Title Long-term warming effects on the microbiome and nitrogen fixation associated with the moss *Racomitrium lanuginosum* in Icelandic tundra

Running title Shifts in a moss microbiome after 20 years warming

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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 the moss microbiome. Here, we studied the total and potentially active bacterial community associated with *Racomitrium lanuginosum*, a common moss species in the Arctic, in response to 20-year *in situ* warming in an Icelandic heathland. We evaluated changes in moss bacterial community composition and diversity. Further, we assessed the consequences of warming for *nifH* gene copy numbers and nitrogen-fixation rates. Long-term warming significantly changed both the total and the potentially active bacterial community structure. The relative abundance of Proteobacteria increased, while the relative abundance of Cyanobacteria and Acidobacteria decreased. While warming did not affect nitrogen-fixation rates and *nifH* gene abundance, we did find 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 and the potentially nitrogen-fixing taxa are sensitive to future warming.

Keywords

Moss, microbiome, climate change, tundra, nitrogen fixation, shrub expansion

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). Dinitrogen (N_2)-fixation by moss-associated Cyanobacteria, the most 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 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). 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 quality and quantity of litter, resulting in a faster turnover of 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. 2020). The increase in labile shrub litter may lead to an increase in copiotrophic taxa and decrease in oligotrophic taxa (Fierer, Bradford, and Jackson 2007; 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. These changes could consequently also affect N_2-fixation rates of moss-associated diazotrophs. In addition, N_2-fixation rates can be expected to increase with temperature, as metabolic processes rate in microorganisms increases with temperature and the enzyme nitrogenase is more active at higher 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 2014; Rousk et al. 2015; Whiteley and Gonzalez 2016; Rousk, Sorensen, and Michelsen 2018). 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 2018). 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 *Betula nana* L..

We hypothesised 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 and a decrease in bacterial diversity and (2) leads to a decrease in oligotrophic taxa and an increase in copiotrophic taxa,. Further, we hypothesised (3) that 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.

To address these hypotheses, we used a warming simulation experiment in the northwest highlands of Iceland that has been running for 20 years (Jonsdottir et al. 2005), coupled with an assessment of the *R. lanuginosum* associated bacterial community structure by 16S rDNA and rRNA amplicon sequencing, measurement of 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), in northwest Iceland and according to Köppen's climate definitions, the sampling site is situated within the lower Arctic. The vegetation has been characterized as a relatively species-rich dwarf shrub heath, with *B. nana* being the most dominant vascular species and the moss *R. lanuginosum* and the lichen *Cetraria islandica* as the dominating cryptogam 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 plots 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 to simulate a warmer summer climate in August 1996 and 1997 (Hollister and Webber 2000; Jonsdottir et al. 2005). They raise the mean daily temperature during summer by 1-2 °C and minimize secondary experimental effects such as differences in atmospheric gas concentration and reduction in ambient precipitation.

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 (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 this study we use data from 2014 on abundance (total number of hits per plot) for *R. lanuginosum*, *B. nana* and litter to test hypotheses 1-3 (Jónsdóttir, unpublished data). 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 (Jónsdóttir, unpublished data).

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 moss samples 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 samples were used for qPCR and subsets of 48 DNA samples (24 from each treatment) and 48 cDNA samples (24 from each treatment) were sequenced. 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 the standard Illumina protocol and 16S rRNA gene V3-V4 primers.

Sequence processing

In order to obtain high-resolution data and to better discriminate ecological patterns, 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 260 bp and reverse reads at 250 bp. Assembled ASVs were assigned taxonomy using the Ribosomal Database Project (RDP) naïve Bayesian classifier (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 and we removed ASVs assigned to chloroplasts and mitochondria, singletons, as well as ASVs present in only one sample. In total, for 85 samples, 3, 598 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 rDNA 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 (10 replicates based on 5 biological samples per plot) 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. Acetylene reduction was used as a proxy for N_2-fixation. 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 72 h in the growth chamber under the same conditions, acetylene reduction and ethylene production were measured by gas chromatography. In order to convert acetylene reduction to N_2-fixation, we used a previously calculated conversion factor for ethylene production to N_2-fixation in a moss heath: 2.48 (Sorensen, Jonasson, and Michelsen 2006). To estimate N_2-fixation rates per square meter, we converted the N_2-fixation estimates per g of moss per hour to N2-fixation rates per Kg of moss per year and we used moss biomass estimates per square meter from Jägerbrand, Jónsdóttir, and Økland 2005 for the control plots to estimate N_2-fixation rates per square meter.

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’ (Oksanen et al. 2013) and ‘phyloseq’ (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 models using a Bayesian method that relies on Markov Chain Monte Carlo (MCMC) iterations. In these models we treated treatment (control or OTC), Betula nana abundance and litter abundance as fixed factors and plot as a random factor to account for the pseudo replication within plots, using the R package 'MCMCglmm' (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.

Distances between the bacterial community composition of the control and OTC samples were based on Bray-Curtis distances. The effect of warming, *B. nana* abundance and litter abundance on the bacterial community composition (hypothesis 1) 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 visualise the relationships between samples from OTC and control 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 a single plot were pooled for this purpose) using the *stat_compare_means* function from the R package 'ggpubr' (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 (Love, Huber, and Anders 2014) on the non-CSS normalised datasets with the R package 'DESeq2' (Love, Huber, and Anders 2014). The adjusted *p*-value cut-off was 0.1 (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' (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 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'. 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. 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 total richness and Shannon diversity of the DNA-based and the cDNA-based bacterial communities did not differ significantly between control and OTC samples (Figure 1A, Figure S1-S4). 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, Figure S3 and S4).

No strong treatment specific clustering of the bacterial community composition could be observed in PCoA plots, although they showed some divergence between the control and the warmed samples (Figure 1B and 1C). This divergence was reflected in the PERMANOVA permutation test of the Bray-Curtis dissimilarity, with: treatment significantly influencing 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 $P < 0.001$; Figure 1B and 1C, Table S1 and S2). 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 S1 and S2).

Taxonomic composition of OTC and control 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%, DNA: 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%) (Figure 2A). The most abundant

Proteobacterial class were Alphaproteobacteria (DNA: 29%, cDNA: 31%) (Figure 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 (Figure S5). 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%) (Figure S6).

The Acidobacteria were dominated by the orders Acidobacteriales (DNA: 17%, cDNA 16%) and Solibacterales (DNA: 11%, cDNA: 7%) (Figure S5). 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 *Ca*. *Solibacter* (DNA: 6%, cDNA: 5%) (Figure S7).

Actinobacteria mainly comprised the orders Solirubrobacterales (DNA: 5%, cDNA: 8%) and Frankiales (DNA: 2%, cDNA: 4%) (Figure S5).

Cyanobacteria were dominated by the genera *Nostoc* (DNA: 5%, cDNA: 2%) and *Stigonema* (DNA: 1%, cDNA 0.1%) (Figure S8).

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 (Figure 2 and Figure S5). On phylum level, Acidobacteria (Wilcoxon rank-sum test, P = 0.008), Cyanobacteria (P = 0.03), and Gemmatimonadetes (P = 0.02), and Cyanobacteria (P = 0.03) decreased in relative abundance in the OTCs, while Proteobacteria (P = 0.04) increased in relative abundance in the DNA-based bacterial communities (Figure 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 (Figure 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 (Figure S5). Acidobacteriales (DNA: P = 0.03, cDNA: P = 0.04) showed a lower relative abundance in the warmed DNA- and cDNA-based bacterial communities (Figure S5). 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 (Figure S5).

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 S4). 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*, *Granulicella*

(Acidobacteria), *Acidiphilium*, *Endobacter*, and *Bradyrhizobium* (Alphaproteobacteria), *Nostoc* (Cyanobacteria), and *Conexibacter* (Actinobacteria) (Figure 3 and Table S4).

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 (Figure 3, Table S5). 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) (Figure 3, Table S5).

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 abundance between the control and warmed samples (Figure 4A). The *nifH* gene abundance was similar in the warmed and control samples (Figure 4B). However, litter abundance negatively affected *nifH* gene abundance (pMCMC = 0.04, Figure S10) and *B. nana* abundance tended to positively influence 16S rRNA gene abundance (pMCMC = 0.072, Figure S9).

We did not find any differences between N_2-fixation rates in the control and warmed plots in June or August 2014 (Figure 4C). However, N_2-fixation in control and warmed plots in August was significantly lower (pMCMC < 0.001, Figure S13) than in June (Figure 4C). We also found a significant negative correlation between *B. nana* abundance and N_2-fixation activities measured in August (pMCMC = 0.04, Figure S12). When assuming a biomass per unit area of 2.99 Kg *R. lanuginosum* m^{-2} (as measured by Jägerbrand, Jónsdóttir, and Økland 2005) in the control plots, we estimate an N_2-fixation rate per unit area of 0.26 g N_2 m^{-2} $year^{-1}$ under ambient conditions.

Relationships between treatment, plant biomass, bacterial community structure and N_2-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) (Figure 5, Table S3). We found that warming was directly associated with changes in bacterial community structure and positively correlated with increased of *B. nana* abundance. 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 (Figure 5, 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 climate change on moss-associated bacterial communities will help to predict the 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 associated with the moss *R. lanuginosum* at a tundra site in the highlands of Iceland by comparing richness, diversity, taxonomic composition, *nifH* gene abundance and N_2-fixation rates, in control and warmed moss samples. Overall our results suggest that moss-associated bacterial communities are sensitive to long-term experimental warming with 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 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) 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). We however 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, the Permanova showed that a small part of the variation of the total bacterial community could also be attributed to litter abundance. In addition, the SEM showed that the bacterial community structure was also indirectly correlated with warming via changes in *B. nana* abundance, and indirectly via the combined effect of *B. nana* and litter abundance. Furthermore, warming induced changes in environmental factors such as lower moss layer thickness, higher soil organic matter content, lower soil moisture (Björnsdóttir 2018; Jonsdottir et al. 2005), or other not measured variables such as leaf nutrient content (Vandenkoornhuyse et al. 2015; Koyama et al. 2018; Sayer et al. 2017) could contribute to the variation in bacterial communities between moss shoots.

Effect of warming on moss-associated bacterial taxa

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. Shifts in individual taxa can affect microbe-microbe and microbe-host interactions and thereby possibly alter functionality or stability of the moss-associated bacterial communities and affect host health and ecosystem functioning (Aydogan et al. 2018; van der Heijden and Hartmann 2016; Agler et al. 2016). Plants might be able to shape their microbial communities (Vorholt 2012; Lebeis 2014) to increase their tolerance to stress like drought (Compant, Van Der Heijden, and Sessitsch 2010) or N limitation (Vries and Bardgett 2012; Bay et al. 2013).

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* (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 (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).

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 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 shown to be 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 2018; 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). Indeed and supporting hypothesis 3, *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 importance of indirect effects of warming on N_2-fixation.

Finally, it is important to note that *R. lanuginosum* biomass tends to decrease in the warmed plots (Björnsdóttir 2018; Jonsdottir et al. 2005). Thus considering that in our study N_2-fixation rates are expressed per gram moss warming would consequently lead to a reduction of N_2-fixation rates 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. Here we focussed on the dominating moss *R. lanuginosum*, in a sub-Arctic-alpine dwarf shrub heath in Iceland. We observed an increase in the relative abundance of Proteobacteria, and a decrease in the relative abundance of Acidobacteria, probably due to their different life strategies (copiotrophic versus oligotrophic) that reflect warming-induced changes in litter quality. Our results also showed no response of N_2-fixation rates and *nifH* gene abundance to warming. However, long-term warming led to 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. The bacterial community of the moss *R. lanuginosum* might thus be sensitive to future warming, with potential implications for moss growth, C seque stration, and N_2-fixation rates.

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Competing Interests

The authors declare that the research was conducted in the absence of any conflict of interest.

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.

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Figures

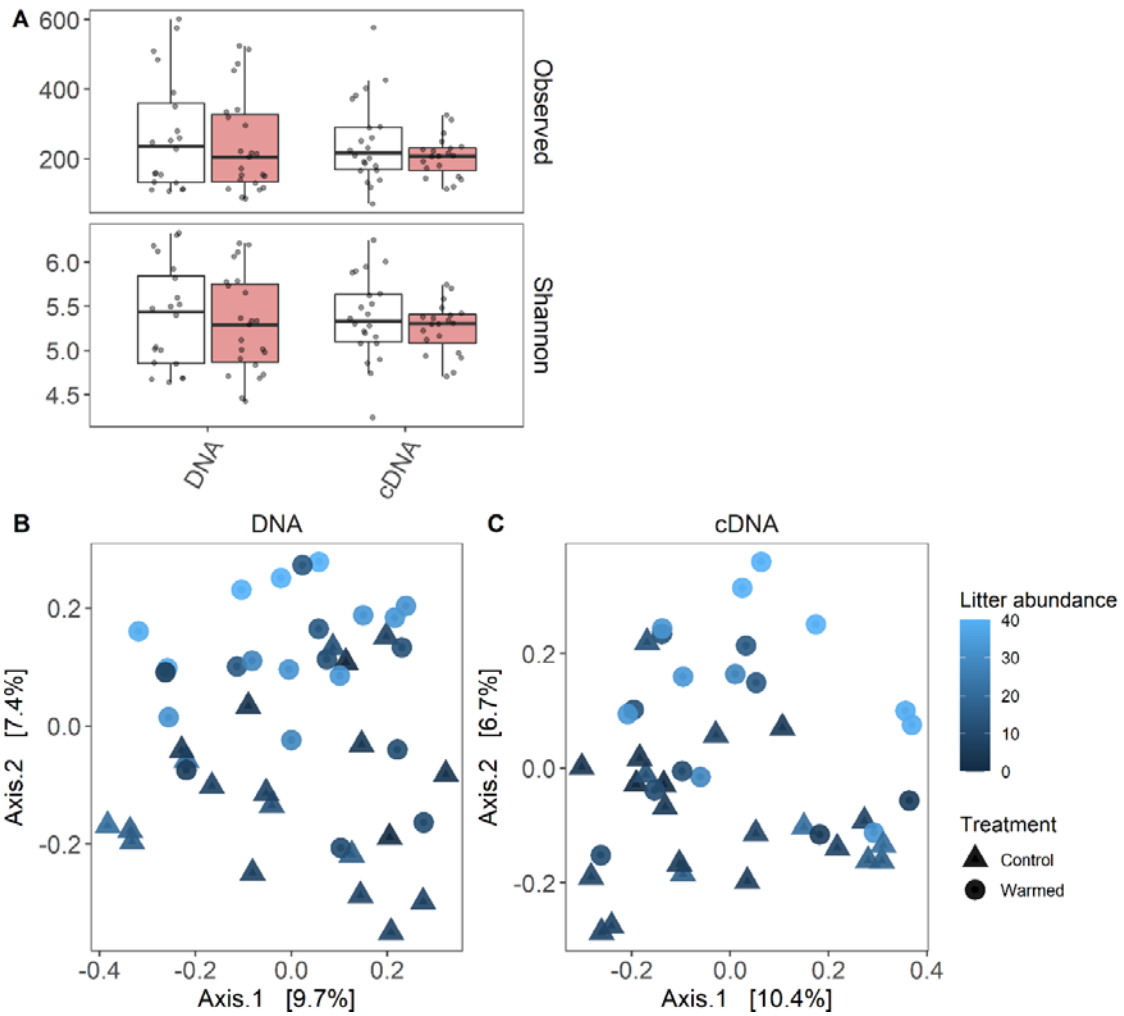

Figure 1 A) Bacterial ASV (amplicon sequence variant) richness (Observed) and Shannon diversity of DNA and cDNA derived bacterial communities associated with the moss *R. lanuginosum*, with control samples represented in white and OTC (warmed) samples in red. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values. Principal coordinate analysis (PCoA) at the ASV level of weighted Bray-Curtis distances between warmed and control samples for (B) DNA- and (C) cDNA- based ASV abundance matrices of the bacterial communities of the moss *R. lanuginosum*. Warmed samples are represented as circles and control samples as triangles. The DNA-based bacterial community composition was significantly correlated with litter abundance (Table S1), thus colour represents correlates to litter abundance; the lighter the blue, the higher the litter abundance.

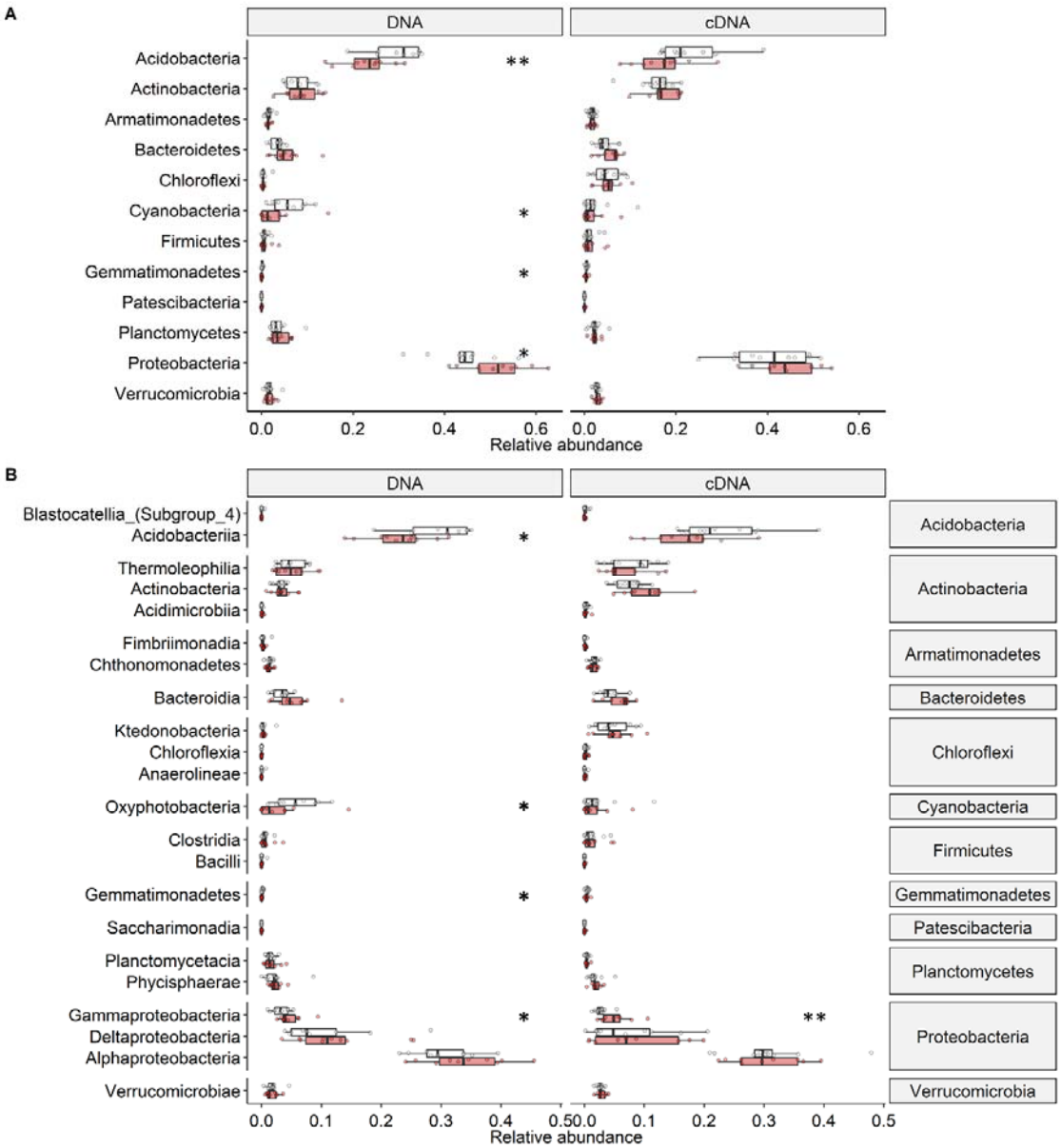

Figure 2 Boxplots of the relative abundances of (A) phyla and (B) classes in DNA and cDNA based bacterial communities associated with the moss *R. lanuginosum*. Controls are shown in white and OTC (warmed) samples are shown in red. Boxplots represent minimum values, first quartiles, medians, third quartiles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

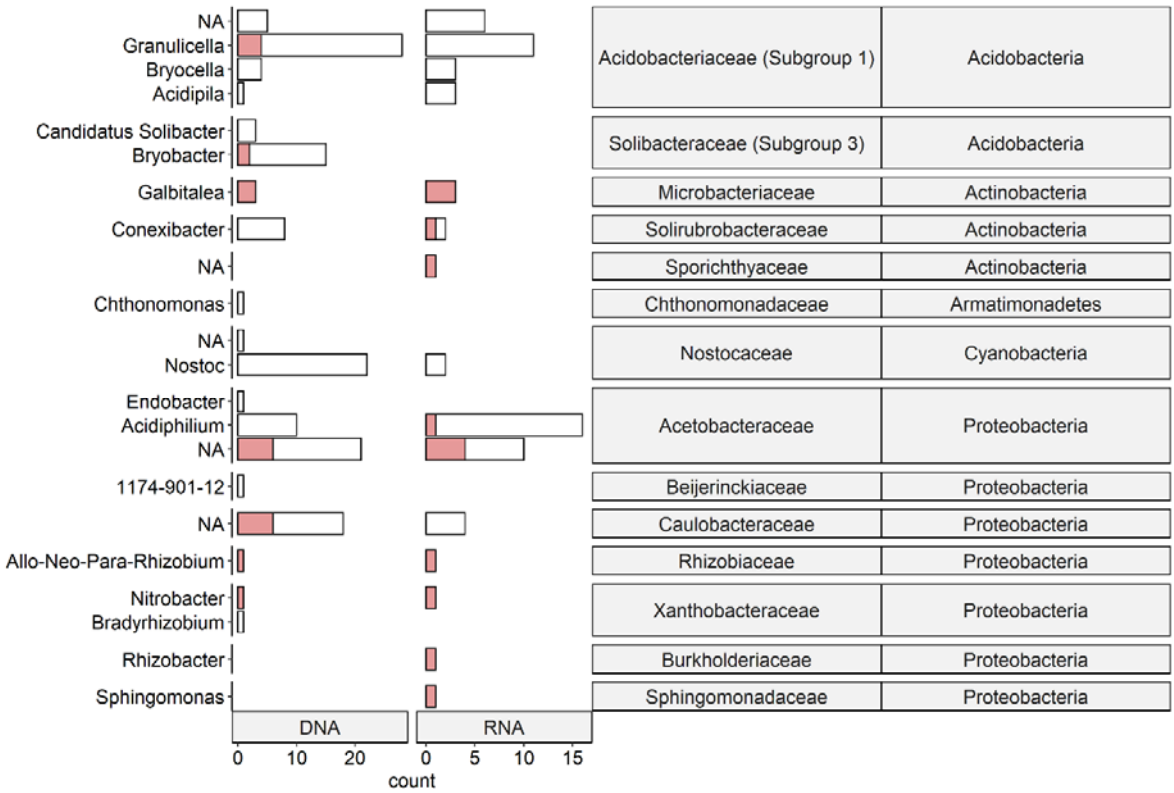

Figure 3 Number of ASVs (amplicon sequence variants) per genus sensitive to warming for DNA and cDNA based bacterial communities associated with the moss *R. lanuginosum.* Sensitivity was determined by differential abundance analysis (DESeq2) and indicator species analysis. ASVs not assigned to genus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

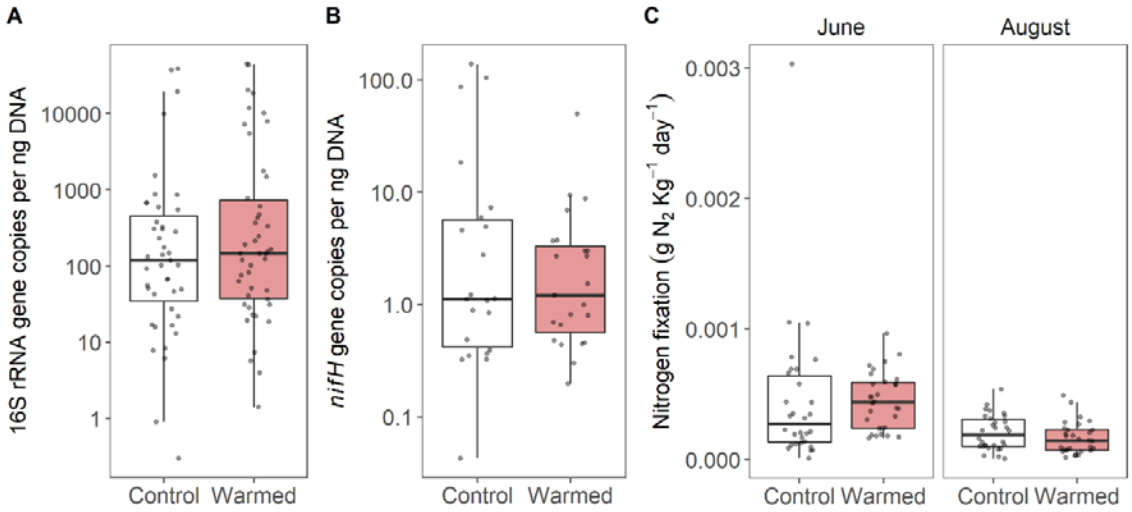

Figure 4 Boxplots of (A) N_2-fixation rates rates (g N_2 Kg dry weight^{-1} day^{-1}) in June and August 2014, (B) 16S rRNA gene copy numbers per ng DNA, (C) nifH gene copy number per ng DNA in *R. lanuginosum* samples from control (in white) and warmed (in red) treatments. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values.

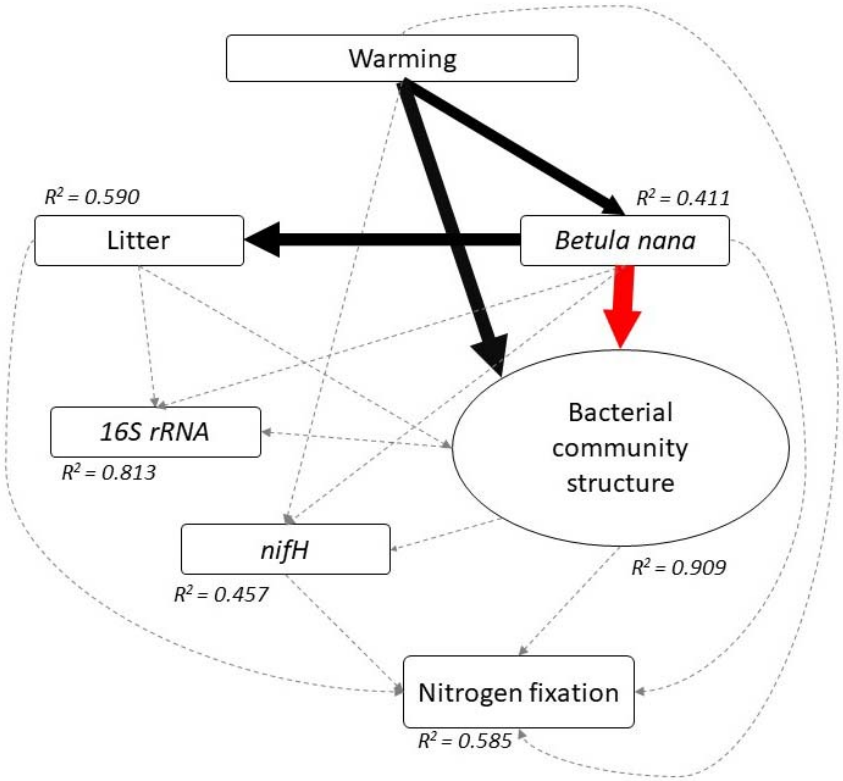

Figure 5 Structural equation model of relationships between warming, *Betula nana* and litter abundance, moss-associated bacterial community and N_2-fixation. A latent variable (bacterial community structure) was computed to represent β-diversity and Shannon diversity index for each plot. $\chi 2 = 2.896$, *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.