Long-term warming effects on the microbiome and *nifH* gene abundance of a common moss species in sub-Arctic tundra

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Summary

- Bacterial communities form the basis of biogeochemical processes and determine plant growth and health. Mosses 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 in these environments.
- 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, and *nifH* gene abundance.
- Warming changed both the total and the potentially active bacterial community structure, while litter abundance only affected the total bacterial community structure. The abundance of *nifH* genes was negatively affected by litter abundance. 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 and potentially nitrogen fixing taxa are sensitive to future warming, partly via changes in litter and shrub abundance.

Keywords

Climate change, moss, microbiome, *nifH*, *Racomitrium lanuginosum*, shrub expansion, tundra

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; 2012; Turetsky *et al.*, 2012; Porada *et al.*, 2013). Most mosses consist of an upper living segment with photosynthetic tissue and a lower decaying dead segment and thus link aboveground and belowground processes (Whiteley & Gonzalez, 2016). Mosses provide a habitat for a range of microbiota, microfauna and mesofauna (Lindo & 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 *et al.*, 2013) and thereby controls C sequestration in moss tissues. Moss-associated diazotrophy is also an important source of N in boreal and Arctic ecosystems (DeLuca *et al.*, 2002; Rousk *et al.*, 2017). The extent and timing to which N is released into these ecosystems is uncertain and may be fast or slow depending on plant N utilization strategies or environmental conditions (Rousk *et al.*, 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 whole ecosystem warming.

The bacterial community composition of mosses is species specific and influenced by environmental factors such as pH and nutrient availability (Bragina *et al.*, 2012a; Tang *et al.*, 2016; Holland-Moritz *et al.*, 2018). While Cyanobacteria have received most of the attention for their N_2-fixing capability (Gentili *et al.*, 2005; Stewart *et al.*, 2011b; Ininbergs *et al.*, 2011; Rousk *et al.*, 2013; Berg *et al.*, 2013; Lindo *et al.*, 2013; Warshan *et al.*, 2016, 2017), mosses harbour diverse bacterial communities. Commonly found phyla associated with mosses include Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Cyanobacteria,

Planctomycetes, Proteobacteria and Verrucomicrobia (Kostka *et al.*, 2016; Tang *et al.*, 2016), and their potential functions include N_2-fixation (Bragina *et al.*, 2012b), anoxygenic phototrophy (Holland-Moritz *et al.*, 2018) and freeze protection (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 *et al.*, 1995). It is a dominant species in many Icelandic ecosystems, forming dense mats where conditions are favourable for colonisation and growth (Tallis, 1958; Bjarnason, 1991; Ingimundardóttir *et al.*, 2014).

Despite the importance of microbial communities for plant functioning and ecosystem processes, long-term effects of warming on moss microbial communities have 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 *in situ* 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; Myers-Smith *et al.*, 2019; Bjorkman *et al.*, 2020). 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.*, 2020). The increase in labile shrub litter may lead to an increase in copiotrophic taxa and decrease in oligotrophic taxa (Wallenstein *et al.*, 2007; Fierer *et al.*, 2007). Copiotrophic taxa could be favoured by *B. nana* litter inputs as they grow faster in nutrient-rich environments, while oligotrophic taxa have lower growth rates, thrive in nutrient-low environments and use difficult to degrade substrates (Koch, 2001; Fierer *et al.*,

2007). Copiotrophs could therefore outcompete oligotrophs under labile litter inputs. 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), for instance via physiological adaptation of diazotrophic communities (Whiteley & Gonzalez, 2016), or shifts to a species composition better suited to the new conditions (Deslippe *et al.*, 2005; Rousk & Michelsen, 2017; Rousk *et al.*, 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 diazotroph abundance, for instance via an increase in shading leading to a decrease in autotrophic nitrogen fixers (Cyanobacteria).

In this study we investigated how two decades of whole ecosystem warming with open top chambers impact the bacterial community and *nifH* gene abundance associated with the prevailing moss *R. lanuginosum* in a subarctic-alpine dwarf shrub heath in northern Iceland, dominated by *B. nana*.

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 with 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 a decrease in bacterial diversity, a shift in community composition and a decrease in *nifH* gene abundance are more strongly driven by the indirect effects of warming via litter and *B. nana* than the direct effect of warming. 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). This site is part of the International Tundra Experiment (ITEX). We assessed the associated bacterial community structure by 16S rRNA gene and rRNA amplicon sequencing and N_2-fixation potential by quantitative PCR (qPCR) of the *nifH* gene encoding the iron-protein component of nitrogenase.

Materials and Methods

Field site and experimental design

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) in June 2017. 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).

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 & Webber, 2000; Jonsdottir *et al.*, 2005). The air temperature in the OTCs was on average 1.4 °C higher in June 2016 to August 2016 and the moss surface temperature was 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 & 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). Since the increase in *B. nana* and litter were already detected in 2000 (Jonsdottir *et al.*, 2005), no drastic changes in plant community were expected within the three years until the moss sampling in June 2017. 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 on average 2.5 times greater in the warmed plots and litter abundance was 2.7 times greater (Table S2).

RNA and DNA extraction, cDNA generation 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. 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 (complementary DNA) was synthesized from the extracted RNA 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). We selected 2-3 samples from each plot based on the highest cDNA and DNA concentrations. 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, 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 (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 *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 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.

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 & Holmes, 2013). Differences in 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 as a factor did not have a significant effect on either the cDNA-based or the DNA-based bacterial community

composition (Table S3 and S4). Even though no effect of plot was found, 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. As PERMANOVA is sensitive to data dispersion, we performed analysis of multivariate homogeneity using *betadisper* from the R package 'vegan' to analyze whether within-group dispersion is similar among the treatments.

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 (Love *et al.*, 2014) on the non-CSS normalised datasets (with pseudoreplicates pooled per plot) with the R package 'DESeq2' (version 1.24.0) (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 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 & 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 *nifH* gene abundance. The structural equation models were fit 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 *nifH* gene abundance (Supplementary Methods 1). Variables included in the

final model, were treatment, litter abundance, *B. nana* abundance, 16S rRNA abundance, *nifH* abundance, and bacterial alpha and beta diversity. The latter two consisted of the average of beta diversity and Shannon diversity index per plot for the combined cDNA and DNA data. Beta 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 *et al.*, 2008).

Results

Treatment effect on bacterial diversity and community structure

Richness and Shannon diversity of the DNA-based and the cDNA-based bacterial communities did not differ significantly between control and OTC samples (Fig. **1a,b,c,d**, Table S4). However, we found a negative effect of *B. nana* abundance on richness and Shannon

diversity of the cDNA-based bacterial community (richness: pMCMC = 0.004; Shannon diversity pMCMC = 0.01, Fig. **1c,d**).

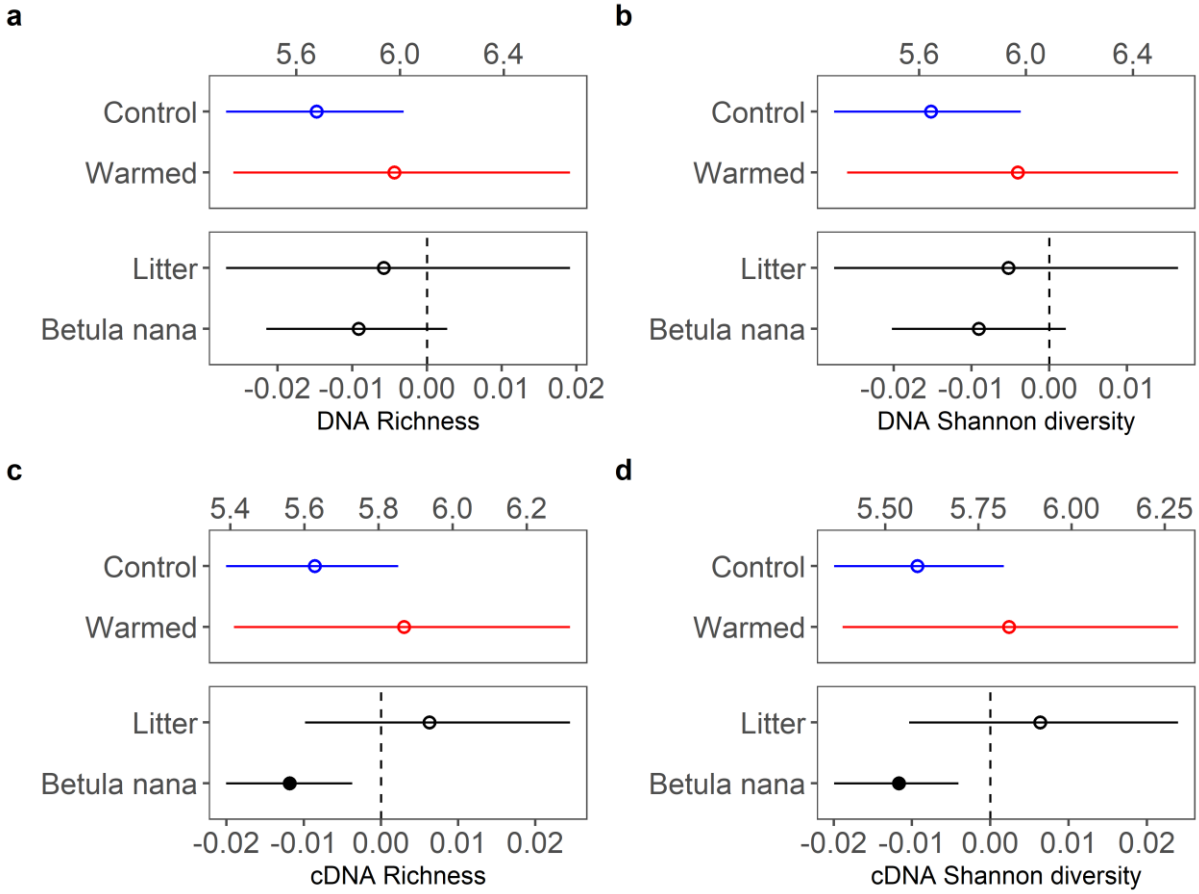

Figure 1 Fixed effect structure of the linear mixed-effect models testing the effect of treatment (warmed and control), *Betula nana* abundance and litter abundance on a) DNA-based richness and b) Shannon diversity, c) cDNA-based richness and d) Shannon diversity. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 95% CrI not overlapping each other (for control versus warming treatment) or 0 (for the litter and *B. nana*) are indicated by closed circles.

The PERMANOVA showed that treatment significantly influenced the DNA- and the cDNA-based community compositions of the moss (DNA: $R^2 = 0.05$, $P < 0.001$, betadisper $P = 0.07$ and cDNA: $R^2 = 0.04$, $P < 0.001$, betadisper $P = 0.77$; Table **1**). 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 **1**).

Table 1. Summary table for the Permanova testing the effect of treatment, *Betula nana* abundance and litter abundance on the DNA-based and cDNA-based bacterial community variation of the moss. Significant effects ($P < 0.05$) are indicated in bold.

	Source	Df	Sum of Squares	Mean Squares	F	R^2	P
DNA	Treatment	1	0.772	0.772	1.957	0.045	**P < 0.001**
	B. nana	1	0.549	0.549	1.392	0.032	0.300
	Litter	1	0.565	0.565	1.434	0.033	**P < 0.05**
	Residuals	39	15.38	0.395		0.891	
	Total	42	17.27			1	
cDNA	Treatment	1	0.694	0.694	1.777	0.041	**P < 0.001**
	B. nana	1	0.707	0.707	1.810	0.042	0.709
	Litter	1	0.500	0.500	1.280	0.030	0.259
	Residuals	38	14.84	0.391		0.886	
	Total	41	16.74			1	

Taxonomic composition of 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. **2**). The most abundant Proteobacterial class were Alphaproteobacteria (DNA: 29%, cDNA: 31%) (Fig. **3**). 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**).

Acidobacteria were dominated by the orders Acidobacteriales (DNA: 17%, cDNA 16%) and Solibacterales (DNA: 11%, cDNA: 7%) (Fig. **S3**). 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. **S4**).

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. **S5**).

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. **2,3** and Fig. **S1,S2**). 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. **2**). 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. **3**).

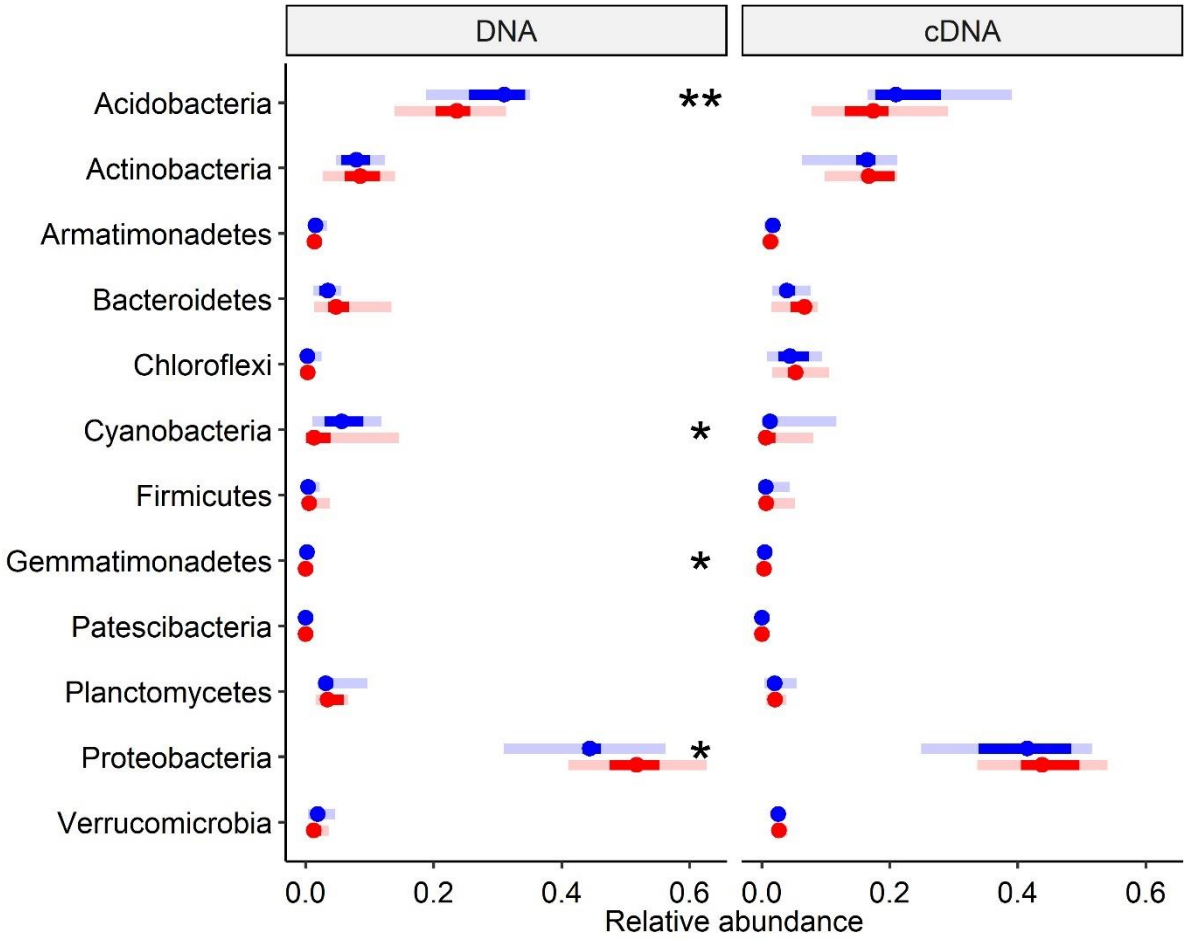

Figure 2 Boxplots of the relative abundances of phyla in DNA and cDNA based bacterial communities associated with the moss *R. lanuginosum*. Boxplots represent minimum values (left end of the light blue and light red), first quartiles (blue and red), medians (filled circle), third quartiles (blue and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

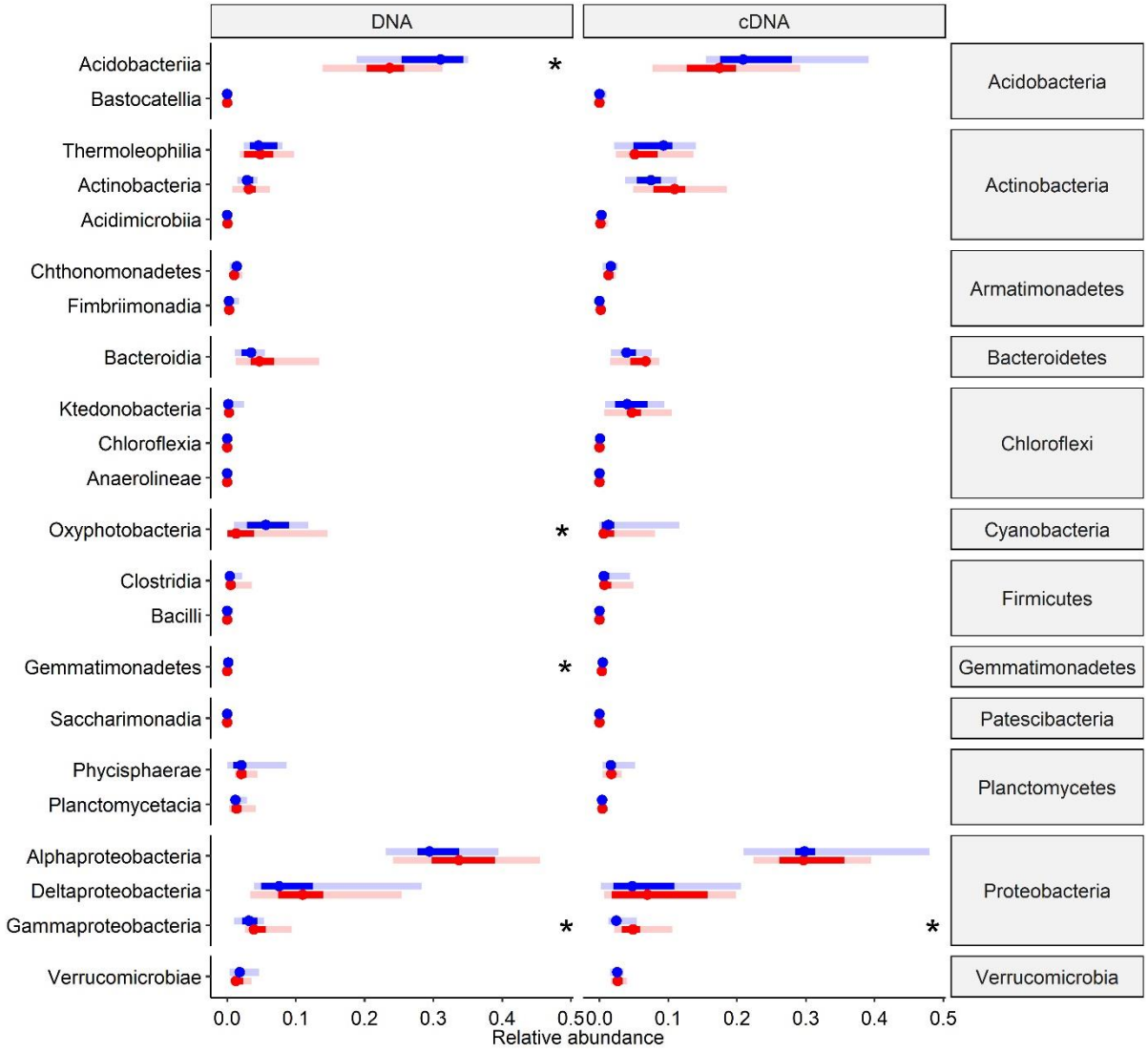

Figure 3 Boxplots of the relative abundances of classes in DNA and cDNA based bacterial communities associated with the moss *R. lanuginosum*. Boxplots represent minimum values (left end of the light blue and light red), first quartiles (blue and red), medians (filled circle), third quartiles (blue and red) and maximum values (right end of the light blue and light red). Significance levels (* < 0.05) are based on Wilcoxon rank sum tests.

At 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. **S2**). 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, S2**).

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 S6). 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. **4** and Table **S6**).

For the bacterial communities in the 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. **4**, Table **S7**). 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. **4**, Table **S7**).

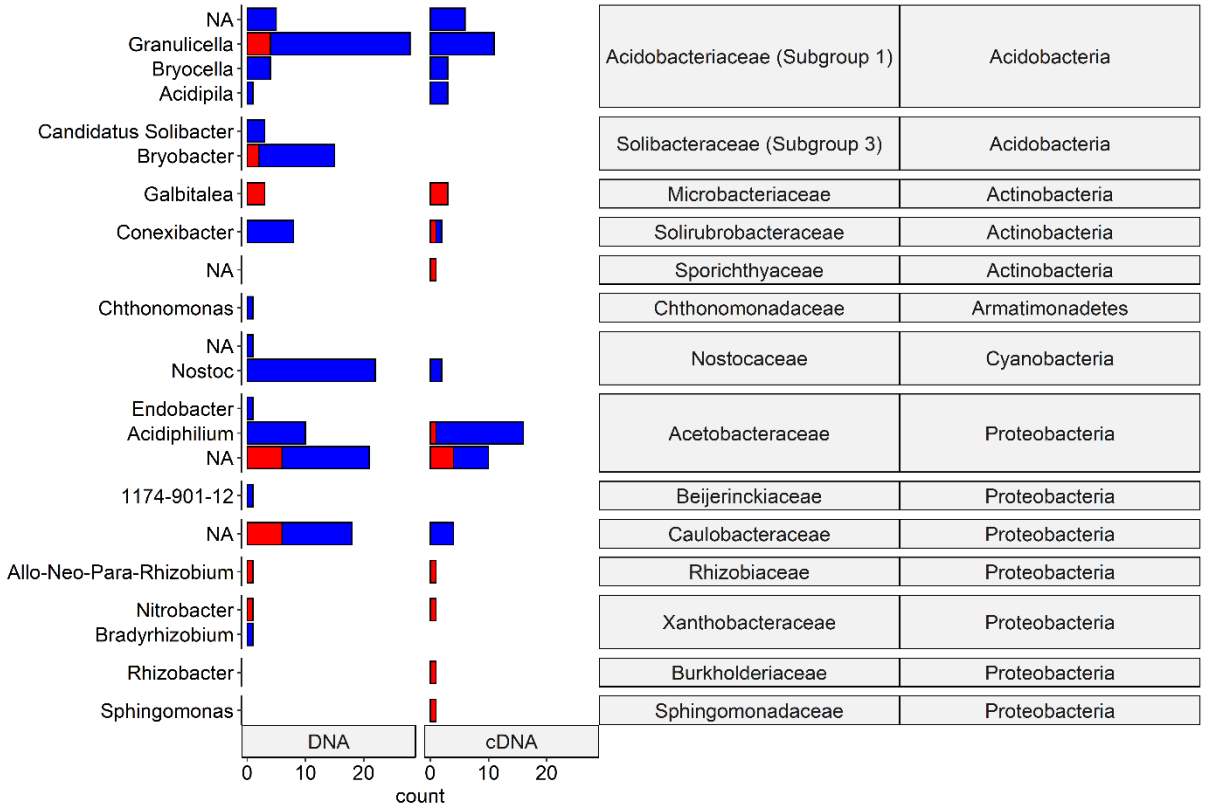

Figure 4 Number of ASVs (amplicon sequence variants) per genus sensitive to warming for DNA and cDNA based bacterial communities associated with the moss *R. lanuginosum.* Sensitivity was determined by combining the results of differential abundance analysis (DESeq2) and indicator species analysis. ASVs 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 **5**, Table S**3**). However, litter abundance negatively affected *nifH* gene abundance (pMCMC = 0.04, Fig. **5b**) and *B. nana* abundance tended to positively influence 16S rRNA gene abundance (pMCMC = 0.072, Fig. **5a**).

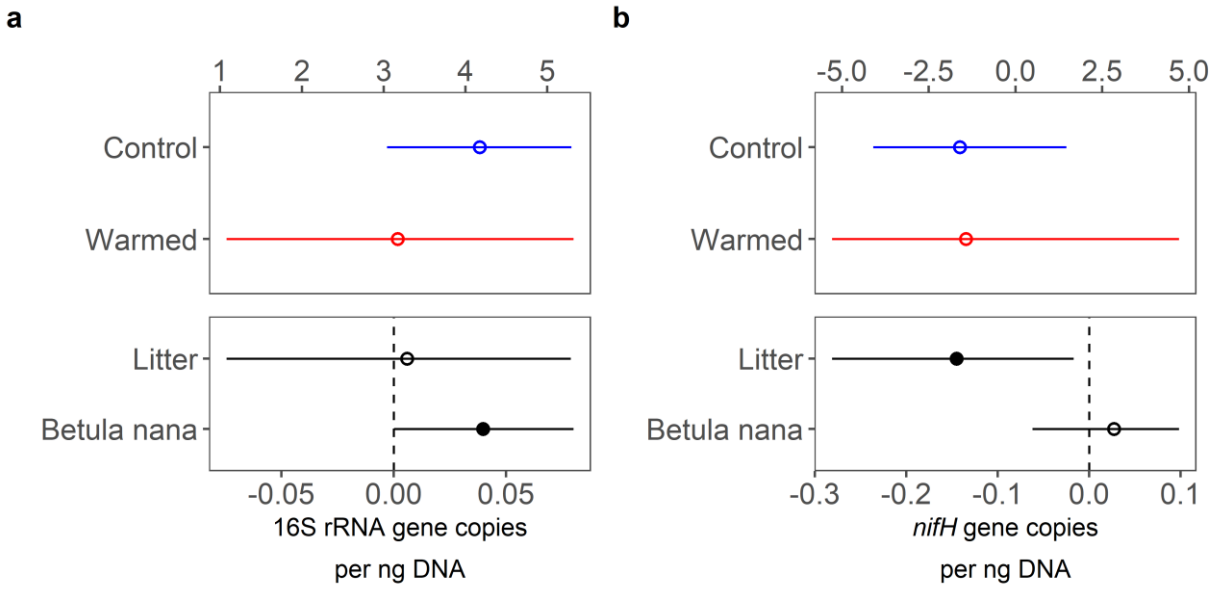

Figure 5 Fixed effect structure of the linear mixed-effect models testing the effect of treatment (warmed and control), Betula nana abundance and litter abundance on a) 16 rRNA gene abundance and b) *nifH* gene abundance. Non-overlapping 95% High Posterior Density Credible Interval (95% CrI) are used to detect significant differences between effects. Parameters with 95% CrI not overlapping each other (for control versus warming treatment) or 0 (for the litter and *B. nana*) are indicated by closed circles.

Relationships between treatment, plant biomass, and bacterial community structure

To explore the relative strengths of direct and indirect effects of warming on bacterial community structure and *nifH* gene abundance, we constructed a structural equation model (SEM) (Fig. **6**, Table **S8**). We found that warming was directly associated with changes in bacterial alpha diversity and positively correlated with increased *B. nana* abundance. The direct negative effect of *B. nana* was stronger than the direct positive effect of treatment on bacterial alpha diversity (-0.71 versus 0.50 standardized regression coefficients). Changes in bacterial community structure were also indirectly negatively associated with warming through variation in *B. nana* abundance (-0.43 standardized regression coefficient).

The SEM also showed that warming was negatively associated with *nifH* gene abundance (-0.59 standardized regression coefficient).

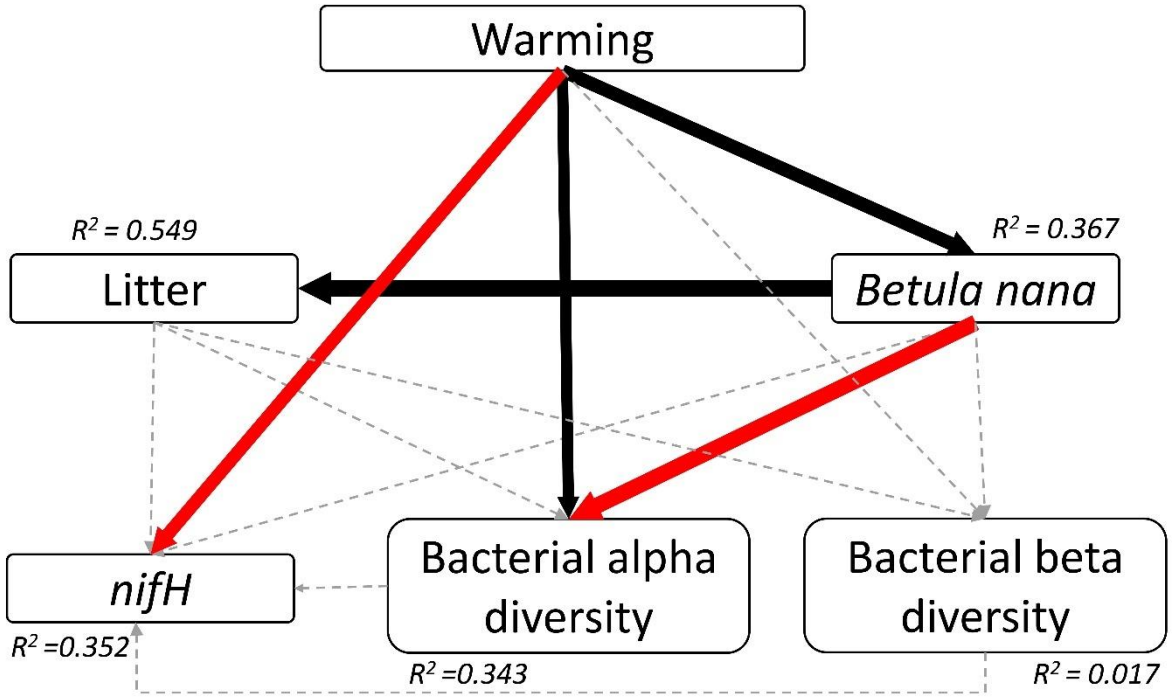

Figure 6 Structural equation model of relationships between warming, *Betula nana* and litter abundance, moss-associated bacterial alpha and beta diversity and *nifH* gene abundance. $\chi 2$ = 1.349, *P*-value = 0.509, df = 2, GFI = 0.989, RMSEA = 0, TLI = 1.091. Positive significant effects are represented 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 S**8**.

Discussion

Mosses form important C and N sinks 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 *nifH* gene abundance 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 diazotrophs appeared to be negatively affected by warming. Bacterial taxa that benefitted from the warming treatment almost exclusively

belonged to groups involved in N_2-fixation and nitrification, 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 OTCs may seem small (1-2°C), but a temperature increase in this range can affect microbial growth rate, respiration, C uptake and turnover (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.

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 under warming 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 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 richness and diversity of the potentially active bacterial community. In addition, the SEM showed that the bacterial community alpha diversity 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 a result of warming was stronger than the direct effect of warming on the bacterial alpha diversity. This indicates that warming-induced changes in vegetation structure can be more important for the bacterial diversity than the warming treatment itself. The associated changes in litter chemistry may be for instance affect bacterial community structure (Creamer *et al.*, 2015).

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 *et al.*, 2021), or other not measured variables such as leaf nutrient content (Vandenkoornhuyse *et al.*, 2015; Sayer *et al.*, 2017; Koyama *et al.*, 2018) could also contribute to the variation in bacterial communities between moss shoots. In addition to these indirect effects of warming, it should be noted that our data represent a single-point observation and that the bacterial community and thus the differences between the control and the OTC treatment may differ throughout time. This may depend on difference in environmental conditions (e.g. precipitation, temperature) between seasons or years.

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 *et al.*, 2012a), *R. lanuginosum* also showed a high abundance of the Proteobacterial order Acetobacterales 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 *et al.*, 2007; Dedysh & Sinninghe Damsté, 2018) and the Alphaproteobacterial genus *Acidiphilium* (Hiraishi & 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 (Gallet *et al.*, 1999; Männistö *et al.*, 2013)). In shrub tundra dominated by *B. nana* and *Salix* species, Proteobacteria dominate the soil bacterial community (Wallenstein *et al.*, 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 *et al.*, 2015) could be involved in degradation of more recalcitrant plant organic matter (Starke *et al.*, 2016; McGenity, 2019). 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 or historical conditions, while the active bacterial community may reflect short-term differences between OTC and controls at the time of sampling (Wang *et al.*, 2020).

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

Our results of the bacterial community structure and composition revealed 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*).

The SEM and the MCMCglmm showed a negative direct effect of warming and a negative effect of litter abundance on *nifH* gene abundance, respectively. These seemingly divergent results may be explained by the link between litter abundance and warming through *B. nana* abundance, even though the SEM did not detect this indirect link between warming and *nifH* gene abundance. If increased litter indeed leads to a decrease in *nifH* gene abundance, this could be due to, among others, increased shading or fertilization. Increased shading could for instance have led to a decrease in abundance of Cyanobacteria, as the community analysis also indicated. The community analysis also showed that other potential N_2-fixing taxa increase in relative abundance, potentially leading to some degree of functional compensation. It is also important to consider some experimental caveats. The primer pair used (PolF/PolR) has a relatively low level of degeneracy (Gaby & Buckley, 2012) and our approach may thus have underestimated the real abundance of *nifH* genes. Whether the decrease in *nifH* gene abundance with warming or increased litter also leads to a decrease in N_2-fixation, could depend on the degree of functional compensation that occurs through the shift in the diazotrophic community.

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 a negative response of *nifH* gene abundance to whole-ecosystem warming. Long-term warming also 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. Our results also showed that warming-induced changes in shrub and litter abundance affect moss-associated bacterial community alpha and beta diversity as well as *nifH* gene abundance. The increase in *B. nana* had a larger negative effect on bacterial diversity than the positive effect of the warming treatment. These results suggest that warming-induced changes in vegetation structure, such as shrubification, are more important for moss-associated bacterial communities than long-term warming itself. 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. Future experiments could focus on measuring N_2-fixation rates and relating these to the decrease in *R. lanuginosum* abundance, measuring bacterial community composition throughout the seasons and investigating the links between diazotrophic community composition and N_2-fixation rates.

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Author contribution

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. Other data supporting the findings of this article are available in the supplementary material of this study.

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