.58	0.04
Proteobacteria	Acetobacteraceae	NA	w	w	0.87	0.38	0.58	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.87	0.38	0.58	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.73	0.71	0.72	0.00

Proteobacteria	Acetobacteraceae	NA	w	w	0.87	0.33	0.54	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.29	0.54	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.74	0.48	0.60	0.03
Proteobacteria	Acetobacteraceae	NA	c	c	0.71	0.83	0.77	0.00
Proteobacteria	Acetobacteraceae	NA	c	c	0.70	0.83	0.76	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.24	0.49	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.63	0.87	0.74	0.03
Proteobacteria	Acetobacteraceae	NA	c	c	0.63	0.87	0.74	0.03
Proteobacteria	Acetobacteraceae	NA	w	w	0.85	0.38	0.57	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.89	0.43	0.62	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.85	0.43	0.61	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.83	0.38	0.56	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.72	0.62	0.67	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.24	0.49	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.81	0.43	0.59	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.84	0.38	0.56	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.80	0.38	0.55	0.03
Proteobacteria	Acetobacteraceae	NA	c	c	0.73	0.78	0.76	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.24	0.49	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.65	0.87	0.75	0.01
Proteobacteria	Acetobacteraceae	NA	c	c	0.65	0.87	0.75	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.84	0.29	0.49	0.04

Proteobacteria	Acetobacteraceae	NA	w	w	0.83	0.48	0.63	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.90	0.43	0.62	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	0.87	0.43	0.61	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	0.74	0.76	0.75	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	0.69	0.67	0.68	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.74	0.62	0.68	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.77	0.43	0.58	0.03
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Acetobacteraceae	NA	c	c	0.71	0.83	0.77	0.00
Proteobacteria	Acetobacteraceae	NA	c	c	0.71	0.83	0.77	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	1.00	0.24	0.49	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.65	0.87	0.75	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	0.64	0.87	0.75	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.82	0.38	0.56	0.02
Proteobacteria	Caulobacteraceae	NA	w	w	0.92	0.38	0.59	0.01
Proteobacteria	Caulobacteraceae	NA	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Caulobacteraceae	NA	w	w	0.94	0.29	0.52	0.02
Proteobacteria	Caulobacteraceae	NA	w	w	0.90	0.24	0.46	0.04
Proteobacteria	Beijerinckiaceae	1174-901-12	w	w	0.90	0.48	0.66	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12	w	w	1.00	0.43	0.66	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12	w	w	0.87	0.33	0.54	0.02
Proteobacteria	Beijerinckiaceae	1174-901-12	w	w	0.84	0.29	0.49	0.04

Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	0.86	0.48	0.64	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	1.00	0.33	0.58	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	0.86	0.43	0.61	0.01
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	0.81	0.43	0.59	0.01
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	0.77	0.38	0.54	0.03
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	1.00	0.48	0.69	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	1.00	0.43	0.66	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	1.00	0.33	0.58	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12								w	w	0.77	0.43	0.57	0.03
Proteobacteria	Beijerinckiaceae	1174-901-12	12.44	6.67	1.93	3.46	0.00	0.03	w		w				
Proteobacteria	Beijerinckiaceae	1174-901-12	10.36	6.40	1.96	3.27	0.00	0.05	w	w	w	1.00	0.43	0.66	0.00
Proteobacteria	Beijerinckiaceae	1174-901-12	11.55	6.56	1.94	3.38	0.00	0.04	w		w				
Proteobacteria	Beijerinckiaceae	1174-901-12	12.32	6.65	1.94	3.43	0.00	0.03	w		w				
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.81	0.48	0.62	0.01
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.77	0.43	0.57	0.03
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.93	0.29	0.52	0.02
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.84	0.43	0.60	0.01
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.77	0.43	0.57	0.03
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.78	0.38	0.55	0.04
Proteobacteria	Beijerinckiaceae	Methylorosula								w	w	0.90	0.29	0.51	0.02
Proteobacteria	Beijerinckiaceae	NA								w	w	1.00	0.19	0.44	0.05
Proteobacteria	Beijerinckiaceae	NA								w	w	1.00	0.19	0.44	0.05

Proteobacteria	Beijerinckiaceae	NA	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Beijerinckiaceae	NA	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Beijerinckiaceae	NA	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Beijerinckiaceae	NA	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Sphingomonadaceae	Novosphingobium	w	w	1.00	0.19	0.44	0.05
Proteobacteria	Sphingomonadaceae	Sphingomonas	w	w	0.68	0.62	0.65	0.03
Proteobacteria	Sphingomonadaceae	Sphingomonas	w	w	0.71	0.62	0.67	0.01
Proteobacteria	Sphingomonadaceae	Sphingomonas	w	w	0.68	0.62	0.65	0.03
Proteobacteria	Sphingomonadaceae	Sphingomonas	w	w	1.00	0.19	0.44	0.04
Proteobacteria	Sphingomonadaceae	Sphingomonas	w	w	0.70	0.57	0.63	0.02
Proteobacteria	Diplorickettsiaceae	NA	w	w	1.00	0.19	0.44	0.04

Paper II

Long-term warming effects on the microbiome and nitrogen fixation of a common moss species in sub-Arctic tundra

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Abstract

Bacterial communities form the basis of biogeochemical processes and determine plant growth and health. Mosses, an abundant plant group in Arctic ecosystems, harbour diverse bacterial communities that are involved in nitrogen fixation and carbon cycling. Global climate change is causing changes in aboveground plant biomass and shifting species composition in the Arctic, but little is known about the response of moss microbiomes.

Here, we studied the total and potentially active bacterial community associated with *Racomitrium lanuginosum*, in response to 20-year *in situ* warming in an Icelandic heathland. We evaluated the effect of warming and warming-induced shrub expansion on the moss bacterial community composition and diversity, *nifH* gene abundance and nitrogen-fixation rates.

Warming changed both the total and the potentially active bacterial community structure, while litter abundance only affected the total bacterial community structure. The relative abundance of Proteobacteria increased, while the relative abundance of Cyanobacteria and Acidobacteria decreased. *NifH* gene abundance and nitrogen-fixation rates were negatively affected by litter and *Betula nana* abundance, respectively. We also found shifts in the potentially nitrogen-fixing community, with *Nostoc* decreasing and non-cyanobacterial diazotrophs increasing in relative abundance. Our data suggests that the moss microbial community including the potentially nitrogen-fixing taxa is sensitive to future warming.

Long-term warming led to a shift in moss-associated bacterial community composition, while the abundance of nitrogen-fixing bacteria and nitrogen-fixation rates were negatively affected by increased litter and *Betula nana* abundance respectively. Warming and increased shrub abundance as a result of warming can affect moss-associated bacterial communities and nitrogen fixation rates in tundra ecosystems.

Introduction

Temperature in high-latitude regions is rising twice as fast as elsewhere (IPCC 2019), which is predicted to have large impacts on Arctic ecosystems, for instance by altering species distributions and interactions (Wookey et al. 2009; Van der Putten 2012). One such interaction that might be affected by warming is the association between mosses and bacterial communities as well as related ecosystem processes such as pedogenesis, carbon (C) cycling, and nitrogen (N) cycling.

Bryophytes, mosses in particular, comprise a large component of the vegetation in many high-latitude ecosystems (Longton 1992). They play important roles in biogeochemical cycles by forming a C sink via their slow decomposition rates, by accounting for up to 7% of terrestrial net primary productivity and by supporting up to half of the terrestrial N_2-fixation (Turetsky 2003; Cornelissen et al. 2007; Turetsky et al. 2012; 2012; Porada et al. 2013). Most mosses consist of a upper living segment with photosynthetic tissue and a lower decaying dead segment and thus link above-ground and belowground processes (Whiteley and Gonzalez 2016). Mosses provide a habitat for a range of microbiota, microfauna and mesofauna (Lindo and Gonzalez 2010). These moss-associated microorganisms are involved in the decomposition of dead moss tissue (Kulichevskaya et al. 2007) and some of them are active diazotrophs (Chen et al. 2019). N_2-fixation by moss-associated Cyanobacteria, the best studied of these diazotrophs, was shown to directly increase moss growth rates (Berg, Danielsson, and Svensson 2013) and thereby control C sequestration in moss tissues. Moss-associated diazotrophy is also an important source of new available N in boreal and Arctic ecosystems (DeLuca et al. 2002; Rousk, Sorensen, and Michelsen 2017). In order to understand the implications of climate change for the role of mosses in ecosystem C and N cycling, we need to understand how moss-associated microbial communities react to elevated temperatures.

The bacterial community composition of mosses is species specific and influenced by environmental factors such as pH and nutrient availability (Holland-Moritz et al. 2018; Bragina, Berg, et al. 2012; Tang et al. 2016). While Cyanobacteria have received most of the attention for their N_2-fixing capability (Lindo, Nilsson, and Gundale 2013; Berg, Danielsson, and Svensson 2013; Gentili et al. 2005; Warshan et al. 2016; 2017; Stewart et al. 2011; Ininbergs et al. 2011; Rousk, Jones, and DeLuca 2013), mosses harbour diverse bacterial communities. Commonly found phyla associated with mosses include Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Cyanobacteria, Planctomycetes, Proteobacteria and Verrucomicrobia (Tang et al. 2016; Kostka et al. 2016), and their potential functions include N_2-fixation (Bragina, Maier, et al. 2012), anoxygenic phototrophy (Holland-Moritz et al. 2018) and freeze protection (J. A. Raymond 2016). The bacterial community composition of mosses has primarily been studied for peat and feather mosses, but we know little about the bacterial communities of other moss species. For instance, little is known about the bacterial community associated with ecologically important moss species such as *Racomitrium lanuginosum* (Hedw.) Brid. This moss species has a wide distribution at high altitudes in temperate regions of the Northern and Southern Hemisphere and at low altitudes in the Arctic (Tallis 1995; Jonsdottir, Callaghan, and Lee 1995). It is a dominant species in many Icelandic ecosystems, forming dense mats where conditions are favourable for colonisation and growth (Ingimundardóttir, Weibull, and Cronberg 2014; Bjarnason 1991; Tallis 1958).

Despite the importance of microbial communities for plant functioning and ecosystem processes, the long-term effect of warming on moss microbial communities has received little attention. Two studies describing the effect of four weeks to two years warming-related changes in peat moss bacterial community composition, reported a decrease in overall bacterial and diazotrophic diversity with higher temperatures *in situ* and under laboratory conditions (Kolton et al. 2019; Carrell et al. 2019). Whether this warming-induced decrease in diversity also holds for bacterial communities associated with other moss species in high latitudes is unknown. Moreover, decades-long-warming effects on moss-associated bacterial communities have yet to be explored.

Nonetheless, the effect of warming on some high-latitude plant communities has been better documented, where for instance ambient and experimental warming (ranging from 5-43 years) in tundra heaths have resulted in shrub expansion (Myers-Smith et al. 2011; Bjorkman et al. 2020; Myers-Smith et al. 2019). The increase in deciduous dwarf shrubs, for example *Betula nana*, led to an increase in the quantity of relatively high quality litter, resulting in a faster turnover of the overall leaf litter C and N (McLaren et al. 2017). This warming-induced change in litter quality and nutrient cycling might also affect the composition of microbial communities (Deslippe et al. 2012). Indeed, changing litter inputs can consequently lead to shifts in moss microbiomes (Jean et al. 2020b). The increase in labile shrub litter may lead to an increase in copiotrophic taxa and decrease in oligotrophic taxa (Fierer, Bradford, and Jackson 2007; Matthew David Wallenstein, McMahon, and Schimel 2007). Warming might thus also, indirectly, via a change in leaf litter quality and quantity resulting from increasing shrub biomass, lead to changes in the bacterial communities associated with the moss layer. Changes in bacterial community composition could consequently affect N_2-fixation rates (Wu et al. 2020). In addition, N_2-fixation rates can be expected to increase with temperature, as metabolic process rate in microorganisms increases with temperature and the enzyme nitrogenase is more active at higher temperatures than average Arctic temperatures (Houlton et al. 2008). Temperature-induced drought, however, can inhibit N_2-fixation rates, especially cyanobacterial N_2-fixation (Zielke et al. 2005; Stewart et al. 2011; Stewart, Coxson, and Grogan 2011; Stewart et al. 2014; Rousk, Jones, and DeLuca 2014a; Rousk et al. 2015; Whiteley and Gonzalez 2016; Rousk, Sorensen, and Michelsen 2018b). Indirect effects of temperature on N_2-fixation rates might also be related to physiological adaptation of diazotrophic communities (Whiteley and Gonzalez 2016), or shifts to a species composition better suited to the new conditions (Deslippe, Egger, and Henry 2005; Rousk and Michelsen 2017; Rousk, Sorensen, and Michelsen 2018b). Warming-induced changes in bacterial species composition could potentially feedback to the abundance, diversity and/or N_2-fixation activity of diazotrophs, through alteration of biotic interactions between bacteria e.g. competition and/or cooperation (Ho et al. 2016). The increase in shrubs might also affect N_2-fixation rates, either negatively via an increase in shading leading to an decrease in N_2-fixation rates, or either inhibit or promote N_2-fixation depending on the nutrient content of the litter (Rousk and Michelsen 2017; Sorensen and Michelsen 2011).

In this study we investigated how two decades of experimental warming with open top chambers impact the bacterial community and N_2-fixation rates associated with the prevailing moss *R. lanuginosum* (Hedw.) Brid in a subarctic-alpine dwarf shrub heath in northern Iceland, dominated by *B. nana*.

We hypothesized that long-term warming directly and/or indirectly via the warming-induced increase in labile *B. nana* litter (1) leads to a shift in bacterial community composition with a decrease in bacterial diversity and (2) leads to a decrease in oligotrophic taxa and an

increase in copiotrophic taxa. Further, we hypothesized (3) that changes in N_2-fixation rates will depend on the combination of the direct effect of warming leading to an increase in N_2-fixation rates and indirect effects of warming. These indirect effects include shading leading to a decrease in N_2-fixation rates; increased litter leading to an increase or a decrease in N_2-fixation rates; and/or changes in the bacterial community that could mediate the effects of warming, shading and/or litter on N_2-fixation rates. To address these hypotheses, we sampled *R. lanuginosum* in a warming simulation experiment in the northwest highlands of Iceland that has been running for 20 years (Jonsdottir et al. 2005). We assessed the associated bacterial community structure by 16S rRNA gene and rRNA amplicon sequencing, N_2-fixation rates with acetylene reduction assays (ARA), and N_2-fixation potential using quantitative PCR (qPCR) of the *nifH* gene encoding the iron-protein component of the nitrogenase.

Methods

Field site and experimental design

The sampling was conducted in permanent plots of a long-term warming simulation experiment at Auðkúluheiði in the northwest highlands of Iceland (65°16'N, 20°15'W, 480 m above sea level). The site is a part of the International Tundra Experiment (ITEX; Henry and Molau 1997) and according to Köppen's climate definitions, the sampling site is situated within the lower Arctic (Köppen 1931). The vegetation has been characterized as a relatively species-rich dwarf shrub heath, with *B. nana* being the most dominant vascular species and *R. lanuginosum* and *Cetraria islandica* as the dominating moss and lichen species (Jonsdottir et al. 2005). The experimental site has been fenced off since 1996 to prevent sheep from disturbing the experiment.

Ten plot pairs of 75x75 cm were selected and one of the plots in each pair was randomly assigned to a warming treatment while the other served as a control. Open top plexiglass chambers (OTCs) were set up in August 1996 and 1997 to simulate a warmer summer climate and have been in place throughout the year ever since (Hollister and Webber 2000; Jonsdottir et al. 2005). The temperature in the OTCs was on average 1.4 °C higher in June 2016 to August 2016 and 0.22 °C higher from August 2018 to June 2019 (Table S1). Relative humidity was -3 % lower in the OTCs in June 2016 to August 2016 (Table S1).

The vegetation responses were monitored by a detailed vegetation analysis after peak biomass at a few year intervals using the point intercept method following standard protocols of the International Tundra Experiment (Molau and Mølgaard 1996): 100 points per plot, all hits (intercepts) per species recorded in each point through the canopy; relates to biomass) (Jonsdottir et al. 2005). In this study we use data from August 2014 on abundance (total number of hits per plot) for *R. lanuginosum*, *B. nana* and litter to test hypotheses 1-3 (Table S2). In 2014 the abundance of *R. lanuginosum* was on average 0.8 times lower in the warmed plots than control plots, but not significantly, while the abundance of *B. nana* was 2.5 times greater in the warm plots on average and litter was 2.7 times greater (Table S2).

RNA and DNA extraction and sequencing

To assess overall bacterial community structure and bacterial diversity (hypothesis 1 and 2) associated with *R. lanuginosum* we collected moss shoots, extracted DNA and RNA and used 16S rRNA gene amplicon sequencing. For RNA and DNA extraction we collected *R.*

lanuginosum moss shoots in June 2017. Per warmed (OTC) and control plot, five moss shoots were collected with sterile tweezers. In total 50 OTC and 50 control samples were collected. The moss shoots were immediately soaked in RNAlater (Ambion) to prevent RNA degradation and kept cool until storage at -80 °C. Prior to extraction, the samples were rinsed with RNase free water to remove soil particles and RNAlater and ground for six minutes using a Mini-Beadbeater and two sterile steel beads. RNA and DNA were extracted simultaneously using the RNeasy PowerSoil Total RNA Kit (Qiagen) and the RNeasy PowerSoil DNA Elution Kit (Qiagen), following the manufacturer's instructions. DNA and RNA concentrations were determined with a Qubit Fluorometer (Life Technologies) and quality was assessed with a NanoDrop (NanoDrop Technologies) and Bioanalyzer (Agilent Technologies). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher) following the manufacturer's instructions and quantified on a Qubit Fluorometer (Life Technologies). All DNA extractions (100 samples) were used for qPCR. From all DNA and cDNA samples, we selected 48 DNA samples (24 from each treatment) and 48 cDNA samples (24 from each treatment) for sequencing based on RNA and DNA quality and quantity. Library preparation and sequencing of the V3-V4 region of the 16S rRNA gene on an Illumina MiSeq platform (2 x 300 bp) was performed by Macrogen, Seoul, using MiSeq v3 reagents and the primer pair 337F/805R and the PCR conditions described in (Klindworth et al. 2013).

Sequence processing

In order to obtain high-resolution data and to better discriminate ecological patterns, we processed the raw sequences using the DADA2 (version 1.12.1) pipeline (Callahan et al. 2016; Callahan, McMurdie, and Holmes 2017), which does not cluster sequences into operational taxonomic units (OTUs), but uses exact sequences or amplicon sequence variants (ASVs). Forward reads were truncated at 260 bp and reverse reads at 250 bp. Assembled ASVs were assigned taxonomy using the Ribosomal Database Project (RDP) naïve Bayesian classifier (Q. Wang et al. 2007) in DADA2 and the SILVA_132 database (Quast et al. 2013). We removed samples with less than 10.000 non-chimeric sequences (11 samples) and we removed ASVs assigned to chloroplasts and mitochondria, singletons, as well as ASVs present in only one sample. In total, for 85 samples, 3598 ASVs remained with an average read size of 448 bp after DADA2. To account for uneven sequencing depths, the data were normalised using cumulative-sum scaling (CSS) (Paulson et al. 2013). The 16S rRNA gene based community is hereafter sometimes referred to as the 'total bacterial community' and the 16S rRNA (cDNA) based community is hereafter referred to as the 'potentially metabolically active bacterial community', acknowledging that 16S rRNA is not a direct indicator of activity but rather protein synthesis potential (Blazewicz et al. 2013). Raw sequences are available in the European Nucleotide Archive under accession number PRJEB40635.

Quantitative real-time PCR of nifH and 16S rRNA genes

We used the DNA samples (100 samples (50 control and 50 OTC samples)) for quantification of *nifH* and 16S rRNA genes (to test hypothesis 3). This was performed by quantitative PCR (Corbett Rotor-Gene) using the primer set PolF/PolR and 341F/534R respectively (Poly, Monrozier, and Bally 2001). The specificity of the *nifH* primers for our samples was confirmed by SANGER sequencing of 10 clone fragments. Standards for *nifH* reactions were obtained by amplifying one cloned *nifH* sequence with flanking regions of the plasmid vector (TOPO TA cloning Kit, Invitrogen). Standard curves were obtained by

serial dilutions (E = 0.9 – 1.1, R^2 = > 0.99 for all reactions). Each reaction had a volume of 20 µL, containing 1x QuantiFast SYBR Green PCR Master Mix (Qiagen), 0.2 µL of each primer (10 µM), 0.8 µL BSA (5 µg/µL), 6.8 µL RNase free water and 2 µL template. The cycling program was 5 min at 95 °C, 30 cycles of 10 s at 95 °C and 30 s at 60 °C.

Acetylene reduction assays

We used acetylene reduction assays (ARA) to estimate N_2-fixation rate to test hypothesis 3. We followed the procedure described in DeLuca et al. (2002) and Zackrisson et al. (2004). We collected three moss shoots of 5 cm length per control plot and OTC in June and August 2014. The three shoots per plot were analysed separately. The moss shoots were placed in 20 mL vials with 2 mL deionized water. Moss shoots were acclimated in a growth chamber for 24 h at 10 °C and 200 µmol m^{-2} s^{-1} PAR. 10% of the headspace was replaced by acetylene. After an additional 24 h of incubation in the growth chamber under the same conditions, acetylene reduction and ethylene production were measured by gas chromatography.

Statistical analysis

All statistical analyses were performed in R (version 3.6.3). Richness (number of ASVs) and Shannon diversity were calculated with the R packages 'vegan' (version 2.5-4) (Oksanen et al. 2013) and 'phyloseq' (version 1.28.0) (McMurdie and Holmes 2013). Differences in N_2-fixation rates, 16S rRNA and *nifH* gene abundance, ASV richness and Shannon diversity (hypothesis 1) between the control and warmed plots were assessed with generalised linear mixed models using a Bayesian method that relies on Markov Chain Monte Carlo (MCMC) iterations. In these models we treated treatment (control or OTC), *B. nana* abundance and litter abundance as fixed factors and plot as a random factor to account for repeated sampling within plots, using the R package 'MCMCglmm' (version 2.29) (Hadfield 2010). For all models, we used as many iterations as necessary to allow for model convergence and an effective sample size of at least 1000. Interferences of differences between the control and warmed estimates were based on the posterior mode estimates and the 95% Highest Posterior Density Credible Intervals.

We tested the effect of treatment, *B. nana* abundance and litter abundance on the bacterial community composition with PERMANOVAs (Anderson 2001). All PERMANOVAs were based on Bray-Curtis distance matrices and were performed using the *adonis* function in the R package 'vegan' (version 2.5-6). We also tested whether samples taken from the same plot were similar to each other using PERMANOVAs. Plot indeed had a significant effect on the cDNA-based bacterial community composition, but not on the DNA-based bacterial community composition (Table S3 and S4). To reduce possible biases related to samples coming from the same plot, we used plot as *strata* in the PERMANOVAs testing the effect of treatment, *B. nana* abundance and litter abundance. In this way we controlled for the variation caused by repeated sampling within plots by limiting permutations within plots.

The relative abundances of taxa on phylum, class and order level between the warmed and the control samples (hypothesis 2) were tested using Wilcoxon rank-sum tests on plot averages (samples from the same plot were pooled for this purpose) using the *stat_compare_means* function from the R package 'ggpubr' (version 0.2.1) (Kassambara 2020).

Two methods were used to determine taxa on ASV level sensitive to warming (hypothesis 2). First, differential abundance of bacterial genera between warmed and control samples was assessed using the DESeq2 procedure (M. I. Love, Huber, and Anders 2014) on the non-CSS normalised datasets (with pseudoreplicates pooled per plot) with the R package 'DESeq2' (version 1.24.0) (M. I. Love, Huber, and Anders 2014). The adjusted *P*-value cut-off was 0.1 (M. I. Love, Huber, and Anders 2014). Differential abundance analysis only uses ASVs present in both the OTC and control samples. The second method we used to find taxa sensitive to warming, was the indicator species analysis. To find bacterial taxa indicative for the warming or the control treatment, correlation-based indicator species analysis was done with all possible site combinations using the function *multipatt* of the R package 'indicSpecies' (version 1.7.6) (De Caceres and Legendre 2009) based on 10^3 permutations. For this, we pooled all samples originating from the same plot. The indicator species analysis takes into account ASVs present in both OTC and control samples, but also ASVs present in only one of the treatments. We combined results of the DESeq2 and indicator species analysis into a final list of ASVs sensitive to warming. Data are presented as the number of significant ASVs identified in DESeq2 and/or indicator species analysis and represented at the genus level.

To test hypothesis 3, we used structural equation modelling to estimate the direct and indirect effects of warming on the bacterial community and the consequences for N_2-fixation. The structural equation models were fitted using the R package 'lavaan' (version 0.6-7). Initial models were constructed using current knowledge and hypotheses of effects of warming on plant-microbe interactions and on N_2-fixation activities. As variables included in the model, we used treatment, litter abundance, *B. nana* abundance, 16S rRNA abundance, *nifH* abundance, N_2-fixation rates and 'bacterial community structure'. The latter is a latent variable which consisted of the average of β-diversity and Shannon diversity index per plot for the combined cDNA and DNA data. β-diversity was derived from the first axis of a PCoA analysis. All variables were averaged per plot. We tested whether the model has a significant model fit according to the following criteria: $\chi 2/df < 2$, P-values ($P > 0.05$), root mean square error of approximation (rmsea) < 0.07 and goodness of fit index (GFI) > 0.9 (Hooper, Coughlan, and Mullen 2008).

Results

Treatment effect on bacterial diversity and community structure

The richness and Shannon diversity of the DNA-based and the cDNA-based bacterial communities did not differ significantly between control and OTC samples (Figs 1a-1d, Table S3). However, we found a negative effect of *B. nana* abundance on the richness and Shannon diversity of the cDNA-based bacterial community (richness: pMCMC = 0.004; Shannon diversity pMCMC = 0.01, Figs 1c-1d).

The PERMANOVA showed that treatment significantly influenced the DNA- and the cDNA-based community compositions of the moss (DNA: $R^2 = 0.05$, and $P < 0.001$ and cDNA: $R^2 = 0.04$, and $P < 0.001$; Table S6 and S7). In addition to the warming treatment, litter abundance also significantly influenced the DNA-based bacterial community composition ($R^2 = 0.03$, $P = 0.05$), but not the cDNA-based bacterial community composition (Table S6 and S7).

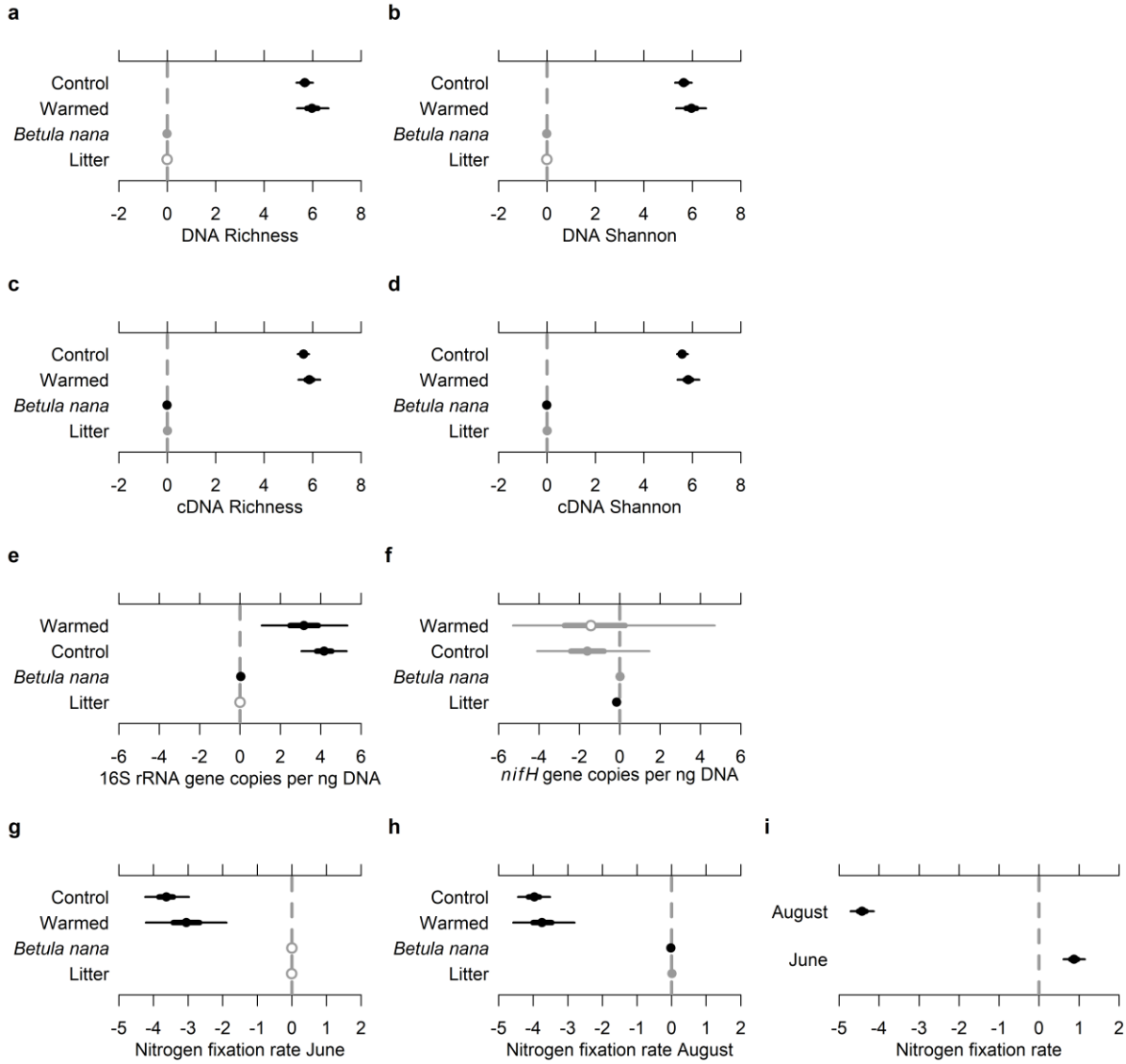

Figure 1 Fixed effect structure of the linear mixed-effect models testing the effect of treatment (warmed and control), *Betula nana* abundance and litter abundance on a) DNA-based richness and b) Shannon diversity, c) cDNA-based richness and d) Shannon diversity, e) 16 rRNA gene abundance, f) *nifH* gene abundance, N_2-fixation rate g) in June, h) in August and i) fixed effect structure of the linear mixed-effect model testing the difference between N_2-fixation rates in June and August. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 50% CrI overlapping 0 are indicated by open circles. Parameters with 50% CrI not overlapping 0, but with 95% CrI overlapping 0 are indicated by closed black circles. Thick lines represent 50% CrI and thin lines represent 95% CrI.

Taxonomic composition R. lanuginosum-associated bacterial communities

In the control samples, where bacterial communities were under ambient environmental conditions, the most abundant phyla in the DNA and cDNA samples included Proteobacteria (44% and 40% average relative abundance across all control DNA and cDNA samples respectively), followed by Acidobacteria (DNA: 29%, cDNA: 23%), Actinobacteria (DNA:

8%, cDNA: 15%), Cyanobacteria (DNA: 7%, cDNA: 2%), Planctomycetes (DNA: 4%, cDNA: 2%), Bacteroidetes (DNA: 4%, cDNA: 4%), Verrucomicrobia (DNA: 2%, cDNA: 3%) and Armatimonadetes (DNA: 2%, cDNA: 2%) (Fig. 2a). The most abundant Proteobacterial class were Alphaproteobacteria (DNA: 29%, cDNA: 31%) (Fig. 2b). Acetobacterales (DNA: 15%, cDNA: 21%), Myxococcales (DNA: 12%, cDNA: 7%), Caulobacterales (DNA: 6%, cDNA 3%) and Rhizobiales (DNA: 6%, cDNA 5%) were the most abundant orders of the Proteobacteria (Fig. S1). The order Acetobacterales was dominated by the genus *Acidiphilium* (DNA: 5%, cDNA 8%), the order Myxococcales was dominated by the genus *Haliangium* (DNA: 4%, cDNA 3%) (Fig. S2).

The Acidobacteria were dominated by the orders Acidobacteriales (DNA: 17%, cDNA 16%) and Solibacterales (DNA: 11%, cDNA: 7%) (Fig. S1). The Acidobacteriales were dominated by the genus *Granulicella* (DNA: 11%, cDNA: 7%). The Solibacterales were dominated by the genera *Bryobacter* (DNA: 5%, cDNA 2%) and *Candidatus* Solibacter (DNA: 6%, cDNA: 5%) (Fig. S3).

Actinobacteria mainly comprised the orders Solirubrobacterales (DNA: 5%, cDNA: 8%) and Frankiales (DNA: 2%, cDNA: 4%) (Fig. S1).

Cyanobacteria were dominated by the genera *Nostoc* (DNA: 5%, cDNA: 2%) and *Stigonema* (DNA: 1%, cDNA 0.1%) (Fig. S4).

Treatment effect on the relative abundances of bacterial taxa on phylum, class and order level

We compared the relative abundances of taxa on phylum, class and order level in the controls with the warmed samples from the OTCs (Fig. 2 and Fig. S1). On phylum level, Acidobacteria (Wilcoxon rank-sum test, $P = 0.008$), Cyanobacteria ($P = 0.03$) and Gemmatimonadetes ($P = 0.02$) decreased in relative abundance with warming, while Proteobacteria ($P = 0.04$) increased in relative abundance in the DNA-based bacterial communities (Fig. 2a). We did not detect significant changes in the cDNA-based bacterial communities on phylum level.

On class level, Acidobacteriia ($P = 0.01$), Gemmatimonadetes ($P = 0.02$), and Oxyphotobacteria ($P = 0.03$) decreased in relative abundance under warming in the DNA-based bacterial communities, while Gammaproteobacteria ($P = 0.04$) increased in relative abundance in the DNA- and the cDNA-based bacterial communities (Fig. 2b).

At the order level, Betaproteobacteriales (DNA: $P = 0.04$, cDNA: $P = 0.005$) and Micrococcales (DNA: $P = 0.007$, cDNA: $P = 0.0007$) had a higher relative abundance in the warmed DNA- and cDNA-based bacterial communities (Fig. S1). Acidobacteriales (DNA: $P = 0.03$, cDNA: $P = 0.04$) showed a lower relative abundance in the warmed DNA- and cDNA-based bacterial communities (Fig. S1). In addition, in the DNA-based bacterial communities, Sphingobacterales ($P = 0.05$) and Cytophagales ($P = 0.02$) increased in relative abundance under warming. Nostocales ($P = 0.03$) decreased in relative abundance under warming. In the cDNA-based bacterial communities, the orders Sphingomonadales ($P = 0.02$) and Rhizobiales ($P = 0.02$) increased in relative abundance under warming, while Acetobacterales ($P = 0.05$) decreased in relative abundance under warming (Fig. S1).

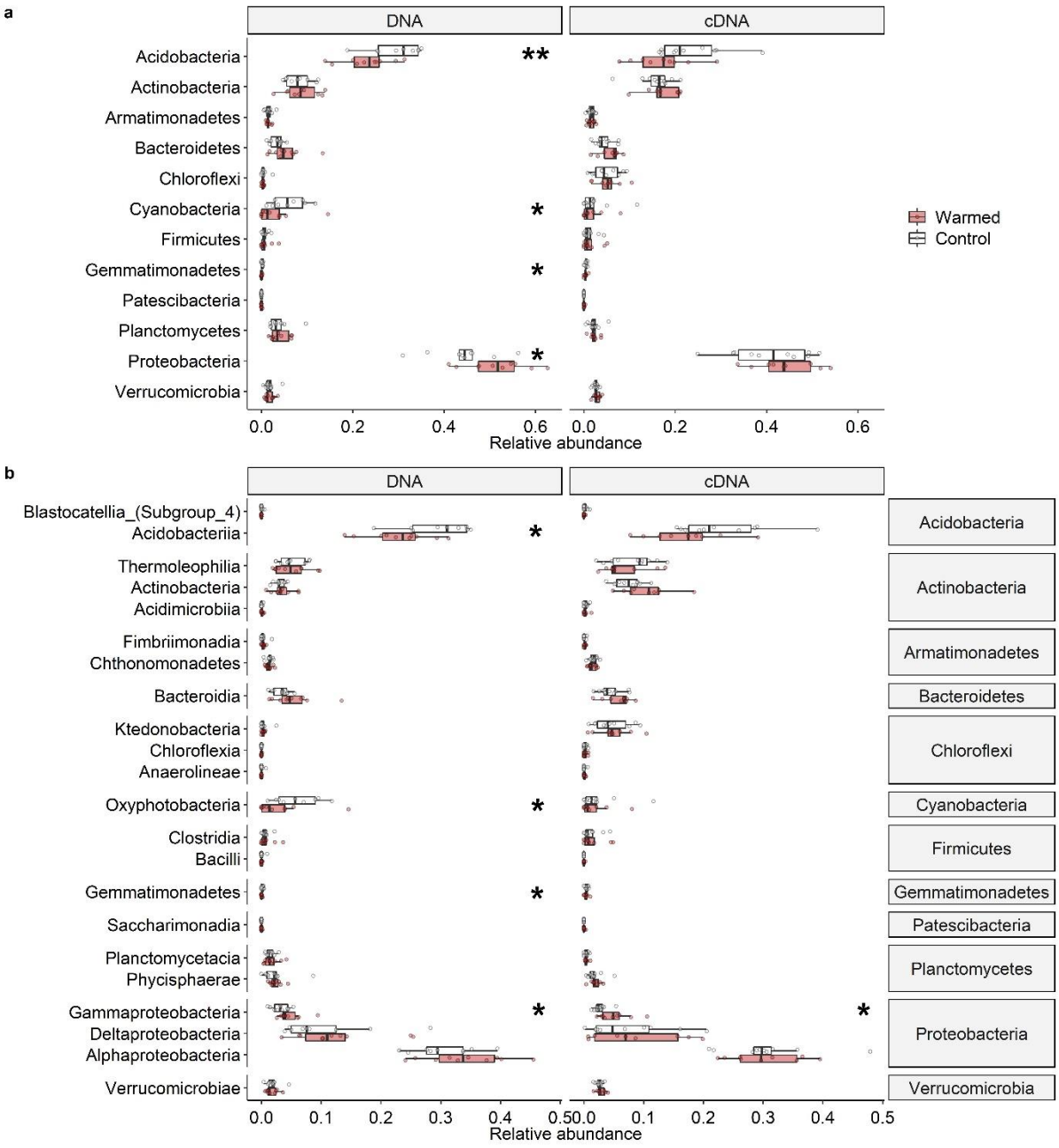

Figure 2 Boxplots of the relative abundances of (A) phyla and (B) classes in DNA and cDNA based bacterial communities associated with the moss R. lanuginosum. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values. Significance levels (* < 0.05, ** < 0.01) are based on Wilcoxon rank sum tests.

Treatment related shifts in the relative abundance of ASVs

For the bacterial communities in the DNA-based analysis, DESeq2 and indicator species analysis combined revealed 23 ASVs significantly higher in relative abundance under warming and 122 ASVs with higher relative abundance in the controls (Table S8). The strongest indicator species for the control plots corresponded to the taxa that were more abundant in the control plots according the DESeq2 analysis. ASVs with increased relative abundance in the warmed samples belonged to the genera *Allorhizobium-Neorhizobium-*

Pararhizobium-Rhizobium, *Nitrobacter* (Alphaproteobacteria), and *Galbitalea* (Actinobacteria). ASVs with increased relative abundance in the controls belonged to the genera *Acidipila*, *Bryocella*, *Bryobacter*, *Candidatus* Solibacter and *Granulicella* (Acidobacteria), *Acidiphilium*, *Endobacter*, and *Bradyrhizobium* (Alphaproteobacteria), *Nostoc* (Cyanobacteria), and *Conexibacter* (Actinobacteria) (Fig. 3 and Table S8).

For the bacterial communities in cDNA-based analysis, we detected 54 potentially active ASVs with higher abundance in the control plots and 14 potentially active ASVs more abundant in the warmed plots (Fig. 3, Table S9). ASVs more abundant in the control plots belonged to the genera *Acidipila*, *Bryocella*, *Granulicella* (Acidobacteria), *Nostoc* (Cyanobacteria) and *Acidiphilium* (Alphaproteobacteria). ASVs more abundant under warming belonged to the genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Nitrobacter*, *Sphingomonas* (Alphaproteobacteria), *Galbitalea* (Actinobacteria), and *Rhizobacter* (Gammaproteobacteria) (Fig. 3, Table S8).

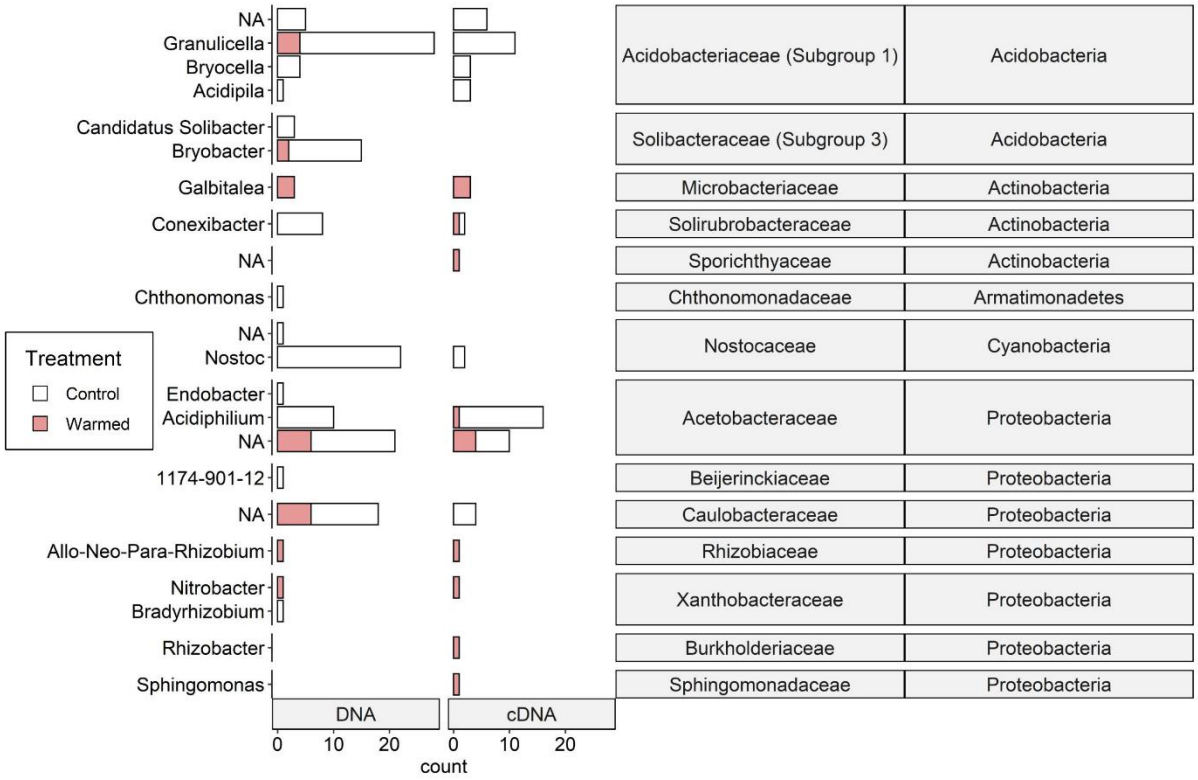

Figure 3 Number of ASVs (amplicon sequence variants) per genus sensitive to warming for DNA and cDNA based bacterial communities associated with the moss R. lanuginosum. Sensitivity was determined by differential abundance analysis (DESeq2) and indicator species analysis. ASVs not assigned to genus level are labelled 'NA' and 'Allo-Neo-Para-Rhizobium' refers to 'Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium'

Treatment effect on 16S rRNA gene and nifH gene copy numbers and nitrogen fixation rates

No significant difference was found in the 16S rRNA gene and *nifH* gene abundance between the control and warmed samples (Figs 1e-1f, Table S3). However, litter abundance

negatively affected *nifH* gene abundance (pMCMC = 0.04, Fig. 1f) and *B. nana* abundance tended to positively influence 16S rRNA gene abundance (pMCMC = 0.072, Fig. 1e).

We did not find any differences between N_2-fixation rates (expressed as produced ethylene) in the control and warmed plots in June or August 2014 (Figs 1g-1h, Table S3). However, N_2-fixation in control and warmed plots in August was significantly lower (pMCMC < 0.001) than in June (Fig. 1i). We also found a significant negative correlation between *B. nana* abundance and N_2-fixation activities measured in August (pMCMC = 0.04, Fig. 1h).

Relationships between treatment, plant biomass, bacterial community structure and N2-fixation

To explore the direct and indirect linkages between warming, *B. nana* and litter abundance, bacterial community structure and N_2-fixation, we constructed a structural equation model (SEM) (Fig. 4, Table S10). We found that warming was directly associated with changes in bacterial community structure and positively correlated with increased of *B. nana* abundance. The direct effect of *B. nana* was stronger than the direct effect of treatment on the bacterial community (-1.2 versus 0.79 standardized regression coefficients). Changes in the bacterial community structure were also indirectly associated with warming through variation in *B. nana* abundance.

The strongest positive effect detected was warming treatment on the bacterial community structure and the strongest negative effect was *B. nana* abundance on bacterial community structure (Fig. 4, Table S10).

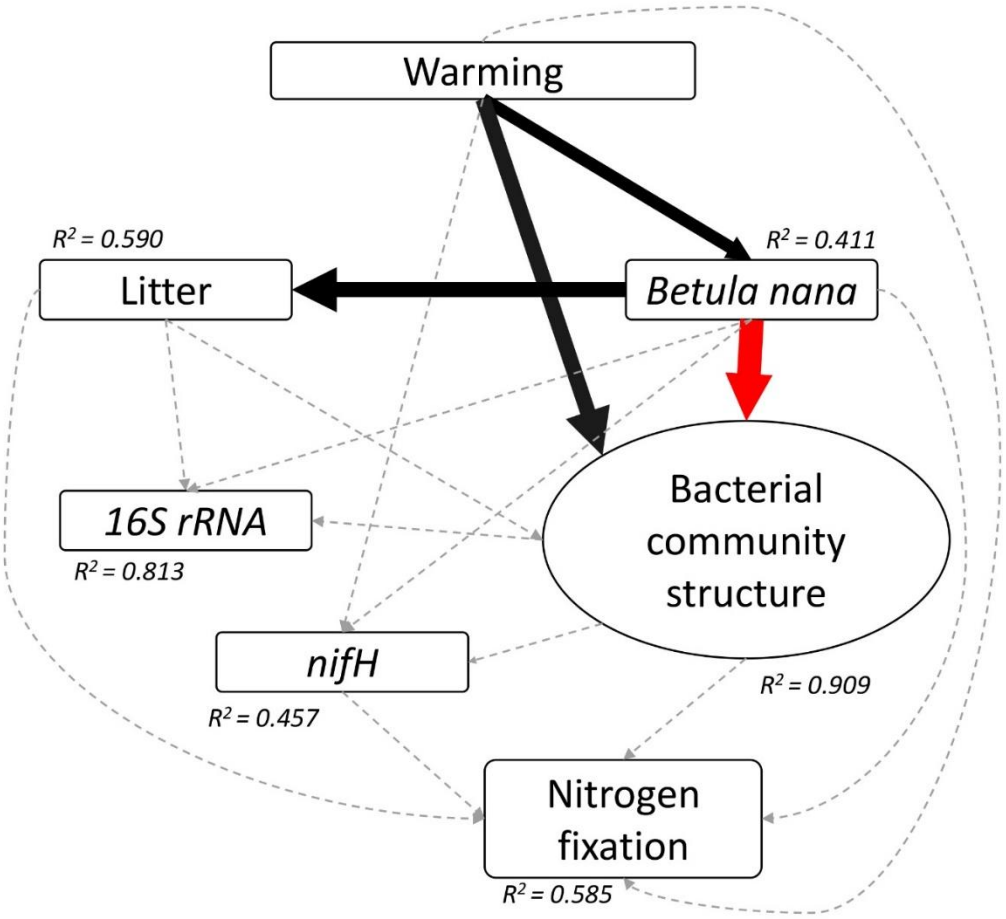

Figure 4 Structural equation model of relationships between warming, Betula nana and litter abundance, moss-associated bacterial community and N_2-fixation. A latent variable (bacterial community structure) was computed to represent β-diversity and Shannon diversity index for each plot. $\chi 2 = 2.896$, P-value = 0.894, df = 7, GFI = 0.982, RMSEA = 0, TLI = 1.202. Positive significant effects are represented in black and negative significant effects in red. The strength of the effect is visualized by the width of the arrow. The R^2-value represents the proportion of total variance explained for the specific dependent variable. Dash-line arrows indicate non-significant effects. Standardized path coefficients are presented in Table S3.

Discussion

Mosses form an important C and N sink in high latitudes and their associated bacterial communities are, to a large extent, responsible for N inputs and organic matter decomposition in these environments. Elucidation of the effect of warming on moss-associated bacterial communities will help to understand how climate change affects C and N cycling driven by the bacterial component of mosses in high-latitude ecosystems. We assessed the effect of long-term (20 years) warming by open-top chambers (OTCs) on bacterial communities and N_2-fixation associated with the moss *R. lanuginosum* at a tundra site in the highlands of Iceland. Overall, our results suggest that moss-associated bacterial communities are sensitive to long-term experimental warming and the associated plant community change, which caused changes in structure and composition. The abundance of bacteria and diazotrophs however, appeared to be unaffected by warming and, consistent

with this finding, no effect on N_2-fixation rates was observed. However, bacterial taxa that benefitted from the warming treatment almost exclusively belonged to groups involved in N-cycling, which might indicate changes in N turnover and usage of this important nutrient for Arctic ecosystem productivity.

Effect of warming on the moss-associated bacterial community structure

The average temperature increase induced by the OTCs may seem small (1-2°C), but a temperature increase in this range can affect microbial growth rate, respiration, C uptake and turnover (T. W. N. Walker et al. 2018). In addition, the effect of the OTC treatment is a long-term (20-year) disturbance, which has shown a clear effect on the vegetation structure and biomass (Jonsdottir et al. 2005) and thereby also leads to indirect effects of warming on the microbial community.

The richness and Shannon diversity of the total and potentially metabolically active bacterial community were not significantly affected by 20 years of warming. These results contrast with our first hypothesis and with trends of decreasing richness and diversity in *Sphagnum* moss observed by Carrell et al. (2017) and by Kolton et al. (2019). *R. lanuginosum* has a much lower water holding capacity than *Sphagnum* (Elumeeva et al. 2011), a different physiology and grows in heathlands and therefore *R. lanuginosum* might react differently to warming. In addition, while our study describes the effect of 20 years warming *in situ*, those previous studies on *Sphagnum* were much shorter such as a four week laboratory (Kolton et al. 2019) and two years *in situ* experimental warming study (Carrell et al. 2019). Nevertheless, we found that warming altered the bacterial community structure, even though only a small part of the variation could be directly explained by the warming treatment. Warming correlated with an increase in shrub and litter abundance and a decrease in moss abundance, as already observed in the site after 3-4 years of warming (Jonsdottir et al. 2005). Indeed, a small part of the variation of the total bacterial community could be attributed to litter abundance, which also negatively affected the richness and diversity of the potentially active bacterial community. In addition, the SEM showed that the bacterial community structure was indirectly correlated with warming via changes in *B. nana* abundance, and indirectly via the combined effect of *B. nana* and litter abundance. The effect of the increase in *B. nana* abundance as result of warming was stronger than the direct effect of warming on the bacterial community structure.

Warming-induced changes in environmental factors such as lower moss layer thickness, higher soil organic matter content, lower soil moisture (Jonsdottir et al. 2005; Björnsdóttir, Barrio, and Jónsdóttir 2021), or other not measured variables such as leaf nutrient content (Vandenkoornhuyse et al. 2015; Koyama et al. 2018; Sayer et al. 2017) could also contribute to the variation in bacterial communities between moss shoots.

We did not find an effect of warming on the 16S rRNA gene abundance, but *B. nana* abundance was correlated with an increase in 16S rRNA gene abundance. However, as we are not sure about the degree of bias towards chloroplast and mitochondrial DNA of the 16S rRNA gene primers in our samples, we cannot conclude that the bacterial load is indeed affected by *B. nana* abundance.

Effect of warming on moss-associated bacterial taxa

The total and potentially active bacterial community of *R. lanuginosum* was dominated by Proteobacteria and Acidobacteria, whereas Actinobacteria, Cyanobacteria, Planctomycetes, Bacteroidetes and Verrucomicrobia were present in lower abundances. In agreement with the bacterial community composition of boreal moss species (Holland-Moritz et al. 2018) and *Sphagnum* species (Bragina, Berg, et al. 2012), *R. lanuginosum* also showed a high abundance of the Proteobacterial order Acetobacerales and the Acidobacterial order Acidobacteriales.

We analysed changes in relative abundances in several ways to better understand the warming response of the moss bacterial community. This revealed changes in the relative abundances of taxa on phylum, class, order and ASV levels. We hypothesized that the warming-induced increase in labile *B. nana* litter (Jonsdottir et al. 2005) would lead to a decrease in slow-growing, more oligotrophic taxa, while fast-growing copiotrophic taxa would increase in relative abundance. Our data show indications for a decrease in the relative abundance of oligotrophic taxa in response to warming, such as Acidobacteria (and more specific ASVs of the genera *Granulicella*, *Solibacter*, *Bryocella*, *Bryobacter* and *Acidipila*) (Fierer, Bradford, and Jackson 2007; Dedysh and Sinninghe Damsté 2018) and the Alphaproteobacterial genus *Acidiphilium* (Akira Hiraishi and Imhoff 2015). Acidobacteria often dominate tundra soils (Männistö et al. 2013), especially environments with high concentrations of phenolic compounds, (for instance in *Sphagnum* peat (Pankratov et al. 2011) and *Empetrum* heath (Männistö et al. 2013; Gallet, Nilsson, and Zackrisson 1999)). In shrub tundra dominated by *B. nana* and *Salix* species, Proteobacteria dominate the soil bacterial community (Matthew David Wallenstein, McMahon, and Schimel 2007). In our study, the increase in the relative abundance of Proteobacteria (more specifically the genera *Rhizobacter*, *Nitrobacter* and *Rhizobium*) associated with *R. lanuginosum* in the warmed plots could thus be due to the increase in dwarf shrub biomass and labile litter, selecting for copiotrophic taxa, such as Rhizobiales (Starke et al. 2016). Some oligotrophic taxa with increased abundance in the warmed conditions such as Sphingomonadales and ASVs of the Caulobacterales (Garrity, Bell, and Lilburn 2015) could be involved in degradation of more recalcitrant plant organic matter (McGenity 2019; Starke et al. 2016). Caulobacterales has for instance been shown to be able to degrade lignin (Wilhelm et al. 2019), which can be found in high concentrations in *B. nana* roots and leaves (McLaren et al. 2017). An increase in *B. nana* litter likely increases the rate of C fluxes (Parker et al. 2018), and this may partly be due to a shift towards faster growing copiotrophic bacterial taxa, at least in the moss layer.

While the overall warming-induced changes in bacterial phylotypes for the total and the potentially active bacterial community were similar, we found that the total bacterial community reacted more strongly to warming than the potentially active bacterial community in terms of changes in relative abundance of the number of phyla, classes and ASVs. This difference may be explained by a difference in drivers for the total and potentially metabolically active bacteria, with changes in total bacterial community structure reflecting long-term drivers, while the active bacterial community may reflect short-term differences between OTC and controls (Y. Wang et al. 2020).

Implications of warming for the moss bacterial community involved in N-cycling

Our results of the bacterial structure and composition revealed that warming induced changes in relative abundances of several taxa potentially involved in N-cycling. Here, it appears that these taxa involved in the first steps of the N-cycle (entrance of new N through N_2-fixation and production of nitrate from nitrite) are altered by warming. Although we did

not explicitly target the N_2-fixing or nitrifying community by sequencing in this study, we found indications for changes in the relative abundance of potentially N_2-fixing and nitrifying taxa. In particular, the relative abundance of Cyanobacteria decreased. At the genus level, this was characterized by the lower abundance of the genus *Nostoc*. The vast majority of taxa that exclusively increased in abundance and had a higher potential metabolic activity under warming belong to groups capable of N_2-fixation (*Sphingomonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Rhizobacter*) and nitrification (*Nitrobacter*). However, neither *nifH* gene abundance nor N_2-fixation rates were directly affected by warming. This is in agreement with the general response of N_2-fixation and abundances of *nifH* genes to warming in cold ecosystems (Salazar et al. 2019), where N_2-fixation and abundances of *nifH* genes are unresponsive and nitrification rates increase under warming treatments. The apparent lack of response in N_2-fixation rates to warming may be due to a combination of several direct and indirect effects of warming on N_2-fixation rates counterbalancing each other: the shift in the potentially N_2-fixing community towards taxa better adapted to the new environment and thereby compensating for the decrease in Cyanobacteria in our study, the negative effect of drier conditions due to the warming treatment (Rousk, Sorensen, and Michelsen 2018b; Whiteley and Gonzalez 2016), the direct positive effect of warming (Rousk and Michelsen 2017) and the negative effect of shading due to increasing shrub cover (Sorensen, Lett, and Michelsen 2012) and the positive or negative effect of fertilization by shrub litter (Rousk and Michelsen 2017; Sorensen and Michelsen 2011). The SEM however did not indicate any links between the warming treatment, the bacterial community structure, litter and *B. nana* abundance to *nifH* gene abundance or N_2-fixation rates. One reason for this could be that a degree of functional compensation occurs through the shift in the diazotrophic community with warming. Nevertheless, the findings are supporting hypothesis 3, and *nifH* gene abundance was negatively affected by litter abundance and N_2-fixation rates in August were negatively affected by *B. nana* abundance, indicating the presence of indirect effects of warming on N_2-fixation. N_2-fixation rates in August were also lower than in June in both OTCs and control plots, maybe due to an increase of the effect of shading in August as indicated by the effect of *B. nana*. It may also have been drier in August, or it could be due to a seasonal shift in the N_2-fixing bacterial community (Warshan et al. 2016).

Finally, it is important to note that *R. lanuginosum* biomass tends to decrease in the warmed plots (Jonsdottir et al. 2005; Björnsdóttir, Barrio, and Jónsdóttir 2021, Table S2). Thus considering that in our study N_2-fixation rates are expressed per gram moss, warming would consequently lead to a reduction of the total amount of N_2 fixed per unit area in this tundra ecosystem.

Our study is among the first to assess the effect of long-term (20 years) experimental warming with OTCs on the bacterial part of a moss microbiome. Our results showed no direct response of N_2-fixation rates and *nifH* gene abundance to warming. However, long-term warming led to changes in the bacterial community composition. On ASV level, these changes were characterized by a decrease in the relative abundance of Cyanobacteria and an increase in abundance and potential metabolic activity of non-cyanobacterial diazotrophs, which may explain the lack of response of N_2-fixation to warming. Our results also showed that warming-induced changes in the surrounding vegetation structure can affect moss-associated bacterial communities, thus underlining the importance of indirect effects of long-term warming. The bacterial community associated with the moss might thus be sensitive to

future warming, with potential implications for N_2-fixation rates, moss growth and C sequestration.

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Author contributions

IJK, AJRC, ISJ and OV designed the study. IJK, AJRC and CK performed the research. IJK analysed the data and wrote the paper with input from AJRC, CK, DW, ADJ, ISJ and OV.

Data availability

Raw sequences are available in the European Nucleotide Archive under accession number PRJEB40635.

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Supplementary Material Paper II

Figure S1. Boxplots of the relative abundances of bacteria orders associated with the moss *R. lanuginosum* at the order level for DNA- and cDNA-based bacterial community samples associated with the moss *R. lanuginosum*. Controls are shown in white and OTC (warmed) samples are shown in red. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values. Significance levels (* < 0.05, ** < 0.01, *** < 0.001) are based on Wilcoxon rank sum tests.

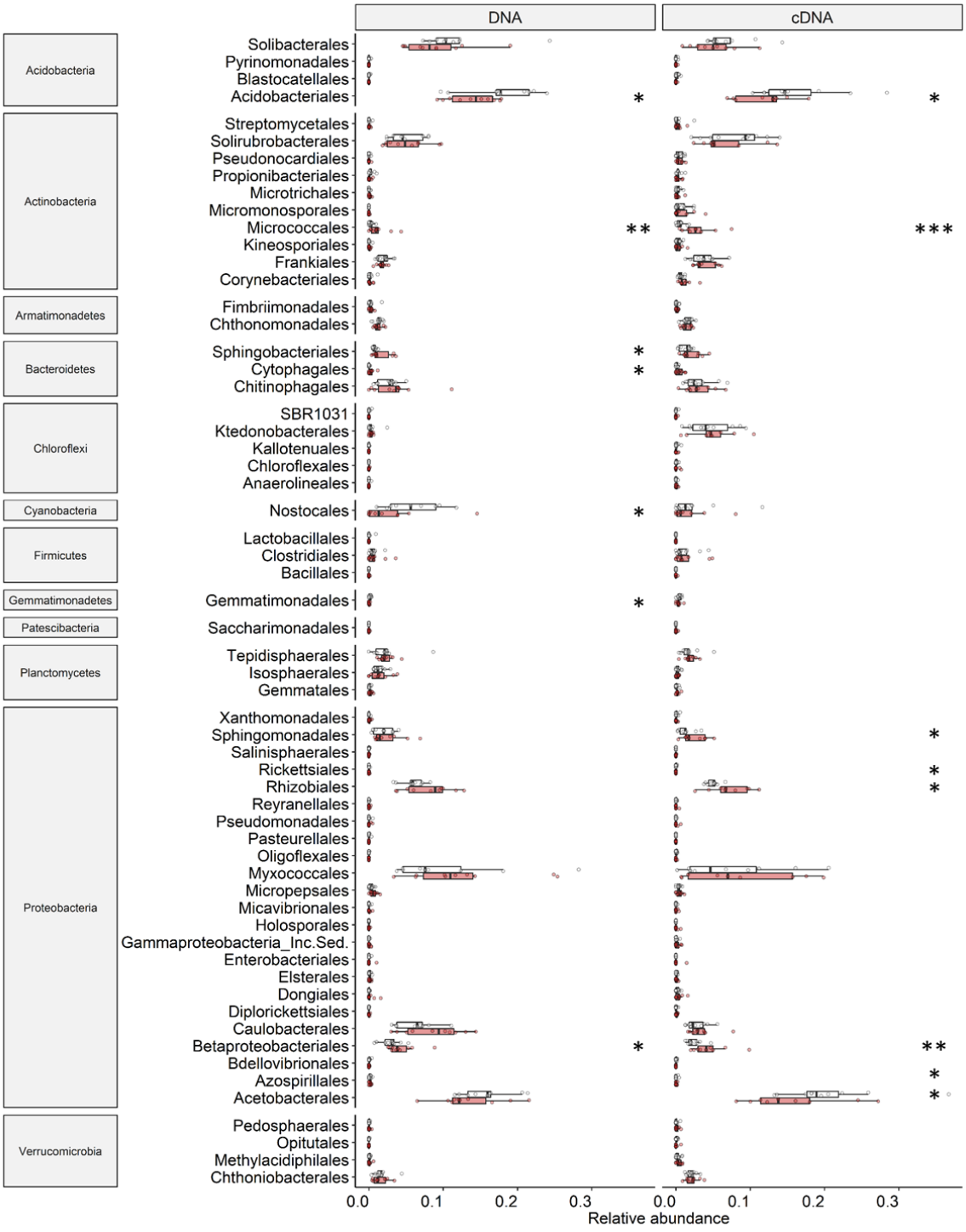

Figure S2 Barplots showing the relative abundance of Alphaproteobacterial genera in warmed and control plots of the cDNA- and the DNA-based bacterial communities associated with the moss *R. lanuginosum*.

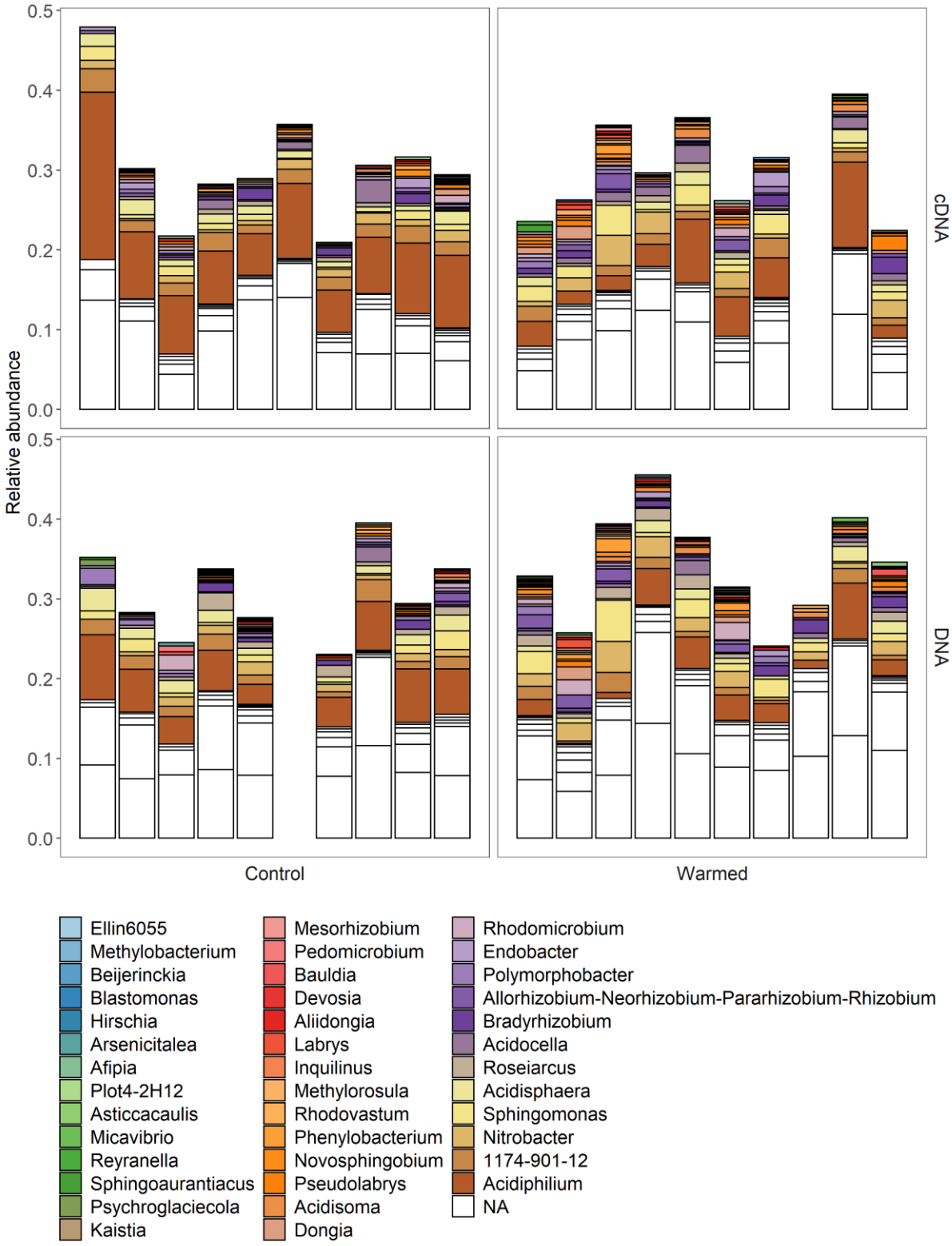

Figure S3 Barplots showing the relative abundance of Acidobacterial genera in warmed and control plots of the cDNA- and the DNA-based bacterial communities associated with the moss *R. lanuginosum*.

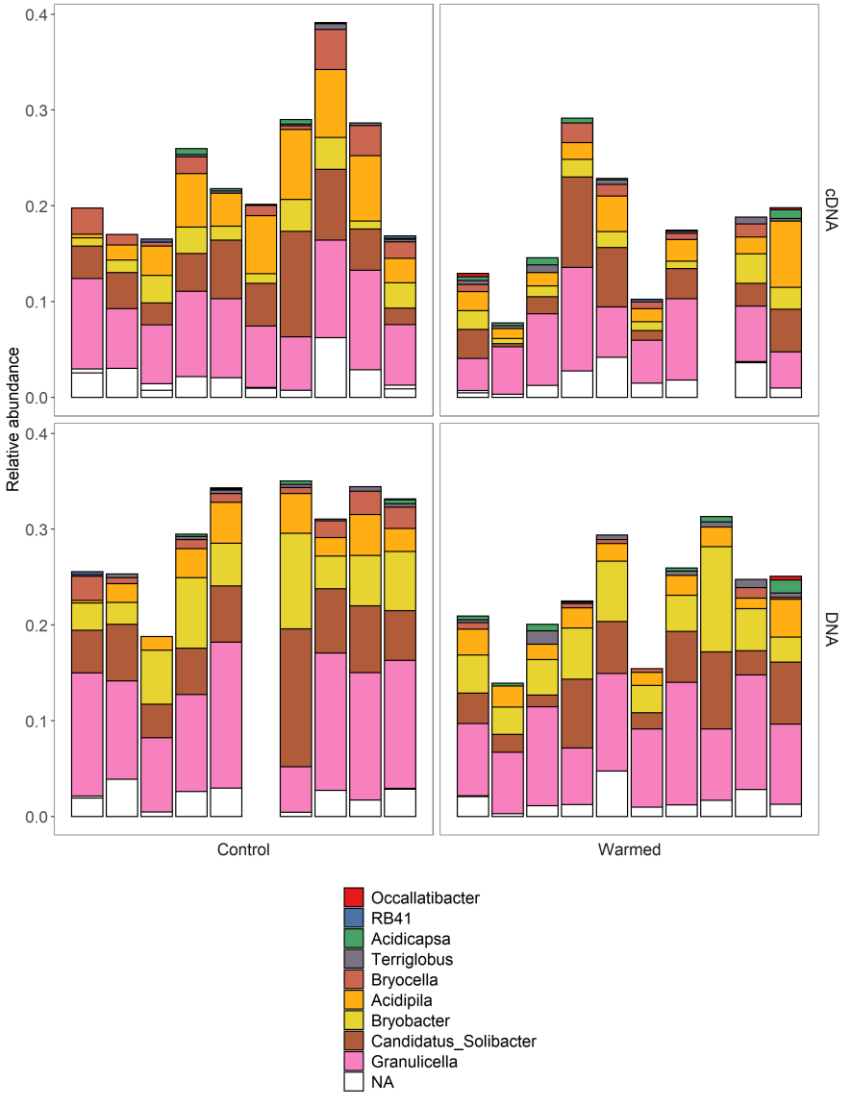

Figure S4 Barplots showing the relative abundance of Cyanobacterial genera in warmed and control plots of the cDNA- and the DNA-based bacterial communities associated with the moss *R. lanuginosum*.

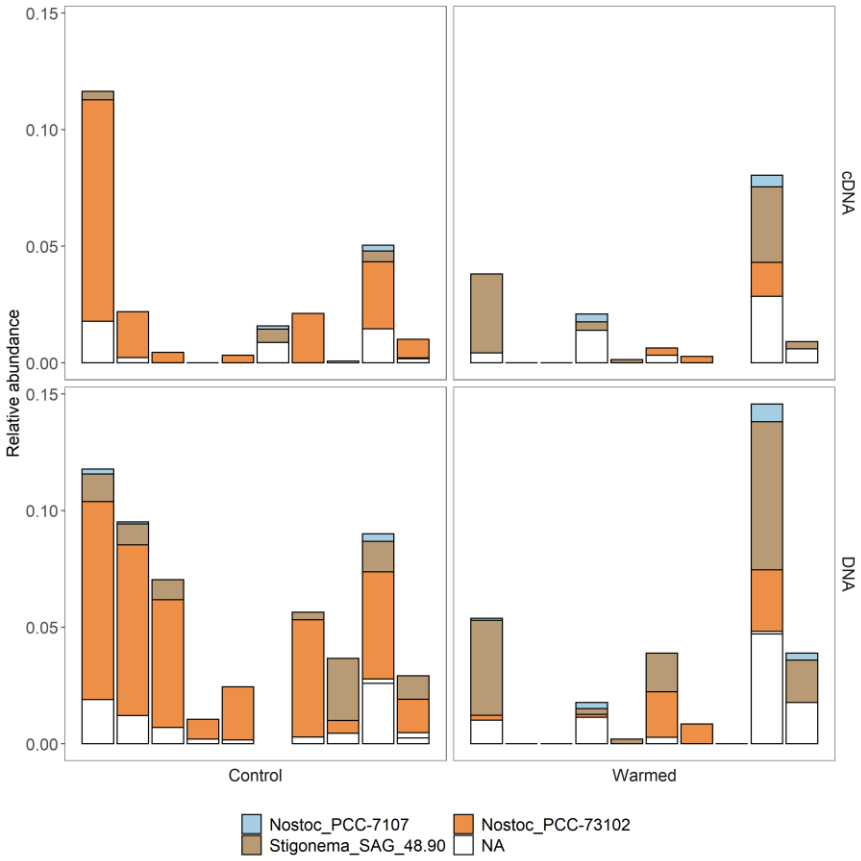

Table S1. Temperature and relative humidity for the OTC (warmed) and control plots measured in June-August 2016 (temperature and relative humidity 10 cm above the moss layer) and August 2018-June 2019 (temperature on the moss surface). Shown are mean ± standard error of the mean. Significant differences (t-test, $P < 0.05$) are indicated in bold.

Air temperature June – August 2016			Moss surface temperature August 2018 – June 2019			Relative humidity Air June – August 2016		
OTC	Control	Δ°C	OTC	Control	Δ°C	OTC	Control	Δ%
11.4 ± 0.1	10.0 ± 0.1	**1.4**	1.28 ± 0.01	1.06 ± 0.01	**0.22**	78.8 ± 0.36	81.8 ± 0.37	**-3**

Table S2. Abundance (total hits) for *Racomitrium lanuginosum*, *Betula nana* and litter in controls and OTCs. Differences between controls and OTCs were tested with paired t-tests. ** p<0.01, *** p<0.001.). Shown are mean ± standard error of the mean. Significant differences (t-test, $P < 0.05$) are indicated in bold.

Racomitrium lanuginosum			*Betula nana*			Litter		
OTC	Control	Δ	OTC	Control	Δ	OTC	Control	Δ
48.1 ± 6.53	58.7 ± 4.68	-10.6	59.7 ± 7.09	23.8 ± 5.54	**35.9**	23.1 ± 4.00	8.7 ± 1.96	**14.4**

Table S3. Bacterial richness and diversity indicators, 16S rRNA and *nifH* gene copy numbers per ng DNA, and N_2-fixation rates in June and August in control and warmed plots (OTC). Shown are mean ± standard error of the mean.

	Control	OTC
DNA richness	267.4 ± 36.6	240.2 ± 29.3
DNA Shannon diversity	5.38 ± 0.13	5.29 ± 0.12
cDNA richness	248.3 ± 25.7	206.1 ± 13.0
cDNA Shannon diversity	5.37 ± 0.10	5.26 ± 0.06
16S rRNA gene copy number per ng DNA	2681 ± 1313	3902 ± 1463
nifH gene copy number per ng DNA	17.4 ± 8.3	4.5 ± 2.1
N_2-fixation rate June	0.040 ± 0.009	0.039 ± 0.004
N_2-fixation rate August	0.018 ± 0.002	0.014 ± 0.002

Table S4. Summary table for the Permanova testing the effect of plot on the DNA-based bacterial community variation of the moss.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Plot	1	0.4982	0.49823	1.2182	0.02886	0.09191
Residuals	41	16.7681	0.40898		0.97114	
Total	42	17.2663			1	

Table S5. Summary table for the Permanova testing the effect of plot on the DNA-based bacterial community variation of the moss.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Plot	1	0.5932	0.59320	1.4692	0.03543	0.01199
Residuals	40	16.1502	0.40375		0.96457	
Total	41	16.7434			1	

Table S6. Summary table for the Permanova testing the effect of treatment, *Betula nana* abundance and litter abundance on the DNA-based bacterial community variation of the moss.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Treatment	1	0.7719	0.77194	1.9574	0.04471	1.00E-04
Betula nana	1	0.5489	0.54891	1.3919	0.03179	0.2978
Litter	1	0.5654	0.56544	1.4338	0.03275	0.0448
Residuals	39	15.38	0.39436		0.89075	
Total	42	17.2663			1	

Table S7. Summary table for the Permanova testing the effect treatment, *Betula nana* abundance and litter abundance on the cDNA-based bacterial community variation of the moss.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Treatment	1	0.694	0.69401	1.7769	0.04145	1.00E-04
Betula nana	1	0.7071	0.70706	1.8103	0.04223	0.7089
Litter	1	0.5	0.50002	1.2802	0.02986	0.2587
Residuals	38	14.8423	0.39059		0.88646	
Total	41	16.7434			1	

Table S8. Indicator and differentially abundant ASVs (every row represents a single ASV) in the DNA-based bacterial communities associated with *Racomitrium lanuginosum*.

Phylum	Family	Genus	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Diffab	Indicator species	comb	A	B	Indstat	Indpvalue
Acidobacteria	Acidobacteriaceae	Acidipila								c	c	0.86	0.56	0.69	0.04
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.83	0.56	0.68	0.04
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	0.86	0.56	0.69	0.02
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	1.00	0.44	0.67	0.04
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	1.00	0.44	0.67	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.67	0.82	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.85	0.56	0.69	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	6.60	-6.36	2.20	-2.90	0.00	0.10	c	c	c				
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.85	0.67	0.75	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.91	0.78	0.84	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.76	0.89	0.82	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.44	0.67	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	6.76	-6.40	1.91	-3.35	0.00	0.04	c	c	c				
Acidobacteria	Acidobacteriaceae	Granulicella	6.67	5.98	2.85	2.10	0.04	0.37	w	w	w				

Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.56	0.75	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.72	0.78	0.75	0.05
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.78	1.00	0.88	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.97	0.56	0.73	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.84	0.78	0.81	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.89	0.67	0.77	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	8.24	6.28	2.47	2.55	0.01	0.15	w	w	w	1.00	0.50	0.71	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.72	0.78	0.75	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								w	w	0.71	0.90	0.80	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.92	0.78	0.84	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.80	0.89	0.84	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.90	0.78	0.84	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.76	0.89	0.82	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Acidobacteriaceae	Granulicella	17.35	-7.37	1.22	-6.07	0.00	0.00	c	c	c				
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Acidobacteriaceae	NA								c	c	0.83	0.78	0.81	0.02
Acidobacteria	Acidobacteriaceae	NA								c	c	0.93	0.67	0.79	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	0.83	0.78	0.81	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	1.00	0.56	0.75	0.02

Acidobacteria	Acidobacteriaceae	NA								c	c	0.92	0.56	0.71	0.02
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.44	0.67	0.02
Acidobacteria	Solibacteraceae	Bryobacter								c	c	0.89	0.56	0.70	0.02
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.44	0.67	0.02
Acidobacteria	Solibacteraceae	Bryobacter	7.08	-6.46	2.34	-2.76	0.01	0.10	c	c	c				
Acidobacteria	Solibacteraceae	Bryobacter	10.75	6.66	1.99	3.34	0.00	0.04	w	w	w	0.91	0.60	0.74	0.03
Acidobacteria	Solibacteraceae	Bryobacter	10.57	4.21	1.92	2.19	0.03	0.30	w	w	w				
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Solibacteraceae	Bryobacter								c	c	0.87	0.67	0.76	0.01
Acidobacteria	Solibacteraceae	Bryobacter								c	c	0.84	0.56	0.68	0.04
Acidobacteria	Solibacteraceae	Bryobacter	8.60	-6.74	2.12	-3.18	0.00	0.05	c	c	c	1.00	0.56	0.75	0.01
Acidobacteria	Solibacteraceae	Bryobacter	7.22	-6.49	2.33	-2.78	0.01	0.10	c	c	c	1.00	0.56	0.75	0.02
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.56	0.75	0.01
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.44	0.67	0.04
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.44	0.67	0.03
Acidobacteria	Solibacteraceae	Bryobacter								c	c	1.00	0.44	0.67	0.04
Acidobacteria	Solibacteraceae	Candidatus Solibacter								c	c	1.00	0.44	0.67	0.04
Acidobacteria	Solibacteraceae	Candidatus Solibacter	15.04	-7.55	1.70	-4.45	0.00	0.00	c	c	c	1.00	0.67	0.82	0.00
Acidobacteria	Solibacteraceae	Candidatus Solibacter								c	c	0.92	0.67	0.78	0.01
Actinobacteria	Microbacteriaceae	Galbitalea								w	w	0.77	0.70	0.73	0.04
Actinobacteria	Microbacteriaceae	Galbitalea								w	w	1.00	0.50	0.71	0.04
Actinobacteria	Microbacteriaceae	Galbitalea								w	w	1.00	0.50	0.71	0.03

Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.75	0.78	0.76	0.03
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.73	0.78	0.75	0.03
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.88	0.56	0.70	0.04
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.85	0.56	0.69	0.03
Actinobacteria	Solirubrobacteraceae	Conexibacter	6.05	-6.24	2.23	-2.80	0.01	0.10	c	c	c				
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.89	0.67	0.77	0.01
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.83	0.67	0.74	0.02
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.79	0.67	0.73	0.03
Armatimonadetes	Chthonomonadaceae	Chthonomonas	6.41	-6.32	2.70	-2.34	0.02	0.25	c	c	c				
Cyanobacteria	Nostocaceae	NA	6.37	-3.15	1.52	-2.07	0.04	0.37	c	c	c	0.85	0.89	0.87	0.00
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.83	0.89	0.86	0.00
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.81	0.89	0.85	0.00
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.89	0.78	0.83	0.01
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.88	0.78	0.83	0.01
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	29.17	-2.68	1.36	-1.98	0.05	0.38	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	28.41	-3.73	1.65	-2.26	0.02	0.28	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	12.38	-3.22	1.63	-1.98	0.05	0.38	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.89	0.89	0.89	0.00
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.88	0.89	0.89	0.00
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.88	0.89	0.88	0.00

Cyanobacteria	Nostocaceae	Nostoc PCC-73102	20.71	-3.13	1.53	-2.05	0.04	0.37	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	22.59	-3.28	1.44	-2.28	0.02	0.28	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	12.20	-3.17	1.63	-1.95	0.05	0.40	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.87	0.89	0.88	0.00
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.83	0.89	0.86	0.01
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	30.64	-2.50	1.37	-1.82	0.07	0.48	c	c	c	0.81	0.89	0.85	0.01
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	24.89	-2.97	1.48	-2.00	0.05	0.38	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	14.60	-2.95	1.62	-1.82	0.07	0.48	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.89	0.78	0.83	0.01
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.89	0.78	0.83	0.01
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	12.30	-3.59	1.61	-2.24	0.03	0.28	c	c	c				
Cyanobacteria	Nostocaceae	Nostoc PCC-73102	12.47	-3.29	1.62	-2.04	0.04	0.37	c	c	c				
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.56	0.75	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.44	0.67	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium	8.21	-6.68	2.13	-3.14	0.00	0.05	c	c	c	1.00	0.89	0.88	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.78	0.89	0.83	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.56	0.75	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium	6.60	-6.36	2.20	-2.89	0.00	0.10	c	c	c	0.86	0.56	0.69	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.44	0.67	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	1.00	0.89	0.94	0.00

Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.78	0.78	0.78	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium	8.95	-6.80	1.83	-3.72	0.00	0.01	c	c	c				
Proteobacteria	Acetobacteraceae	Endobacter								c	c	0.87	0.56	0.69	0.02
Proteobacteria	Acetobacteraceae	NA								c	c	0.74	0.89	0.81	0.02
Proteobacteria	Acetobacteraceae	NA								c	c	0.74	0.89	0.81	0.04
Proteobacteria	Acetobacteraceae	NA								c	c	0.81	0.78	0.79	0.01
Proteobacteria	Acetobacteraceae	NA	14.48	-2.63	1.44	-1.82	0.07	0.48	c		c				
Proteobacteria	Acetobacteraceae	NA								w	w	0.79	0.80	0.79	0.02
Proteobacteria	Acetobacteraceae	NA								w	w	0.91	0.60	0.74	0.02
Proteobacteria	Acetobacteraceae	NA								w	w	0.78	0.70	0.74	0.04
Proteobacteria	Acetobacteraceae	NA								w	w	0.91	0.50	0.67	0.05
Proteobacteria	Acetobacteraceae	NA								c	c	0.79	0.78	0.78	0.03
Proteobacteria	Acetobacteraceae	NA								c	c	0.71	0.78	0.75	0.05
Proteobacteria	Acetobacteraceae	NA								c	c	0.79	0.67	0.73	0.04
Proteobacteria	Acetobacteraceae	NA								c	c	1.00	0.44	0.67	0.04
Proteobacteria	Acetobacteraceae	NA								c	c	1.00	0.67	0.82	0.00
Proteobacteria	Acetobacteraceae	NA								c	c	0.85	0.56	0.69	0.04
Proteobacteria	Acetobacteraceae	NA	6.16	-6.26	2.39	-2.63	0.01	0.13	c	c	c				
Proteobacteria	Acetobacteraceae	NA								c	c	1.00	0.44	0.67	0.04
Proteobacteria	Acetobacteraceae	NA								w	w	0.90	0.60	0.73	0.03
Proteobacteria	Acetobacteraceae	NA								c	c	0.76	0.89	0.82	0.03
Proteobacteria	Acetobacteraceae	NA								c	c	0.83	0.78	0.80	0.01

Proteobacteria	Acetobacteraceae	NA	14.20	-2.70	1.51	-1.79	0.07	0.50	c	c	c						
Proteobacteria	Acetobacteraceae	NA								w	w	1.00	0.60	0.78	0.01		
Proteobacteria	Caulobacteraceae	NA								w	w	1.00	0.50	0.71	0.04		
Proteobacteria	Caulobacteraceae	NA								c	c	0.81	0.78	0.80	0.02		
Proteobacteria	Caulobacteraceae	NA								c	c	0.70	0.89	0.79	0.03		
Proteobacteria	Caulobacteraceae	NA								c	c	1.00	0.56	0.75	0.01		
Proteobacteria	Caulobacteraceae	NA								c	c	1.00	0.44	0.67	0.04		
Proteobacteria	Caulobacteraceae	NA	6.51	-6.34	2.37	-2.68	0.01	0.12	c	c	c						
Proteobacteria	Caulobacteraceae	NA								w	w	0.88	0.60	0.73	0.04		
Proteobacteria	Caulobacteraceae	NA								c	c	0.69	0.89	0.78	0.04		
Proteobacteria	Caulobacteraceae	NA								c	c	0.76	0.78	0.77	0.04		
Proteobacteria	Caulobacteraceae	NA								c	c	0.75	0.78	0.77	0.04		
Proteobacteria	Caulobacteraceae	NA								w	w	1.00	0.60	0.78	0.01		
Proteobacteria	Caulobacteraceae	NA								c	c	1.00	0.56	0.75	0.01		
Proteobacteria	Caulobacteraceae	NA								c	c	1.00	0.44	0.67	0.04		
Proteobacteria	Caulobacteraceae	NA								w	w	1.00	0.50	0.71	0.04		
Proteobacteria	Caulobacteraceae	NA								c	c	0.79	0.89	0.84	0.01		
Proteobacteria	Caulobacteraceae	NA								w	w	1.00	0.50	0.71	0.03		
Proteobacteria	Caulobacteraceae	NA								c	c	1.00	0.44	0.67	0.03		
Proteobacteria	Caulobacteraceae	NA	6.69	5.98	2.12	2.82	0.00	0.10	w	w	w	1.00	0.60	0.78	0.01		
Proteobacteria	Beijerinckiaceae	1174-901-12								c	c	1.00	0.44	0.67	0.03		
Proteobacteria	Rhizobiaceae	Allorhizobium-								w	w	0.91	0.60	0.74	0.03		

		Neorhizobium-Pararhizobium-Rhizobium						
Proteobacteria	Xanthobacteraceae	Bradyrhizobium	c	c	1.00	0.44	0.67	0.03
Proteobacteria	Xanthobacteraceae	Nitrobacter	w	w	0.73	0.90	0.81	0.03

Table S9 Indicator and differentially abundant ASVs (every row represents a single ASV) in the cDNA-based bacterial communities associated with *Racomitrium lanuginosum*.

Phylum	Family	Genus	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Diffab	Indicator species comb		A	B	Indstat	Indpvalue
Acidobacteria	Acidobacteriaceae	Acidipila								c	c	0.95	0.60	0.75	0.02
Acidobacteria	Acidobacteriaceae	Acidipila								c	c	0.75	0.80	0.77	0.04
Acidobacteria	Acidobacteriaceae	Acidipila								c	c	0.77	0.80	0.79	0.02
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	1.00	0.50	0.71	0.03
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	1.00	0.60	0.78	0.01
Acidobacteria	Acidobacteriaceae	Bryocella								c	c	1.00	0.60	0.78	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.50	0.71	0.04
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.84	0.90	0.87	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.78	0.90	0.84	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.50	0.71	0.03
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.50	0.71	0.02
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.90	0.95	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.70	0.84	0.01
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	1.00	0.90	0.95	0.00
Acidobacteria	Acidobacteriaceae	Granulicella								c	c	0.90	0.80	0.85	0.00

Acidobacteria	Acidobacteriaceae	NA								c	c	0.90	0.80	0.85	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	0.84	0.70	0.77	0.01
Acidobacteria	Acidobacteriaceae	NA								c	c	0.90	0.60	0.74	0.02
Acidobacteria	Acidobacteriaceae	NA								c	c	1.00	0.60	0.78	0.02
Acidobacteria	Acidobacteriaceae	NA								c	c	0.82	0.70	0.76	0.02
Acidobacteria	Acidobacteriaceae	NA	11.31	-6.92	2.03	-3.41	0.00	0.35	c	c	c	0.86	0.60	0.72	0.04
Acidobacteria	Acidobacteriaceae	Granulicella	8.12	-6.44	1.86	-3.46	0.00	0.35	c		c				
Acidobacteria	Acidobacteriaceae	Granulicella	8.60	-6.52	1.82	-3.59	0.00	0.35	c		c				
Actinobacteria	Sporichthyaceae	NA								w	w	0.81	0.67	0.74	0.03
Actinobacteria	Microbacteriaceae	Galbitalea								w	w	0.85	0.89	0.87	0.01
Actinobacteria	Microbacteriaceae	Galbitalea								w	w	0.87	0.78	0.82	0.01
Actinobacteria	Microbacteriaceae	Galbitalea								w	w	0.86	0.78	0.82	0.01
Actinobacteria	Solirubrobacteraceae	Conexibacter								w	w	1.00	0.44	0.67	0.04
Actinobacteria	Solirubrobacteraceae	Conexibacter								c	c	0.73	0.80	0.76	0.03
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.92	0.60	0.74	0.02
Cyanobacteria	Nostocaceae	Nostoc PCC-73102								c	c	0.90	0.60	0.74	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.76	1.00	0.87	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.74	0.80	0.77	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.87	0.70	0.78	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium								w	w	1.00	0.44	0.67	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium								c	c	0.82	1.00	0.91	0.00

Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	0.84	0.80	0.82	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.60	0.78	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.60	0.78	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	0.78	1.00	0.88	0.00
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	0.78	0.80	0.79	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	0.75	0.80	0.78	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.60	0.78	0.02
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.60	0.78	0.01
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Acetobacteraceae	Acidiphilium	c	c	1.00	0.60	0.78	0.02
Proteobacteria	Acetobacteraceae	NA	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Acetobacteraceae	NA	w	w	0.84	0.78	0.81	0.01
Proteobacteria	Acetobacteraceae	NA	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Acetobacteraceae	NA	c	c	0.90	0.60	0.73	0.03
Proteobacteria	Acetobacteraceae	NA	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Acetobacteraceae	NA	c	c	1.00	0.50	0.71	0.04
Proteobacteria	Acetobacteraceae	NA	c	c	0.83	0.80	0.82	0.01
Proteobacteria	Acetobacteraceae	NA	w	w	0.92	0.78	0.84	0.00
Proteobacteria	Acetobacteraceae	NA	w	w	0.92	0.56	0.72	0.02
Proteobacteria	Acetobacteraceae	NA	w	w	0.91	0.44	0.63	0.04
Proteobacteria	Caulobacteraceae	NA	c	c	0.88	0.60	0.73	0.04

Proteobacteria	Caulobacteraceae	NA	c	c	0.96	0.50	0.69	0.04
Proteobacteria	Caulobacteraceae	NA	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Caulobacteraceae	NA	c	c	1.00	0.50	0.71	0.03
Proteobacteria	Rhizobiaceae	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	w	w	1.00	0.44	0.67	0.04
Proteobacteria	Xanthobacteraceae	Nitrobacter	w	w	0.73	0.78	0.76	0.04
Proteobacteria	Sphingomonadaceae	Sphingomonas	w	w	1.00	0.44	0.67	0.03
Proteobacteria	Burkholderiaceae	Rhizobacter	w	w	0.85	0.56	0.69	0.03

Table S10. Summary statistics of the structural equation model of direct and indirect effects of warming on N_2-fixation shown in Figure 6. Shown are the standardized path coefficients (Std. est.), the standard error of regression weight (se), the z-value (z) and the significance level for the regression weight (p).

Parameter	Std. est.	se	z	p
Microbial community				
(Shannon diversity and position on first PCoA axis)				
Treatment	**0.79**	**0.25**	**3.18**	**0.00**
B. nana	**-1.21**	**0.31**	**-3.85**	**0.00**
Litter	-0.04	0.35	-0.12	0.90
B. nana				
Treatment	**0.64**	**0.09**	**7.11**	**0**
Litter				
B. nana	**0.77**	**0.07**	**11.21**	**0**
16S rRNA gene abundance				
B. nana	-1.97	3.21	-0.61	0.54
Litter	0.44	0.67	0.66	0.51
Treatment	1.07	2.11	0.51	0.61
Microbial community	-1.94	2.46	-0.79	0.43
nifH gene abundance				
B. nana	-1.75	1.92	-0.91	0.36
Litter	-0.04	0.57	-0.08	0.94
Treatment	0.68	1.27	0.53	0.60
Microbial community	-1.59	1.43	-1.11	0.27
N_2-fixation rate (June)				
Treatment	1.57	4.05	0.39	0.70
Microbial community	-2.67	5.33	-0.50	0.62
B. nana	-3.19	6.60	-0.48	0.63
Litter	-0.24	0.94	-0.26	0.80

nifH gene abundance	-0.70	0.82	-0.86	0.39
Indirect effects on the microbial community and on N_2-fixation				
Treatment → Microbial community +				
Treatment → B. nana → Microbial community +	-0.01	0.28	-0.02	0.98
Treatment → B. nana → litter → Microbial community				
Treatment → B. nana → Microbial community +				
Treatment → B. nana → litter → Microbial community	**-0.80**	**0.20**	**-4.02**	**0.00**
Treatment → B. nana → Microbial community	**-0.77**	**0.26**	**-2.98**	**0.00**
Treatment → B. nana → Litter → Microbial community	-0.02	0.17	-0.12	0.90
Treatment → N_2-fixation +				
Treatment → Microbial community → N_2-fixation +				
Treatment → B. nana → Microbial community → N_2-fixation +				
Treatment → B. nana → N_2-fixation +				
Treatment → B. nana → Litter → N_2-fixation +				
Treatment → B. nana → Litter → Microbial community → N_2-fixation +	-0.28	0.39	-0.70	0.48
Treatment → nifH gene abundance → N_2-fixation +				
Treatment → B. nana → Litter → Microbial community → nifH gene abundance → N_2-fixation +				
Treatment → B. nana → Litter → nifH gene abundance → N_2-fixation +				
Treatment → B. nana → nifH gene abundance → N_2-fixation				
Treatment → Microbial community → N_2-fixation +				
Treatment → B. nana → Microbial community → N2-fixation +				
Treatment → B. nana → N_2-fixation +				
Treatment → B. nana → Litter → N_2-fixation +				
Treatment → B. nana → Litter → Microbial community → N_2-fixation +	-1.85	4.12	-0.45	0.65
Treatment → nifH gene abundance → N_2-fixation +				
Treatment → B. nana → Litter → Microbial community → nifH gene abundance → N_2-fixation +				
Treatment → B. nana → Litter → nifH gene abundance → N_2-fixation +				
Treatment → B. nana → nifH gene abundance → N_2-fixation				

Treatment → Microbial community → N_2-fixation	-2.11	4.39	-0.48	0.63
Treatment → B. nana → Microbial community → N_2-fixation	2.07	4.31	0.48	0.63
Treatment → B. nana → N_2-fixation	-2.05	4.24	-0.48	0.63
Treatment → B. nana → Litter → N_2-fixation	-0.12	0.46	-0.26	0.80
Treatment → B. nana → Litter → Microbial community → N_2-fixation	0.06	0.47	0.12	0.90
Treatment → nifH gene abundance → N_2-fixation	-0.48	1.28	-0.37	0.711
Treatment → B. nana → Litter → Microbial community → nifH gene abundance → N_2-fixation	-0.02	0.19	-0.12	0.90
Treatment → B. nana → Litter → nifH gene abundance → N2-fixation	0.02	0.20	0.08	0.94
Treatment → B. nana → nifH gene abundance → N_2-fixation	0.79	1.58	0.50	0.62

Paper III

Nitrogen fixation and bacterial communities of *Racomitrium* mosses and underlying substrate in an Icelandic glacier forefield

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Abstract

Mosses are among the first colonizing organisms after glacier retreat and can develop into thick moss mats during later successional stages. They are key players in N_2 fixation through their microbiome, which is an important process for nutrient build-up during primary succession. How these moss-microbe interactions develop during succession is not well-studied and is relevant in the light of climate change and increased glacier retreat.

We examined the bacterial communities associated with two moss species of the genus *Racomitrium* and the underlying substrate along a successional gradient in the glacier forefield of Fláajökull in southeast Iceland. In addition, we measured N_2-fixation rates and abundance of N_2-fixing bacteria, as well as moss functional traits, such as carbon (TC) and nitrogen contents (TN).

Although time since deglaciation did not affect TN and moisture content, TC and shoot length increased with time since deglaciation. Soil bacterial community structure was driven by time since deglaciation and moss C/N ratio, while the moss microbiome was affected by time since deglaciation and moisture content. Moss N_2-fixation rates were affected by changes in the bacterial community composition and *nifH* gene abundance rather than moss TN or time since deglaciation. This was accompanied by a shift from autotrophic to heterotrophic diazotrophs. In addition, we found little evidence for lateral transfer between moss and soil bacterial communities.

Overall our results suggest that moss and underlying soil bacterial community structures are affected by moss traits and time since deglaciation. In addition, moss N_2-fixation rates are determined by bacterial community structure, rather than moss traits or time since deglaciation.

Introduction

Formerly ice-covered terrains are becoming increasingly exposed as glaciers retreat due to climate change (Roe, Baker, and Herla 2017). Such glacier forefields are subject to rapid ecosystem development, with microbial communities as the first colonizers. These early colonizing microbial communities are responsible for the first stages of soil development, which often involves the formation of a Biological Soil Crust capable of stabilizing the soil and of fixing carbon (C) and nitrogen (N) (Bradley, Singarayer, and Anesio 2014; Breen and Lévesque 2008). The subsequent increase in C and N availability facilitates the colonization of other organisms, such as mosses (Vilmundardóttir, Gísladóttir, and Lal 2015b). Mosses can develop into thick moss mats during succession, especially in regions with high precipitation and little competition from higher plants (Tallis 1958). Moss establishment further enhances soil development in newly exposed terrain, by contributing to N (Arróniz-Crespo et al. 2014; Bowden 1991; Vilmundardóttir, Gísladóttir, and Lal 2015b), retaining moisture, and contributing to organic matter build-up (Wietrzyk-Pełka et al. 2020; Vilmundardóttir, Gísladóttir, and Lal 2015b), which additionally promotes soil microbial growth (Bardgett and Walker 2004). Thus, while microbial communities create the conditions necessary for plant establishment, plants influence microbial communities, for instance via litter inputs (Fanin, Hättenschwiler, and Fromin 2014). Despite an increasing number of studies linking the development of soil microbial communities to establishment of plants in glacier forefields (Arróniz-Crespo et al. 2014; Knelman et al. 2012; 2018; Bueno de Mesquita et al. 2017), we have a very limited understanding of the dynamics of moss-associated bacterial communities during ecosystem development in these environments.

Due to their diazotrophic microbiome (Ininbergs et al. 2011), mosses are the most important source for new N in Arctic ecosystems (Rousk, Sorensen, and Michelsen 2017). As glacier forefields are typically nutrient limited, moss microbiomes may be key players in biogeochemical N cycling during primary succession (Arróniz-Crespo et al. 2014). N_2-fixation rates are variable and can be influenced by moss species (Stuart et al. 2020; Jean et al. 2020b), N availability (Arróniz-Crespo et al. 2014), moisture (Rousk, Sorensen, and Michelsen 2018a), temperature (Rousk, Pedersen, et al. 2017), diazotroph composition (Ininbergs et al. 2011), diazotroph abundance (Arróniz-Crespo et al. 2014) and diazotroph activity (Warshan et al. 2016) throughout succession.

Moss-associated bacterial community composition may also be driven by host identity (Holland-Moritz et al. 2018). Moss traits such as C and N content, may affect moss-associated bacterial community composition, similarly to how phyllosphere microbial taxa are linked to leaf traits (Yunshi Li et al. 2018; Laforest-Lapointe et al. 2017). These moss traits can change during succession. For instance, Sphagnum and bryophyte C/N ratio increased with peatland succession (Laine et al. 2021) and time since deglaciation in Chilean glacier forefields (Arróniz-Crespo et al. 2014). These changes might subsequently affect the composition of the moss-microbiome.

The development of a plant-microbiome during succession may furthermore depend on where the microbes are inherited from (Poosakkannu et al. 2017). Plant-associated microbial communities are thought to be mainly inherited from the surrounding soil which is also referred to as horizontal transfer (Compant et al. 2019). While mosses might not have a large

rhizosphere, some have rhizoids and are thus connected to the soil. In higher plants, microorganisms can also be transferred vertically, via the seed (Hardoim et al. 2012). For mosses, microbial organisms might indeed be transferred between the sporophyte and the gametophyte (Bragina, Berg, et al. 2012) or via vegetative regeneration (Tallis 1959). Depending on which source is more important for the composition of moss-associated bacterial communities, successional changes in soil microbial communities can be reflected in the moss microbiome, or alternatively the moss microbiome may stay relatively stable throughout succession.

As moss cover increases the amount of organic carbon, moisture and nutrient content in soil (Bragazza et al. 2019; Breen and Lévesque 2008; Bardgett and Walker 2004), it may also indirectly influence the underlying soil bacterial communities (Juottonen et al. 2020).

Here we examine the bacterial communities of two moss species of the genus *Racomitrium* and the underlying substrate along a successional gradient in the glacier forefield of Fláajökull, in southeast Iceland. Mosses of the genus *Racomitrium* are important colonizers in Icelandic glacier forefields (Vilmundardóttir, Gísladóttir, and Lal 2015a; Glausen and Tanner 2019).

We hypothesized that: (i) moss total N (TN) and moss total C (TC) increase with time since deglaciation; (ii) changes in moss functional traits (such as TN and TC) and time since deglaciation lead to shifts in moss-associated bacterial communities and the underlying soil bacterial community; and (iii) that moss-associated N_2-fixation rates and *nifH* gene abundance will depend on time since deglaciation, moisture content, TN and/or bacterial community composition.

Materials and methods

The chronosequence we studied lies in the pro-glacial area of Fláajökull glacier (64.328124°; -15.527791°), which is an outlet glacier on the south-eastern side of the Vatnajökull icecap (Figure 1). The Fláajökull glacier forefield is characterized by a number of moraine ridges and other landforms such as drumlins and eskers (Evans, Ewertowski, and Orton 2016; Jónsson et al. 2016). The oldest moraine dates from the glacier's furthest advance towards the end of the Little Ice Age in 1894 (Hrafnhildur Hannesdóttir et al. 2015). The extent of the glacier in the last 120 years has been estimated using multiple dating methods, including glaciological methods, lichenometry and historical records (Evans, Ewertowski, and Orton 2016; Dabski 2002; Icelandic Glaciological Society 2018). Our furthest sampling point in 2018 lays more than 3000 m from the front of the glacier.

The two closest weather stations are located in Fagurhólsmýri (72 km) and Höfn (18 km), which have a mean annual temperature of 4.8 °C and 4.6 °C respectively and a mean annual precipitation of 1814 mm and 1381 mm respectively. The climate can be described as sub-polar oceanic (Einarsson 1984).

The substrate in the glacier forefield is characterised by gravel, silt and sand (Jónsson et al. 2016) and the soils are classified as cambic vitrisols and further from the glacier as andosols (Arnalds and Óskarsson 2009).

The area closest to the glacier is mostly unvegetated, with some scattered mosses and lichens. Moss cover (mainly *Racomitrium* sp.) increases with distance from the glacier with 25-50% cover on the oldest moraine (Wojcik et al. 2020).

Study site

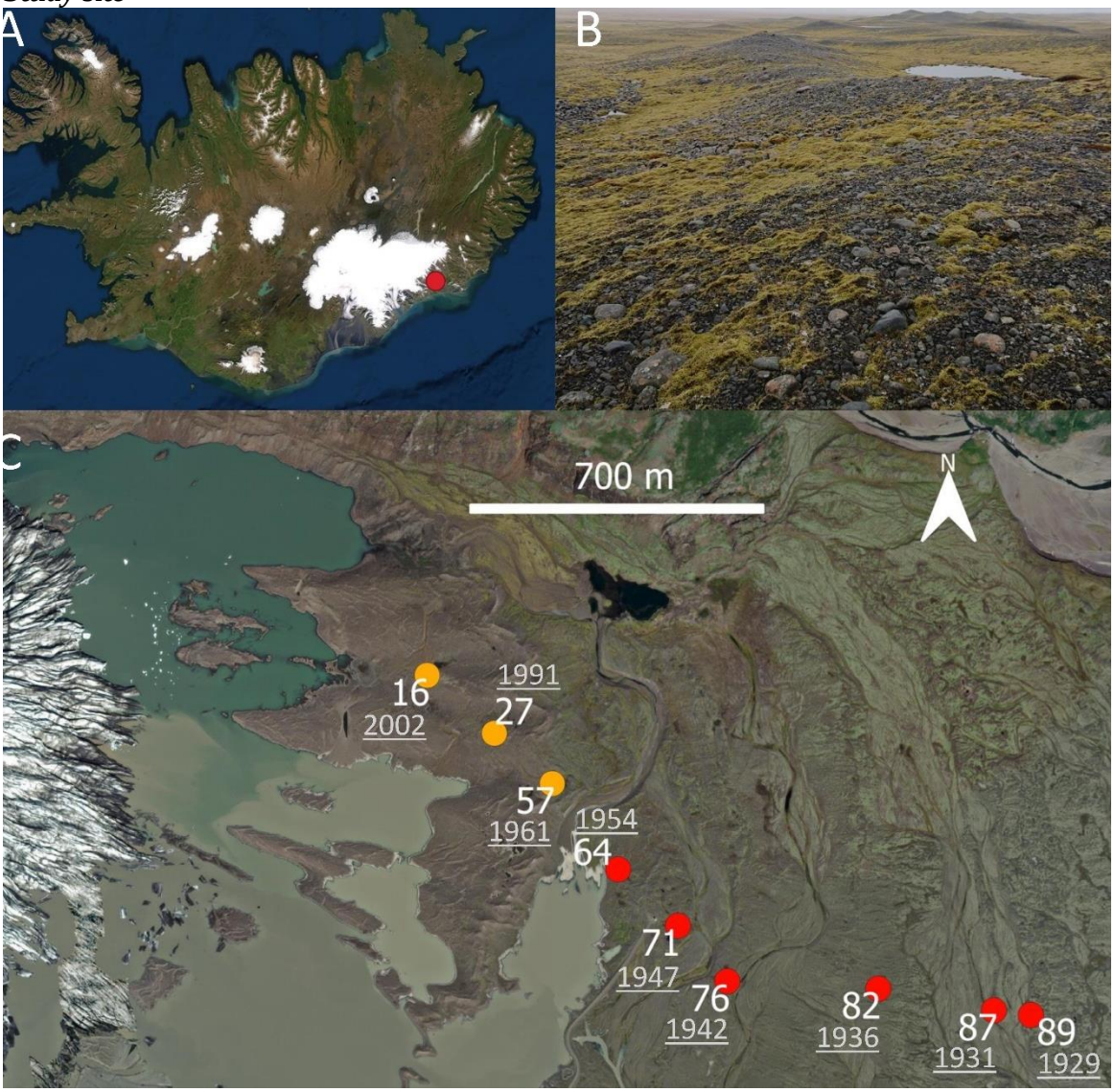

Figure 1 Overview of A) the location of the Fláajökull glacier forefield, B) Racomitrium spp. at the sampling site and C) the sampling locations along the chronosequence. The sites with *R. ericoides* are depicted in yellow and the sites with *R. lanuginosum* in red. At each sampling location the time since deglaciation in 2018 and year of deglaciation are indicated. Time since deglaciation was determined after Evans et al. 2016 and the Icelandic Glaciological Society, 2018.

Sampling

We collected samples of moss and underlying substrate in May 2018. Samples were taken in triplicate along one transect on moraine ridges, at the same locations where Wojcik et al. (2020) collected soil samples (Figure 1).

Moss samples were collected aseptically with a tweezer. Soil samples of 10 cm depth were taken just below the moss cover with a sterilized (with ethanol) hand corer. We collected a total of 27 moss and 27 soil samples. After collection, samples were transported on ice packs for one day and stored at -20 °C until further analyses.

The moss samples were homogenized and split in three parts, one for moss species determination and acetylene reduction assays, one for biogeochemical analysis and one for DNA extraction. The moss species were determined by light microscopy.

Additional soil samples were taken in late April 2021, to measure pH and moisture content. These soil samples were collected at the same coordinates as the samples taken in 2018 and where taken of soil under moss cover and additionally of bare soil. Methods and results of these measurements can be found in Supplementary Methods 1.

Moss shoot length, moisture content and chemical analysis

Moss shoot length was measured for five shoots of each sample. Moss samples were dried at 70°C for 24h and analyzed for field moisture content. Samples were subsequently milled to a fine powder and the total nitrogen (TN) and total carbon (TC) contents and the carbon isotopic composition ($\delta^{13}C$) were analyzed content. The analysis was carried out at GFZ Potsdam using a mass spectrometer (DELTAplusXL, ThermoFisher) coupled via a ConFlowIII interface with an elemental analyzer (Carlo-Erba NC2500). The analytical precision for $\delta^{13}C$ was 0.2% and for TC and TN it was 0.01% and replicate determinations showed a standard deviation < 0.02%.

Moss N2-fixation rates

Moss N_2-fixation rates were assessed using the acetylene reduction assay (ARA) method (Hardy et al. 1968). The upper 5 cm of five shoots of each moss sample were weighed and wetted until saturated and then acclimated for 24h at 15 °C in 22 ml vials. Then, we replaced 10% of the headspace (2.2 ml) with acetylene and incubated the samples at 15 °C, under 60 µmol m^{-2} s^{-1} Photosynthetically Active Radiation (PAR) for 24h in a growth chamber (Termaks series 8000, Bergen, Norway). Ethylene and acetylene were quantified by gas chromatography.

Acetylene reduction rates were expressed as ethylene per gram dry weight (field weight) of the moss per day (as in Hardy et al. 1968).

DNA extraction

DNA was extracted for quantification of *nifH* and 16S rRNA gene abundance and 16S rRNA gene sequencing. Before nucleic acid extraction, moss samples were ground in liquid N. DNA from the soil and the moss samples was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. DNA concentrations were assessed with a NanoDrop (NanoDrop Technologies, Wilmington, USA).

Quantitative real-time PCR of nifH genes

Quantification of *nifH* genes was performed by quantitative PCR (Corbett Rotor-Gene) using the primer set PolF/PolR. We confirmed the specificity of the *nifH* primers for our samples by Sanger sequencing of 10 clone fragments. Standards for *nifH* reactions were obtained by amplifying one cloned *nifH* sequence with flanking regions of the plasmid vector using the M13 primer sites on the plasmid (TOPO TA cloning Kit, Invitrogen). Standard curves were obtained by serial dilutions (10^6 to 10^1 copies per reaction; E = 0.9 – 1.1, R2 = > 0.99 for all reactions). Each reaction had a volume of 20 µL, containing 10 µL of 2x QuantiFast SYBR Green PCR Master Mix (QIAGEN), 0.2 µL of each primer (10 µM), 0.8 µL of BSA (5 µg/µL), 6.8 µL of RNase free water and 2 µL of template. The cycling program was 5 min at 95 °C, 30 cycles of 10 s at 95 °C and 30 s at 60 °C. Samples with less than 10 *nifH* gene copies per µL and less than 100 16S rRNA gene copies per µL reaction were considered negative.

Sequencing and bioinformatics

Library preparation and paired-end (2 x 300 nt) sequencing of the V3-V4 region of the 16S rRNA gene on an Illumina HiSeq 2500 platform was performed by the Beijing Genomics Institute, using 338F/806R primer pair (Klindworth et al. 2013) and the standard Illumina protocol. We processed the raw sequences using the DADA2 pipeline (Callahan et al. 2016; Callahan, McMurdie, and Holmes 2017), which does not cluster sequences into operational taxonomic units (OTUs), but uses exact sequences or amplicon sequence variants (ASVs). Forward reads were truncated at 250 bp and reverse reads at 220 bp. Assembled ASVs were assigned taxonomy to the SILVA_132 database (Quast et al. 2013) using the Ribosomal Database Project (RDP) naïve Bayesian classifier (Q. Wang et al. 2007) in DADA2. We removed ASVs assigned to chloroplasts and mitochondria and singletons. In total, for 47 samples, 2 972 ASVs remained. To account for uneven sequencing depths, the data were normalized using cumulative-sum scaling (CSS) (Paulson et al. 2013).

Statistics

We used linear models (*lm* from the R package 'stats') to investigate the responses of TC, TN, C/N ratio, and moss tissue $\delta^{13}C$, N_2-fixation rates, *nifH* gene abundance, and richness and diversity of the soil and moss associated bacterial communities to moss species and time since deglaciation. We used a post-hoc Tukey test to analyze differences in richness and diversity of bacterial communities in moss species and underlying soil.

To test the effect of time since deglaciation and moss traits on the bacterial community composition of the mosses and the underlying soil, we used PERMANOVAs on weighted Unifrac distance matrices (*adonis* from the R package 'vegan'). We used moss species as strata in the PERMANOVAs to check whether time since deglaciation and moss traits could explain variation in the bacterial communities in the whole dataset, but we also ran PERMANOVAs on the two moss species separately. To avoid multicollinearity in the linear regression, we only included explanatory factors in the PERMANOVAs with correlation coefficients lower than 0.7 (Table S1).

To identify soil- and moss-associated bacterial taxa whose relative abundance changes with time since deglaciation, we used the R-package 'DESeq2' (M. I. Love, Huber, and Anders 2014). We used the non-normalised data and an adjusted P-value cut-off of 0.1. For the moss-associated taxa, we included moss species and time since deglaciation in the model (species + time since deglaciation), to correct for moss species, similarly to the linear models.

To explore the direct and indirect relationships between time since deglaciation, moisture content, TN, moss-associated bacterial composition and N_2-fixation, we constructed a structural equation model (SEM) (using the R package 'lavaan' (Rosseel 2012)). For moss-associated bacterial community composition we used the position on the first PCoA axis. As controlling for moss species was not possible, we only used the data from *R. lanuginosum* for the SEM.

Results

Moss functional traits and N2-fixation in the glacier forefield

Moss shoot length increased with time since deglaciation, from 16.5 to 46.9 mm ($P = 0.02$) (Figure 2A, Table S2 and S3). Moss moisture content, TC, TN, C/N ratio and δ13C did not change (Figure 2B-E, Table S2,S4-8), and *nifH* gene abundance decreased ($P = 0.01$) with time since deglaciation (Figure 2G, Table S2 and S9). Moss $\delta^{13}C$ was significantly higher in *R. lanuginosum* than in *R. ericoides* ($P < 0.001$) (Figure 2f, Table S8). Moss-associated N_2-

fixation rate (expressed as acetylene reduction rate) showed considerable variation along the chronosequence, but no significant trend with time since deglaciation (Figure 2H, Table S2 and S10). The average acetylene reduction rate in the forefield was 0.00769 µmol C_2H_2 Kg^{-1} day^{-1}.

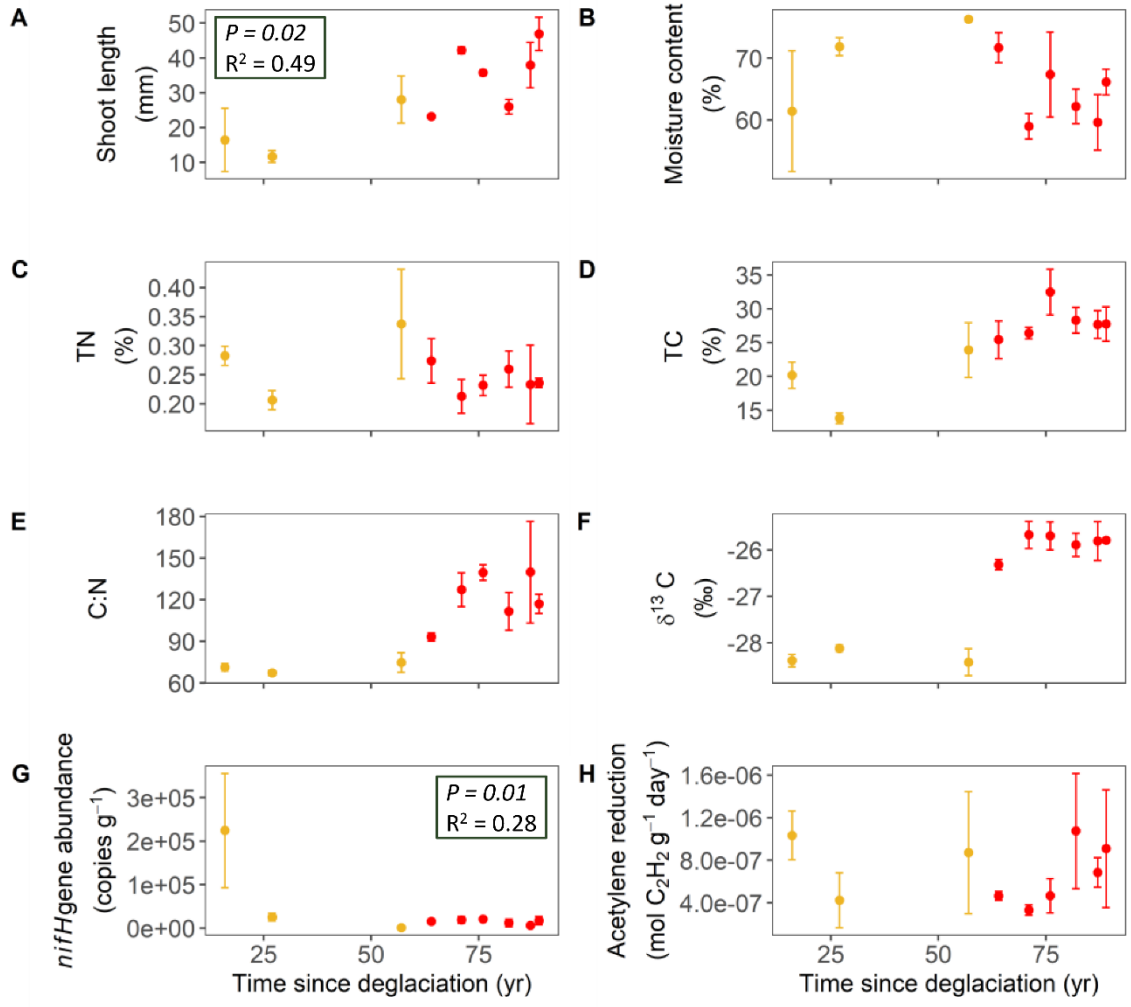

Figure 2 Variations in A) moss shoot length, B) moss moisture content, C) TC, D) TN, E) C/N ratio and F) $\delta^{13}C$ content of moss shoots, G) *nifH* gene abundance in the mosses and H) moss-associated N_2-fixation rates (measured by acetylene reduction) with time since deglaciation, in two moss species: *R. ericoides* (yellow) and *R. lanuginosum* (red). Shown are mean ± standard error of triplicate samples from each sampling location. *P*-values and R^2 are shown in the top left corner of each plot when $P < 0.05$.

Richness and diversity of moss and soil bacterial communities

There was no difference in soil or moss microbial diversity between the farthest and oldest edge of our sampling scheme and the closest and more recent land exposed by the glacier retreat (Figure S1, Table S11, S13 and S15). All of the diversity indicators were higher for the soil compared to the two moss species ($P < 0.001$ for both phylogenetic diversity, richness and Shannon diversity; Figure S1, Table S12, S14 and S16).

Moss and soil bacterial community structure

Moss and soil bacterial community composition differed from each other (Permanova R^2 = 0.41, P < 0.001; Figure 3A, Table S17). The soil bacterial community changed significantly with time since deglaciation (Permanova R^2 = 0.10, P = 0.04; Figure 3C, Table S18). Part of the variation in the structure of the soil bacterial communities was related to moss C/N ratio (Permanova R^2 = 0.09. P = 0.04; Figure 3C, Table S17). The moss bacterial community (regardless of moss species) was also affected by time since deglaciation (Permanova R^2 = 0.38, P < 0.001) and additionally by moisture content (Permanova R^2 = 0.12, P < 0.001; Table S19).

When moss species were analyzed separately, the Permanova showed that the *R. ericoides* bacterial community changed with time since deglaciation (Permanova R^2 = 0.22, P = 0.004), but was also affected by moss C/N ratio (Permanova R^2 = 0.18, P = 0.02) and moss moisture content (Permanova R^2 = 0.24, P = 0.003; Table S20). The *R. lanuginosum* bacterial community was not affected by time since deglaciation, but varied with moss moisture content (Permanova R^2 = 0.19, P = 0.003; Figure 3B, Table S21).

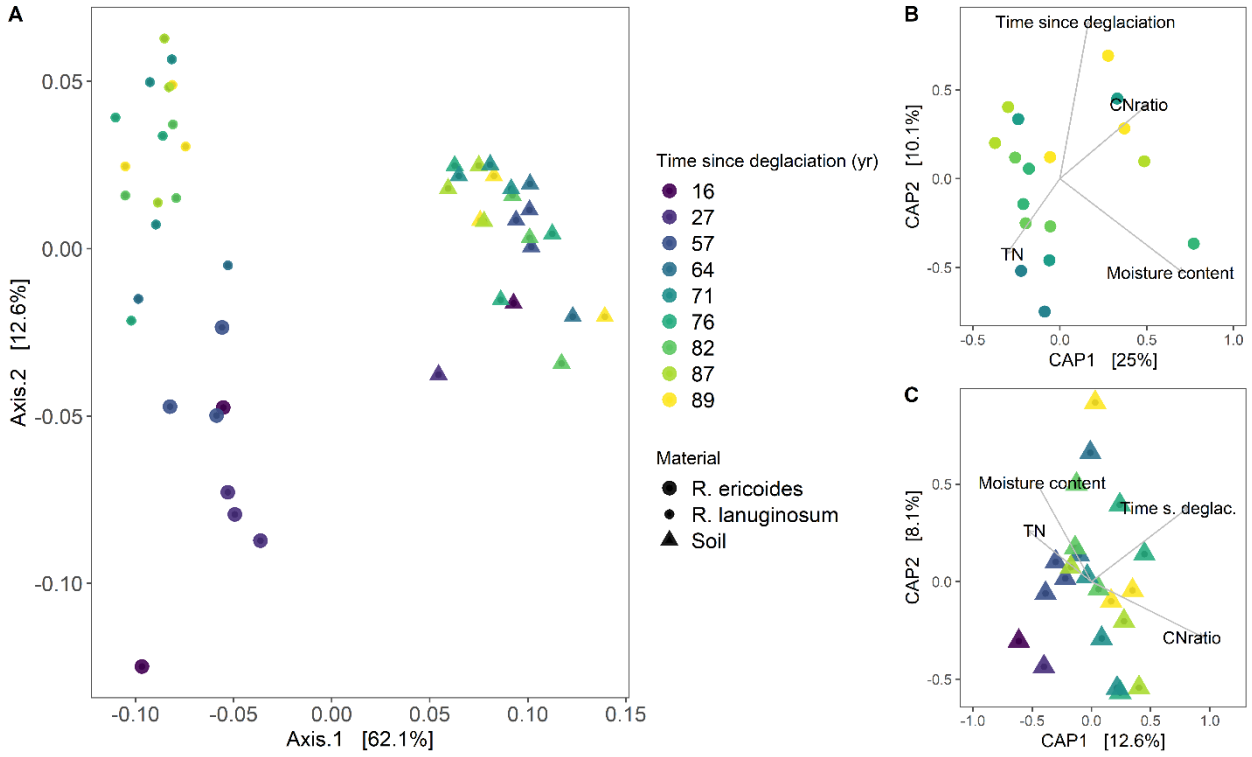

Figure 3 A) Principal coordinate analysis (PCoA) biplot of the bacterial communities of the mosses *R. ericoides* and *R. lanuginosum* and the underlying soil on ASV level based on weighted unifrac distances and B) CAP analysis of the bacterial community of the mosses R. lanuginosum and environmental factors and C) CAP analysis of the underlying soil bacterial community environmental factors.

Bacterial community composition

The bacterial communities of the mosses in the Fláajökull glacier forefield were characterised by Proteobacteria (35% and 28% on average in *R. ericoides* and *R. lanuginosum* respectively), Acidobacteria (15% and 23%), Bacteroidetes (25% and 24%), Verrucomicrobia (7% and 7%), Chloroflexi (1% and 5%) and Actinobacteria (10% and 7%), on phylum level (Figure 4). Cyanobacteria abundance was relatively low in the moss species (3% and 2%).

Alphaproteobacteria were the most abundant class within the Proteobacteria (22% and 19%). The Alphaproteobacteria were dominated by the families Acetobacteraceae (7% and 8%) and Sphingomonadaceae (7% and 4%) (Figure S2). Acidobacteria were dominated by the families Acidobacteriaceae (6% and 13%) and Solibacteraceae (6% and 8%) (Figure S3). There were no dominant families within the Actinobacteria (Figure S3). Within this phylum, we identified Frankiaceae (1% in both *R. ericoides* and *R. lanuginosum*), Ilumatobacteraceae (1% in both *R. ericoides* and *R. lanuginosum*), Nakamurellaceae (2% and <1%) and Pseudonocardiaceae (1% in both *R. ericoides* and *R. lanuginosum*) in all successional stages (Figure S4). Bacteroidetes were dominated by the family Chitinophagaceae (14% and 15%) (Figure S5) and Cyanobacteria were dominated by the genus *Nostoc* (3% and 2%) (Figure S6).

The soil bacterial communities were dominated by the phyla Proteobacteria (36%), Acidobacteria (19%), Actinobacteria (7%) and Bacteroidetes (19%) (Figure 4).

The classes Alpha- and Gammaproteobacteria shared similar abundances in all soil samples along the chronosequence (15% and 16% for Alpha- and Gammaproteobacteria respectively). The Alphaproteobacteria were dominated by Xanthobacteraceae (5%) and Sphingomonadaceae (2%) (Figure S2). The family Nitrosomonadaceae (5%) dominated the Gammaproteobacteria (Figure S7). The Acidobacteria were dominated by the families Solibacteraceae_(Subgroup_3) (7%), Pyrinomonadaceae (7%) and Blastocatellaceae (4%) (Figure S3). The Actinobacteria were similar to their abundance in the moss samples, relatively variable and lacking a clearly dominating taxon. The Ilumatobacteraceae (1%) occurred in all soil samples (Figure S4). The Bacteroidetes were dominated the family Chitinophagaceae (11%) (Figure S5).

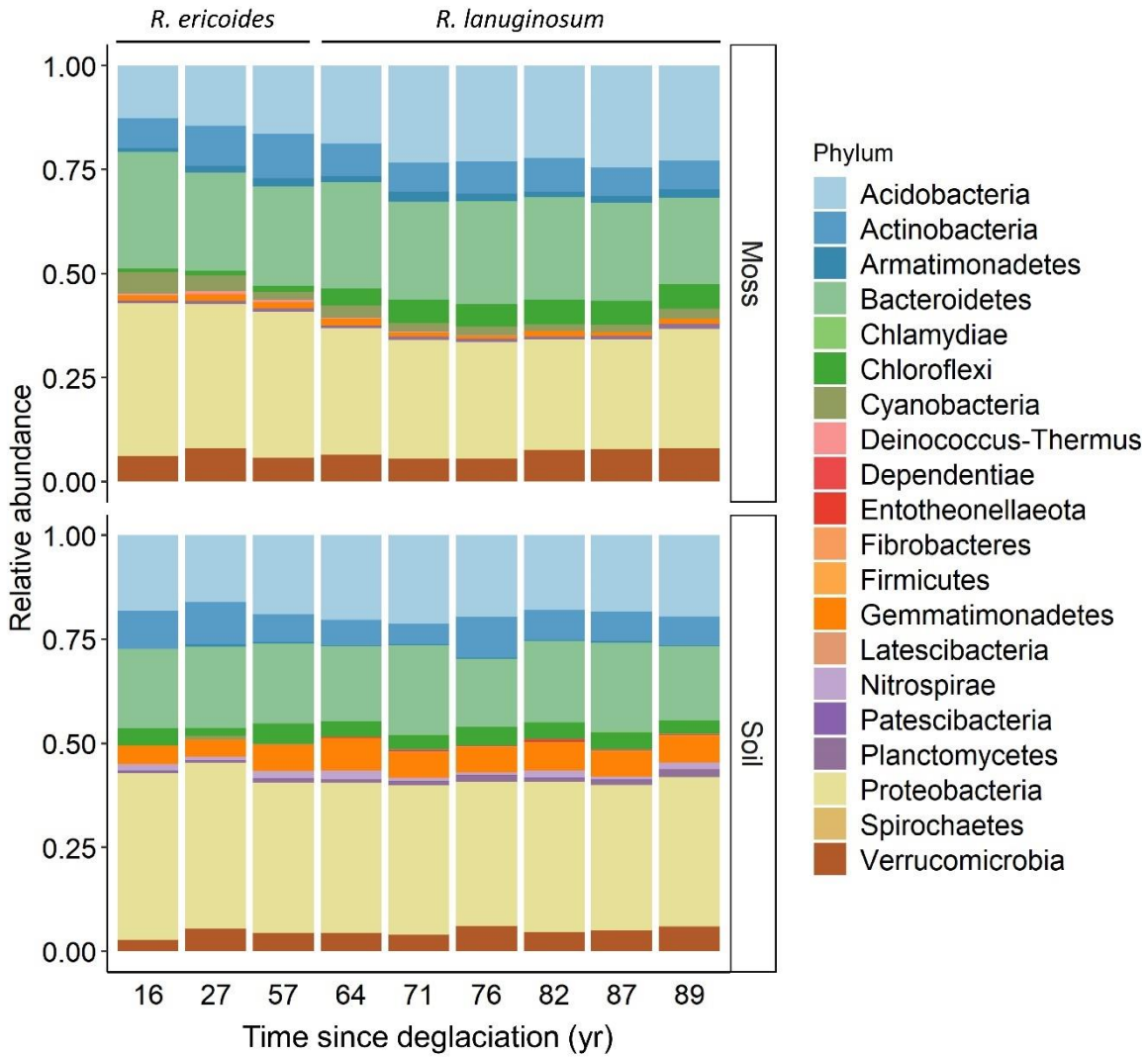

Figure 4 Phyla-level composition of the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

Bacterial community composition across time since deglaciation and moss species

On phylum level, the relative abundance of Chloroflexi increased across our chronosequence, both in moss and soil; while Proteobacteria, Cyanobacteria and Bacteroidetes decreased in the moss (Figure 4, Figure S8 ad S9).

On ASV level, we detected more ASV changing in relative abundance with time since deglaciation in the soil than in the moss (Figure 5). All detected ASVs increased in relative abundance across the chronosequence. Most of these ASVs belonged to the Proteobacteria. The two ASVs that increased with time since deglaciation belonged to the candidate genus Solibacter and the family Acetobacteraceae. The ASVs showing the strongest increase in relative abundance with time since deglaciation in the soil belonged to the families

Acetobacteraceae, Micropepsaceae and Chinitophagaceae and the genera *Parafilimonas* and *Nocardioides*.

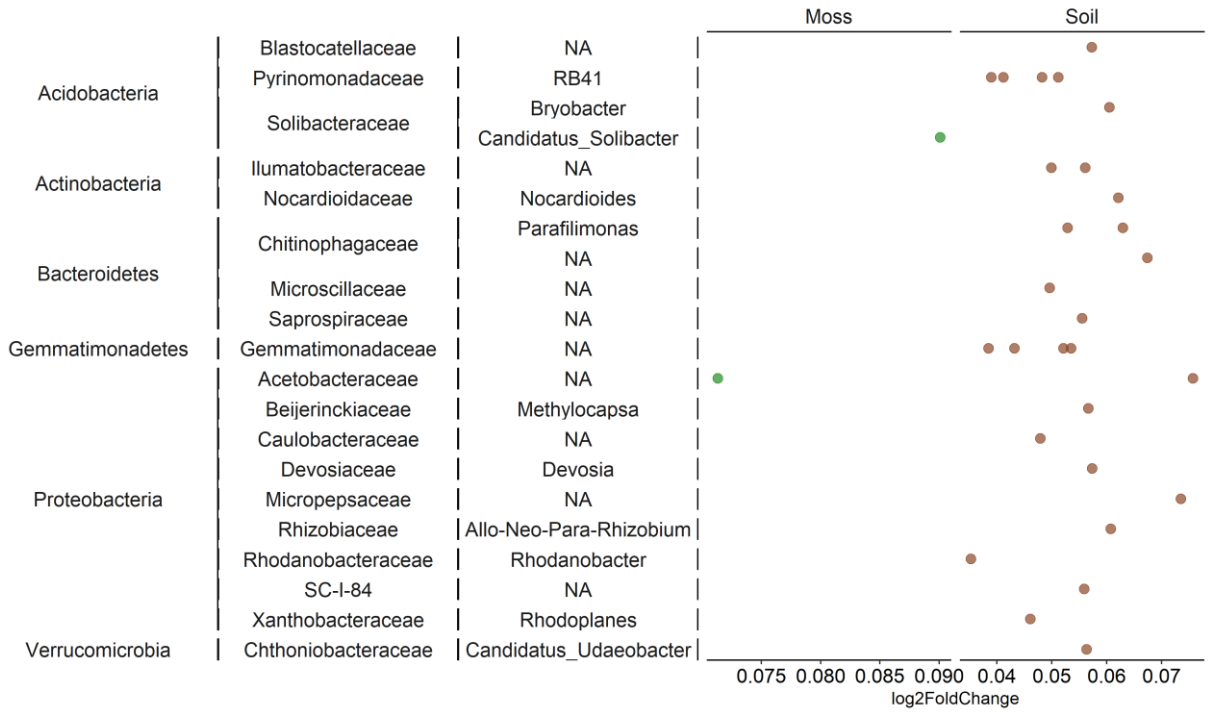

Figure 5 Changes in relative abundance of microbial groups (at ASV level) across the chronosequence in *Racomitrium* moss species (green) and underlying soil (brown).

Linkages between N2-fixation, time since deglaciation, moisture content, TN and bacterial community structure

We used structural equation modelling to investigate the direct and indirect linkages between time since deglaciation, moisture content, moss TN and TC, the *R. lanuginosum*-associated bacterial community structure, *nifH* gene abundance and N_2-fixation (Figure 6 and Table S22). Bacterial community structure was positively affected by TN (standardized path coefficient 0.41; $P < 0.01$) and negatively affected by moisture content (standardized path coefficient -0.63; $P < 0.001$). *nifH* gene abundance was negatively affected by the structure of the bacterial community (standardized path coefficient -0.87; $P < 0.001$) and N_2-fixation rate was negatively affected by *nifH* gene abundance (standardized path coefficient -0.68; $P = 0.01$). This indirect effect of the bacterial community structure on N_2-fixation rates via changes in *nifH* gene abundance was also significant (standardized path coefficient 0.60; $P = 0.047$; Table S22).

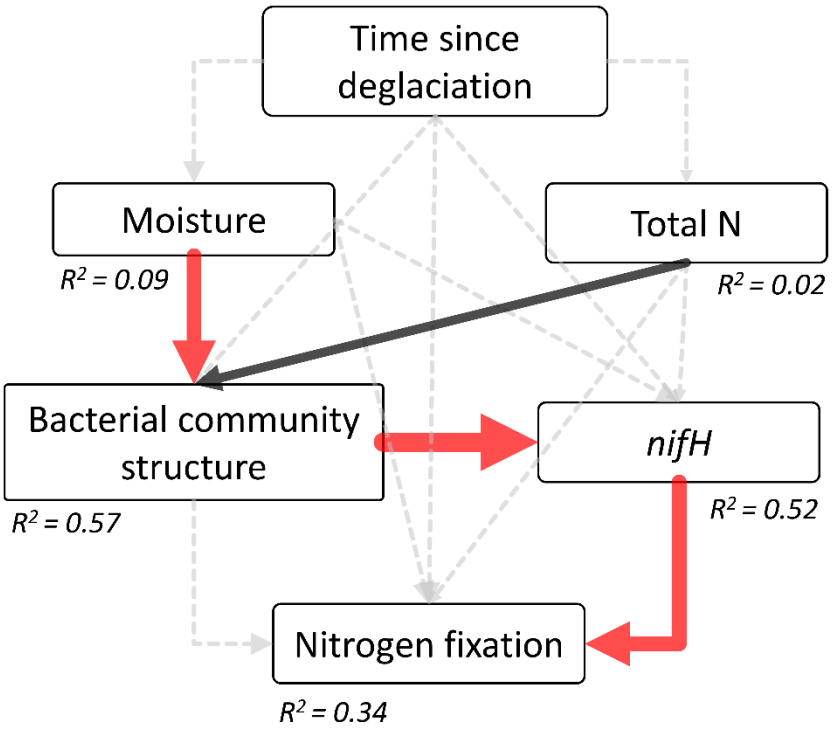

Figure 6 Structural equation model (SEM) showing linkages between time since deglaciation, moss moisture content and moss total N, moss bacterial community, *nifH* gene abundance and N_2-fixation. Note that this SEM is only based on the moss *R. lanuginosum*. χ^2 = 2.59, P = 0.459, df = 3.00, GFI = 0.975, RMSEA = 0, TLI = 1.031. Positive significant effects are represented in black and significant negative significant effects in red. Non-significant effects are indicated with dash-line arrows. The strength of the effect is visualized by the width of the arrow. The R^2-value represents the proportion of total variance explained for the specific dependent variable. Standardized path coefficients are presented in Table S22.

Discussion

Mosses are among the first colonising plants on newly exposed substrates following glacier retreat. Mosses and their bacterial communities play important roles in the C and N cycle, which are crucial during ecosystem development in glacier forefield. But it is unclear how moss bacterial communities develop during primary succession. Here, we studied moss traits, moss-associated bacterial communities and N_2-fixation as well as the bacterial communities of the underlying substrate along a chronosequence in the Fláajökull glacier forefield in Iceland. We found links between time since deglaciation and moss traits and bacterial community composition of the mosses and the underlying soil. We also found that soil and moss bacterial community composition are related to certain moss traits. N_2-fixation rates were linked to bacterial community structure, but not time since deglaciation. Our new data set on primary succession as a driver of moss-associated bacterial community composition and associated processes contributes to the understanding of biogeochemical cycling in newly exposed ice-free substrates.

Changes in moss functional traits with time since deglaciation

We hypothesized that moss shoot traits would change with time since deglaciation. Most of the changes occurred in the earlier stages of the successional gradient and stabilised in the later

stages. We expected TC to increase with succession, and the overall pattern pointed in that direction albeit not significantly, at least until the 76 year old soil on the 1942 moraine. This TC trend in the moss partly agrees with the patterns of soil organic carbon content (SOC) in a parallel study in the same forefield (Wojcik et al. 2021), where authors showed that TC content increased until the 1936 moraine and that TC was lower on the 1931 and 1929 moraines, probably due to soil disturbance via geomorphological events or due to the heterogeneous nature of the soil substrates in the Fláajòkull forefield.

We also expected moss TN to increase with time since deglaciation, but our data did not show any successional trends in moss N content. In the early successional stages of the Fláajökull forefield soil TN increases (Wojcik et al. 2020), but moss shoot TN didn't correlate with the soil TN trend. There are several potential reasons for the discrepancy between moss and soil TN. Moss N may for instance be lost via denitrification or via leeching to deeper soil layers (Johnson, Neuer, and Garcia-Pichel 2007). Moss N has also been found to be more rapidly lost from moss litter than C during decomposition (Philben et al. 2018). Nevertheless, as moss mat coverage and shoot length increased with succession, moss TN per m^2 will increase. Overall moss C/N showed an increasing trend along the chronosequence, probably driven by the increasing C content, but again with lower values on the three oldest, potentially disturbed soils. The increase is similar to the increase in C/N found in the bryophytes with succession in glacier forefields in Tierra del Fuego in Chile (Arróniz-Crespo et al. 2014).

$\delta^{13}C$ can reflect the signal of multiple environmental factors (Waite and Sack 2011) and often increases in moss tissue with ecosystem age (Bansal, Nilsson, and Wardle 2012; Jonsson et al. 2015b). Our data did show an increase in moss shoot $\delta^{13}C$ with time since deglaciation. This however could also be due to differences between moss species, with lower values in *R. ericoides* (-28.3 ‰ ± 0.x) and more negative values in *R. lanuginosum* (-27.8 ‰ ± 0.1). This confirms the importance of moss species for $\delta^{13}C$ values (Bramley-Alves et al. 2015; Waite and Sack 2011). Our average $\delta^{13}C$ value for *R. lanuginosum* (-25.8 ± 0.1) is comparable to those found in *R. lanuginosum* on Mauna Loa, Hawaii (-26.3% ± 0.4) (Waite and Sack 2011).

Potential drivers of the moss-associated and underlying soil bacterial community structure

The bacterial community structure of the mosses and the soil were both affected by time since deglaciation. As moss species is considered to be an important factor in determining the composition of the bacterial communities (Holland-Moritz et al. 2018; Bragina, Berg, et al. 2012), the shift in moss species along the chronosequence may also have contributed to the effect of time since deglaciation on the moss bacterial communities.

Moss moisture content turned out to be an important factor contributing to variation in the bacterial community structure of both *R. ericoides* and *R. lanuginosum*. Moisture content is an important driver of microbial decomposition (Schimel et al. 1999) and may thereby also affect bacterial community structure, especially in the decomposing part of the moss shoots. Interestingly, moisture has also been found to affect the occurrence of Antarctic moss-associated fungi (Hirose et al. 2016). In our study time since deglaciation and C/N ratio affected the bacterial community composition of *R. ericoides*, but not *R. lanuginosum*. The discrepancy in factors structuring the bacterial communities of the two mosses may also be caused by the smaller sample size of *R. ericoides* versus *R. lanuginosum*, but factors driving moss bacterial communities may also change with succession. Our results indicate that time since deglaciation and C/N ratio are important in the earlier stages of succession (eg. in *R. ericoides*), potentially because C/N ratio becomes more stable in the later stages (eg. in *R. lanuginosum*).

The soil bacterial community structure below the mosses showed variation with succession, but less than the overall moss bacterial community. Interestingly, moss C/N ratio was also driver of the soil bacterial community composition. Moss C/N ratio may influence soil C/N ratio, without directly affecting the soil bacterial community, but plant traits such as leaf N can influence soil bacterial community composition (Vries et al. 2012) and moss chemical traits may thus also affect bacterial community in the underlying substrate.

The bacterial communities of *Racomitrium* moss species and underlying soil were clearly distinct at the ASV level, indicating that there might be little or no lateral transmission of the soil bacterial communities to the moss bacterial communities and/or vice versa. The bacterial community of *R. lanuginosum* in the Fláajökull glacier forefield was also similar to the bacterial community of *R. lanuginosum* from a subarctic-alpine heathland in northwest Iceland (Klarenberg et al. 2021). Many taxa are shared in similar proportions, such as the orders Acetobacterales, Acidobacterales and Solibacterales, while Bacteroidetes were more abundant in the mosses in the glacier forefield and Planctomycetes more abundant in the heathland. Contrary to what one would expect, Cyanobacteria were less abundant in the moss in the glacier forefield than in the heathland. One possibility is that the Fláajökull glacier forefield receives more nitrogen from deposition as the forefield is in the vicinity of farmlands that could provide the moss with N and reduce the need for Cyanobacteria as diazotrophic symbionts. Generally, the moss-associated bacterial communities are dominated by presumptively acidophilic bacteria often associated with ombrotrophic or other oligotrophic environments and comparable to other mosses (Holland-Moritz et al. 2021).

Taxa specific trajectories in of moss-associated and underlying soil bacterial communities with succession

Most of the bacterial phyla that shifted in relative abundance during succession were found in the moss. The phylum Chloroflexi increased in relative abundance with succession in both the soil and the mosses. An increase in Chloroflexi has also been detected with succession in the soil and rhizosphere of *S. oppositifolia* in a glacier forefield in the high Arctic (Mapelli et al. 2018). In the moss, we found decreasing abundance of Proteobacteria, Cyanobacteria and Bacteroidetes. These taxa often become less abundant as succession progresses in glacier forefields in soils (Bajerski and Wagner 2013; Jiang et al. 2018; Fernández-Martínez et al. 2017; Bradley et al. 2016) and our results show that similar patterns are found in the moss microbiome, but less so in the moss-covered soil.

On ASV level, most changes with soil age were detected in the soil bacterial community. All of these ASVs increased in relative abundance with soil age. Many of them were classified as genera known to be able to degrade plant-organic matter, such as *Ca. Solibacter* (Ward et al. 2009), *Nocardioides* (Guo et al. 2021), Chitinophagaceae (Yong Li et al. 2011) and Micropepsaceae (Harbison et al. 2016), indicating increased moss abundance with succession also increases the potential for degradation of dead moss material. While Cyanobacteria decreased in relative abundance with soil age in the moss, heterotrophic N_2-fixers became more abundant with soil age (for instance *Devosia* (Rivas et al. 2002), Rhizobiaceae (Dobbelaere, Vanderleyden, and Okon 2003), Methylocapsa (Dedysh et al. 2002) and *Rhodoplanes* (Buckley et al. 2007) in soil, and Acetobacteriaceae (Saravanan et al. 2008) in the mosses), probably because of increased substrate availability. An increase in potential denitrifiers (*Ca. Solibacter* (Ward et al. 2009) and *Rhodanobacter* (Kostka et al. 2012)) suggests an increase in nitrates and/or nitrites with succession and loss of N via denitrification with succession. Some of the taxa increasing along the chronosequence are known to be acidophilic (Chitinophagaceae and Gemmatimonadaceae (Cline and Zak 2015), Acetobacteraceae in moss (Kersters et al. 2006),

Micropepsaceae (Harbison et al. 2016)), and may be linked to decreased soil pH with soil age in the Fláajökull glacier forefield (Wojcik et al. 2020 and Table S24).

Moss N_2-fixation and diazotroph abundance during succession

We did not detect any trends in N_2-fixation rates with soil age. *nifH* gene abundance however, showed an overall decrease with soil age, indicating a decreasing abundance of diazotrophs with succession. Moss-associated N_2-fixation rates were not affected by moss N content, soil age or moisture, but rather by the abundance of diazotrophs and bacterial community composition, at least in *R. lanuginosum*. The negative link between *nifH* gene abundance and N_2-fixation rates, could indicate that not all bacteria taxa with *nifH* genes are actively involved in N_2-fixation, or that with succession, a shift in N_2-fixing taxa takes place. The last explanation is supported by the indirect connection found between bacterial community composition and N_2-fixation rates via *nifH* gene abundance. We for instance detected a decrease in the relative abundance of Cyanobacteria in the mosses with soil age and an increase in an ASV of the Acetobacteraceae, which contain nitrogen fixing members (Saravanan et al. 2008). Additionally, past research has shown that shifts in *nifH* gene diversity with succession occur in soil in glacier forefields (Duc et al. 2009) and our study suggests that these shifts may also take place in mosses.

Moss N_2-fixation may be and stay an important source of N in glacier forefields, with increasing importance as moss cover increases with succession. The relative importance of N_2-fixation and mineralization for the N content of the moss and the soil along the chronosequence may be better understood when ^{15}N depletion is taken into account in future studies.

Conclusion

We studied the development of *Racomitrium* moss bacterial communities as well as those of the underlying substrate in relation to moss functional traits along a chronosequence in the glacier forefield of Fláajökull in southeast Iceland. While moss functional traits such as TN and moisture content did not show clear trends along the chronosequence, moss shoot length increased with succession. Time since deglaciation as well as moss C/N ratio and moss moisture content were related to moss bacterial community structure, showing for the first time how moss functional traits are important drivers for moss-associated bacterial communities. The bacterial communities of the underlying soil were also affected by time since deglaciation and by moss C/N ratio, highlighting the influence of moss cover on soil development. Moss and underlying soil bacterial communities differed strongly from each other, suggesting that little lateral transfer between them takes place. We did not detect any trends in moss-associated N_2-fixation rates with time since deglaciation or moss TN, but N_2-fixation rates were linked to bacterial community structure and negatively linked to *nifH* gene abundance. This may indicate a shift in diazotrophic taxa with different N_2-fixing efficiencies along the chronosequence and our data indeed show a proportional decrease in Cyanobacteria and an increase in heterotrophic N_2-fixing taxa.

Our study underlines the importance of moss functional traits as potential drivers for moss bacterial community structure, but also links moss functional traits to bacterial communities in the underlying substrate. This is one way in which mosses can enhance soil development in glacier forefields, but our results also shows that moss-associated N_2-fixation takes place along the whole chronosequence and thereby likely contributes to N availability. Our study contributes to the understanding of the role of mosses in ecosystem development, which will be increasingly important in a future warmer climate leading to increased glacier retreat.

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Author contributions

IJK designed the study, OV and IJK collected the samples. IJK, CK and AS performed the laboratory analysis. IJK analysed the data and wrote the paper with input from OV, CK, AS and LGB.

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Supplementary Material Paper III

Figure S1. ASV (Amplicon Sequence Variant) richness, Shannon diversity and Faith's phylogenetic diversity of the bacterial communities of the mosses *R. ericoides* and *R. lanuginosum* and underlying soil along the chronosequence. Shown are mean ± standard error for three replicates from each moss species and soil collected at each sampling point.

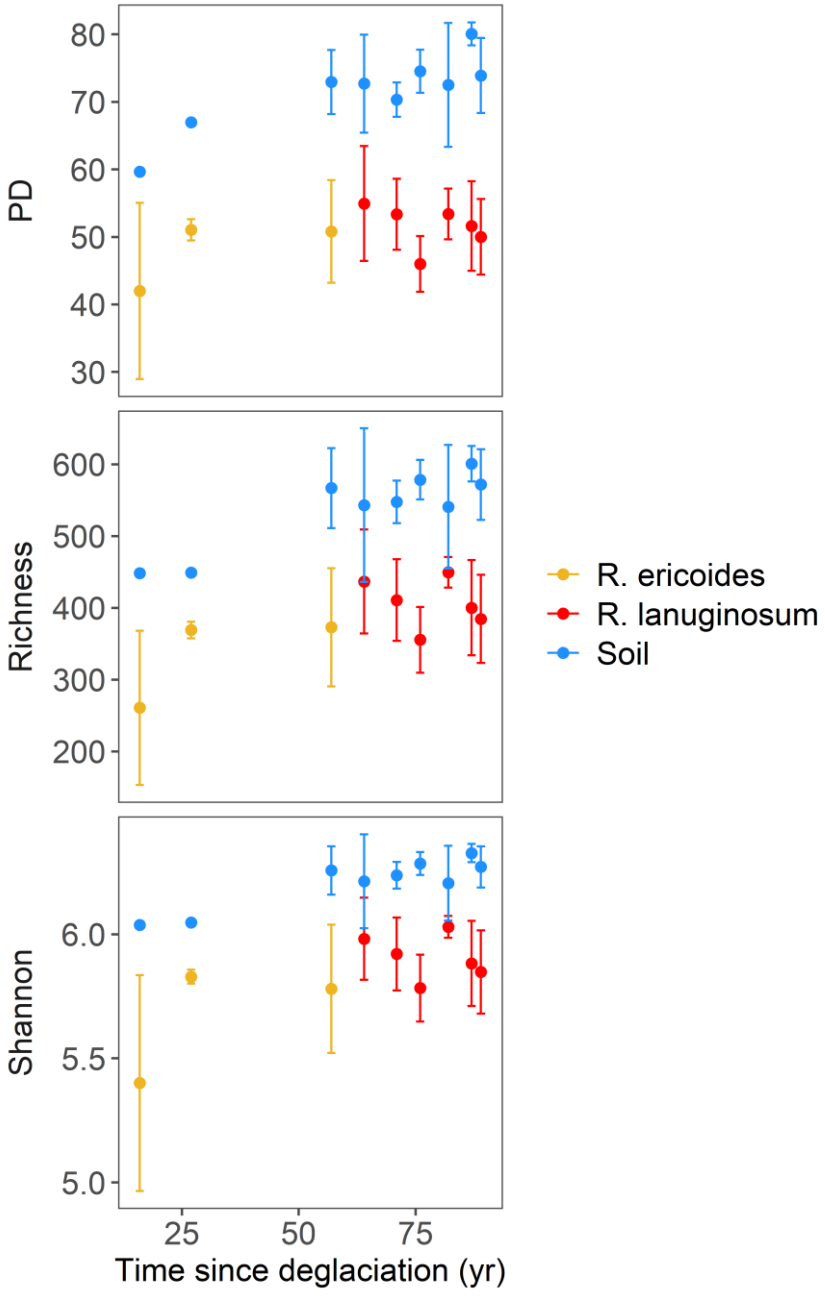

Figure S2. Family level composition of the Alphaproteobacteria within the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

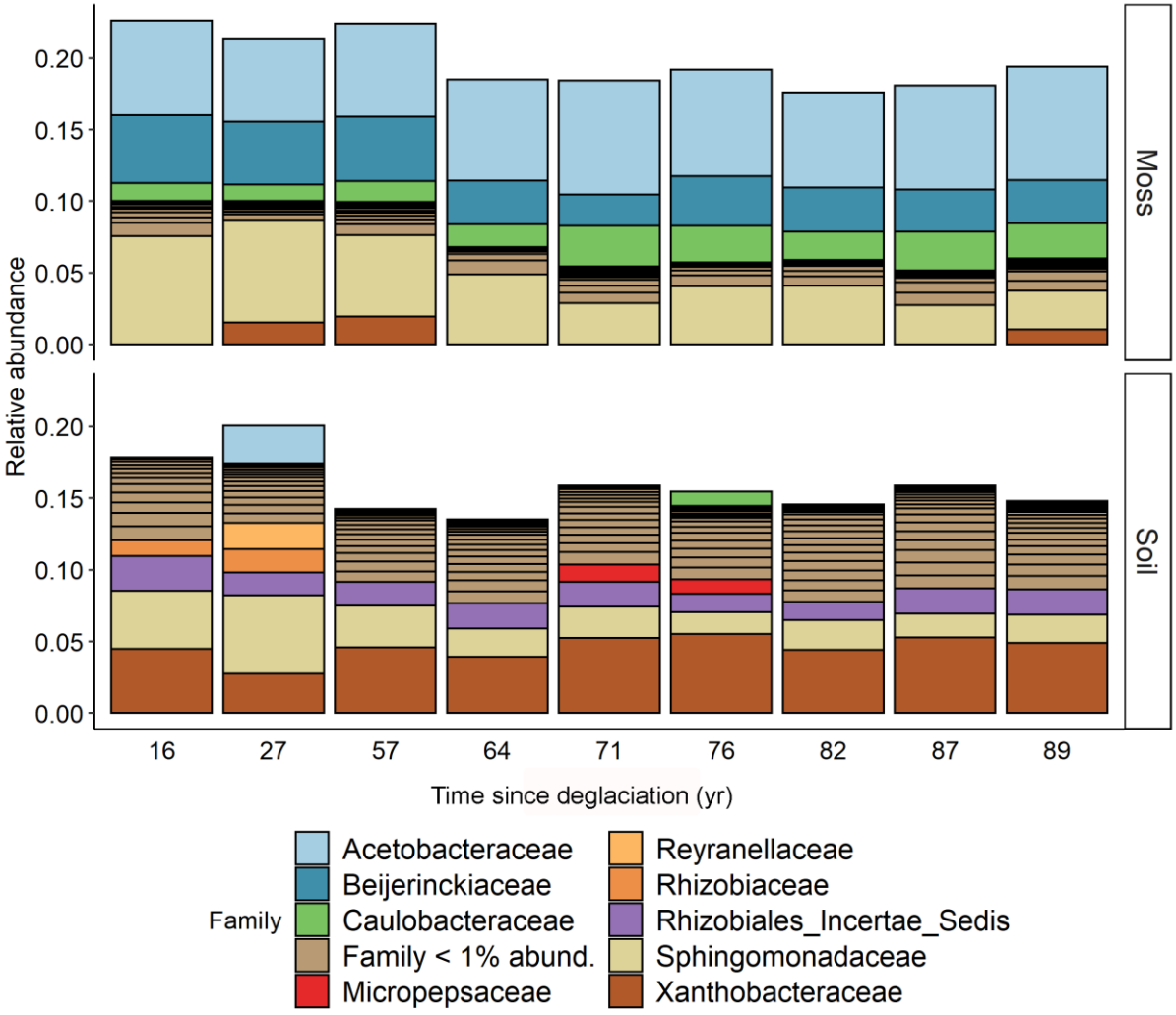

Figure S3. Family level composition of the Acidobacteria within the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

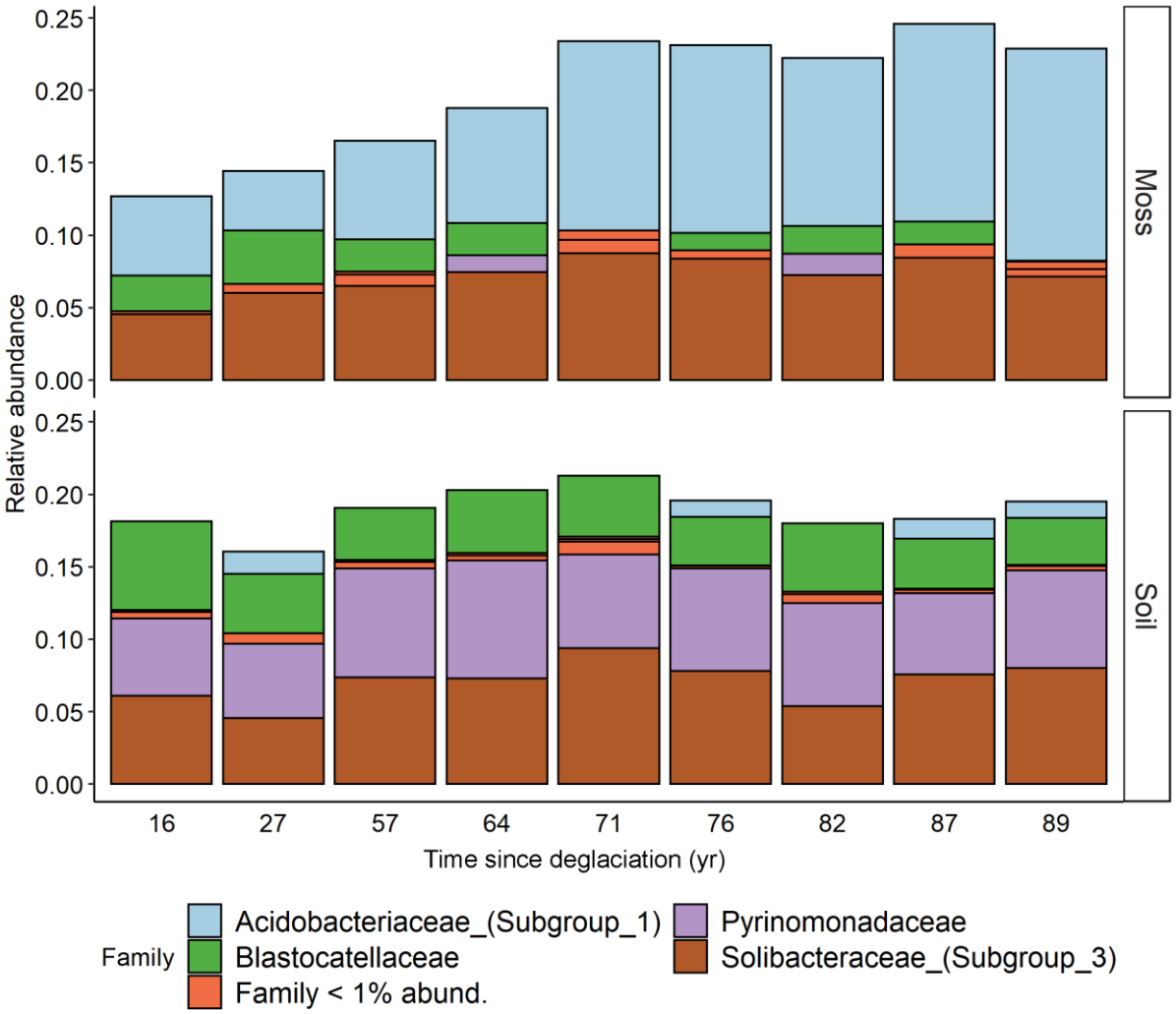

Figure S4. Family level composition of the Actinobacteria within the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

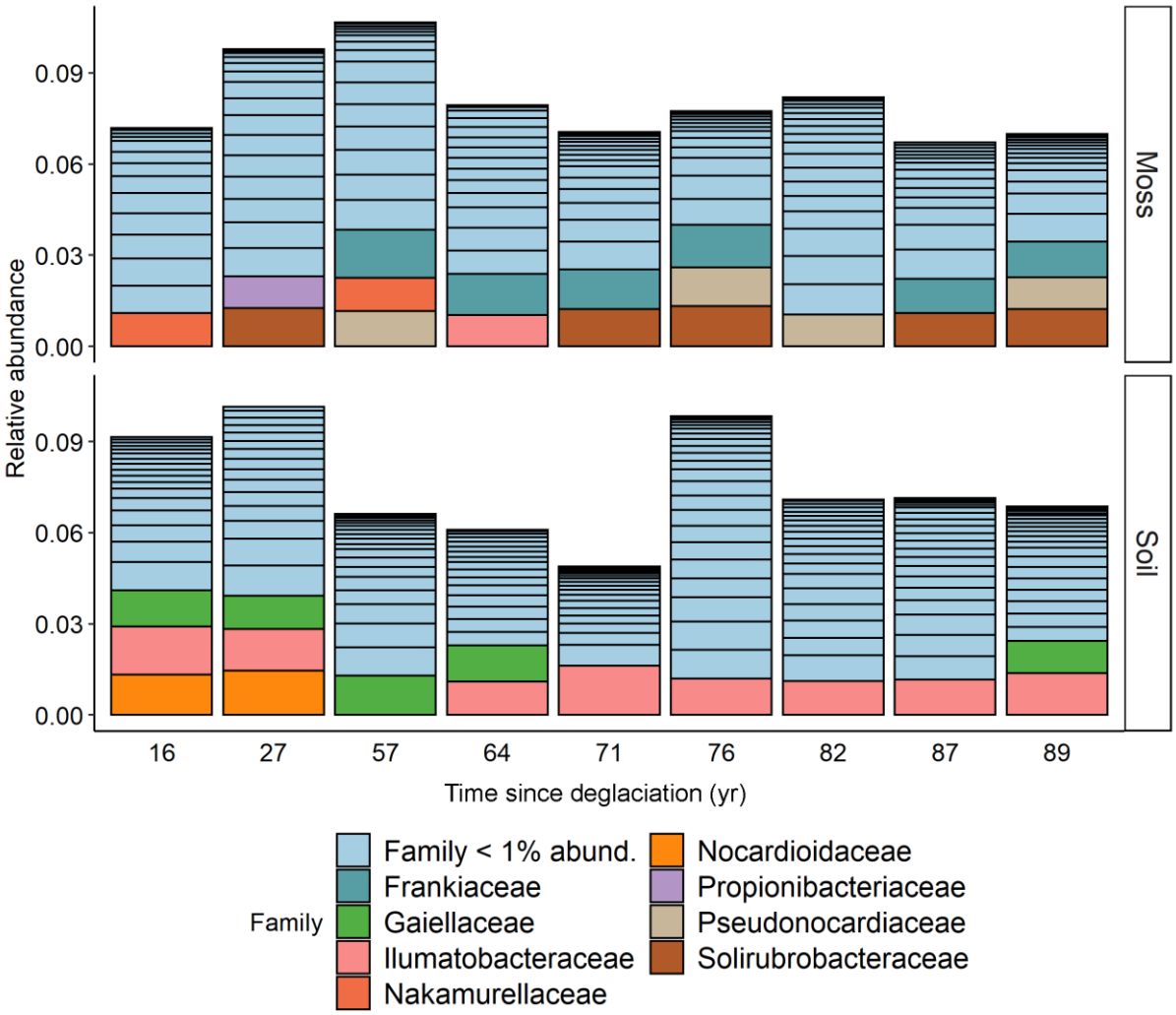

Figure S5. Family level composition of the Bacteroidetes within the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

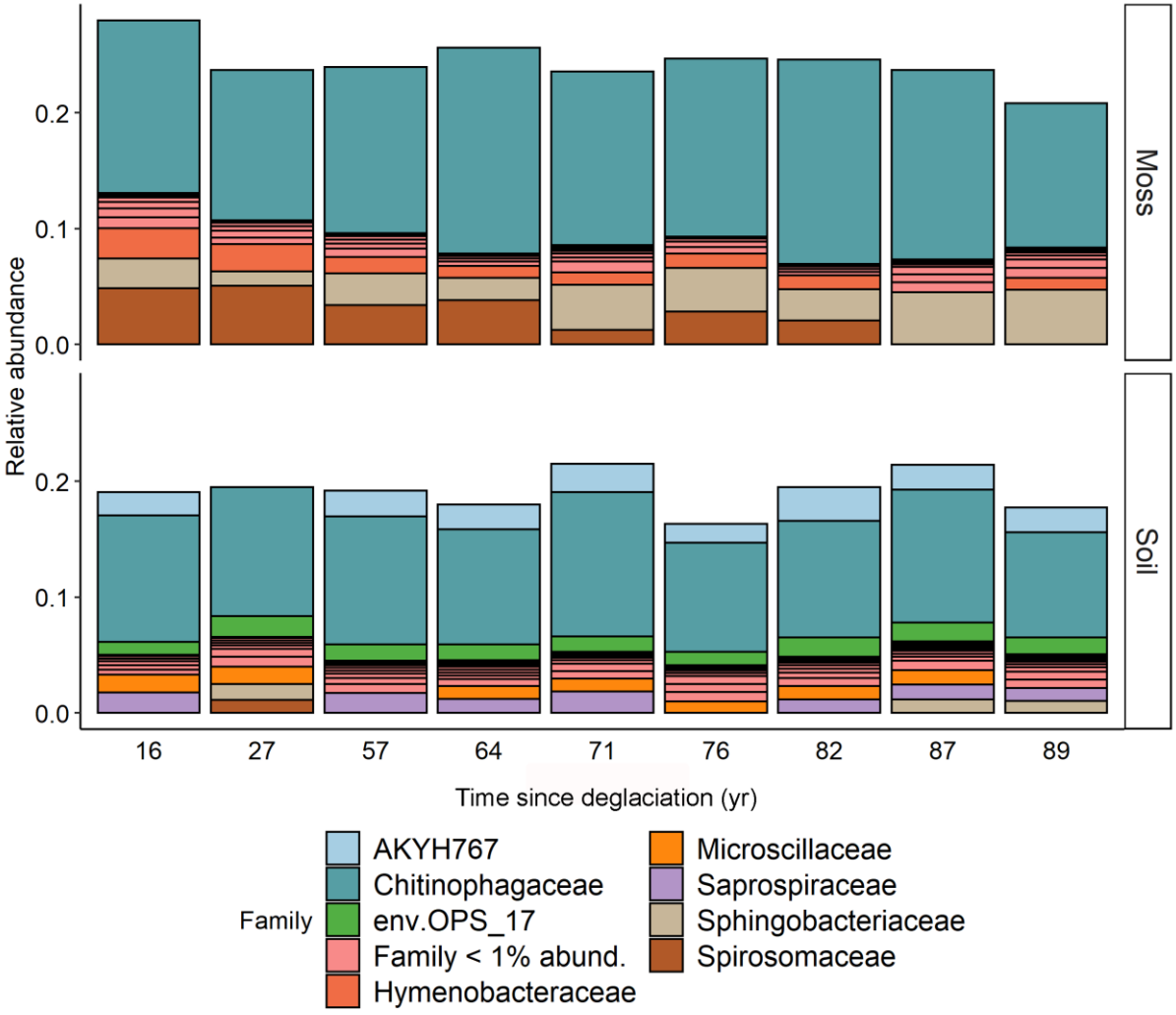

Figure S6. Genus level composition of the Cyanobacteria within the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

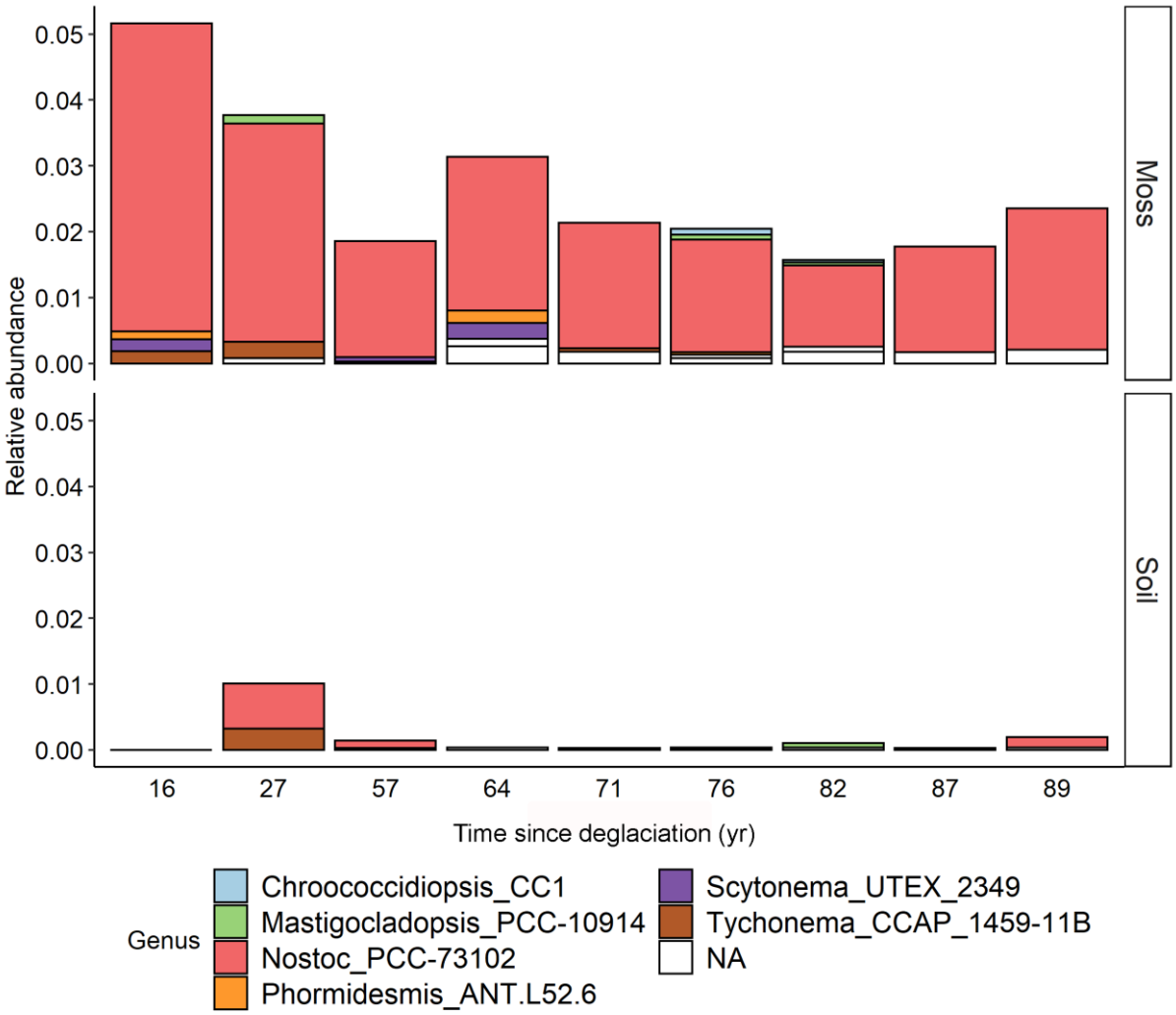

Figure S7. Family level composition of the Gammaproteobacteria within the bacterial communities of *Racomitrium* mosses and underlying soil along a chronosequence in the Fláajökull glacier forefield.

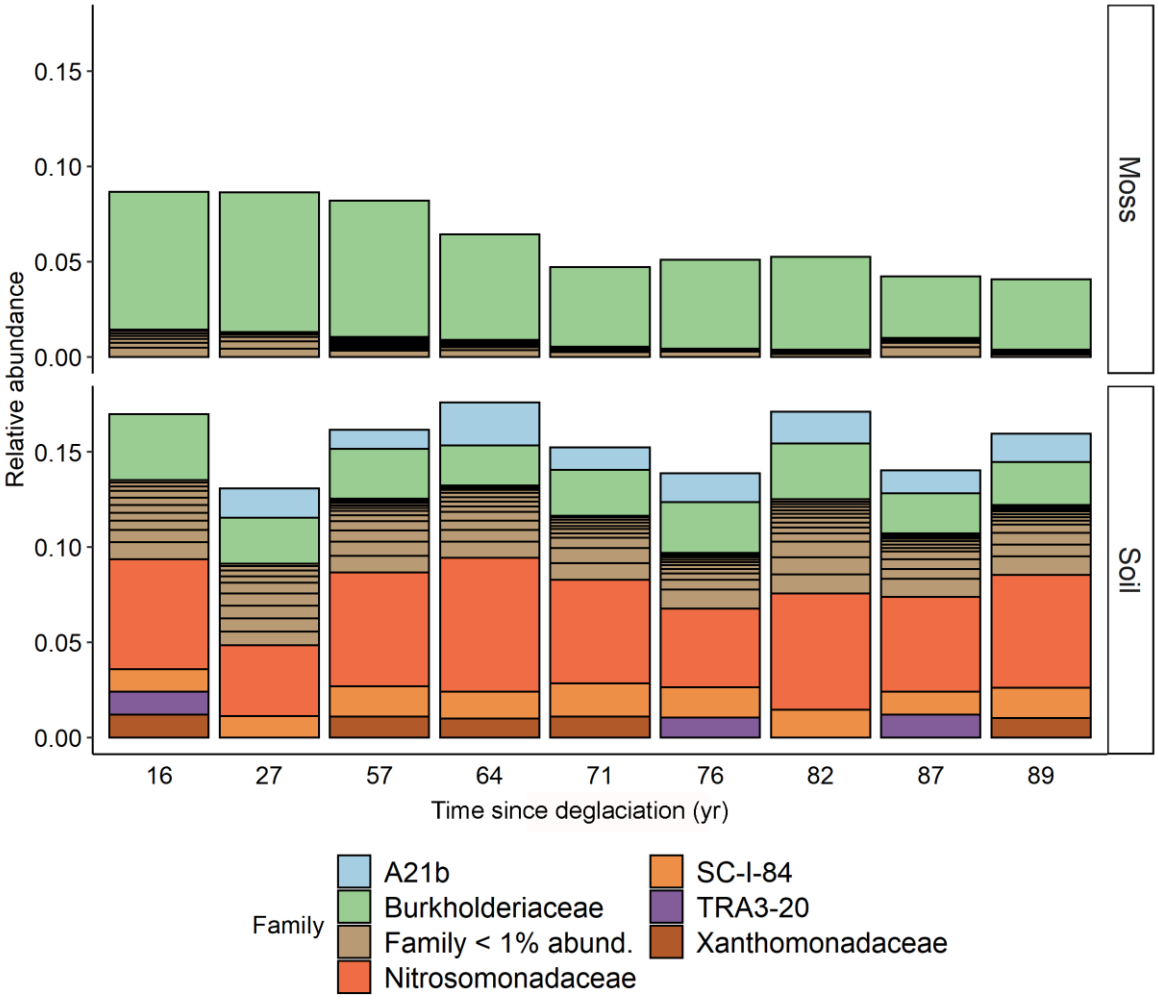

Figure S8. Bacterial phyla changing in relative abundance with soil age in the moss detected by DESeq2.

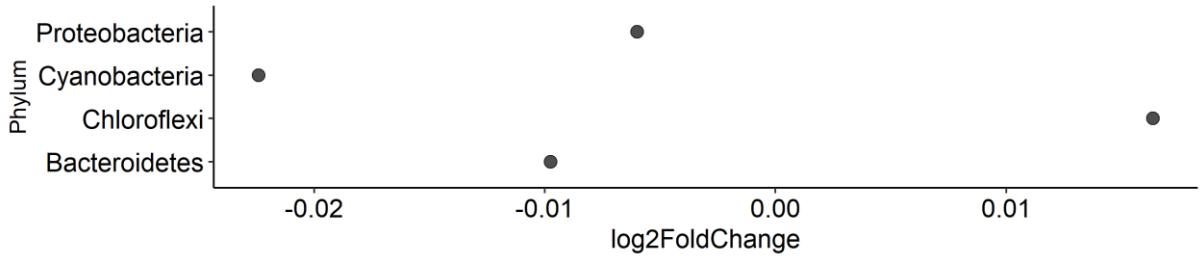

Figure S9. Bacterial classes changing in relative abundance with soil age in the soil detected by DESeq2.

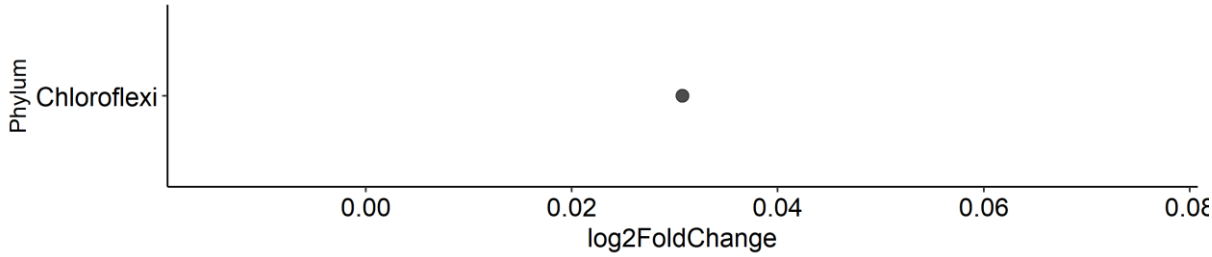

Figure S10 Soil moisture content (%) of bare soil and moss-covered soil along the chronosequence in the Fláajökull glacier forefield.

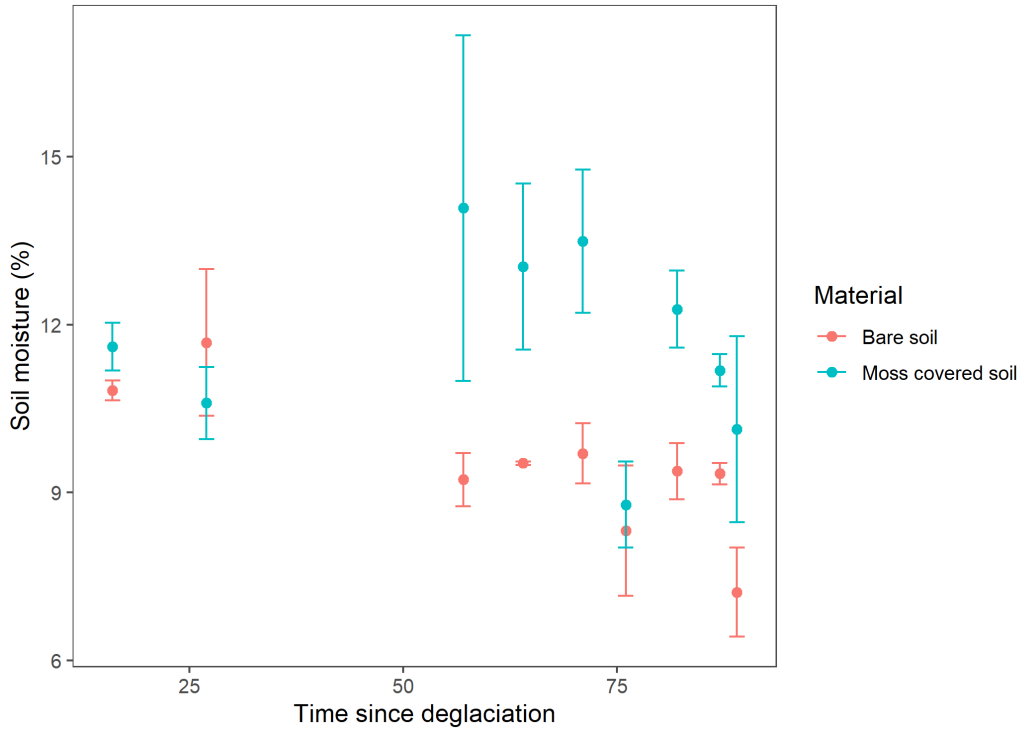

Figure S11 pH of bare soil and moss-covered soil along the chronosequence in the Fláajökull glacier forefield.

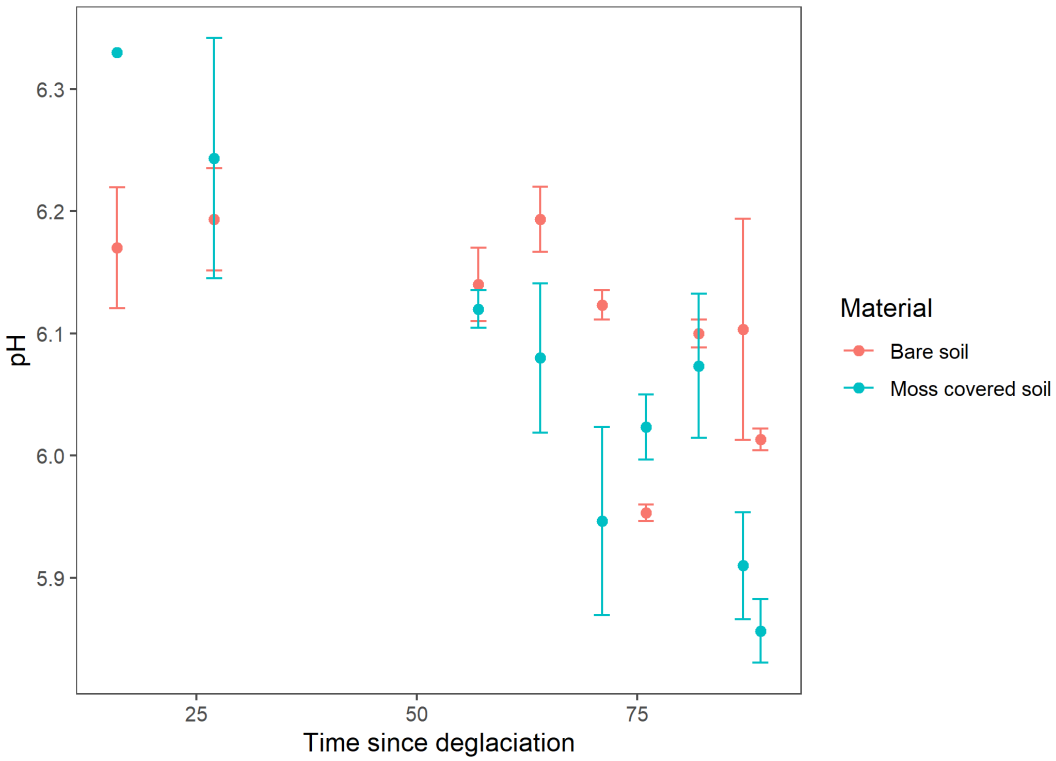

Table S1. Correlation matrix of edaphic factors.

	Time since deglaciation	Species	TC	TN	C:N ratio	Shoot length
Species	0.862					
TC	0.711	0.676				
TN	-0.074	-0.221	0.287			
C:N ratio	0.661	0.704	0.582	-0.543		
Shoot length	0.728	0.628	0.549	-0.347	0.728	
Moisture content	-0.250	-0.414	-0.233	0.252	-0.402	-0.401

Table S2. Moss functional traits, moss-associated *nifH* gene abundance and acetylene reduction rates along the chronosequence. For all measured variables mean ± S.E. are shown.

	Sampling site (time since deglaciation)								
	16	27	57	64	71	76	82	87	89
TC (%)	21.6 ± 1.8	13.8 ± 0.8	23.9 ± 4.0	24.4 ± 1.9	26.4 ± 0.9	32.5 ± 3.4	28.3 ± 1.9	27.7 ± 2.1	27.8 ± 2.5
TN (%)	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
C:N ratio	70.8 ± 7.7	67.3 ± 2.0	74.8 ± 7.1	81.2 ± 12.2	127 ± 12.2	139.6 ± 5.6	112 ± 13.6	140 ± 36.7	117 ± 6.9
$\delta^{13}C$ (‰)	-28.4 ± 0.1	-28.1 ± 0.1	-28.4 ± 0.3	-26.5 ± 0.2	-25.7 ± 0.3	-25.7 ± 0.3	-25.9 ± 0.3	-25.8 ± 0.4	-25.8 ± 0.04
Moisture content (%)	67.6 ± 8.3	71.9 ± 1.5	76.3 ± 0.3	71.2 ± 1.5	58.9 ± 2.1	67.4 ± 6.9	62.2 ± 2.8	59.6 ± 4.5	66.1 ± 2.1
Shoot length (mm)	17.1 ± 5.3	11.7 ± 1.7	28.1 ± 6.7	23.5 ± 0.4	42.2 ± 1.0	35.8 ± 0.9	26.0 ± 2.1	37.9 ± 6.5	46.9 ± 4.8
nifH gene abundance (copies g^{-1})	184992 ± 85656	25421 ± 8832	931 ± 318	13337 ± 3620	19059 ± 8014	20439 ± 2083	11842 ± 8822	6167 ± 1161	17269 ± 9588
Acetylene reduction (mol C^2H^2 Kg^{-1} day^{-1})	1.0e-06 ± 2.3e-06	4.2e-07 ± 2.6e-07	5.7e-06 ± 4.8e-06	4.6e-07 ± 4.2e-08	3.3e-07 ± 4.8e-08	4.7e-07 ± 1.6e-07	1.1e-06 ± 5.4e-07	6.9e-07 ± 1.4e-07	9.1e-07 ± 5.5e-07

Table S3. Model summary for the linear relationship between shoot length and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	30.617	5.620	5.448	1.79e-05
R. lanuginosum	-0.002	7.807	0.000	0.999
Time since deglaciation	9.296	3.679	2.527	0.019
Residual standard error	9.222 on 22 degrees of freedom			
Multiple R2	0.531			
Adjusted R2	0.488			
F-statistic	12.44 on 2 and 22 DF			
p-value	2.426e-04			

Table S4. Model summary for the linear relationship between moss moisture content and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	75.034	4.545	16.509	7.04e-14
R. lanuginosum	-13.068	6.314	-2.070	0.050
Time since deglaciation	3.314	2.975	1.114	0.277
Res standard error	7.459 on 22 degrees of freedom			
Multiple R^2	0.215			
Adjusted R^2	0.144			
F-statistic	3.019 on 2 and 22 DF			
p-value	0.069			

Table S5. Model summary for the linear relationship between moss TN and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	0.317	0.046	6.932	5.84e-07
R. lanuginosum	-0.097	0.063	-1.533	0.140
Time since deglaciation	0.034	0.030	1.136	0.268
Res standard error	0.075 on 22 degrees of freedom			
Multiple R^2	0.101			
Adjusted R^2	0.020			
F-statistic	1.242 on 2 and 22 DF			
p-value	0.308			

Table S6. Model summary for the linear relationship between moss TC and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	23.070	2.782	8.292	3.24e-08
R. lanuginosum	3.284	3.865	0.850	0.405
Time since deglaciation	3.108	1.821	1.707	0.102
Res standard error	4.565 on 22 degrees of freedom			
Multiple R^2	0.521			
Adjusted R^2	0.477			
F-statistic	11.96 on 2 and 22 DF			
p-value	3.052e-04			

Table S7. Model summary for the linear relationship between moss CN ratio and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	80.23	15.72	5.104	4.1e-05
R. lanuginosum	38.63	21.84	1.769	0.091
Time since deglaciation	7.33	10.29	0.712	0.484
Res standard error	25.8 on 22 degrees of freedom			
Multiple R^2	0.507			
Adjusted R^2	0.462			
F-statistic	11.32 on 2 and 22 DF			
p-value	4.161e-04			

Table S8. Model summary for the linear relationship between moss $\delta^{13}C$ and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	-28.282	0.256	-110.522	< 2e-16
R. lanuginosum	2.442	0.356	6.870	6.7e-07
Time since deglaciation	0.013	0.168	0.077	0.939
Res standard error	0.419 on 22 degrees of freedom			
Multiple R^2	0.895			
Adjusted R^2	0.886			
F-statistic	93.79 on 2 and 22 DF			
p-value	1.705e-11			

Table S9. Model summary for the linear relationship between moss *nifH* gene abundance and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	-14549	36418	-0.399	0.693
R. lanuginosum	67479	50590	1.334	0.196
Time since deglaciation	-64786	23839	-2.718	0.013
Res standard error	59760 on 22 degrees of freedom			
Multiple R^2	0.341			
Adjusted R^2	0.281			
F-statistic	5.679 on 2 and 22 DF			
p-value	1.02e-02			

Table S10. Model summary for the linear relationship between moss acetylene reduction and time since deglaciation in the two moss species. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	1.018e-06	4.015e-07	2.536	0.020
R. lanuginosum	-4.594e-07	5.529e-07	-0.831	0.416
Time since deglaciation	2.126e-07	2.474e-07	0.859	0.400
Res standard error	5.658e-07 on 20 degrees of freedom			
Multiple R^2	0.037			
Adjusted R^2	-0.060			
F-statistic	0.381 on 2 and 20 DF			
p-value	0.689			

Table S11. Model summary for the linear relationship between ASV richness and time since deglaciation in the two moss species and the soil. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	390.548	39.921	9.783	1.67e-12
R. lanuginosum	-2.822	50.782	-0.056	0.956
Soil	159.511	45.396	3.514	0.001
Time since deglaciation	32.114	17.616	1.823	0.075
Res standard error	85.82 on 43 degrees of freedom			
Multiple R^2	0.547			
Adjusted R^2	0.515			
F-statistic	17.29 on 3 and 43 DF			
p-value	1.636e-07			

Table S12. Summary for the post-hoc Tukey test between the ASV richness of two moss species and the soil.

Comparison	Mean difference	Lower	Upper	P adjusted
R. lanuginosum-R. ericoides	60.985	-30.584	152.555	2.499e-01
Soil-R. ericoides	211.250	123.0735	152.555	1.896e-06
Soil-R. lanuginosum	150.265	81.297	219.232	1.105e-05

Table S13. Model summary for the linear relationship between Shannon diversity and time since deglaciation in the two moss species and the soil. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	5.804	0.104	55.860	< 2e-16
R. lanuginosum	0.064	0.132	0.484	0.631
Soil	0.426	0.118	3.603	0.001
Time since deglaciation	0.068	0.046	1.491	0.143
Res standard error	0.223 on 43 degrees of freedom			
Multiple R^2	0.506			
Adjusted R^2	0.472			
F-statistic	14.7 on 3 and 43 DF			
p-value	9.928e-07			

Table S14. Summary for the post-hoc Tukey test between the Shannon diversity of two moss species and the soil.

Comparison	Mean difference	Lower	Upper	P adjusted
R. lanuginosum-R. ericoides	0.200	-0.036	0.435	1.104e-01
Soil-R. ericoides	0.536	0.309	0.763	2.476e-06
Soil-R. lanuginosum	0.336	0.159	0.513	1.060e-04

Table S15. Model summary for the linear relationship between Faith's phylogenetic diversity and time since deglaciation in the two moss species and the soil. The intercept is for *R. ericoides*.

term	estimate	std.error	T value	p value
Intercept	53.250	3.967	13.423	< 2e-16
R. lanuginosum	-3.509	5.046	-0.695	0.491
Soil	19.247	4.511	4.267	**0.000**
Time since deglaciation	3.105	1.750	1.774	0.083
Res standard error	8.528 on 43 degrees of freedom			
Multiple R^2	0.663			
Adjusted R^2	0.640			
F-statistic	28.24 on 3 and 43 DF			
p-value	3.003e-10			

Table S16. Summary for the post-hoc Tukey test between the Faith's phylogenetic diversity of two moss species and the soil.

Comparison	Mean difference	Lower	Upper	P adjusted
R. lanuginosum-R. ericoides	2.660	-6.422	11.742	7.587e-01
Soil-R. ericoides	24.249	15.504	32.994	8.610e-08
Soil-R. lanuginosum	21.589	14.749	28.429	3.783e-09

Table S17. Summary for the Permanova testing the effect of material (moss versus soil) on the bacterial community variation of the mosses and the soil. Here we used time since deglaciation as strata.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Material (moss versus soil)	1	3.405	3.405	31.371	0.411	**9.999e-05**
Residuals	45	4.885	0.109		0.589	
Total	46	8.290			1	

Table S18. Summary for the Permanova testing the effect of time since deglaciation and moss characteristics on the structure of the soil bacterial community. Here we used moss species as strata.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Time since deglaciation	1	0.009	0.009	2.444	0.104	**0.042**
CN ratio	1	0.007	0.008	2.01	0.086	**0.044**
TN	1	0.002	0.002	0.57	0.024	0.758
Moisture content	1	0.005	0.005	1.418	0.060	0.176
Residuals	17	0.066	0.004		0.725	
Total	21	0.091			1	

Table S19. Summary for the Permanova testing the effect of time since deglaciation and moss characteristics on the structure of the moss bacterial community variation. Note that we used moss species as strata here.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Time since deglaciation	1	0.099	0.099	18.230	0.381	**9.99e-05**
CN ratio	1	0.009	0.009	1.662	0.035	0.214
TN	1	0.011	0.011	1.980	0.041	0.065
Moisture content	1	0.033	0.033	5.939	0.124	**9.999e-05**
Residuals	20	0.110	0.005		0.418	
Total	24	0.262			1	

Table S20. Summary for the Permanova testing the effect of time since deglaciation and moss characteristics on the structure of the bacterial community of the moss *R. ericoides*.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Time since deglaciation	1	0.006	0.006	2.876	0.216	**0.004**
CN ratio	1	0.005	0.005	2.491	0.188	**0.022**
TN	1	0.003	0.003	1.787	0.134	0.093
Moisture content	1	0.006	0.006	3.134	0.236	**0.004**
Residuals	3	0.006	0.002		0.226	
Total	7	0.026			1	

Table S21. Summary for the Permanova testing the effect of time since deglaciation and moss characteristics on the structure of the bacterial community of the moss *R. lanuginosum*.

Source	Df	Sum of Squares	Mean Squares	F	R^2	P
Time since deglaciation	1	0.005	0.005	1.923	0.094	0.079
CN ratio	1	0.004	0.004	1.658	0.081	0.128
TN	1	0.003	0.003	1.123	0.055	0.330
Moisture content	1	0.009	0.009	3.844	0.187	**0.003**
Residuals	12	0.029	0.002		0.584	
Total	16	0.050			1	

Table S22. Statistics of the structural equation model of direct and indirect effects of warming on N_2-fixation as shown in Figure 7. We show the standardized path coefficients (Std. est.), the standard error of regression weight (se), the z-value (z) and the significance level for the regression weight (p). Significant effects are shown in bold.

Parameter	Variable	Std. est.	se	z	P-value
Moss bacterial community	Time since deglaciation (tm)	-0.33	0.17	-1.88	0.06
	TN (Nm)	0.41	0.16	2.55	**0.01**
	Moisture (Mm)	-0.63	0.16	-4.01	**6.21E-05**
nifH	axis1 (mn)	-0.87	0.22	-3.97	**7.18E-05**
	Time since deglaciation (tn)	-0.28	0.20	-1.38	0.17
	TN (Nn)	0.05	0.20	0.23	0.82
	Moisture (Mn)	-0.23	0.25	-0.92	0.36
N_2-fixation	axis1 (mnfix)	-0.63	0.39	-1.61	0.11
	nifH (nnfix)	-0.68	0.27	-2.54	**0.01**
	Time since deglaciation (tnfix)	0.11	0.25	0.42	0.67
	TN (Nnfix)	0.11	0.24	0.45	0.65
	Moisture (Mnfix)	-0.27	0.29	-0.92	0.36
TN	Time since deglaciation (tC)	-0.13	0.25	-0.52	0.61
Moisture	Time since deglaciation (tM)	-0.30	0.23	-1.31	0.19
Indirect effects on N_2-fixation		Std. est.	se	z	P-value
Total_effect_nfix	tnfix+(tM*Mnfix)+(tM*Mn*nnfix)+(tM*Mm*mnfix)+(tM*Mm*mn*nnfix)+(tN*Nnfix)+(tN*Nn*nnfix)+(tN*Nm*mnfix)+(tN*Nm*mn*nnfix)+(tm*mnfix)+(tm*mn*nnfix)+(tn*nnfix)	0.33	0.22	1.47	0.14
Indirect_time_nfix	(tM*Mnfix)+(tM*Mn*nnfix)+(tM*Mm*mnfix)+(tM*Mm*mn*nnfix)+(tN*Nnfix)+(tN*Nn*nnfix)+(tN*Nm*mnfix)+(tN*Nm*mn*nnfix)+(tm*mnfix)+(tm*mn*nnfix)+(tn*nnfix)	0.22	0.18	1.22	0.22
Indirect_time_moisturen_nfixall	(tM*Mnfix)+(tM*Mn*nnfix)+(tM*Mm*mnfix)+(tM*Mm*mn*nnfix)	0.03	0.08	0.38	0.71

Indirect_time_moisturen_nfix1	(tM*Mnfix)	0.08	0.11	0.75	0.46
Indirect_time_moisturen_nfix2	(tM*Mn*nnfix)	-0.05	0.07	-0.71	0.48
Indirect_time_moisturen_nfix3	(tM*Mm*mnfix)	-0.12	0.12	-0.96	0.34
Indirect_time_moisturen_nfix4	(tM*Mm*mn*nnfix)	0.11	0.11	1.03	0.31
Indirect_time_TN_nfixall	(tN*Nnfix)+(tN*Nn*nnfix)+(tN*Nm*mnfix)+(tN*Nm*mn*nnfix)	-0.00	0.03	-0.24	0.81
Indirect_time_TN_nfix1	(tN*Nnfix)	-0.01	0.04	-0.34	0.74
Indirect_time_TN_nfix2	(tN*Nn*nnfix)	0.00	0.02	0.21	0.84
Indirect_time_TN_nfix3	(tN*Nm*mnfix)	0.03	0.07	0.48	0.63
Indirect_time_TN_nfix4	(tN*Nm*mn*nnfix)	-0.03	0.06	-0.48	0.63
Indirect_microb_nfix1	(Nm*mn*nnfix)	0.25	0.16	1.55	0.12
Indirect_microb_nfix2	(mn*nnfix)	0.60	0.30	1.99	**0.05**
Indirect_microb_nfix3	(Mm*mn*nnfix)	-0.38	0.22	-1.74	0.08
Indirect_time_microb_nfixall	(tm*mnfix)+(tm*mn*nnfix)	0.01	0.12	0.09	0.93
Indirect_time_microb_nfix1	(tm*mnfix)	0.21	0.17	1.23	0.22
Indirect_time_microb_nfix2	(tm*mn*nnfix)	-0.20	0.14	-1.38	0.17
Indirect_time_nifh_nfix	(tn*nnfix)	0.19	0.15	1.25	0.21

Table S23. Model summary for the linear relationship between soil moisture and time since deglaciation in bare and moss-covered soil. The intercept is for *bare soil.*

term	estimate	std.error	T value	p value
Intercept	2.394	0.074	32.529	< 2e-16
Time since deglaciation	-0.002	0.001	-2.398	< 0.05
Moss-covered soil	0.198	0.050	3.928	< 0.001
Res standard error	0.184 on 50 degrees of freedom			
Multiple R^2	0.296			
Adjusted R^2	0.268			
F-statistic	10.49 on 2 and 50 DF			
p-value	< 0.001			

Table S24. Model summary for the linear relationship between soil pH and time since deglaciation in bare and moss-covered soil. The intercept is for *bare soil.*

term	estimate	std.error	T value	p value
Intercept	1.845e+00	7.051e-03	261.692	< 2e-16
Time since deglaciation	-5.605e-04	9.956e-05	-5.630	< 0.001
Moss-covered soil	-8.999e-03	4.599e-03	-1.957	0.0561
Res standard error	0.017on 49 degrees of freedom			
Multiple R^2	0.434			
Adjusted R^2	0.411			
F-statistic	18.79 on 2 and 49 DF			
p-value	8.773e-07			

Supplementary Methods 1 Soil parameters of bare and moss-covered soils in the glacier forefields

Samples were collected in late April 2021 along the same transect as the previous sample collection in 2018 had taken place. The same coordinates were visited. At each sampling point, three bare soil samples and three moss-covered soil samples were taken, both the upper 10 cm of the soil, but without the moss mat.

The soil samples were stored cool until processed. Field-moist soil samples were sieved to 2 mm. For soil moisture content, samples were weighed before and after drying at 70 °C for 24 h. pH was measured after mixing 5 g of soil and 15 ml deionized water for 1 h and left to stand overnight.

One soil moisture measurement was left out as the weight after drying was registered as higher than before drying. And two pH measurements were ignored as the pH meter was not calibrated and the samples were discarded.

Multiple linear regressions with time since deglaciation and material (bare soil versus moss-covered soil) as independent variables were used to test whether soil moisture, pH and organic matter content change with time since deglaciation and differ between material.