Title Long-term warming effects on the microbiome and nitrogen fixation associated with the moss *Racomitrium lanuginosum* in a subarctic alpine heathland

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 many Arctic ecosystems, harbour diverse bacterial communities that are for instance involved in nitrogen fixation. 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 study the bacterial community associated with the moss *Racomitrium lanuginosum*, a common species in the Arctic, in a 20-year *in situ* warming experiment in an Icelandic heathland. We evaluate changes in bacterial community composition and diversity. Further, we assess the consequences of warming for *nifH* gene copy numbers and nitrogen fixation rates. Our findings indicate an increase in the relative abundance of Proteobacteria and a decrease in the relative abundance of Cyanobacteria and Acidobacteria with warming. The *nifH* gene copy number decreases, while the rate of nitrogen fixation is not affected. This contradiction could be explained by a shift in the nitrogen fixing bacterial community. Although climate warming might not change the contribution of *R. lanuginosum* to nitrogen input in nitrogen-limited ecosystems, the microbial community resilience and the nitrogen fixing taxa may shift.

36 ## Introduction

37 Temperature in high-latitude regions is rising twice as fast as elsewhere [1], which
38 has large impacts on Arctic ecosystems, for instance by altering species distributions and
39 interactions among species [2, 3]. One such interaction that might be affected by warming
40 is the interaction between mosses and their associated bacterial communities and related
41 ecosystem processes.

42 Mosses comprise a large component of the vegetation in many high-latitude
43 ecosystems and play important roles in biogeochemical cycles by forming a carbon (C)
44 sink via their slow decomposition rates, by accounting for up to 7% of terrestrial net
45 primary productivity and half of the terrestrial nitrogen (N_2) fixation [4–8]. Most mosses
46 consist of a upper living segment with photosynthetic tissue and a lower decaying dead
47 segment and thus link above-ground and belowground processes [9]. They provide a
48 habitat for a range of microbiota, micro- and mesofauna, which together with the alive and
49 dead moss tissue have been defined as the 'bryosphere' [10]. Moss-associated
50 microorganisms are involved in the decomposition of dead moss tissue and responsible for
51 N_2 fixation. N_2 fixation by Cyanobacteria associated with mosses directly increases moss
52 growth rates [11] and thereby controls C sequestration in moss tissues. These
53 Cyanobacteria are also an important source of new available N in boreal and Arctic
54 ecosystems [12, 13]. In order to understand the implications of climate change for the role
55 of mosses in ecosystem C and N cycling, we need to understand how moss-associated
56 microbial communities react to elevated temperatures.

57 The bacterial community composition of mosses is species specific and influenced
58 by environmental factors such as pH and nutrient availability [14–16]. Bacterial associates
59 can increase mosses survival and growth under extreme conditions [17], for instance by

protecting moss tissue from freeze damage [18]. Commonly found phyla associated with mosses are Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Armatimonadetes, Verrucomicrobia, Planctomycetes and Cyanobacteria [16, 19]. While Cyanobacteria have received most of the attention for their N_2-fixing capability [11, 20–26], mosses harbour many other putative N_2-fixing (diazotrophic) taxa such as members of the Alphaproteobacteria [14, 27–29]. 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.

N_2 fixation rates of moss-associated diazotrophic bacteria can be expected to increase with temperature, as metabolic processes in microorganisms increase with temperature and the enzyme nitrogenase is more active at higher temperatures [30]. Temperature induced drought however, can inhibit N_2 fixation rates, especially cyanobacterial N_2 fixation [9, 24, 31–36]. Temperature effects on N_2 fixation rates might also be influenced by physiological adaptation of diazotrophic communities, or shifts to a species composition better suited to the new conditions [31, 37, 38]. Despite the importance of microbial communities for plant functioning and ecosystem processes, the effect of warming on moss microbial communities has received little attention. Two studies describing 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 [39, 40]. Whether this warming induced decrease in diversity also holds for bacterial communities associated with other moss species in high latitudes is unknown. Moreover, long-term warming effects on moss-associated bacterial communities have yet to be explored.

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 moss *Racomitrium lanuginosum* (Hedw.) Brid in a subarctic-alpine dwarf shrub heath in northern Iceland. *R. lanuginosum* has a wide distribution at high altitudes in temperate regions of the Northern and Southern Hemisphere and at low altitudes in the Arctic [41]. Projected temperature increases for Iceland as a whole are estimated between 2.1 to 4.0 degrees °C by 2100 [42].

We assessed the bacterial community composition by 16S rRNA gene amplicon sequencing and we hypothesised (1) that long-term warming would reduce the bacterial richness and diversity and affect community composition as compared to non-warmed control plots. N_2 fixation rates were measured with acetylene reduction assays (ARA) and N_2 fixation potential was quantified by quantitative PCR (qPCR) of nitrogenase (*nifH*) genes. We hypothesised (2) that N_2 fixation would be either positively or negatively influenced by long-term warming depending on the warming-induced changes in the abundance of potential N_2-fixing taxa.

Methods

Field site

The sampling was conducted in permanent plots of a long-term warming simulation experiment, part of the International Tundra Experiment (ITEX), in northwest Iceland [43]. According to Köppen's climate definitions, the sampling site, called Auðkúluheiði (65°16'N, 20°15'W, 480 m above sea level) is situated in the lower Arctic. The vegetation has been characterized as a relatively species-rich dwarf shrub heath, with *Betula nana* being the most dominant vascular species and the moss *R. lanuginosum* and the lichen

106 *Cetraria islandica* as the dominating cryptogam species [44, 45]. The area has been fenced
107 off since 1996 to prevent sheep from disturbing the experiment.

108 Ten open top plexiglass chambers (OTCs) were set up to simulate a warmer
109 summer climate in August 1996 and 1997 [44, 46]. They raise the mean daily temperature
110 during summer by 1-2 °C, and minimize secondary experimental effects such as
111 differences in atmospheric gas concentration and reduction in ambient precipitation.
112 Control plots were established next to the OTCs, but without any treatment, thus exposed
113 to ambient temperatures.

114 ***Acetylene reduction assays***

115 For N_2 fixation rate measurements, we collected three moss shoots of 5 cm length
116 per control plot and OTC in June and August 2014. Acetylene reduction was used as a
117 proxy for N_2 fixation [47]. Moss shoots were acclimated in a growth chamber for 24 h at
118 10 °C and 200 µmol m^{-2} s^{-1} PAR. 10% of the headspace was replaced by acetylene. After
119 an additional 72 h in the growth chamber under the same conditions, acetylene reduction
120 and ethylene production were measured by gas chromatography.

121 ***RNA and DNA extraction and sequencing***

122 For RNA and DNA extraction we collected moss shoots in June 2017. Per warmed
123 (OTC) and control plot, five shoots were collected with sterile tweezers. The samples were
124 immediately soaked in RNAlater (Ambion) to prevent RNA degradation and kept cool
125 until storage at -80 °C. Prior to extraction, the samples were rinsed with RNase free water
126 to remove soil particles and RNAlater and ground for six minutes using a Mini-Beadbeater
127 and two sterile steel beads. RNA and DNA were extracted simultaneously using the
128 RNeasy PowerSoil Total RNA Kit (Qiagen) and the RNeasy PowerSoil DNA Elution Kit
129 (Qiagen), following the manufacturer's instructions. DNA and RNA concentrations were

130 determined with a Qubit Fluorometer (Life Technologies) and quality was assessed with a
131 nanodrop and bioanalyzer. cDNA was synthesized using the High-Capacity cDNA
132 Reverse Transcription Kit (Thermofisher) following the manufacturer's instructions and
133 quantified on a Qubit Fluorometer. 48 DNA samples (24 from each treatment) and 48
134 cDNA samples (24 from each treatment) were sequenced. Library preparation and
135 sequencing of the V3-V4 region of the 16S rRNA gene on an Illumina MiSeq platform
136 was performed by Macrogen, Seoul, using the standard Illumina protocol and 16S rRNA
137 gene V3-V4 primers.

138 ***Sequence processing***

139 In order to obtain high-resolution data and to better discriminate ecological
140 patterns, we processed the raw sequences using the DADA2 pipeline [48, 49], which does
141 not cluster sequences into operational taxonomic units (OTUs), but uses exact sequences
142 or amplicon sequence variants (ASVs). Forward reads were truncated at 260 bp and reverse
143 reads at 250 bp. Assembled ASVs were assigned taxonomy using the Ribosomal Database
144 Project (RDP) naïve Bayesian classifier [50] in DADA2 and the SILVA_132 database
145 [51]. We removed samples with less than 10.000 non chimeric sequences and we removed
146 ASVs assigned to chloroplasts and mitochondria, singletons and ASVs abundant in only 1
147 sample. In total, for 85 samples, 3 598 ASVs remained. To account for uneven sequencing
148 depths, the data were normalised using cumulative-sum scaling (CSS) [52]. The 16S rDNA
149 based community is hereafter sometimes referred to as the 'total bacterial community' and
150 the 16S rRNA (cDNA) based community is hereafter referred to as the 'potentially
151 metabolically active bacterial community', acknowledging that 16S rRNA is not a direct
152 indicator of activity but rather protein synthesis potential [53].

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

154 We used all DNA extractions (50 replicates per treatment) for quantification of
155 *nifH* and 16S rRNA genes, which was performed by quantitative PCR (Corbett Rotor-
156 Gene) using the primer set PolF/PolR and 341F/534R respectively [54]. The specificity of
157 the *nifH* primers for our samples was confirmed by SANGER sequencing of 10 clone
158 fragments. Standards for *nifH* reactions were obtained by amplifying one cloned *nifH*
159 sequence with flanking regions of the plasmid vector (TOPO TA cloning Kit, Invitrogen).
160 Standard curves were obtained by serial dilutions (E = 0.9 – 1.1, R^2 = > 0.99 for all
161 reactions). Each reaction had a volume of 20 µL, containing 1x QuantiFast SYBR Green
162 PCR Master Mix (Qiagen), 0.2 µL of each primer (10 µM), 0.8 µL BSA (5 µg/µL), 6.8 µL
163 RNase free water and 2 µL template. The cycling program was 5 min at 95 °C, 30 cycles
164 of 10 s at 95 °C and 30 s at 60 °C.

165 ***Statistical analysis***

166 Richness (number of ASVs) and Shannon diversity were calculated with the R
167 packages 'vegan' [55] and 'phyloseq' [56]. Differences in N_2 fixation rates, 16S rRNA and
168 *nifH* gene abundance, ASV richness and Shannon diversity were assessed with MCMC
169 generalised linear models using the R package 'MCMCglmm'[57] with treatment as a
170 fixed and plot as a random factor. The relative abundances between the warmed and the
171 control samples were tested using Wilcoxon rank sum tests on plot averages.

172 Distances between the community composition of the control and OTC samples
173 were based on Bray-Curtis distances. The warming effect of the OTCs on the bacterial
174 community composition was tested by permutational-MANOVA (PERMANOVA) [58]
175 analysis of the Bray-Curtis distance matrices using the *adonis* function in the R package
176 'vegan' with plot as a random factor. Principal coordinate analysis was used to ordinate

177 the Bray-Curtis distance matrices and to visualise the relationships between samples from
178 OTC and control plots.

179 Two methods were used to determine taxa sensitive to warming. First, differential
180 abundance of bacterial genera between warmed and control samples was assessed using
181 the DESeq2 procedure [59] on the non-CSS normalised datasets with the R package
182 'DESeq2' [59]. The adjusted *p*-value cut-off was 0.1 [59]. Differential abundance analysis
183 only uses ASVs present in both the OTC and control samples. The second method we used
184 to find taxa sensitive to warming, was the indicator species analysis. To find bacterial taxa
185 indicative for the warming or the control treatment, correlation-based indicator species
186 analysis was done with all possible site combinations using the function *multipatt* of the R
187 package 'indicSpecies' [60] based on 10^3 permutations. The indicator species analysis
188 takes into account ASVs present in both OTC and control samples, but also ASVs present
189 in only one of the treatments. We combined results of the DESeq2 and indicator species
190 analysis for a final list of ASVs sensitive to warming.

191 Results

192 *Treatment effect on bacterial richness and diversity*

193 The richness and Shannon diversity of the DNA and cDNA based bacterial
194 communitues did not differ significantly between control and OTC samples (Figure 1).
195 However, the ASV abundance the phylum Acidobacteria and the class
196 Gammaproteobacteria showed significant differences between the control and OTC
197 samples (Figure 1). The Shannon diversity of Gammaproteobacteria (which include the
198 order Betaproteobacteriales) was higher in the warmed samples for both the DNA (p =
199 0.072) and cDNA based bacterial communities. The richness of Acidobacteria was

200 significantly lower in the warmed samples for both the cDNA based bacterial communities.
201 The Shannon diversity of Acidobacteria was lower ($p = 0.096$) in the warmed samples of
202 the cDNA based bacterial communities.

203 ***Treatment effects on bacterial community composition***

204 No strong treatment specific clustering of the bacterial community composition
205 could be observed in PCoA plots, although they showed some divergence between the
206 control and the warmed samples (Figure S1). This divergence was reflected in the
207 MANOVA permutation test of the Bray-Curtis dissimilarity: treatments significantly
208 influenced the DNA and the cDNA based community compositions of the moss (DNA: R^2
209 $= 0.05$ and $P < 0.001$ and cDNA: $R^2 = 0.04$ and $P < 0.001$).

210 When looking only at the potential diazotrophic community composition, the
211 control and warmed communities were significantly different, both for the DNA and the
212 cDNA. The treatment explained more variation of the DNA derived diazotrophic
213 communities than the cDNA derived diazotrophic community and the total cDNA and
214 DNA derived bacterial communities (PERMANOVA: DNA: $R^2 = 0.08$ and $P < 0.001$ and
215 cDNA: $R^2 = 0.04$ and $P = 0.002$).

216 ***Taxonomic composition of control and OTC communities***

217 In the control samples, where bacterial communities were under ambient
218 environmental conditions, the most abundant phyla made up 97% of the total abundance
219 of ASVs and included Proteobacteria (44% average relative abundance across all control
220 DNA samples), followed by Acidobacteria (29%), Actinobacteria (8%), Cyanobacteria
221 (7%), Planctomycetes (4%), Bacteroidetes (4%), Verrucomicrobia (2%) and
222 Armatimonadetes (2%) (Figure 2A). The most abundant Proteobacterial class were
223 Alphaproteobacteria (29%) (Figure 2B). Acetobacterales (15%), Myxococcales (12%),

224 Caulobacterales (6%) and Rhizobiales (6%) were the most common orders of the
225 Proteobacteria (Figure S2). The order Acetobacterales was dominated by the genus
226 *Acidiphilium* (5%), the order Myxococcales was dominated by the genus *Haliangium*
227 (4%). The order Rhizobiales was mainly represented by the genera *Nitrobacter* (0.08%)
228 and *1174-901-12* (2%). The Acidobacteria were dominated by the orders Acidobacteriales
229 (17%) and Solibacterales (11%) (Figure S2). The Acidobacteriales were dominated by the
230 genus *Granulicella* (11%). The Solibacterales were dominated by the genera *Bryobacter*
231 (5%) and *Ca. Solibacter* (6%). Actinobacteria mainly comprised the orders
232 Solirubrobacterales (5%) and Frankiales (2%) (Figure S2). The genera *Jatrophihabitans*
233 (1%), *Acidothermus* (0.05%) and *Nakamurella* (0.02%) accounted for the largest share of
234 the order Frankiales. Planctomycetes were dominated by the orders Isosphaerales (1%) and
235 Tepidisphaerales (2%) (Figure S2). The orders Fimbriimonadales (0.03%) and
236 Chthonomonadales (1%) dominated the phylum Armatimonadetes (Figure S2). The
237 phylum Bacteroidetes was dominated by the orders Chitinophagales (3%) and
238 Sphingobacteriales (0.7%) (Figure S2). Chthoniobacterales (2%) was the most abundant
239 order within the phylum Verrucomicrobia. Cyanobacteria were dominated by the genera
240 *Nostoc* (5%) and *Stigonema* (1%).

241 We compared the relative abundances of taxa on phylum, class and order level in
242 the control with the warmed samples from the OTC (Figure 2 and Figure S2).
243 Acidobacteria, Gemmatimonadetes and Cyanobacteria decreased in relative abundance,
244 while Proteobacteria increased in relative abundance in the DNA based bacterial
245 communities (Figure 2A). No significant changes were detected in the cDNA based
246 bacterial communities on phylum level. On class level, Acidobacteriia,
247 Gemmatimonadetes and Oxyphotobacteria decreased relative abundance in the DNA

248 based bacterial communities and Gammaproteobacteria increased in relative abundance in
249 the DNA and the cDNA based bacterial communities (Figure 2B). On order level
250 Betaproteobacteriales and Micrococcales were higher in relative abundance for the
251 warmed DNA and cDNA based bacterial communities (Figure S2). Acidobacteriales had
252 a lower relative abundance in the warmed DNA and cDNA based bacterial communities
253 (Figure S2). In addition, in the DNA based bacterial communities, Sphingobacterales and
254 Cytophagales increased in relative abundance under warming, while Nostocales decreased
255 under warming. In the cDNA based bacterial communities, the orders Sphingomonadales
256 and Rhizobiales increased in relative abundance under warming, while Acetobacterales
257 decreased in relative abundance under warming (Figure S2).

258 The relative abundance of potential diazotrophic Proteobacteria was higher in the
259 warmed plots than in the control plots for both the DNA and cDNA based bacterial
260 communities (Figure 2C). Cyanobacteria in the DNA based bacterial communities had a
261 lower relative abundance in the warmed plots (Figure 2C).

262 ***Treatment related shifts in the relative abundance of ASVs***

263 The DESeq2 differential abundance analysis showed that 33 ASVs were
264 differentially abundant in the DNA based moss bacterial communities, of which 28 ASVs
265 were significantly enriched in the control samples and 5 ASVs were significantly enriched
266 in the warmed samples (Table S1). The indicator species analysis revealed 122 ASVs
267 indicative for the control plots (Table S1). 23 ASVs were found indicative for the warmed
268 plots (Table S1). The strongest indicator species for the control plots corresponded to the
269 taxa that were less abundant in the warmed plots according the Deseq2 analysis. DESeq2
270 and indicator species analysis combined revealed 23 ASVs higher in relative abundance
271 under warming and 122 ASVs with higher relative abundance in the controls (Table S1).

272 For the bacterial communities in the DNA based analysis, ASVs with increased
273 relative abundance in the warmed samples belonged to the genera *Allorhizobium-*
274 *Neorhizobium-Pararhizobium-Rhizobium, Nitrobacter* (Alphaproteobacteria) and
275 *Galbitalea* (Actinobacteria). ASVs with increased relative abundance in the controls
276 belonged to the genera *Acidipila*, *Bryocella*, *Bryobacter*, *Candiatus Solibacter,*
277 *Granulicella* (Acidobacteria), *Acidiphilium*, *Endobacter* and *Bradyrhizobium*
278 (Alphaproteobacteria), *Nostoc* (Cyanobacteria) and *Conexibacter* (Actinobacteria) (Figure
279 3 and Table 1).

280 For the bacterial communities in cDNA based analysis, indicator species analysis
281 revealed 51 potentially active ASVs indicative for the control plots and 14 potentially
282 active ASVs indicative for the warmed plots (Figure 3, Table S2). DESeq2 detected 3
283 ASVs with a higher relative abundance in the control plots. ASVs more abundant in the
284 control plots belonged to the genera *Acidipila*, *Bryocella*, *Granulicella* (Acidobacteria),
285 *Nostoc* (Cyanobacteria) and *Acidiphilium* (Alphaproteobacteria). ASVs more abundant
286 under warming belonged to the genera *Allorhizobium-Neorhizobium-Pararhizobium-*
287 *Nitrobacter*, *Sphingomonas* (Alphaproteobacteria), *Galbitalea* (Actinobacteria) and
288 *Rhizobacter* (Gammaproteobacteria) (Figure 3, Table S2).

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

291 We did not find any differences between N_2 fixation in the control and warmed
292 plots in June or August 2014 (Figure 4A), but N_2 fixation in August was significantly lower
293 (Wilcoxon rank sum test, $p = < 0.0002$) than in June (Figure 4A). No significant difference
294 was found in the 16S rRNA gene copy number per ng of DNA between the control and
295 warmed samples (Figure 4B). The *nifH* gene copy number per ng DNA was similar in the

296 warmed and control samples (Figure 4C). However, *nifH* copy numbers in the warmed
297 samples were significantly lower when expressing the *nifH* gene copy number per 16S
298 rRNA gene copy (Figure 4D).

299

300 Discussion

301 Mosses form an important C and N sink in high latitudes, and their associated
302 bacterial communities are to a large extent responsible for N inputs and organic matter
303 decomposition in these environments. Elucidation of the effect of climate change on moss-
304 associated bacterial communities will help to predict the C and N cycling driven by the
305 bacterial component of the bryosphere in high latitude ecosystems. We assessed the effect
306 of long-term (20 years) warming by open-top chambers (OTCs) on bacterial communities
307 associated with the moss *R. lanuginosum* by comparing richness and diversity, community
308 structure, taxonomic composition, N_2 fixation rates *nifH* gene abundance, in control and
309 warmed moss samples. Overall our results suggest that moss-associated bacterial
310 communities are sensitive to long-term experimental warming.

311 *Effect of warming on the moss associated bacterial community structure*

312 While the overall ASV richness and Shannon diversity of the total community was
313 not significantly affected by the 20 years of warming as compared to the non-warmed
314 controls, when assessed for individual phyla and classes, significant differences emerged
315 for both the total and potentially metabolically active bacterial communities. Specifically,
316 richness and diversity increased under warming for Gammaproteobacteria (including
317 Betaproteobacteriales), whereas both richness and diversity of Acidobacteria decreased.
318 These results contrast with our hypothesis and with trends of decreasing richness and

319 diversity in *Sphagnum* moss observed by Carrell et al. (2017) and by Kolton et al. (2019).
320 *R. lanuginosum* has a much lower water holding capacity than *Sphagnum* and grows in
321 heathlands and therefore *R. lanuginosum* might react differently to warming. In addition,
322 while our study describes the effect of 20 years warming *in situ*, those previous studies on
323 *Sphagnum* were much shorter such as a four week laboratory [39] and one year *in situ*
324 experimental warming study [40]. Long-term warming studies on soils have yielded
325 various results ranging from a change in bacterial community structure in temperate forest
326 soils [61], no effect on the microbial community structure in a sub-arctic peatland [62] to
327 changes in the microbial community composition of soils in a subarctic heath and an arctic
328 soil [63, 64]. In our study, the effect of warming on the moss bacterial community
329 characterized by shifts in selected taxa.

330 Warming correlates with an increase in vascular plant canopy height and litter
331 abundance and a decrease in bryophyte abundance [44]. In fact, in the OTC treatment,
332 moss layer thickness was lower and soil organic matter content was higher, and a trend
333 towards lower soil moisture was found compared to the controls [44, 65]. Temperature-
334 induced changes in the amount of litter, surrounding plant species composition, moss traits
335 such as leaf nutrient content, soil organic matter content and soil moisture are
336 environmental factors that could potentially influence the moss bacterial community
337 composition [66–68]. The large amount of unexplained variation in the bacterial
338 community structure might thus be due to small-scale variation in moisture content,
339 nutrient availability, temperature and shading of vascular plants.

340 Furthermore, bacterial communities and thereby their potential functions can be
341 controlled by top-down factors such as predation which might change with the effects of
342 long-term warming [69–72]. For instance, in a sub-Arctic peatland, year-round warming

343 led to an increase in richness of the decomposing and fungivory oribatid mites, as well as
344 a decrease in abundance of the predatory mesostigmatid mites [73]. While these mite taxa
345 not necessarily feed on bacteria, it illustrates that foodwebs within the bryosphere are
346 affected by warming. Micro- and mesofauna might also act as a source and distribution
347 mechanism of bryophyte-associated bacteria [74]. The candidate genus *Xiphinematobacter*
348 in our study is for instance likely to be nematode-born [75], but was not affected in relative
349 abundance by warming in our study. Further studies could elucidate whether top-down
350 controls via trophic cascades affect the moss bacterial community structure and whether
351 warming induced changes at higher trophic levels could influence for example N_2 fixation
352 rates in *R. lanuginosum*, as has already been demonstrated for *Sphagnum* [69].

353 ***Effect of warming on moss-associated bacterial taxa***

354 We analysed changes in relative abundances in several ways to better understand
355 the warming response of the moss bacterial community. This revealed changes in the
356 relative abundances of taxa on all taxonomic levels. Shifts in individual taxa can affect
357 microbe-microbe and microbe-host interactions and possibly alter functionality or stability
358 of the moss-associated bacterial communities and affect host health and ecosystem
359 functioning [76–78]. Plants might be able to shape their microbial communities [79, 80] to
360 increase their tolerance to stress like drought [81] or nitrogen limitation [82, 83]. Many of
361 the taxa responding to warming in our study are potentially plant-growth promoting, such
362 as Rhizobiales.

363 The response of bacterial taxa to disturbances has been proposed to be due to
364 specific traits or their life-histories. Several life strategy conceptual frameworks have been
365 proposed for microbial organisms, such as the r-K-spectrum, where *r* strategists or
366 copiotrophs have high growth rates and low substrate affinities and oligotrophs or *K*

367 strategists low growth rates and high resource use efficiency [84, 85]. As the warming
368 treatment was been in place for 20 years at the time of sampling, it is a press or long-term
369 disturbance leading slowly to a different system with more nutrients available. The average
370 relative abundance of Proteobacteria increased with warming and most proteobacterial
371 indicator species were indicative for warming. Alphaproteobacteria showed multiple types
372 of responses to warming. The positive effect of warming on Alphaproteobacteria has been
373 linked to a copiotrophic growth strategy and dynamic response of this phylum to increasing
374 substrate concentrations [84, 86–88]. The N_2 fixing order Rhizobiales was more abundant
375 under warming in our study, which has also been found in soils [61, 89], but not in peat
376 mosses [40]. Negative responses of Alphaproteobacteria to warming might be due to
377 certain taxa having a more oligotrophic lifestyle, such as members of the Caulobacteraceae
378 that were more often indicative for ambient conditions in the controls than for warming.
379 The Gammaproteobacteria (which include the order Betaproteobacteriales) increased
380 relative abundance and in richness, but only one ASV indicative of warming was detected,
381 an ASV that was classified as *Rhizobacter*. The phylum Acidobacteria decreased in relative
382 abundance under warming. Acidobacteria are adapted to a wide range of temperatures in
383 tundra soils [90] and its members have shown either negative or positive responses in
384 warming experiments. Negative responses of Acidobacteria to increasing temperatures
385 have been linked to their more oligotrophic lifestyle and inability to compete with more
386 copiothrophic species in nutrient richer conditions resulting from the warming field
387 conditions [84, 87, 88, 91].

388 *K* strategists have been hypothesized to be more resistant but less resilient to
389 climate change related pulse disturbance than *r* strategists [85], if on the long-term
390 resources may become limited [92]. Our results show an increase in copiotrophic taxa or *r*

391 strategists and decrease in oligotrophic taxa or *K* strategists, probably because resources
392 are not limited in the warmed conditions but rather more diverse due to an increase in
393 vascular plant biomass and litter types. This also suggests that the bacterial communities
394 under warming that are characterised by a decrease in *K* strategists, are less resistant and
395 more resilient to short-term or pulse disturbances such as drought. In other words, a future
396 short-term disturbance will potentially have a bigger impact on the bacterial community
397 under warming, but the warmed bacterial community has more potential return to its
398 original state before the disturbance than the control bacterial community.

399 ***Implications of warming for the nitrogen-fixing moss microbiome in the Arctic***

400 The second hypothesis we tested was that N_2-fixing communities differ in species
401 composition in the warmed plots compared to the control plots. Indeed, overall the relative
402 abundance of Cyanobacteria decreased, and in particular the genus *Nostoc*. In a study on
403 the effect of warming on peat moss microbiomes by Carell et al. [40], the relative
404 abundance of Cyanobacteria decreased as well, but the relative abundance of *Nostoc*
405 increased, potentially because peat mosses and the *R. lanuginosum* are typical for very
406 different ecosystems (moist peat bogs versus dry tundra heath), and compared to our study
407 they used up to 9 °C of warming. The other Cyanobacterial genus in our study, *Stigonema*,
408 was not affected by warming. Patova et al. [94] found *Nostoc* in biological soil crusts in
409 wet habitats and *Stigonema* in biological soil crusts in drier habitats. A difference in
410 drought tolerance might thus explain the different response to warming between *Stigonema*
411 and *Nostoc* in our study, since the OTCs generally decrease soil moisture [65]. Moss-
412 associated *Stigonema* was also found to be more abundant than *Nostoc* in older sites with
413 more developed soils, dwarf shrubs and forests during primary succession [94], so the
414 decrease in *Nostoc* in our study could also be due to the richer conditions.

415 *Stigonema* has been suggested to be a more efficient N_2-fixer than *Nostoc*
416 associated with boreal forest feathermosses, because it was responsible for most of the N_2
417 fixation while being less abundant than *Nostoc* [22]. Interestingly, while N_2 fixation rates
418 were not affected by the warming, the *nifH* gene abundance was lower under warming.
419 While we cannot rule out yearly variations in the difference between control and warmed
420 N_2 fixation rates or diazotrophic communities, this suggests that *Nostoc* might not add
421 much to N_2 fixation and that *Stigonema* or other non-cyanobacterial diazotrophs are
422 responsible for the N_2 fixation. The decrease in *nifH* gene copy numbers could also be due
423 to a difference in *nifH* gene copies in the genomes of the taxa present in the control plots
424 and the warmed plots, or because these taxa possess multiple genomes [95]. An overlooked
425 nitrogenase is the 'alternative' nitrogenase that uses vanadium (V) instead of molybdenum
426 (Mo) as the most common nitrogenase (*nifH*) does [96]. Plant and lichen cyanobionts often
427 possess this V-nitrogenase gene (*vnf*) [96, 97] and it has been suggested that it performs
428 better at lower temperatures [98]. Our qPCR did not target the *vnf* gene, but it is possible
429 that the presence of this gene influenced the acetylene reduction assays. Our results suggest
430 that long-term warming can shift nitrogen fixing taxa with potential effects for N_2 fixation
431 rates.

432 Our study is among the first to assess the effect of long-term (20 years)
433 experimental warming with OTCs on the bacterial part of a moss microbiome. Here we
434 focussed on the moss *R. lanuginosum*, in a sub-Arctic-alpine dwarf shrub heath in Iceland.
435 We observed an increase in the relative abundance of Proteobacteria, and a decrease in the
436 relative abundance of Acidobacteria, probably due to their different life strategies
437 (copiotrophic versus oligotrophic) that reflect warming-induced changes in nutrient
438 availability. Our results also show that long-term warming leads to a shift in cyanobacterial

genera, from *Nostoc* to *Stigonema* dominated and a decrease in the relative abundance of Cyanobacteria. At the same time, the *nifH* gene abundance tended to be lower under warming, while the N_2 fixation rates did not differ. The bacterial community of the moss *R. lanuginosum* might thus be sensitive to future warming, with potential implications for moss growth, C sequestration, and N_2 fixation rates.

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Competing Interests

None.

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699 # Figure legends

700 **Figure 1** Bacterial ASV (amplicon sequence variant) richness (Observed) and Shannon
701 diversity of DNA and RNA derived bacterial communities associated with the moss *R.*
702 *lanuginosum*, with control samples in white and OTC or warmed samples in red. Total
703 richness and diversity as well as phyla and classes with significant differences between the
704 treatments are shown. Boxplots represent minimum values, first quartiles, medians, third
705 quartiles and maximum values. Significance levels: * < 0.05, ** < 0.01.

706 **Figure 2** Boxplots of the relative abundances of (A) Phyla, (B) Classes and (C) potential
707 nitrogen fixing taxa on phylum level in DNA and cDNA based bacterial communities
708 associated with the moss *R. lanuginosum*. Controls are shown in white and OTC (warmed)

samples are shown in red. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values. Significance levels (* < 0.05, ** < 0.01, *** < 0.001) are based on Wilcoxon rank sum tests.

Figure 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 called 'NA'. The number of ASVs indicative for the OTC (warmed) treatment is indicated in red and the number of ASVs indicative for the control treatment in indicated in white. Note that a division was made between non N2-fixing and N2-fixing taxa.

Figure 4 Boxplots of (A) acetylene reduction rates (µmol C_2H_2 g^{-1} day^{-1}) in June and August 2014, (B) 16S rRNA gene copy numers per ng DNA, (C) nifH gene copy number per ng DNA and (D) nifH gene copy number relative to 16S rRNA gene copy number 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.

Supplementary Figure 1 Principal coordinate analysis (PCoA) of weighted Bray-Burtis distances between warmed and control 16S rDNA (A) and 16S rRNA based relative OTU abundance matrices (B) of the moss *R. lanuginosum*. Warmed samples are represented as black circles and control samples as grey circles.

Supplementary Figure 2 Boxplots of the relative abundances of orders in DNA and cDNA based bacterial communities. Controls are shown in white and OTC (warmed) samples are shown in red. Boxplots represent minimum values, first quartiles, medians, third quartiles and maximum values. Significance levels (* < 0.05, ** < 0.01, *** < 0.001) are based on Wilcoxon rank sum tests.

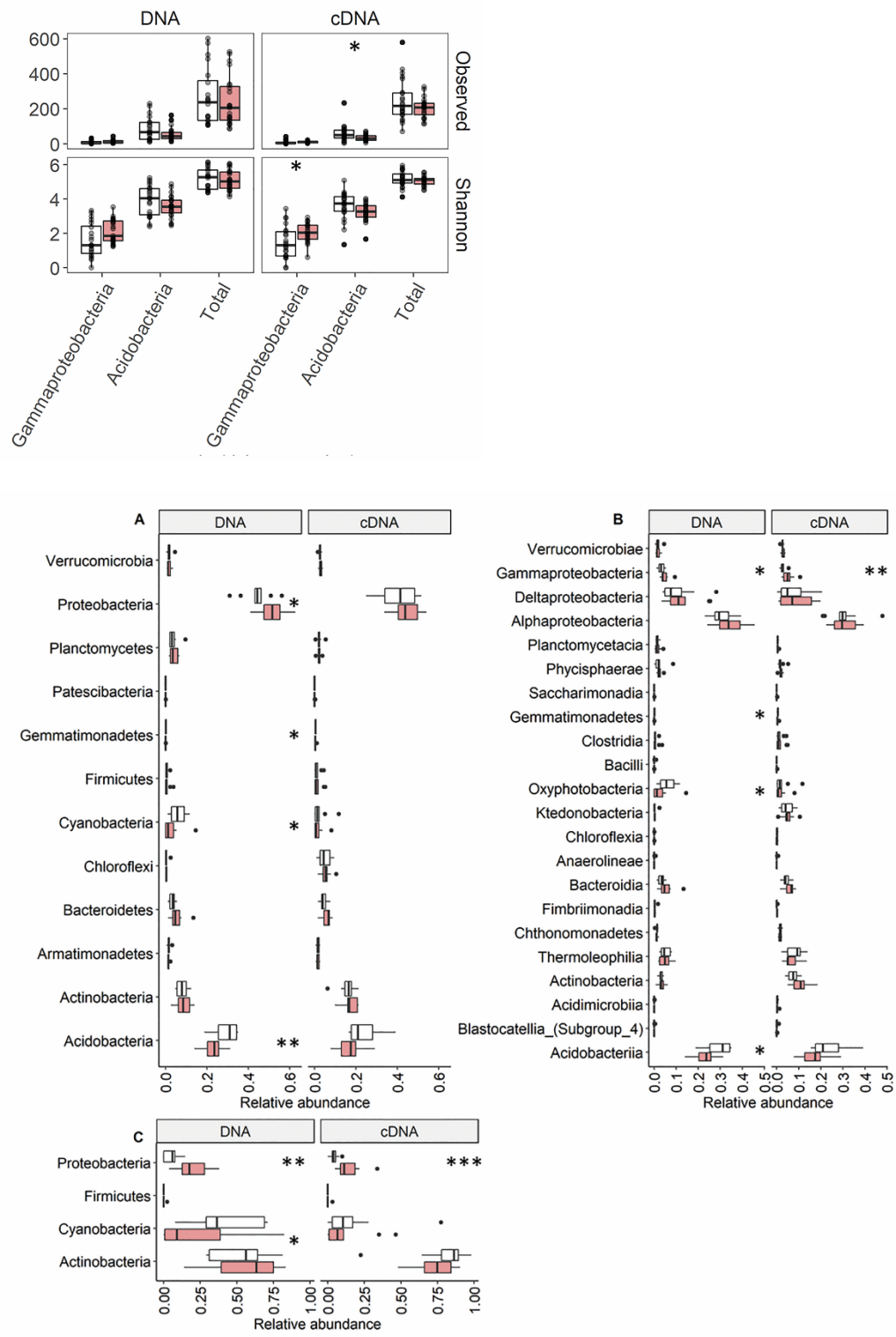
Tables

Supplementary Table 1 List of DESeq2 and indicator species for the warmed and the control conditions of the DNA based bacterial communities, .xlsx

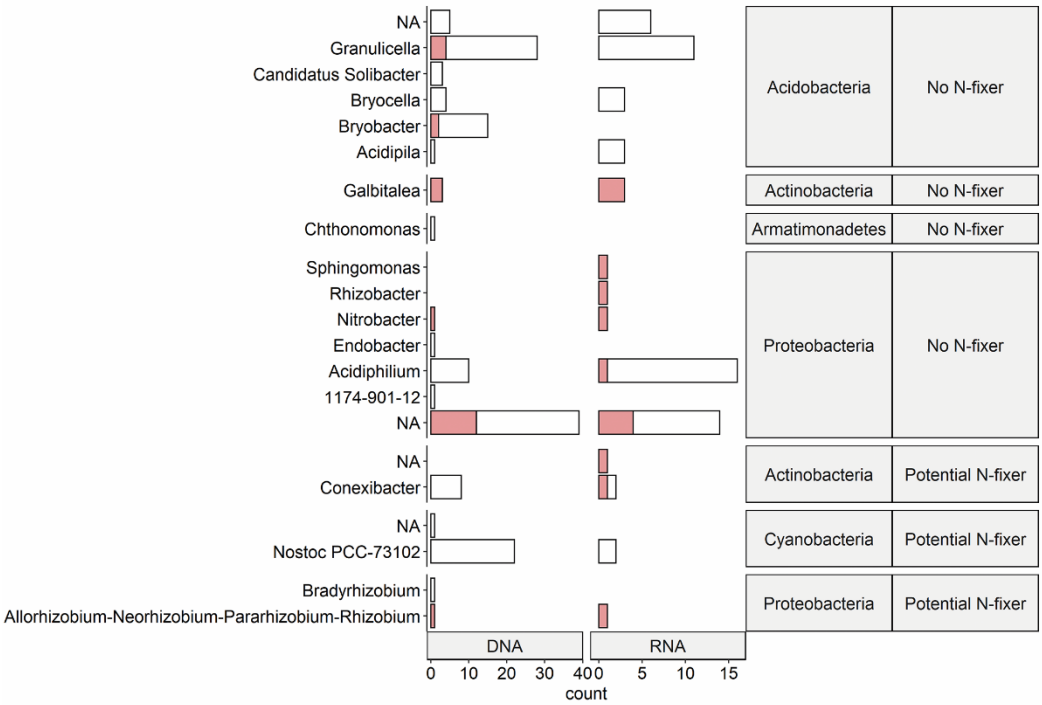
Supplementary Table 2 List of DESeq2 and indicator species for the warmed and the control conditions of the cDNA based bacterial communities, .xlsx

Figures

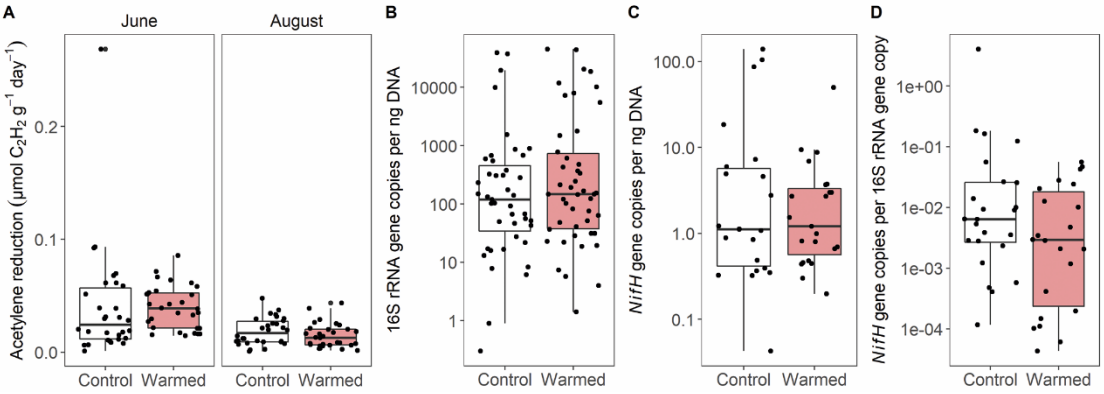
742


DNA
cDNA
Observed
Shannon
Gammaproteobacteria
Acidobacteria
Total
A
Verrucomicrobia
Proteobacteria
Planctomycetes
Patescibacteria
Gemmatimonadetes
Firmicutes
Cyanobacteria
Chloroflexi
Bacteroidetes
Armatimonadetes
Actinobacteria
Acidobacteria
Relative abundance
B
Verrucomicrobiae
Gammaproteobacteria
Deltaproteobacteria
Alphaproteobacteria
Planctomycetacia
Phycisphaerae
Saccharimonadia
Gemmatimonadetes
Clostridia
Bacilli
Oxyphotobacteria
Ktedonobacteria
Chloroflexia
Anaerolineae
Bacteroidia
Fimbriimonadia
Chthonomonadetes
Thermoleophilia
Actinobacteria
Acidimicrobiia
Blastocatellia_(Subgroup_4)
Acidobacteriia
Relative abundance
C
Proteobacteria
Firmicutes
Cyanobacteria
Actinobacteria
Relative abundance

743


NA
Granulicella
Candidatus Solibacter
Bryocella
Bryobacter
Acidipila
Galbitalea
Chthonomonas
Sphingomonas
Rhizobacter
Nitrobacter
Endobacter
Acidiphilium
1174-901-12
NA
NA
Conexibacter
NA
Nostoc PCC-73102
Bradyrhizobium
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Acidobacteria
No N-fixer
Actinobacteria
No N-fixer
Armatimonadetes
No N-fixer
Proteobacteria
No N-fixer
Actinobacteria
Potential N-fixer
Cyanobacteria
Potential N-fixer
Proteobacteria
Potential N-fixer
DNA
RNA
0 10 20 30 40
0 5 10 15
count

744


A
June
August
Acetylene reduction (µmol C_2H_2 g^{-1} day^{-1})
0.0
0.1
0.2
Control Warmed
Control Warmed
B
16S rRNA gene copies per ng DNA
1
10
100
1000
10000
Control Warmed
C
NifH gene copies per ng DNA
0.1
1.0
10.0
100.0
Control Warmed
D
NifH gene copies per 16S rRNA gene copy
1e-04
1e-03
1e-02
1e-01
1e+00
Control Warmed

745