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Research Article

RESEARCH ARTICLE

Prolonged exposure does not increase soil microbial community compositional response to warming along geothermal gradients

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One sentence summary: A unique geothermal warming gradient on Iceland reveals that long-term effects of soil warming on microbial community composition may be overestimated by short-term studies.

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ABSTRACT

Global change is expected to affect soil microbial communities through their responsiveness to temperature. It has been proposed that prolonged exposure to elevated temperatures may lead to progressively larger effects on soil microbial community composition. However, due to the relatively short-term nature of most warming experiments, this idea has been challenging to evaluate. The present study took the advantage of natural geothermal gradients (from +1°C to +19°C above ambient) in two subarctic grasslands to test the hypothesis that long-term exposure (>50 years) intensifies the effect of warming on microbial community composition compared to short-term exposure (5–7 years). Community profiles from amplicon sequencing of bacterial and fungal rRNA genes did not support this hypothesis: significant changes relative to ambient were observed only starting from the warming intensity of +9°C in the long term and +7°C/+3°C in the short term, for bacteria and fungi, respectively. Our results suggest that microbial communities in high-latitude grasslands will not undergo lasting shifts in community composition under the warming predicted for the coming 100 years (+2.2°C to +8.3°C).

Keywords: soil microorganisms; geothermal gradients; soil warming; 16S; ITS1; Illumina

INTRODUCTION

Given the recent concern regarding the stability of ecological systems under a changing climate, there have been numerous studies investigating the effects of warming on microbial community composition (Zogg *et al.* 1997; Zhang *et al.* 2005; Weedon *et al.* 2012; Karhu *et al.* 2014; Luo *et al.* 2014; Rui *et al.* 2015; Xu *et al.* 2015). However, a general conclusion as to how soil microbial community composition will be affected by global warming remains elusive. In some studies, short-term warming of approximately 1–2 years induced significant shifts in soil microbial community composition (Xiong *et al.* 2014; Zhang *et al.* 2016), while others did not find a significant change after substantially longer (4–9 years) warming

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treatments (Allison, McGuire and Treseder 2010; Schindlbacher et al. 2011; Weedon et al. 2017). Rinnan et al. (2007) concluded that more than a decade of warming (+1.2 to +2°C) was needed to detect significant changes in microbial communities in a manipulation experiment in sub-arctic heathlands. Similarly, DeAngelis et al. (2015) reported that microbial community composition in a temperate forest was affected by a +5°C temperature increase only after 20 years of experimental warming. Warming can affect microbial community composition both directly, since microbial physiological activity and growth are temperature sensitive (Ratkowsky et al. 1982), or indirectly: through changes in plant communities and soil properties (Xiong et al. 2016). The microbial community responses to warming may therefore be delayed despite the fact that microorganisms typically have capacity for fast responses to the environmental changes (Shade et al. 2012) because the indirect effects of warming (e.g. the change in quality/quantity of substrate utilized by microbes (Rinnan et al. 2007)) might take a longer time to affect microbial community composition. It has therefore been proposed that long-term warming experiments (on the scale of decades, rather than years) might be needed to detect the consequent changes in microbial community composition (Rinnan et al. 2007; Contosta, Frey and Cooper 2015; DeAngelis et al. 2015; Melillo et al. 2017; Romero-Olivares, Allison and Treseder 2017). It can be assumed that the effect of warming on microbial communities over time would also depend on the magnitude of warming given that direct effect of warming on microbial communities should be stronger and indirect effects should occur faster with increasing warming intensity.

Few studies have directly compared the effects of short-term (in this study defined as a period shorter than 10 years which coresponds to the typical exposure period in most manipulative warming studies) and long-term warming (longer than a decade) on soil microbial community composition. Such a comparison is important to assess whether the change in microbial community composition lags behind the change in climate (Blonder, Moulton and Blois 2017), and to what extent community compositional responses observed in short-term experiments can be extrapolated to longer time scales (Rustad 2001; De Boeck et al. 2015; Romero-Olivares, Allison and Treseder 2017). The present study tests the hypothesis that long-term exposure (of several decades) intensifies the impact of warming on microbial community composition so that a detectable change occurs at lower warming intensities compared to short-term exposure (Fig. 1). If this is the case, the results observed over short timescales would potentially underestimate the extent of microbial community changes over the long term.

To test this hypothesis, we took advantage of a 'natural laboratory' afforded by a geothermal system in south-western Iceland that contains gradients in soil temperature of varying age (Sigurdsson et al. 2016). Geothermal gradients provide a wide range of soil temperatures while avoiding the problems of both small-scale manipulative experiments, which are limited to a small number of temperature treatments, and large-scale latitudinal/altitudinal studies, which often involve difficulties in disentangling the effects of biogeography and temperature (O'Gorman et al. 2014). Given that the geothermal gradients are contained within a relatively small area, the variation in different environmental factors is less pronounced than in comparable large-scale gradient studies, allowing the effects of temperature (both direct and indirect) to be isolated, while at the same time retaining the complexity of the natural system (Woodward et al. 2010; O'Gorman et al. 2014).

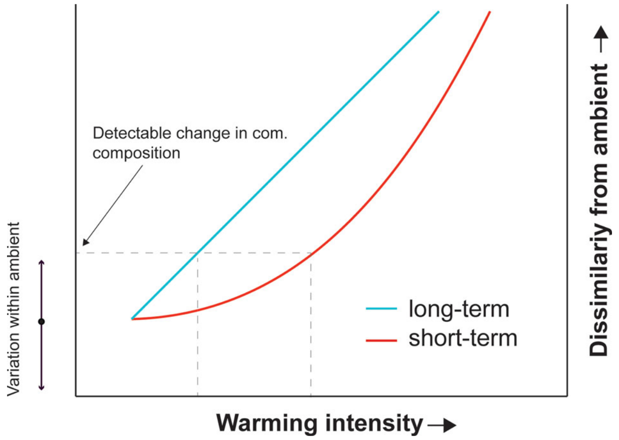

Figure 1. Conceptual figure showing the change in dissimilarity of microbial community composition with increasing warming intensities for long-term warming (blue line) and short-term warming (red line). Microbial communities exposed to increasing temperature elevations are expected to become increasingly different from the ambient communities. However, natural and sampling variation, as well as a possible delay in the response of the soil environment (substrate, vegetation) are expected to dampen this effect in the short-term and detectable effects of warming (where the community composition change surpasses the variation within ambient communities) are expected to occur at higher warming intensities than after a long period of warming where the soil environment is already significantly altered. At very high-temperature elevations, the curves for the short- and long-term response are expected to converge, given that severe warming should produce fast responses. This model assumes the linear initial increase in dissimilarity between communities at increasing temperatures; however, similar logic is applicable to other types of response curves (e.g. exponential and sigmoidal).

MATERIALS AND METHODS

Site description and soil sampling

The study was conducted at the ForHot research sites (Sigurdsson et al. 2016) close to the town of Hveragerði (64°00′01″N, 21°11′09″W, 83–168 m a.s.l.) in south-western Iceland. The sites represent unmanaged grasslands dominated by the grass *Agrostis capillaris* L. We studied grasslands in two valleys with two distinct geothermal systems; one has been geothermally heated for more than 50 years, (hereafter 'long-term warmed grassland' or LWG), the other has been exposed to warming since an earthquake on the 29th May 2008 (hereafter 'short-term warmed grassland' or SWG). After the earthquake, the locations of the LWG and SWG systems were mapped and, although geothermal manifestations in the LWG area were partly altered, the geothermal activity in the part of the LWG region where our study took place was not affected (Þorbjörnsson et al. 2009). Geothermal activity in the area around LWG has most likely been persistent for several centuries, but the presence of multiple geothermal clay layers in soil profiles at the LWG site indicate that warming may have fluctuated somewhat in the past centuries. At both sites, the geothermal systems have resulted in gradients of soil temperature ranging from ambient (mean annual soil temperature of ≈5.1°C), to approximately 20°C above ambient (at a soil depth of 10 cm) over a distance of 50–100 m (see Sigurdsson et al. (2016) for more details on the temperature gradients).

The main soil characteristics of the two grasslands at ambient temperatures were comparable: soil type of both is Silandic Andosol; soil texture is silt loam; soil pH: 5.6 and 5.7; bulk density: 70 g/cm^3 and 55 g/cm^3 in SWG and LWG, respectively. However, it was observed that soil pH increased significantly along the gradients in SWG, while in LWG, changes in pH were not correlated with a temperature increase (Sigurdsson *et al.* 2016; Fig. S1, Supporting Information).

In both grasslands, five replicate transects were established in autumn 2012. Each transect consists of six permanent plots (2 m × 2 m) located to span a range of different warming intensities (expressed relative to ambient soil temperatures in portions of the site unaffected by geothermal warming): A $\approx +0^\circ$C (ambient), B $\approx +1^\circ$C, C $\approx +3^\circ$C, D $\approx +5^\circ$C, E $\approx +10^\circ$C and F $\approx +20^\circ$C. This system of plots was created in an attempt to make a comparable replicated warming gradient with matching temperature elevations in the two grasslands. However, because only instantaneous temperature was known when the plots were established, the mean annual temperature deviated somewhat between matched plots across transects over longer time periods. Therefore, we re-classified the replicated plots based on their realized temperature differences. The range of warming in each of these new groups (based on hourly measurements averaged over the period from May 2013 to May 2015 (Sigurdsson *et al.* 2016)) was as follows: $W_0 = 0$ to $+1^\circ$C ($n = 11/10$), $W_{low} = +2$ to $+3^\circ$C ($n = 5/4$), $W_{med} = +3$ to $+5^\circ$C ($n = 4/6$), $W_{high} = +6$ to $+9^\circ$C (in LWG; $n = 5$) and $W_{high}{}' = +7$ to $+11^\circ$C (in SWG; $n = 5$), $W_{extr} = +15$ to $+19^\circ$C ($n = 5/5$ for LWG and SWG, respectively). There is thus a slight difference between the grasslands in that LWG is slightly less warmed than SWG for W_{high} plots, whereas for other groups the two grasslands span the same range of warming intensities. This grouping was chosen in order to reflect the original experimental design as closely as possible, but we additionally confirmed the robustness of the main results using temperature groups determined empirically using K-means of the realized temperatures (Table S6 and Fig. S5, Supporting Information).

Soil samples were collected from all 60 plots in May 2013 for fungi and in August 2015 for bacteria, from 5 to 10 cm soil depth, using a corer (2.5 cm diameter). The fungal samples were collected and analyzed in the framework of a study examining warming effects on SOM dynamics; the bacterial samples were collected as baseline measurements for an on-going multiyear fertilization experiment. Sampling protocols were identical on both sampling occasions. Soil samples were homogenized and stored at -20°C prior to further analyses.

Library preparation and sequencing

DNA was extracted from approx. 0.25 g of soil using the PowerSoil DNA Isolation Kit according to the manufacturer's protocol (MoBio, Carlsbad, CA, USA). For bacterial analyses, the V3 region of the 16S rRNA gene was amplified using modified 341F and 518R primers (Bartram *et al.* 2011) with unique 6bp indices on the reverse primers. Each 25 μl reaction contained 1.5 μl of the sample, 1 μl each of forward and reverse primers (10 μM) and 12.5 μl of Phusion High-Fidelity PCR Master Mix with HF Buffer (ThermoFisher Scientific, Waltham, MA, USA). PCR conditions were as follows: initial denaturation step at 98°C for 1 min, followed by 25 cycles of: 98°C for 10 s, 50°C for 30 s and 72°C for 30 s, finishing with the extension step at 72°C for 4 min. For fungal analyses, the ITS1 fungal region was amplified using the primers ITS1f and ITS2 augmented with multiplexing barcodes (Smith and Peay 2014). Each 25 μl reaction contained 1 μl of sample, 1 μl of forward primer (5 μM) and 12.5 μl Phusion High-Fidelity PCR Master Mix. PCR conditions were as follows: initial step at 98°C for 30 s, 30 cycles of: 98°C for 30 s, 55°C for 30 s, 72°C for 30 s, and final step of 72°C for 10 min.

Samples that failed to produce PCR product were subject to repeated soil extraction and PCR. However, seven samples for bacteria (1 from W_0 and W_{med}, 2 from W_{high} and W_{extr} in LWG; 1 from W_{high} in SWG) and six samples for fungi (1 from W_{high}, 2 from W_{extr}, in LWG, 3 from W_{high} in SWG) still failed to produce usable PCR and these were excluded from further analyses. Successful amplification products were purified using the AmpureXP method (Beckman Coulter, Brea, CA, USA) and normalized to equimolar concentrations before pooling into a single library, for fungi and bacteria separately. Gel extraction of the pooled library was performed for size selection and additional purification using QIAquick Gel Extraction Kit (Qiagen, Venlo, the Netherlands). Libraries were quantified with real-time PCR (KAPA Library Quantification Kits, Kapa Biosystems, Wilmington, MA, USA).

The libraries were sequenced on the Illumina MiSeq platform (Illumina Inc; San Diego, CA, USA) with 150 cycles for forward and reverse reads for bacteria and 300 cycles (forward read only) for fungi. The reproducibility of sample preparation and sequencing was tested by sequencing a small number of technical replicates (Fig. S2, Supporting Information).

Quality filtering and bioinformatics analysis

The first part of bioinformatics analysis on bacterial sequences was performed using the USEARCH software (Edgar 2013). After merging paired end reads and removing primer sequences, the reads were quality filtered (maximum expected error 0.05) leaving a total of 6.4 M high-quality sequences. Following singleton removal a set of OTU representative sequences (97% similarity) was constructed using the UPARSE-OTU algorithm (Edgar 2013). After chimera removal, all original reads were mapped to the non-chimeric OTUs with the identity threshold of 0.97, yielding an OTU table with a total of 13 620 OTUs (mean number of OTUs per warming level is shown in Table S1, Supporting Information). OTUs were aligned to the Green Genes February 2011 database (DeSantis *et al.* 2006) using the PyNAST algorithm (Caporaso *et al.* 2010). The number of reads per sample was then rarefied to 7000. This rarefaction depth included all but two samples (from W_0 and W_{high} group in LWG) that had a substantailly lower amount of sequences than other samples. Taxonomic identity of each OTU was identified based on the 97% Green Genes database (release 13_8) using the RDP classifier (Wang *et al.* 2007).

Fungal sequences were analyzed using the USEARCH software following the UPARSE pipeline (Edgar 2013). After trimming to 200 bp, the sequences were quality filtered (maximum expected error 0.01), leaving a total of 4.15 M sequences. The reverse primers were removed, and when resulting sequence was shorter than 200 bp N's were added to the end of the sequence to ensure that all sequences have the same length for effective clustering of OTUs as suggested by Edgar (2013). Singleton sequences were removed and all others were clustered to 97% similarity. Chimeras were filtered *de novo* as well as through the UNITE database of ITS1 sequences as implemented in UCHIME. Representative sequences for each OTU were aligned to all fungal representative species in the UNITE database (Kõljalg *et al.* 2005)(release date 01.08.2015), using the BLAST algorithm with default settings and assembled in an OTU table with the total of 1460 OTUs (Table S1, Supporting Information). The resulting hits were assigned to taxa by selecting hits with the lowest E-value and a minimum alignment length of 75 bp. OTUs were classified to taxonomic levels depending on identity percentages with UNITE taxa as in Tedersoo *et al.* (2014). OTUs were subsequently assigned to functional groups (filamentous saprotrophs, yeasts, white rot saprotrophs, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, plant pathogens, animal pathogens, mycoparasites) if the genus was successfully matched with one

Table 1. The results of pairwise PERMANOVA analyses between the communities from ambient soil temperatures and the communities from increased soil temperatures, in the long-term warmed (LWG) and the short-term warmed (SWG) grassland.

	Pairs	N	F model	R^2	P adj.
LWG bacteria	W_0–W_{low}	9–5	1.012	0.078	1
	W_0–W_{med}	9–3	0.833	0.077	1
	W_0–W_{high}	9–2	1.034	0.103	1
	W_0–W_{extr}	**9–3**	**4.596**	**0.315**	**0.020**
LWG fungi	W_0–W_{low}	11–5	1.024	0.068	1
	W_0–W_{med}	11–4	1.379	0.096	0.212
	W_0–W_{high}	11–4	1.383	0.096	0.144
	W_0–W_{extr}	**11–3**	**2.502**	**0.173**	**0.008**
SWG bacteria	W_0–W_{low}	10–4	1.279	0.096	0.588
	W_0–W_{med}	10–6	1.915	0.120	0.088
	W_0–W_{high}'	**10–4**	**2.438**	**0.169**	**0.008**
	W_0–W_{extr}	**10–5**	**4.945**	**0.276**	**0.008**
SWG fungi	W_0–W_{low}	10–4	1.535	0.113	0.072
	W_0–W_{med}	**10–6**	**1.845**	**0.116**	**0.020**
	W_0–W_{high}'	**10–5**	**2.948**	**0.185**	**0.004**
	W_0–W_{extr}	10–2	2.340	0.190	0.060

Warming levels: W_0 – ambient (+0°C to +1C°), W_{low} (+2°C to +3°C), W_{med} (+3°C to +5°C), W_{high} (+6°C to +9°C), W_{high}' (+7°C to +11°C), W_{extr} (+15°C to +19°C). Significant results are indicated in bold.

of the genera with known lifestyles in Tedersoo *et al.* (2014). In the cases when genus level was unknown, lifestyle was assigned at family level if more than 80% of genera within that family (represented by more than three genera) belonged to the same lifestyle. All original sequences were mapped against these OTUs with a similarity threshold of 97% and assembled in an OTU table. The number of reads per sample was then rarefied to the minimum number of reads of 5274.

Statistical analyses

Nonmetric multidimensional scaling (NMDS) was performed to visualize the overall differences in microbial community composition. The differences between microbial communities from the soil exposed to different warming levels (W_{low}, W_{med}, W_{high}/W_{high}', W_{extr}) and the unwarmed soil (W_0) were evaluated using pair-wise PERMANOVA analysis (Anderson 2001). Bonferroni corrections were applied to adjust P values for multiple testing. The correlation between soil temperature/pH and microbial community dissimilarities was quantified using a (partial) Mantel test. Constrained Analysis of Principal (CAP) Coordinates was used to examine and visualize the effect of soil temperature and pH on microbial community composition. All multivariate analyses were based on Bray–Curtis distances to allow comparability between bacterial and fungal datasets, but the results were robust to the choice of distance metric (data not shown). For all statistical tests, OTU abundances were log transformed.

The change in the relative abundance of dominant bacterial high-level taxa/fungal functional groups (defined as those making up more than 2% of the total number of reads) at different warming levels was tested using ANOVA (followed by posthoc Tukey tests in the cases of significant results). When necessary, the data were transformed with log or Box–Cox transformations. P values were in all cases adjusted for multiple testing using Bonferroni correction.

All statistical analyses were conducted using R statistical software (R Core Team 2015) using base packages and *vegan* (Oksanen *et al.* 2015).

RESULTS

Overall community composition along warming gradients

In LWG, bacterial community composition was significantly affected only by the highest temperature elevations W_{extr} (+15°C to +19°C) (Table 1, Fig. 2a and c). It should be noted that group W_{high} (+6°C to + 9°C) had only two observations and the comparison with this group has a higher uncertainty (Table 1). In SWG, bacterial communities from W_{high}' (+7°C to +11°C) and W_{extr} (+15°C to +19°C) differed significantly from the ambient group (R^2 = 10%, $P < 0.01$ and R^2 = 17%, $P < 0.01$, respectively), while the communities at lower temperature elevations were not significantly different to those from ambient soils (Table 1, Fig. 2b and c).

For fungal communities in the LWG, significant changes were also found only for the W_{extr} (Table 1, Fig. 2d and f). In SWG, fungal communities were significantly affected by warming levels W_{med} (+3°C to +5°C) and W_{high}' (+7°C to +11°C) (R^2 = 12%, $P = 0.02$ and R^2 = 19%, $P = 0.004$; respectively), while the difference for W_{low} (+1°C to +3°C) and W_{extr} (+15°C to +19°C) was near significant (R^2 = 11%, $P = 0.07$ and R^2 = 19%, $P = 0.06$; respectively) (Table 1, Fig. 2e and f). The absence of a significant effect in the latter likely results from low statistical power due to small sample size ($n = 2$), especially considering that the effect size was relatively high and that they were clearly separated from the ambient communities based on NMDS ordination (Fig. 2e).

A Mantel test indicated that community dissimilarities between different samples were significantly related to the differences in soil temperatures both in LWG and SWG (Table 2). Variation in soil pH was significantly related to the differences in community composition in SWG but not in LWG (Table 2). However, when the highest temperatures from W_{extr} (that contributed disproportionally to the observed temperature effect) were excluded from the analysis, the effect of temperature was non-significant in LWG ($P > 0.05$), while the effect of pH was significant ($R_M = 0.37$, $P < 0.01$, both for bacteria and fungi). In SWG, after exclusion of W_{extr}, variation of microbial

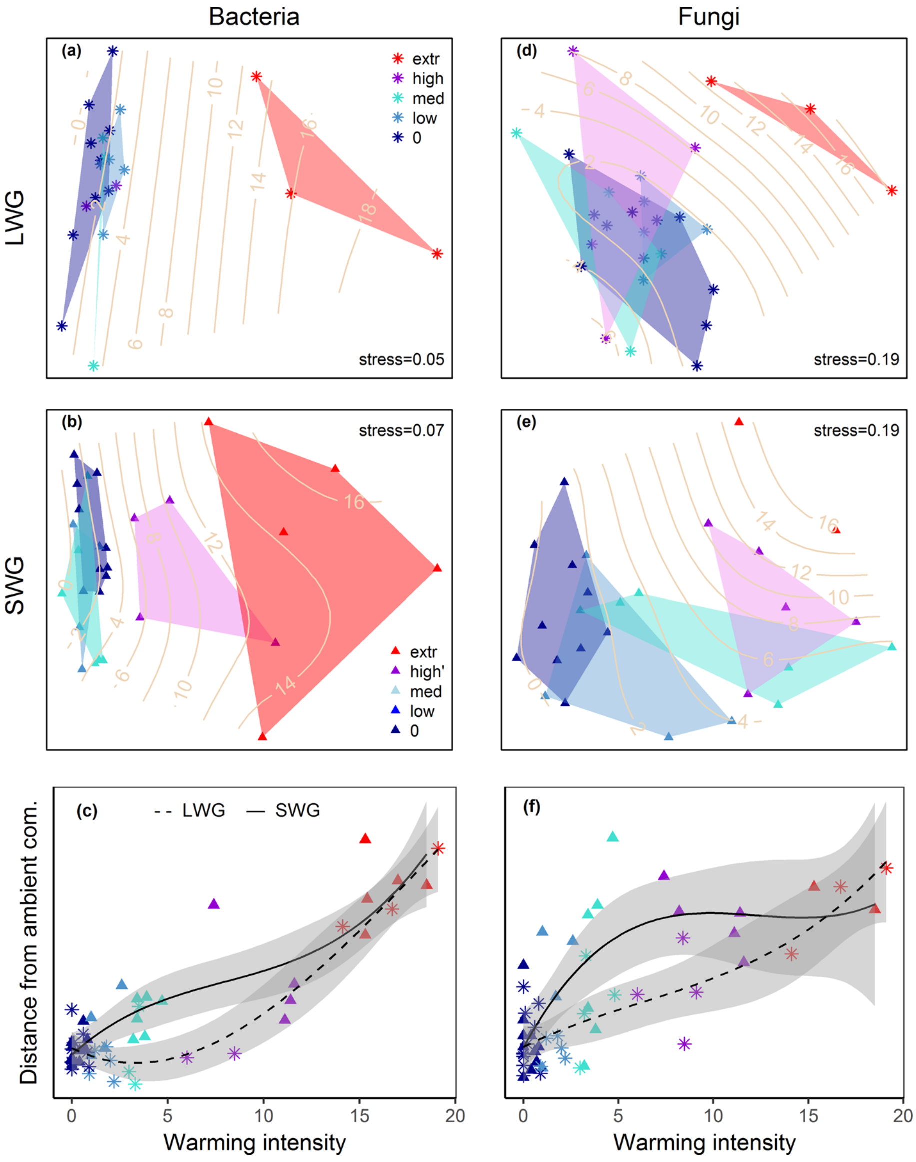

Figure 2. Top: NMDS ordination plots for: bacterial community composition in (a) the long-term warmed grassland (LWG) and (b) the short-term warmed grassland (SWG); and fungal community composition in (d) LWG and (e) SWG. Points (samples) and the corresponding polygons are colored according to warming levels W[0 – ambient (+0°C to +1C°), low (+2°C to +3°C), med (+3°C to + 5°C), high (+6°C to +9°C), high′ (+7°C to +11°C), extr (+15°C to +19°C)]. Isolines represent fitted smooth surface of different warming intensities. Based on a generalized additive model, there was a significant relationship ($P < 0.001$) between the ordination axes in NMDS and soil temperature: in LWG $R^2 = 86\%$ and $R^2 = 88\%$, for bacteria and fungi, respectively, and in SWG $R^2 = 84\%$, $R^2 = 83\%$, for bacteria and fungi, respectively. Bottom: Mean distances (based on BC dissimilarity) between the communities from each sample and the ambient communities along the temperature gradient for (c) bacterial communities and (f) fungal communities in LWG and SWG. The lines represent smoothing splines (third-order polynomial model). Dashed line = LWG, full line = SWG. Shaded area represents 95% confidence intervals of the fitted lines calculated using the predict method.

communities remained significantly correlated with variation in both pH ($R_M = 0.37$) and temperature ($R_M = 0.36$ and $R_M = 0.37$, for bacteria and fungi, respectively). When controlling for the effect of pH (using a partial Mantel test), the effect of temperature in SWG remained significant (Table 2) and the Mantel correlation coefficients decreased only slightly ($R_M = 0.30$ and $R_M = 0.29$ for bacteria and fungi, respectively) suggesting that the relationship between temperature increase and changes in microbial communities was largely independent of the variation in pH. CAP analysis demonstrated that variation in pH was related to the variation in microbial community composition mostly within the same warming levels (i.e. the temperature and pH vectors are almost completely orthogonal), but some degree of correlation between the pH and temperature effects could still be observed in SWG (Fig. S3, Supporting Information).

Microbial taxa/functional-groups along warming gradients

The most abundant bacterial phyla in both LWG and SWG were Proteobacteria (Alphaproteobacteria 13%/15% of the reads; Betaproteobacteria 8%/5%; Deltaproteobacteria 6%/6%; Gammaproteobacteria 2%/2%), Acidobacteria (25%/18%), Actionobacteria (11%/21%) and Chloroflexi (6%/6%) in LWG and SWG, respectively. The list of all bacterial phyla with their relative abundances can be found in Table S2 (Supporting Information).

Table 2. The relationship between microbial composition dissimilarities and dissimilarities in soil properties: soil temperature and soil pH in the long-term warmed (LWG) and the short-term warmed (SWG) grassland; based on a Mantel/Partial Mantel test.

	Soil T		Soil pH		Soil T[a]		Soil pH[a]		Soil T[a] (controlled for pH)	
	P	R	P	R	P	R	P	R	P	R
LWG bacteria	**0.003**	0.70	0.170	0.17	0.793	−0.13	**0.002**	0.63	–	–
SWG bacteria	**0.001**	0.60	**0.001**	0.46	**0.001**	0.36	**0.001**	0.37	**0.008**	0.30
LWG fungi	**0.001**	0.54	0.120	0.18	0.060	0.20	**0.010**	0.37	–	–
SWG fungi	**0.001**	0.42	**0.001**	0.33	**0.002**	0.37	**0.003**	0.37	**0.005**	0.29

[a] W_{extr} excluded. Significant results are indicated in bold.
Significant results are indicated in bold.

Filamentous saprotrophs were by far the most dominant fungal functional group (66% and 62% in LWG and SWG, respectively), followed by arbuscular mycorrhizal (AM) fungi (6% and 5%) and yeasts (2% and 7%). Other functional groups accounted for less than 2% of total number of sequences (Table S3, Supporting Information).

There was one dominant bacterial high-level taxon (comprising more than 2% of all sequences) that differed significantly among different warming levels in LWG (Betaproteobacteria) and there were three in SWG (Betaprotobacteria, Bacteroidetes and Chloroflexi) (Table S4, Supporting Information). A post hoc test (Table S5, Supporting Information) showed that in LWG, the relative abundance of Betaproteobacteria significantly decreased only at W_{extr} ($P = 0.01$) compared to the ambient (Fig. 3). In SWG, the relative abundance of Betaproteobacteria and Bacteroidetes was significantly decreased at W_{high}' and W_{extr} ($P < 0.001$), while the relative abundance of Chloroflexi was significantly increased at W_{extr} ($P < 0.001$) compared to the ambient.

For fungal communities, a significant increase in the relative abundance of AM fungi compared to the ambient was found for the warming level W_{extr} ($P < 0.001$) in LWG and for warming levels W_{high}' and W_{extr} ($P < 0.05$) in SWG. The relative abundance of filamentous saprotrophs decreased significantly at W_{extr} ($P < 0.001$) in LWG and at W_{high}' and W_{extr} ($P = 0.01$ and $P < 0.001$, respectively) in SWG (Fig. 3). The third dominant group (yeasts) did not show a prominent pattern along the warming gradient (Table S4, Supporting Information).

DISCUSSION

Long-term versus short-term effects of warming

Community profiles from amplicon sequencing of bacterial and fungal rRNA genes showed that significant changes in community composition relative to ambient were only observed above +9°C in the long-term and at +7°C to +11°C/+3°C to +5°C in the short-term for bacteria and fungi, respectively (Fig. 2). Collectively, these results do not support our hypothesis that long-term exposure intensifies microbial community responses to warming since there was no evidence that detectable changes relative to ambient communities occurred at lower warming intensities in LWG than in SWG. It is therefore unlikely that the extent of changes in microbial community composition observed in short-term warming experiments (5–7 years) in high-latitude grasslands would be underestimated in the long-term. On the other hand, against our expectations, our data suggested that changes in microbial community composition occurred at lower warming intensities in SWG compared to LWG (Fig. 2c and f). While this pattern cannot be unequivocally asserted for bacteria (due to the slightly lower warming range in LWG (+6°C to + 9°C) than in SWG (+7°C to +11°C), and the fact that there was a substantial gap in data within this warming range), fungal communities differed more clearly in their warming responses between the two grasslands (Fig. 2f).

We propose the following potential explanations for the observed patterns: (i) after an initial shift in response to warming, microbial communities reverted toward their pre-disturbance composition over the years, except at very high temperatures, where a new (stable) state was established; (ii) the inconsistencies between warming effects in the two grasslands may be a consequence of site-specific effects.

The proposition that microbial community shifts could be transient after a long period of warming contradicts many studies that reported that the effects of warming were detected even after many years (up to 20) of continuous exposure (Allison and Martiny 2008; Frey *et al.* 2008; Deslippe *et al.* 2012; Luo *et al.* 2014; DeAngelis *et al.* 2015, Melillo *et al.* 2017). However, none of these studies examined a warming period as long as the one found in LWG (more than 50 years, possibly several centuries). It is possible that the warming had initially altered some components of the soil ecosystem (e.g. the availability of nutrients, C quality, abundance of predators, plant exudation patterns, plant composition and root density), which then led to a substantial change in soil microbial community composition. However, if shifts in these intermediate drivers were transient over longer time scales (e.g. predator or plant communities re-established due to the colonization of warmed areas by more tolerant species with similar ecological functions), microbial community composition may have changed back toward the original state accordingly. In line with this, at the ForHot site, it has been observed that while warming did not substantially affect the abundance of dominant plant species in either of grasslands (except at the highest warming intensities which caused a strong decrease in plant abundance), short-term warming induced the disappearance of certain herbaceous species along the gradient (Leblans; pers.comm.). Similarly, it has been observed that collembola and nematode communities were affected more strongly by short-term than long-term warming (Holmstrup *et al.* subm., Ilieva-Makulec; pers. comm.). Moreover, soil pH was slightly but significantly increased along the gradients in SWG, but not in the LGW (Fig. S1, Supporting Information). The change in soil pH in SWG might be related to changes in root biomass or exudation patterns along the warming gradient, or they might be a consequence of changes in plant community composition along the gradient in SWG (Sigurdsson *et al.* 2016) since leaf litter pH can vary substantially between different plant species possibly influencing soil biogeochemical properties (Cornelissen *et al.* 2006). The increase in soil pH along the warming gradient might have contributed to the observed differences between microbial communities exposed to different warming intensities since the

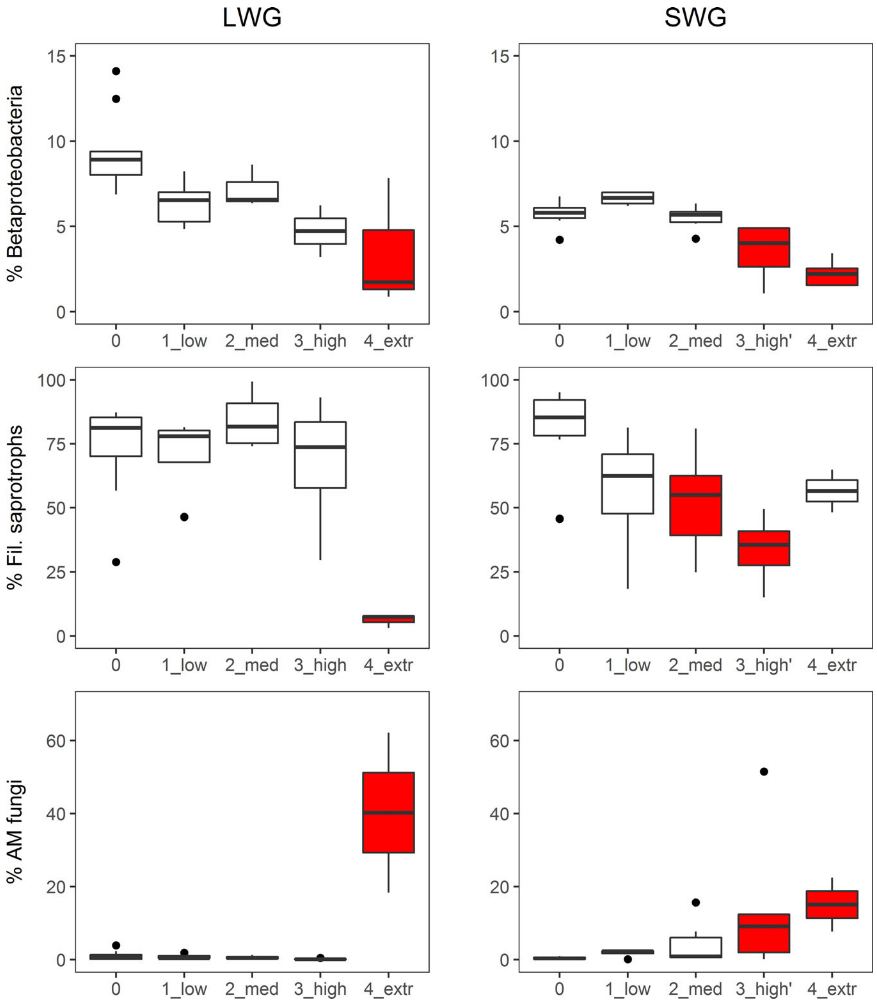

Figure 3. The change in the relative abundance (percentage of the total amount of sequences in a sample) of Betaproteobacteria and fungal functional groups (AM fungi and filamentous saprotrophs) in the long-term warmed grassland (LWG; left) and the short-term warmed grassland (SWG; right). Only the microbial groups that differed significantly between different warming levels in both grasslands are shown. Warming levels: W [0 – ambient (+0°C to +1C°), 1_low (+2°C to +3°C), 2_med (+3°C to +5°C), 3_high (+6°C to +9°C), 3_high′ (+7°C to +11°C), 4_extr (+15°C to +19°C)]. Red box plots indicate warming levels where the relative abundance of a given microbial group was significantly different from the relative abundance at ambient soil temperature based on the Tukey test.

effects of temperature and pH were somewhat correlated (Figs S1 and S3, Supporting Information). The lack of microbial community response to warming in LWG (except at the highest temperatures) may then be explained by the fact that the pH in this grassland did not increase consistently with increasing temperature (Fig. S1, Supporting Information), possibly due to the lack of consistent changes in vegetation along the warming gradient (Leblans; pers.comm.).

It is thus possible that direct effects of elevated soil temperatures are not the single most important factors structuring microbial communities, but rather that a number of other warming-induced changes in biotic and/or abiotic environmental factors may play a substantial role. Indeed, a growing body of evidence supports the assumption that certain factors that are typically influenced by warming, such as substrate quality (Rinnan *et al.* 2007; Conant *et al.* 2011), nutrient availability (Koyama *et al.* 2014), plant productivity or diversity (Zak *et al.* 2003; Zhang *et al.* 2005; Rinnan *et al.* 2007) or abundance and activity of other food web members such as nematodes (Ruess *et al.* 1999) oribatid mites (Maraun, Visser and Scheu 1998) and collembola (Tiunov and Scheu 2005), have an important impact on soil microbial communities.

Indeed, it has been observed previously that the indirect effects of warming on microbial community composition (via warming-induced changes in plant communities and soil properties) can be stronger than the direct effects (Xiong *et al.* 2016). It has recently been suggested that microbial community composition in soils exposed to warming can undergo multiple multi-year phases driven by complex fluctuations in environmental drivers such as soil carbon quality (Melillo *et al.* 2017). If these were to operate in the system presented here this could imply that communities in the long-term warmed grassland might have not yet reached a ‘stable state’. Future study should thus focus on disentangling the direct and indirect effects of warming to clarify the underlying mechanisms that shape microbial community composition under increased temperatures over time.

When considering the apparent differences in microbial community responses between LWG and SWG, it should be emphasized that our study did not follow one single system through time, but two different systems with different exposure periods to warming. Even though the grasslands are comparable in many aspects, the microbial communities at ambient temperatures in LWG differed significantly from the corresponding communities in SWG (Fig. S4, Supporting Information), which is not surprising given that microbial communities can vary substantially in space due to the small-scale variations in different environmental factors. It is theoretically possible that these different microbial communities (or their site-specific drivers) respond differently to the same temperature elevation, in which case this could preclude attributing the observed differences between them to warming period per se. However, it is unclear which biological mechanism should produce this spatial variation in temperature-response, especially given the high degree of similarity between the sites with regard to soil, climate and vegetation (Sigurdsson *et al.* 2016).

The response of high-level microbial groups to warming

In general, the responses of individual bacterial OTUs were not consistent within higher taxonomic classifications since few high-level taxonomic groups showed a significant trend along the temperature gradient (Table S4, Supporting Information). Therefore, the changes in bacterial community composition with warming were probably a result of shifts at lower taxonomic levels. However, the decrease in relative abundance of members of the Betaproteobacteria subphylum corresponded to the pattern in overall bacterial community composition along the gradient in both grasslands (Figs 2c and 3). This implies that the observed patterns in bacterial community composition may, to a certain extent, be explained by the decrease in abundance of Betaproteobacteria. Several other studies have reported a consistent response to environmental drivers, including temperature elevation, for Betaproteobacteria (Fierer, Bradford and Jackson 2007; Rui *et al.* 2015; Zhang *et al.* 2016) indicating that members of this group may largely share environmental requirements.

For soil fungi, the significant changes in abundance of filamentous saprotrophs and AM fungi roughly coincide with the changes in overall fungal community composition along the two gradients (Figs 2f and 3). Since filamentous saprotrophs and AM fungi together represent the majority of the total amount of fungal sequences found in the soil samples (72% and 67% for LWT and SWT, respectively), it can be concluded the changes in overall fungal community composition are largely the consequence of changes in these two groups along the gradient. These results suggest that warming may induce shifts from free-living saprotrophs toward AM fungi that could alter some ecosystem functions, such as soil carbons storage (Treseder and Lennon 2015). It has been demonstrated that winter warming can induce a disproportional increase in carbon provisioned by plants to AM compared to other fungi (Birgander, Rousk and Olsson 2017), which could possibly explain the increase in relative abundance of AM fungi during spring. Some studies also reported decrease in relative abundance of AM fungi under experimental warming (Rudgers *et al.* 2014), while others reported no effect of temperature increase on this functional group (Heinemeyer *et al.* 2004).

Sensitivity of microbial communities to climate warming

Our results provide insights into the sensitivity of soil microbial community composition under predicted climate warming in high-latitude systems (+2.2°C to +8.3°C, by the end of the century (IPCC 2013)) while taking into account different exposure periods and warming intensities. We argue that low-intensity warming, at levels typically applied in static warming experiments (between +1°C and +5°C), is not likely to significantly alter bacterial and fungal community composition in this subarctic grassland system over the long term. In the short-term, fungi were affected already by warming intensities of +3°C, suggesting that future temperature increases may transiently alter fungal community composition. This differential response between fungi and bacteria could potentially be interpreted as reflecting differing temperature sensitivities and/or recovery times of the respective communities; however given that fungal communities were sampled in spring, it is possible that the earlier onset of the growing season observed in warmed plots (Leblans *et al.* 2017) affected fungi at this time of the year more strongly than bacteria that were sampled later in summer. Further research, including sampling at different seasons, is needed to better understand the interactions between warming effects and the effects of seasonality on microbial community composition.

The lack of consistent conclusions regarding the sensitivity of soil microbial communities to warming in different studies could arise both from ecosystem dependency (Allison and Martiny 2008; Pold and DeAngelis 2013; Cregger *et al.* 2014; Weedon *et al.* 2017) and inconsistencies in methodological approaches. This study is unique in that it focuses on the effect of warming on both fungal and bacterial communities along temperature gradients, unlike most other studies that use single-temperature manipulation treatments and are thus not able to evaluate the effect of different warming magnitudes (Thompson *et al.* 2013). Given that high-latitude systems will potentially be subject to a higher intensity of warming than most other regions (Serreze *et al.* 2000; MacDonald 2010; IPCC 2013), it is particularly important to get a better mechanistic understanding of the effect of different temperature elevations in these ecosystems. On the other hand, it must be emphasized that geothermal soil warming is not entirely comparable to the warming resulting from climate change (Sigurdsson *et al.* 2016). This is, however, a common issue for many studies investigating the effects of warming on soil systems. In terms of differences arising from community profiling techniques, we utilized a high-resolution analysis of molecular markers that allows detection of changes in low taxonomic levels, unlike commonly used biochemical approaches.

CONCLUSION

Our results show that, contrary to expectations, long-term exposure did not intensify the effect of warming on microbial community composition. We thus conclude that the effects of warming on microbial community composition observed in short-term experiments (with the duration of 5–7 years), in subarctic grasslands, are not likely to underestimate effects over larger time scales—in contrast, our results suggest a risk of overestimation. The significant influence of warming was observed at medium (+3°C to +5°C) and upper (>+7°C) ranges of predicted temperature increase in next 100 years (+2.2°C to +8.3°C), for fungi and bacteria, respectively. Given that these effects may not persist in the longer term, microbial community composition in high-latitude ecosystems could be less sensitive to climate warming than previously expected.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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