Universiteit Antwerpen

Faculty of Science, Department of Biology

Structure of soil microbial communities along a geothermal gradient in Iceland

Master Project submitted to obtain the degree of Master in Biology, specialisation Biodiversity: conservation and restoration

Student:

Dajana Radujkovic

Promoter:

Dr. James Weedon

Academic year 2015/2016

Table of Contents

SUMMARY

Soil microorganisms play a crucial role in processes of nutrient cycling which potentially makes them important factors in predicting the consequences of on-going climate change. However, microbial responses to climate warming are still poorly understood which is why microbes in general are often treated as a black-box in contemporary climate models. To address this issue, it is very important to gain a better understanding of warming effects on soil microbial community composition.

Our study took advantage of natural geothermal gradients (including a 7 year old and >50 year old gradient) and recently established artificial nutrient gradient in south-western Iceland to investigate long-term and short-term effects of soil warming of different magnitudes, as well as direct and indirect temperature effects on bacterial community composition. This was accomplished by using the advantages of high throughput next generation sequencing of 16s rRNA genes.

The results showed that bacterial communities were resistant to low magnitude warming and clear effects were detected only at temperature elevations above +5°C from the 7 year old and above +10°C from the long-term warming gradient. These results, together with ones obtained in a previous study in the 4 year old warming gradient, indicate that bacterial communities might be resilient to warming over longer time scales. However, at a certain temperature (between +10°C and +20°C) they seem to lose the ability to return to their original, pre-warming state. Two high-level taxa were shown to be particularly vulnerable to increased temperatures: Betaproteobacteria and Bacteroidetes, while Chloroflexi mostly exhibited a positive response to elevated temperatures.

We found no apparent influence of increased nitrogen concentrations on bacterial community structure, probably because exposure period to N addition was too short. Therefore, we have no conclusive result as to how the increase in nitrogen availability may correlate with the effects of warming. However, we demonstrated that some other indirect temperature effects (especially temperature-driven change in soil organic carbon stocks and plant biomass) might have an important influence on bacterial community composition.

LAYMAN'S SUMMARY

For several decades now scientists have been trying to understand the effects of climate change on natural ecosystems. However, in most contemporary climate models the importance of soil microorganisms has been neglected, even though they play a crucial role in nutrient cycling processes. This comes as a result of numerous difficulties in investigating microbial diversity and ecology.

In this study, we used recently developed sequencing technologies to investigate the composition of bacterial communities sampled from different soil temperatures. The soil samples were taken from two naturally warmed grasslands in Iceland; one that has been warmed for 7 and the other for more than 50 years. In addition, we investigated bacterial community structure along artificially created nitrogen gradient in order to compare the effects of warming to the effects of increased nitrogen concentrations. Finally, since warming can affect some other environmental factors, we tried to relate these indirect temperature effects to the changes in bacterial composition.

Our results showed that bacterial communities exhibit observable changes between +5°C and +10°C in short-term warmed grassland and between +10°C and +20°C in longer-term warmed grassland. Based on this result, we argue that, after a certain time of exposure to warming, initially altered microbial communities are able to adapt to elevated temperature conditions and go back to their original state. However, there is a certain temperature threshold (between +10°C and +20°C) above which the communities cannot adapt any more. Nitrogen addition did not have an influence on bacterial communities (probably due to short exposure period), but temperature-induced changes in some other environmental factors (in particular, soil organic carbon and plant biomass) were shown to play a role in determining soil bacterial community composition.

1. INTRODUCTION

Earth's climate is becoming increasingly warmer and this is intensified by both biophysical and biogeochemical (e.g. carbon cycle) feedbacks (IPCC, 2013). IPCC reports that there has been a steady rise in global mean temperatures since 1850 and that carbon dioxide concentrations have increased by 40% since pre-industrial times, primarily from fossil fuel emissions and secondarily from net land use change emissions. Continued emissions of greenhouse gases will cause further warming and changes in all components of the climate system. Most models investigating global warming predict that the global surface temperature will rise at least by another 0.3 – 4.8 C^{o} (based on different emission scenarios) by the end of this century (Stocker et al., 2013). Clear ecological responses to warming have already been observed, but for most ecosystems it is still unknown how they will react to these changes. That is why understanding and predicting the effect of global warming on the structure and functioning of natural ecosystems is one of the biggest challenges confronting scientists today (Gorman et al., 2014).

Soil environment is probably the most complex biological community with extremely high diversity at small scales (Tiedje et al., 2001). Since the soil is extremely heterogeneous system with multiphase nature which includes gases, water, and solid material, it provides many diverse microhabitats for soil organisms (Daniel, 2005). Of all soil organisms, prokaryotes (bacteria and archaea) are the most abundant and they form the largest component of soil biomass (Gans et al., 2005). One gram of soil may contain up to 10 billion microorganisms (Roselló-Mor & Amann, 2001) and some calculations suggest that number of distinct bacterial genomes ranges from 2,000 to 18,000 (Daniel, 2005).

The importance of all levels of biodiversity for ecosystem functioning has been increasingly recognized for several decades now, hence much of the research has been focused on different aspects of biodiversity (Loreau, et al., 2002). However, this kind of research is highly biased towards relatively large organisms, leaving the importance of microorganisms in this context largely unknown (Fierer & Jackson, 2006; Prosser & al, 2007). On the other hand, it is precisely these organisms that have an invaluable role in the functioning of every type of ecosystem since prokaryotes (which contain a major portion of the total nitrogen, phosphorus and up to half of the carbon stored in living organisms (Whitman et al., 1998)) perform bulk of the processes related to carbon, nitrogen and sulphur cycles, decomposition and energy flow (Allison & Martiny, 2008). Bacteria and archaea have an essential role in earth system

processes; they are ubiquitous, possess enormous metabolic and physiological diversity and they drive almost all Earth's biogeochemical cycling processes (Prosser et al., 2007; Falkowski et al., 2008).

Considering all the processes they are involved in, there are numerous ways in which soil microbes could be important factors in the on-going climate change (Melillo et al., 2002, Bardgett et al., 2008).
Soil stores a great quantity of organic carbon, which makes it highly vulnerable to changes in microbial respiration under projected climate scenarios, especially elevated temperatures (Nazaries et al., 2015). Therefore, one of the most important contributions of soil microbes to climate change is their role in soil organic matter decomposition and the notion that global warming will accelerate soil respiration rates thereby further increasing CO_2 emissions (Davidson & Janssens, 2006; Bardgett et al., 2008). On the other hand, it might happen that the net carbon inputs to the soil through increased plant biomass exceed the losses of carbon to the atmosphere through decomposition; in which case the net feedback to global warming would be negative (Davidson & Janssens, 2006). This issue is complicated further by the fact that soil microbes can also have an influence on plant community diversity and productivity and hence the quantity of carbon input to soil (De Deyn et al., 2008; Bardgett et al., 2008).

While our knowledge of the effects of warming on primary productivity is quite advanced, there are considerable gaps in understanding how exactly temperature increase affects soil organic matter decomposition (Pold & DeAngelis, 2013). To understand the contributions of soil microbes to global warming requires consideration of both direct and indirect impacts of increased temperatures on soil microorganisms (Bardgett et al., 2008), which then lead to other feedback loops (Figure 1). The direct influence of warming comes as a consequence of the fact that microorganisms and the processes they drive are temperature sensitive (Classen et al., 2015). However, warming can also affect plant primary productivity which alters soil physicochemical conditions, the supply of carbon to soil in the form of root exudates and litter, and hence it can indirectly alter structure and activity of microbial communities (Bardgett et al., 2011). Furthermore, temperature driven increase in decomposition rates leads to higher nutrient availability (Rustad et al., 2001; Aerts, 2010; Koyama et al., 2014) which could feedback to microbes both directly or through the influence on plant productivity. For this reason, fertilisation has commonly been used to simulate the effects of warming even though there is some disagreement if fertilisation experiments necessarily lead to the same responses as warming experiments (Koyama et al., 2014). Also, temperature induced change in other soil properties, such as soil humidity, can have an influence on these complex plant-microbe feedbacks (Classen, et al., 2015).

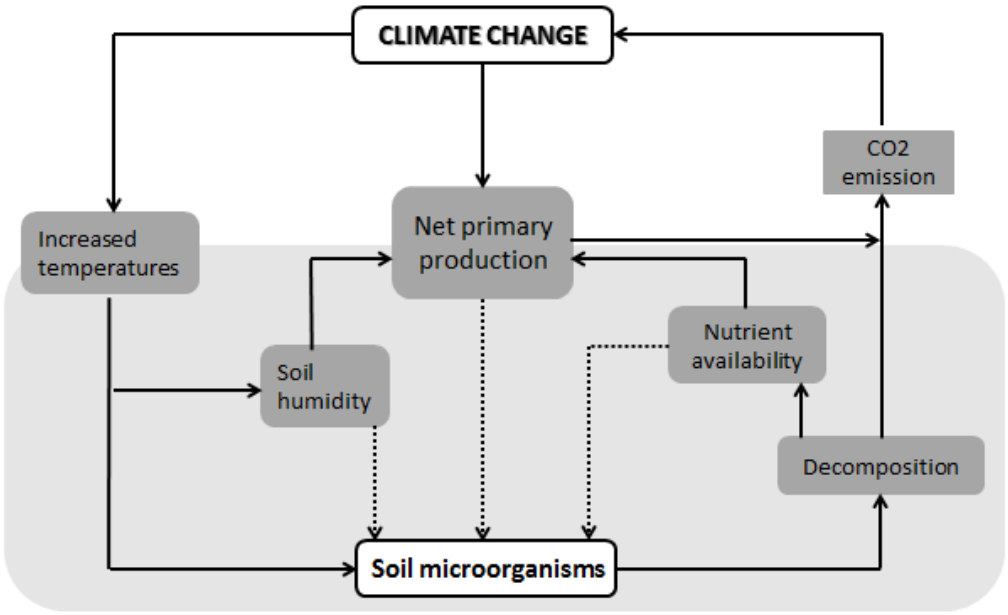

Figure 1. Representation of direct and indirect effects (dashed lines) of climate change on soil microbial communities and feedback to the climate change. Modified from Bardgett et al., 2008.

Despite of their pivotal role in ecosystem functioning, so far, soil biota have had a negligible influence on the development of contemporary ecological theories and climate models (Lynch et al., 2004, Treseder et al., 2012). The responses of soil microbial populations to climate warming have been studied less intensively than the effects on animals and plants due to difficulties in quantifying their overwhelming phylogenetic and physiological diversity and the fact that their interactions with other organisms are considerably more complex (Daniel, 2005). Apart from these methodological limitations, another major problem is the lack of taxonomic knowledge. It is difficult to study diversity and functioning of certain organisms when it is not possible to categorize or identify the species present (Kirk et al., 2004). As a consequence, most ecosystem models simplify the influence of microbial communities, although they are arguably the most complex ones. Therefore, better understanding of microbial ecology could have an essential importance for improvement of current climate models (Wu et al, 2015; Duhamel & Peay, 2015).

For several years now, with improved technologies of DNA sequencing, it has been possible to explore in more detail what kind of organisms live in soil and much of the on-going research is focused exactly on

the responses of microbial communities to the changes of different environmental factors (especially those related to climate change). These studies often look at the influence of precipitation (Zhao et al., 2016), CO_2 elevation (He & al, 2012), warming (Zogg et al., 1997; Zhang et al., 2005; Karhu et al., 2014; Xu, et al., 2015), increased nutrient concentration (Nemergut et al.,2008; Ramirez et al., 2010; Koyama et al., 2014) or the combination of different factors (Castro et al., 2010) on microbial community structure, microbial respiration, carbon utilization etc.

To overcome the limitations of cultivation approaches that are traditionally used for investigation of soil microorganisms (only a small portion of bacteria are actually culturable (Torsvik & Ovreas, 2002)), indirect methods based on isolation and analysis of nucleic acids from soil samples have been developed. Phylogenetic analyses can be accomplished by PCR amplification of 16S rRNA genes from soil DNA using universal primers for bacteria (Winsley et al., 2012). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most commonly used genetic marker for number of reasons: it is present in all bacteria; it contains both highly conserved regions and interspersed hypervariable regions (which allow discrimination at low taxonomic levels); the 16S rRNA gene (1,500 bp) is sufficiently long for bioinformatic purposes (Janda & Abbott, 2007). Moreover, with the development of high throughput next generation sequencing it has become possible to simultaneously sequence millions of reads from the 16S rRNA gene (Caporaso et al., 2010; Bartram et al., 2011). This type of sequencing has become an invaluable tool for microbial ecologists, since it allows for an extremely high level of detail in the analysis of complex microbial communities. Studies have demonstrated the reproducibility of high throughput sequencing platforms (e.g. Illumina HiSeq and MiSeq platforms) which were able to successfully recapture known biological patterns (Caporaso et al., 2012; Sinclair et al., 2015). These molecular methods in microbial ecology have provided enormous quantity of data so far and have helped us understand much more about extremely diverse and complex soil microbial communities. However, they are not without their own limitations (see Discussion below).

Traditional research into the effects of warming on composition and functioning of soil organisms relies on manipulative experiments, which are often criticized as being over simplistic and small scale (Gorman et al., 2014). On the other hand, the large scale studies investigating the effects of warming on natural systems usually involve observations spanning large variations in altitude and latitude. Changes in community composition with latitude and altitude occur for many reasons other than differences in temperature: the interaction between additional environmental gradients (e.g. nutrient concentrations),

hydrology, geology, dispersal constraints etc., which all might influence soil microbial composition (Woodward et al., 2009). This difficulty in disentangling ecological responses to warming from the effects of biogeographical factors is often a serious drawback of studies relying on large-scale natural gradients (Gorman et al., 2014). Ecological systems that can avoid the problems of both small-scale manipulative experiments and large-scale systems are natural geothermal gradients. Since these gradients are typically contained within a relatively small area, other environmental factors stay more or less constant and ecological effects of temperature can be quantified without the added complications of change in altitude and latitude and without any loss of realism (Gorman et al., 2014).

The potential of one such area, containing natural geothermal gradients, in south-western Iceland was recognised by scientists interested in effects of warming on natural ecosystems, which resulted in the creation of the ForHot project. This natural experiment gives rare opportunity for scientists to study how various ecosystem processes are affected by warming both within the range of predicted temperature conditions and more extreme temperatures. Given that these gradients are a stable feature of landscape, they can provide a good insight into long-term ecological and evolutionary responses to warming because microorganisms in these soils have continuously been exposed to elevated temperatures for many generations. Important advantage of the ForHot site is that it includes geothermal gradients that have existed for several centuries now, as well as recently formed temperature gradients. These systems are hence convenient for studying the relation between the short-term and the long-term effects of warming within the same ecosystem type. This topic is particularly interesting because it is still fairly unknown how microbial responses to warming change through time (Allison & Martiny, 2008).

Besides these natural temperature gradients, the ForHot site contains artificial nitrogen gradients which were established in spring 2015. The purpose of this experiment was to investigate to what extent increased nitrogen availability, as one of the most important indirect temperature effects on microorganisms and plant productivity (Figure1), contributes to the ecosystem responses to warming. Therefore, this experiment is designed to complement the studies investigating the effects of warming in natural geothermal systems.

Aims and hypotheses

The overall aim of this study was to investigate the long-term and short-term effects, as well as direct and indirect effects of warming on soil bacterial community composition (from ForHot short-term and long-term warmed geothermal gradients and a nitrogen gradient) using next generation sequencing of 16s rRNA genes.

A previous study conducted in 2012 in the short-term warmed grassland (at a time 4 years old) has shown that bacterial communities were sensitive to temperature elevations higher than +6^{o}C (Weedon et al., in prep). Based on this result, two possible scenarios were expected regarding the effects of long-term versus short-term warming:

- The first scenario assumes that, as the warming period is prolonged, the communities at different temperatures become increasingly distinct until they are completely adapted to altered conditions. This means that the oldest temperature gradient would contain the most differentiated communities at different temperatures and that responses to warming might be found at temperatures lower than +6°C. This would be in concordance with some studies which have shown that the effects of low-magnitude warming on microbial communities is apparent only after a relatively long period of exposure to elevated temperatures (Rinnan, et al., 2007; Frey, et al., 2008).
- The second scenario assumes that bacterial communities are resilient to elevated temperatures, implying that they might return to their pre-warming state after a certain period of warming (Allison & Martiny, 2008). In this case we would see that, through time, the responses to elevated temperatures become weaker or disappear altogether.

As for the indirect effects, we expected to find a certain correlation between microbial responses from naturally warmed grasslands and fertilised grasslands, since it is commonly assumed that warming-induced increase in nutrient availability is important factor in shaping microbial community structure (Rustad et al., 2001; Aerts, 2010).

In order to examine the validity of these hypotheses, the following questions were addressed in the study:

1. Is there a significant difference between bacterial communities along the temperature gradients in the 7 year old and longer-term warmed grassland? Is there a clear point where a shift in community structure can be observed?
2. How do the responses of bacterial communities change over time? In other words, how do results from the two grasslands relate to each other and to the responses observed in the study by Weedon at al. in 2012?
3. Are the observed patterns driven by the responses of certain taxa along the temperature gradient?
4. Is there a significant difference between soil bacterial communities along the nutrient gradient and can the increase in nutrient availability explain the effects of warming on bacterial communities?
5. To what extent are the other possible indirect temperature effects (such as: change in soil humidity, plant biomass and soil organic carbon stocks) correlated with bacterial community responses to warming?

2. MATERIALS AND METHODS

Site description and sampling

The ForHot site is located in the valleys surrounding the town Hveragerdi (64°00'01"N, 21°11'09"W, alt: 53m) in south-western Iceland. The Hveragerdi geothermal field is located on the eastern margin of the western rift zone and the western margin of the South Iceland Seismic Zone (Geirsson & Arnórsson, 1995). The area is highly geothermally active and surface manifestations such as hot springs, mud spots and hot ground are very common (Tharanga & Munasinghe, 2014). These hotspots are formed in places where the heat from the ground is able to reach the surface (Arnórsson, 1995). The highest temperatures are found next to the hotspots, but further away the temperature gradually decreases thereby forming a geothermal gradient. The ForHot site (Figure 2) contains two different geothermal systems: one has been geothermal heated for more than 50 years (Sæmundsson, K., pers. comm, 1965), most likely for several centuries (Magnússon & Vídalín, 1918-1921)(hereafter referred to as 'old

grassland' or GO), the other has been exposed to warming since the earthquake in 2008 (further referred to as 'new grassland' or GN). It has been shown that the earthquake from 2008 altered geothermal manifestations in this area, but geothermal activity and soil warming in the old grassland was not affected (Thorbjörnsson et al., 2009).

In both grasslands 5 replicate transects were established in 2013. Each transect consists of 6 plots spanning a range of different temperatures: a control plot (+0 °C, ambient temperature), and 5 plots with increasing temperatures (+1, +3, +5, +10 and +20 °C). The location of replicate plots was selected in such way to make them as comparable as possible (in terms of soil temperature, moisture content, elevation, slope etc.).

It is important to note that the real temperatures found in these plots do not correspond exactly to the indicated temperatures. The measured average temperatures for the period between May 2013 and May 2015 can be found in the Appendix I. These soil temperatures are not constant over the course of the year (and between different years); on the contrary, they can vary substantially. However, since the magnitude of variation is more or less similar for different plots, on average the temperature differential corresponds to the nominal temperatures indicated above (see Appendix, Figure A_I).

In addition to these two temperature gradients, a nitrogen gradient (hereafter referred to as site N) was artificially created in April 2015, in unwarmed grassland adjacent to the new grassland (Figure 2). This site consists of 5 replicate transects, each with 5 nitrogen treatments (+0, +5, +25, +50, +150 kg/ha.y) at the ambient temperature. One additional transect which consists of 5 plots with elevated temperature (+10°C) was also artificially fertilised with the highest doze of nitrogen (+150 kg/ha.y). The fertiliser (NH_4NO_3 containing 34.5% of N) was added in form of slow released capsules, which were manually distributed over the plots.

Altogether 90 plots were installed; 30 for each site. In July 2015, soil samples were taken from each of these plots using a corer with diameter 2.5 cm, sampling from the depth of 5-10 cm. From every plot 2 samples were taken: one that was used for the analysis of microbial community composition and another that was used for the water content analysis. Individual soil samples were homogenized and stored at -20^{o}C prior to further analyses.

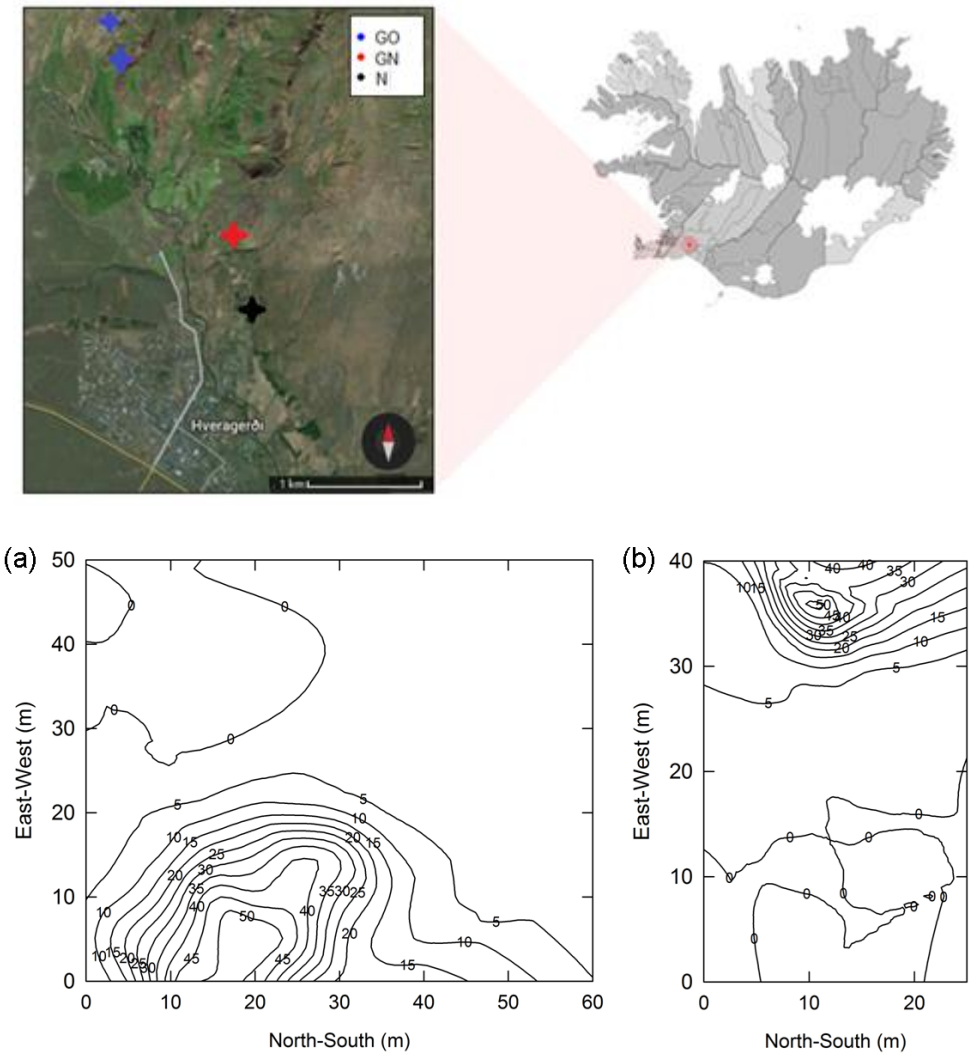

Figure 2: Top – the location of the town Hveragerdi and three investigated sites (Google Earth image). Bottom – spatial distribution of geothermal soil warming at the ForHot site. Isolines show differences in soil temperature (°C) at 10 cm depth between unaffected and warmed areas. (a) Grassland site which has been exposed to geothermal warming since an earthquake in 2008; (b) Grassland site that has been geothermally heated for at least 50 years; from Gorman et al., 2014

For the water content analysis, a subsample (from 15-25 g) was weighed and placed in the oven to dry at the temperature of 105^{o}C for 24h and then it was weighed again so that the percentage of water in the soil could be derived. For microbial community composition analysis, subsamples of approximately 2g were taken for every plot and transferred to Belgium where they were analysed at the University of Antwerp.

Additional data

Some data used in the analysis were provided from other studies conducted at the ForHot site.
Samples for the study by Weedon et al. were taken in July 2012 in the new grassland from the depth of 5-10 cm. At the time, permanent plots with defined temperatures were not yet installed and the sampling was done at the following temperatures: ambient, +1^{o}C, +2^{o}C, +4^{o}C, +6^{o}C, +8^{o}C, +20^{o}C and +40^{o}C in four replicates. Sample preparation and sequencing procedures were almost identical as the one used in this study which makes these two datasets comparable. Samples from this study are further referred to as GN* samples.

The data for soil organic carbon stocks and nitrogen concentrations (measured in spring 2015 using PSR probes installed in each plot) were provided by Niki Leblans, a PhD student working on the effects of climate change on vegetation, carbon and nutrient dynamics. The data for plant and root biomass were measured in July 2015 and provided by Stephanie van Loock, a masters student working on the effect of warming on plant productivity.

DNA extraction

Total community DNA was extracted from the soil samples with the PowerSoil DNA isolation kit according to the protocol of the manufacturer (MoBio, Carlsbad, CA, USA). This method is based on, so called, direct lysis, where microorganisms are lysed directly in the soil after which DNA is extracted (Ogram, et al., 1987). Following cell lysis and nucleic acid extraction, the DNA purification is performed.

The whole process of DNA extraction was done on 0.25 to 0.35 g of soil from each sample, which was first subjected to mechanical cell disruption using bead beating. Subsequent steps included the use of different solutions that sequentially remove different cell components and interfering compounds (soil contains a number of compounds that can decrease the sensitivity of following PCR analysis, e.g. humic acids, phenolic compounds and heavy metals (de Lipthay et al., 2004)) until the pure DNA was obtained.

After the extraction procedure, out of 90 samples, 16 had a low amount of DNA measured by Qubit (see below), so the extraction was repeated for the samples concerned. This time an altered protocol for extraction was applied, where some steps were prolonged and doubled amounts of certain solution

were added as an additional effort to extract higher amounts of DNA. Despite these modifications to the protocol, 9 samples still showed low amounts of DNA and thus could not be used in the further analysis. These samples are: one GN sample (10°C), two sample from N (+25kg/ha.y and +150kg/ha.y) and six GO samples (2 samples +20°C, 2 samples +10°C, 1 sample +5°C, 1 sample +1°C).

DNA library preparation

Before the PCR procedure, DNA concentrations were quantified using a Qubit 3.0 Fluorometer according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA, USA). Based on the measured concentrations, all samples were diluted to the concentration of approximately 5 ng/µl of DNA. This is necessary in order to avoid biases due to different amounts of starting DNA.

The V3 region of the 16S rRNA gene was amplified using modified 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') primers (Bartram et al., 2011). These primers also contain regions complementary to Illumina forward, reverse and indexing sequencing primers, as well as adapters necessary for hybridization to the Illumina flow cell (Appendix II). While the forward primer is the same for every sample, different samples get different reverse primes (each of them has a specific 6bp region – index), which can be recognized by the Illumina sequencer and based on which samples can be separated after the sequencing. Each reaction mixture contained 1.5 µl of the sample, 1µl of forward and reverse primer and 12.5 µl of Phusion High-Fidelity PCR Master Mix with HF Buffer (ThermoFisher Scientific, Waltham, MA, USA).
The PCR conditions were as follows: denaturation step at 98°C for 1 minute, followed by 25 cycles of denaturation step at 98°C for 10 seconds, annealing step at 50°C for 30 seconds and elongation step at 72°C for 30 seconds; finishing with the extension step at 72°C for 4 minutes.

In order to test the success of PCR, samples were run on a 2% w/v agarose gel and electrophoresis was performed. After a successful PCR process, amplified bacterial DNA, (or more specifically V3 region of 16S rRNA gene) was obtained. These DNA sequences were purified from the primers and primer-dimers (artefacts of PCR) using AmpureXP method (Beckman Coulter, Brea, CA, United States). This method uses special magnetic beads which can bind DNA (typically longer DNA fragments), so that everything except the DNA (impurities) can be washed away.

The concentration of DNA for purified samples was measured again using the Qubit fluorometer. Based on these concentrations, samples were diluted in order to obtain equimolar ratios when pooled into a single library. The gel extraction of pooled library was performed as additional step in cleaning the library from impurities using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Final quantification of pooled library was done using KAPA Library Quantification Kits (Kapa Biosystems, Wilmington, MA, USA) to perform real time PCR. Real time PCR is a very sensitive method that can detect very low quantities of DNA. It is important to know the exact concentration of DNA in the library in order to obtain optimal cluster density during sequencing.

DNA sequencing

Soil PCR products with unique indices were sequenced using Illumina paired-end MiSeq multiplex sequencing (Illumina Inc; San Diego, CA, USA). Since the V3 region of bacterial 16S rRNA is typically around 200 bp long and Illumina MiSeq can sequence 150 bp, having both DNA ends sequenced means that entire V3 region is covered including an overlap region.
After the completion of the process, sequences belonging to different samples are separated based on the indices introduced during the sample preparation. The results of sequencing come in a FASTQ format; where for each base a quality (Phred) score is given. This score indicates the probability that a certain base was called incorrectly by the sequencer.
The total number of sequences obtained was approximately 9.6M.

The reproducibility of sample preparaton and sequencing was tested by using technical replicates (DNA isolated from same samples subjected to separate PCR reactions and sequencing procedure) (see Appendix, Figure A_III).

Quality control and bioinformatic analysis

The first part of bioinformatics analysis was performed using USEARCH tool that provides algorithms for relatively fast local and global search of large sequence databases (Edgar C. , 2010).

The initial steps included merging the paired-end reads and removing primer sequences. The sequences were then quality filtered with the maximum expected error of 0.05 and any reads that exceeded this threshold were discarded. Removing the reads with high probability of inaccurate base calls is a vital step in in the analysis of sequencing data because the more the errors there are, the more difficult it is to distinguish them from the true biological sequences and between-sample variation. For instance, sequencing errors can cause mis-clustering of otherwise similar reads, artificially increasing the number of retrieved Operational Taxonomic Units (OTUs) thereby leading to the overestimation of microbial diversity. Sequencing errors will also result in formation of OTUs that are not correct reconstructions of the true biological sequences (Puente-Sánchez et al., 2015).
93% of reads passed this quality filtering step leaving approximately 6M good quality sequences.

Following dereplication and singleton removal (3.1% of high quality sequences were thereby discarded) a set of OTU representative sequences (97% similarity) was constructed using the UPARSE-OTU algorithm (Edgar, 2013). OTUs are defined as clusters of sequences that should represent some (relatively high) degree of taxonomic relatedness. Nevertheless, one should be cautious when identifying OTUs with actual bacterial species because they are based on 97% similarity between genes (in this particular case, a part 16s rRNA gene), which does not always correspond with species level. Determining exactly how OTUs should be defined, and what they represent, is an active area of research (Kuczynski et al., 2011). After the completion of this step, the number of OTUs defined was 20.1K.

After the chimeras had been removed (2.3% Of sequences) all reads were mapped to the newly defined OTUs using USEARCH algorithm with global alignments (Edgar, 2010). An identity threshold was set to 0.97. Mapped reads were then assembled in an OTU table.

From this point on, all subsequent steps were performed using QIIME (Quantitative Insights Into Microbial Ecology) software (Caporaso et al., 2010c).

The OTU sequences were aligned to the Greeengenes database (DeSantis et al., 2006) using PyNAST algorithm (Caporaso et al., 2010b). Based on the aligned OTUs phylogenetic tree was constructed using FastTree (Price et al., 2009). The taxonomic identity of each OTU was identified based on the Greengenes database using the RDP classifier (Wang et al., 2007).

To avoid sample size-biased artefacts, 10 subsampled (rarefied) OTU tables were created by random sampling of the original OTU table. In this step, samples that contained fewer sequences than the requested depth (here 7000) were omitted from the output OTU tables. This rarefaction depth included all but two samples which had significantly lower amount of sequences than other samples (similar numbers found in the negative control) whose reliability was therefore questionable (these two samples are from GO: +10°C and +1°C).

Rarefied tables were used for computing the between community diversity for each of the samples using the UniFrac method. This is a method for computing differences between microbial communities based on phylogenetic information. It measures the phylogenetic distance between sets of taxa in a phylogenetic tree and can be used to determine whether communities are significantly different and to compare many communities simultaneously using clustering and ordination techniques (Lozupone & Knight, 2005). As a result, we obtained 10 UniFrac distance matrices containing a dissimilarity value for each pairwise comparison.

Sequencing data from the study by Weedon et al. were processed in the same manner as described above, only that the filtered reads were first merged to the OTUs from our study, thereby creating a new OTU table. This OTU table was merged with the one containing our GN samples and was then rarefied to 1199 which was the lowest amount of sequences found in any sample. Beta diversity was then calculated between the samples coming from GN from 2012 and 2015.

Since the standard procedure of creating phylogenetic trees (using QUIIME commands *make phylogeny.py* and *assign taxonomy.py*) normally results in a tree that is not entirely in concordance with the taxonomy (e.g. members of the same phyla are not always placed in the same part of the phylogenetic tree), a different method was used for tree construction. This method uses Greengenes curated taxonomy for transferring group names to a tree topology that has overlapping terminal node name (McDonald, et al., 2012). In this case the subset of OTUs was aligned to the Greengenes reference set and the tree was constructed using RaXML (Randomized Axelerated Maximum Likelihood) software (Stamatakis, 2014). The resulting tree was visualized using iTOL (Interactive Tree of Life) online tool (Letunic & Bork, 2011).
The phylogenetic trees were constructed for GO and GN based on 500 most abundant OTUs (which comprise 70% and 67% of all sequences from each site, respectively).

Statistical analysis

The first part of the analysis was performed on randomly sub-sampled databases rarefied to 7000 sequence per samples. Both weighted and unweighted UniFrac distance matrices were used in the analyses. Weighted UniFrac is a type of quantitative measure that takes into account the relative abundance of OTUs thereby down-weighing the importance of rare OTUs and is thus suitable for revealing community differences that are due to changes in relative taxon abundance. Unweighted UniFrac is a qualitative measure that only takes into account the presence/absence data, so it is more informative when communities differ primarily by what can live in them and less by their abundance, in which case abundance information could conceal significant patterns of variation (Lozupone et al., 2007). For this particular study, weighted UniFrac is arguably more important since we mainly wanted to look at overall differences between the samples and in this context dominant OTUs are more relevant than the rare ones. For comparison between the GN data from 2012 and 2015, only weighted UniFrac distances were used.

Principal coordinates analysis (PCoA) was performed to evaluate the overall differences in microbial community composition using the *cmdscale* (Classical multidimensional scaling) function from the '*vegan*' package (Oksanen et al., 2015). Function *betadisper* was used to test if the dispersions (variances) of different groups are significantly different.

The contribution of different temperatures and different nutrient treatments to variation in microbial community composition was quantitatively evaluated using PERMANOVA (permutational multivariate analysis of variance) analysis (Anderson, 2001). For this purpose the *adonis* function in the '*vegan*' package was used. Different temperatures/nutrient treatments were considered as fixed (continuous) variables while replicates served as random variables. For the interaction between nitrogen and temperature the test was performed using the data for +0°C and +10°C without nitrogen addition and +0°C and +10°C with 150kg/ha.y of added nitrogen. These analyses were performed on all 10 rarefied subsets and their mean p and R2 values are reported.

Indicator species for different temperature conditions were determined using the function *indval* from the package '*labdsv*' (Roberts, 2016) which combines OTU abundance with its relative frequency of occurrence in predefined temperature clusters (Dufrêne & Legendre, 1997). Clustering was not based on

all the different temperatures, but on groups of temperatures showing similar impact on microbial community structure as determined by analysing UniFrac distance matrices (see Results below). Hence, two clusters were used for GO (+20°C and the rest), three for GN (+20°C, +10°C and the rest) and two for GN* (one from 0°C to +4°C and the other from +6°C to +40°C). The test was performed using the dataset based on 500 most abundant OTUs from GO, GN and GN*. Rare OTUs were omitted because rare species could erroneously indicate specialized taxa.

The differences in the relative abundance of dominant phyla (classes) at different temperatures were tested using one-way ANOVA and Tukey test and the most relevant results were visualized using ggplot2 boxplots.

The influence of different environmental factors on microbial community structure was determined using the *envfit* function from the '*vegan*' package. This function fits the environmental factors onto ordination and can compute whether every separate factor plays a significant role on the differentiation of the samples based on their microbial community composition.

Simple linear regression was used to test the relation between relative abundance of the dominant high-level taxa (with the abundance greater than 2%) and different environmental factors and the most important results were visualized using splines in ggplot2.

All statistical analyses were conducted using R statistical software (R Core Team, 2015).

3. RESULTS

3.1 Bacterial community composition at different sites

When looking at the overall community composition, the long-term and the short-term warmed grassland do not show a particular divergence from each other (Figure 3). Although the PERMANOVA test shows that a highly significant difference exists between these sites ($p < 0.001$), relatively low R^2 values (11.5 % weighted UniFrac, 5.7% unweighted UniFrac) reveal that only a small portion of variance is explained by overall differences between the sites.

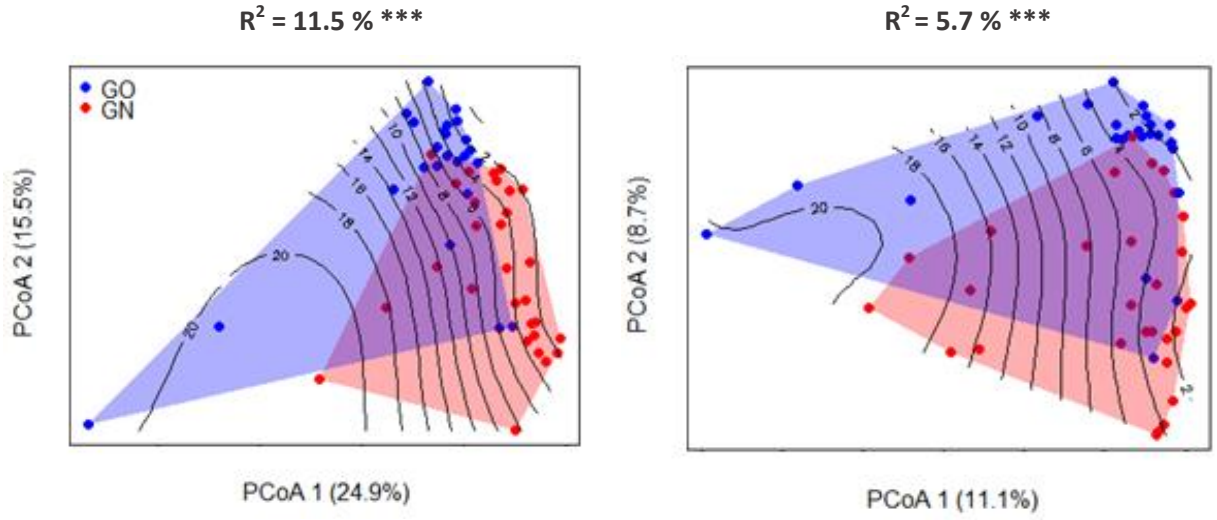

Figure 3: PCoA plots based on: left – weighted and right – unweighted UniFrac distance matrices; points (samples) and the corresponding polygons are coloured according to the sites they come from. Isolines represent fitted smooth surface of soil temperatures (based on the *ordisurf* function). *** p<0.001.

3.2 Bacterial community composition along temperature gradients

a) Old grassland

The analysis of sample beta-diversities (both abundance weighted and unweighted) from the old grassland show that temperature elevation up to +10°C produces no effect on bacterial community composition (Figure 4a).

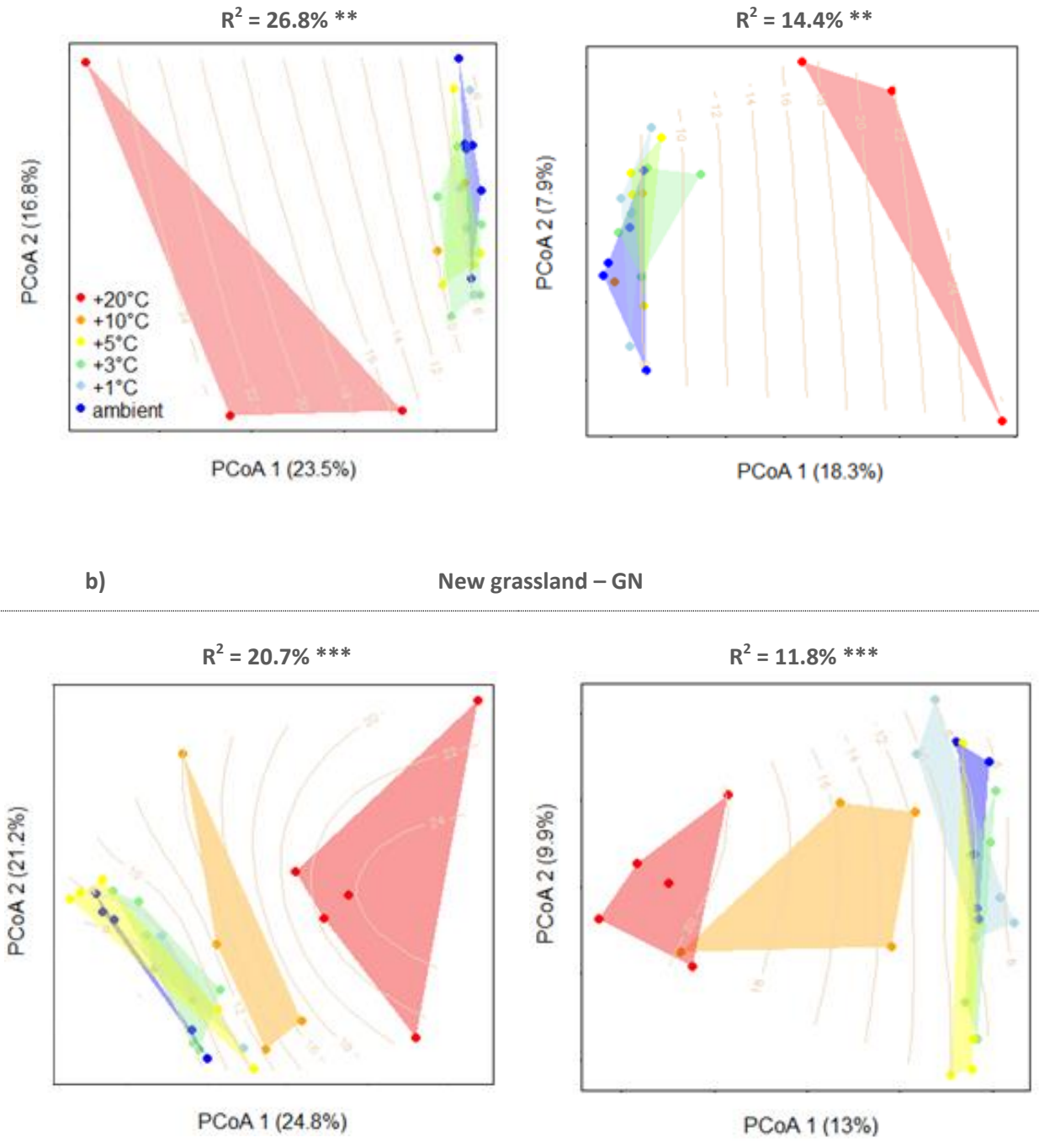

Figure 4: PCoA plots based on: left – weighted and right – unweighted UniFrac distance matrices for a) GO and b) GN; points (samples) and the corresponding polygons are coloured according to soil temperatures. Isolines represent fitted smooth surface of real (measured) temperatures for different plots (based on ordisurf function). ** p<0.01; *** p<0.001.

However, communities sampled from the +20°C plots are clearly distinct from the others which is why a significant difference between bacterial communities from different temperatures was found ($p<0.01$), with a relatively high R^2 value (27% for weighted distances). The samples from +20°C show significantly higher variability relative to those coming from lower temperatures ($p<0.001$, based on betadisper test).

b) New grassland

When considering beta diversity of the samples from the new grassland, no visible response to warming up to +5°C can be detected. However, temperatures of +10°C produce a noticeable change in bacterial communities which can be differentiated from communities at lower temperatures. In addition, communities from +10°C are distinct from those on +20°C (Figure 4b). PERMANOVA results confirm that the differences between communities at different temperatures are significant ($p<0.001$) with relatively high percentage of variance explained by the model (weighted UniFrac R^2 = 21%). The variability of the samples within the temperature group +20°C is significantly higher than for the other samples ($p<0.01$)

c) Integration – temperature influence through time

When comparing bacterial communities from GN sampled in 2012 and 2015, we notice that they are almost entirely separated by the second axis on the PCoA plot, but perfectly comparable along the first axis (Figure 5). The results show that GN and GN* exhibit a similar general pattern in the sense that all the samples coming from temperatures higher than +6°C are distinct from those at lower temperatures. However, GN samples have a somewhat more constricted distribution compared to GN*.

In the GN* study, microbial communities showed no response to warming up to +4°C, but already at +6°C a shift in community composition could be detected, which culminated at +8°C and above this point there was no significant compositional change all the way to +20°C (Figure 6a). It is not possible to compare the shift points of GN* and GN since in the latter study the samples were not taken from the temperature +6°C (the shift happens at temperatures between +5°C and +10°C). However, it can be observed that, in GN, the communities from +10°C are clearly different from those at +20°C, while in GN* community composition did not show noticeable difference going from +8°C up to +20°C (Figure 5). At the temperature of +20°C, GN and GN* contain rather comparable bacterial communities (Figure 6a).

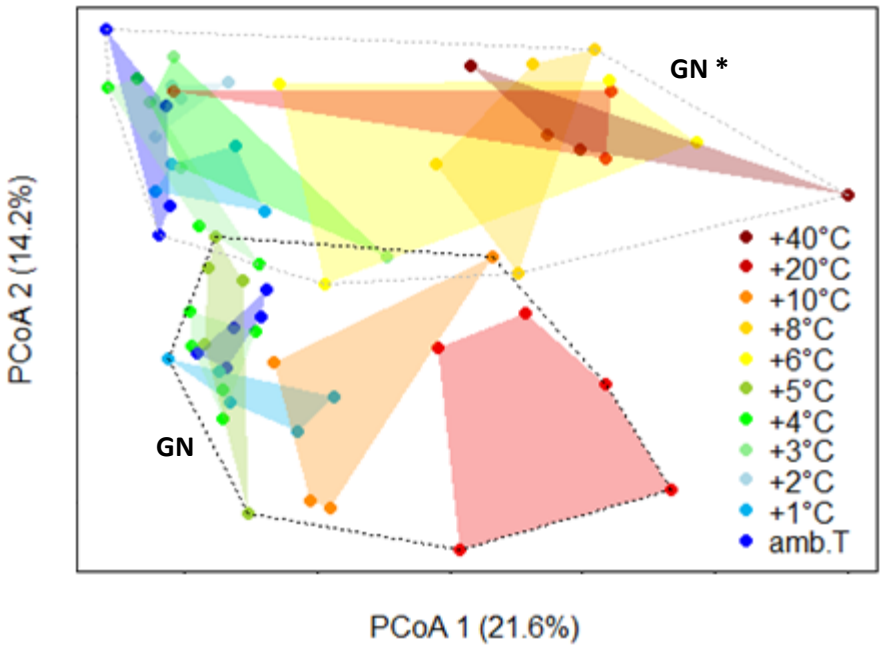

Figure 5. PCoA plots based on weighted UniFrac distance matrices for the samples taken in the new grassland in 2012 (GN*) and 2015 (GN). Points (samples) and the corresponding polygons are coloured according to soil temperatures.

When the GO site is included in the comparison, we observe that, compared to the GN sites, shift to new community composition happens at much higher temperatures, somewhere between +10°C and +20°C (Figure 6b).

If we take the temperature of +10°C as a reference point, the following pattern can be observed: GN* samples (the sampling was not done at this temperature in GN*, but it can be assumed that communities are stable going from +8°C all the way to +40°C) show more prominent distinction from the control than GN, while GO shows no distinction from the control (Figure 6b).

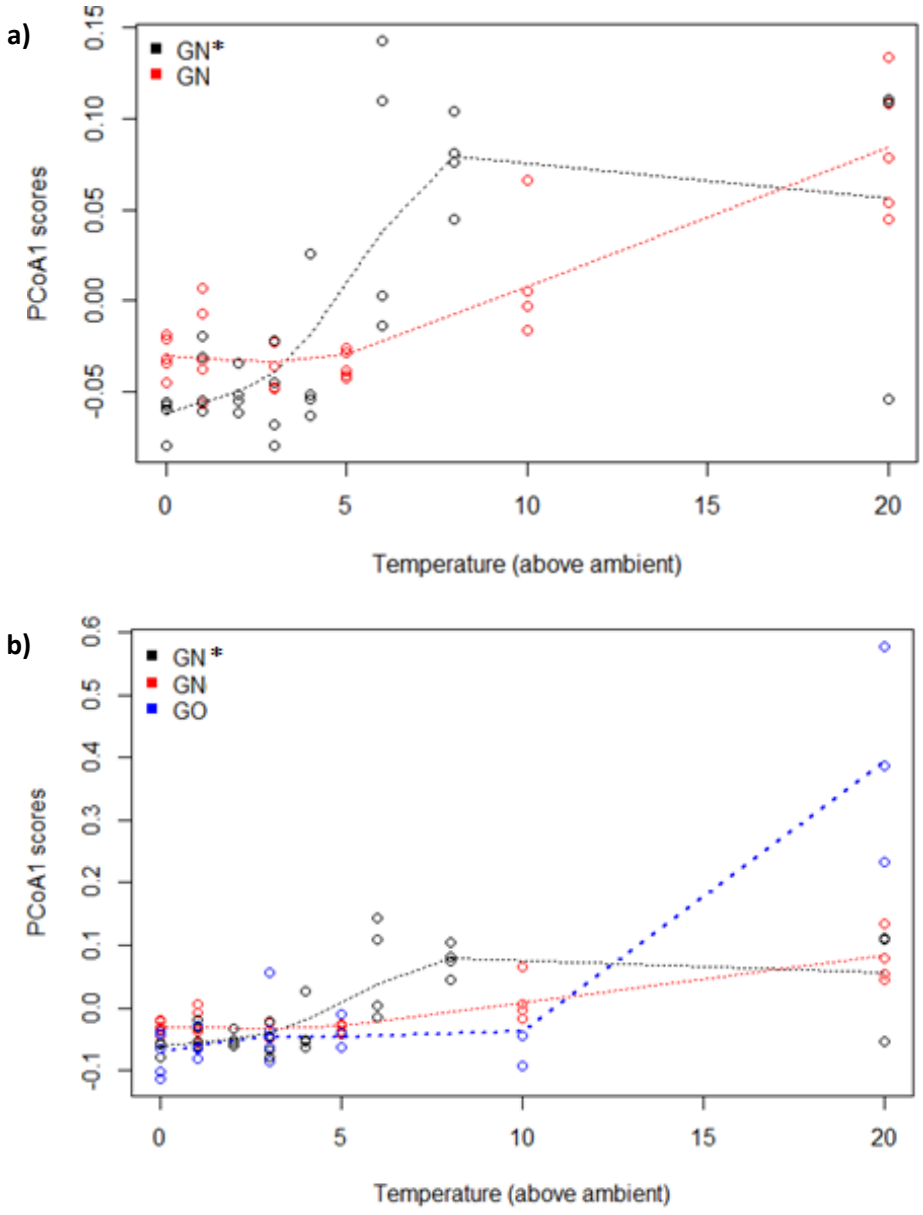

Figure 6: Change in PCoA scores (dissimilarity index) along the temperature gradient. a) Comparison between two different studies in the new grassland; b) Comparison between the old grassland GO (>50 years old) and the new grassland (4 and 7 years old for GN* and GN, respectively). The lines represent smoothing splines fitted to PCoA scores and temperature data

3.3 The effect of warming on different taxa

a) Phylum-based composition of soil samples

The most dominant phyla both in GO and GN were: Proteobacteria, Acidobacteria, Actionobacteria and Chloroflexi. These four phyla accounted for more than 70% sequences of the whole bacterial community in all the samples (Figure 7). 29% of OTUs from GN and GO samples were assigned to Proteobacteria, out of which class Alphaproteobacteria was the most dominant with 12.5/15 % of sequences, followed by Beta-proteobacteria (7.5/5 %), Deltaproteobacteria (6/5.5 %) and Gammaproteobacteria (2/2 %) in GO and GN, respectively. The percentage of Chloroflexi reads found in these two sites was also very similar – 7% in GO and 6% in GN. Acidobacteria had considerably higher abundance in GO than in GN (25% versus 18%, respectively), in contrast to Actinobacteria which were more abundant in GN (11% in GO versus 21% in GN).

Other phyla (see Appendix, Table A_IV) that accounted for 2-5% of sequences in GO and GN were: Verrucomicrobia, Gemmatimonadetes, Bacteroidetes, Firmicutes and Nitrospirae. The remainder of sequences were assigned to phyla or candidate divisions that contributed to less than 2% of sequences in both sites (WS3, Planktomycetes, TM6, TM7, Chlamydiae, Elusimicrobia, OD1, Armatimonadetes, Cyanobacteria, Chlorobi, AD3, Fibrobacteres, Tenericutes, Spirochaetes) or were not successfully assigned to any known phylum (5% in GO and 4% in GN).

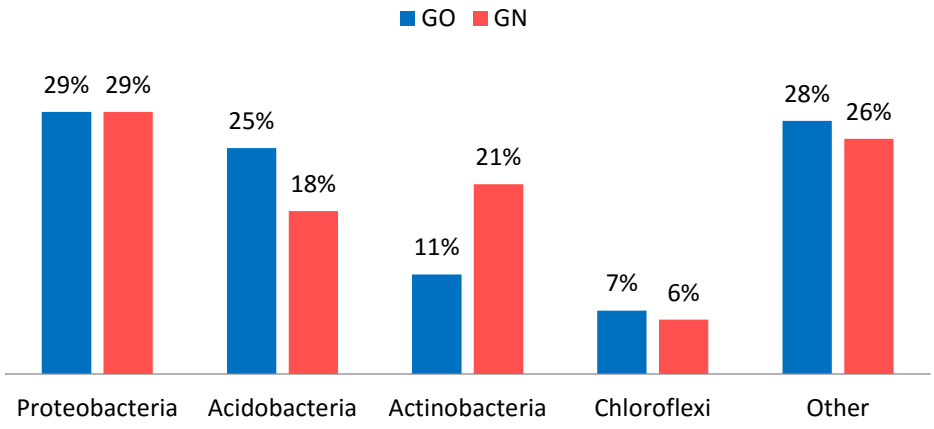

Figure 7: Comparison of relative abundances of 4 most dominant phyla between the two sites (GO and GN)

b) Phylum-level responses to warming in GO

Based on relative abundances of OTUs within the two temperature groups (low = below +20°C and high = above +20°C), we observe that the taxa related to high temperatures are more commonly found in some phyla (e.g. Alphaproteobacteria, Chloroflexi and parts of Acidobacteria clade) than the others (Betaproteobacteria, Gemmatimonadetes, Bacteroidetes), which mainly contain OTUs with high abundances at lower temperatures (Figure 8).

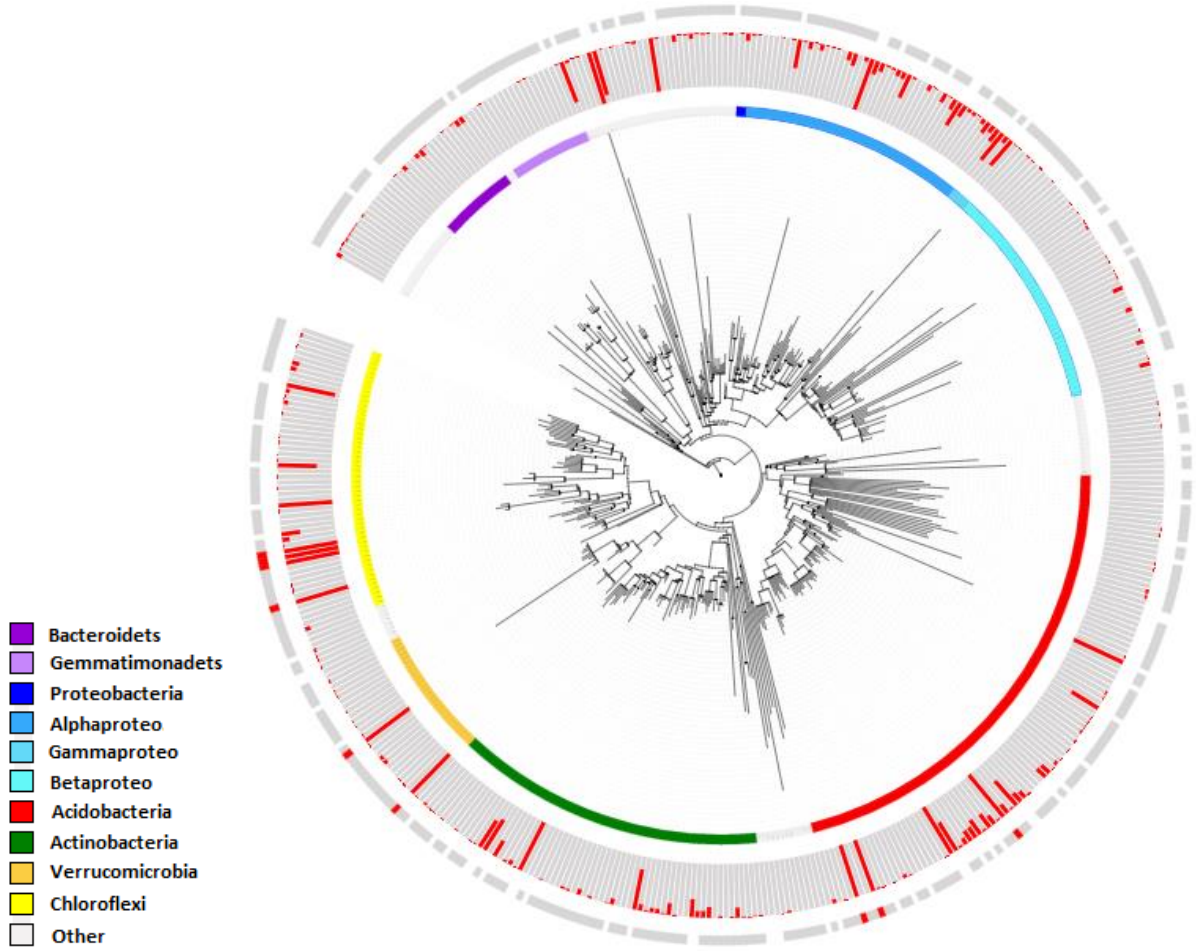

Figure 8: Phylogenetic tree based on 500 most abundant OUTs from the old grassland. First inner circle – phylum-level assignment for each OTU; second inner circle – relative abundance of each OTU in: grey – the low (from +0°C to +10°C), red – high (+20°C) temperature clusters; outer circle – indicator OTUs for the low (grey) and high (red) temperature clusters. Indicator OTUs were selected based on their relative abundance and relative frequency in a cluster.

From indicator species analysis, 397 indicator OTUs were determined, out of which 387 were indicators of lower temperatures and 10 were indicators of +20°C. The indicators for +20°C fall within the phyla: Chloroflexi, Verrucomicrobia and Acidobacteria. The indicators of lower temperatures can be found in every phylum with Bacteroidetes and Gemmetimonadetes having the highest percentage of these indicators (almost 100%).

c) Phylum-level responses to warming in GN

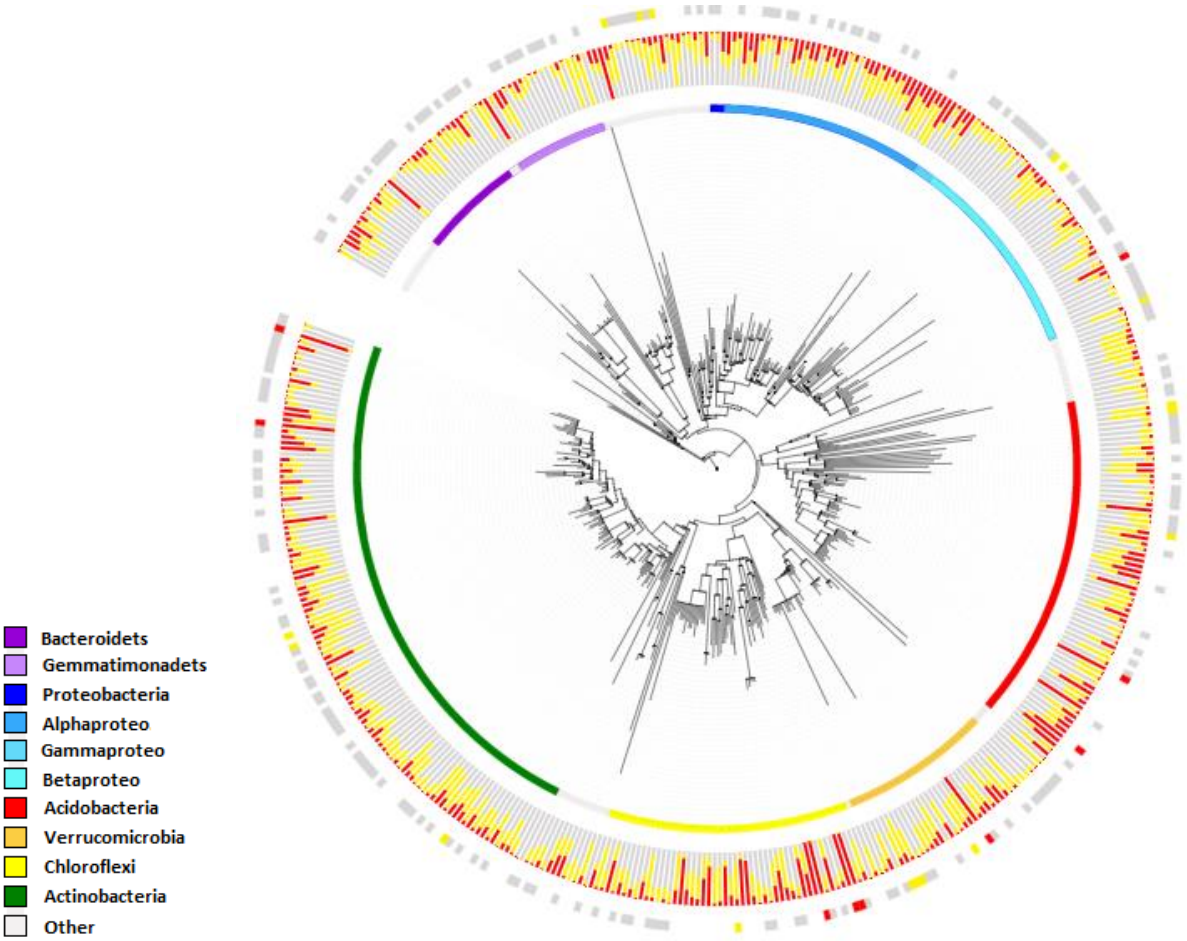

Figure 9: Phylogenetic tree based on 500 most abundant OTUs from the new grassland. First inner circle – phylum-level assignment for each OTU; second inner circle – relative abundance of each OTU in: grey – the low- (from +0°C to +5°C); yellow – medium (+10°C); red – high (+20°C) temperature clusters; outer circle – indicator OTUs for the low (grey), medium (yellow), high (red) temperature clusters.

Some patterns that can be noted from the GN phylogenetic tree (Figure 9) are that OTUs specific for the high and medium-temperature group (+20°C and +10°C) occur predominantly in lineages: Chloroflexi, Acidobacteria and Alphaproteobacteria, while Bacteroidetes, Gemmatimonadetes and Betaproteobacteria contain mostly OTUs specific for lower temperatures. Phylum Verrucomicrobia contains particularly many OTUs with high abundance at +10°C. Actinobacteria, as one of the most abundant phyla, contains OTUs that can be strongly related to both high and low temperature, without evident pattern within the clade.

Within 257 indicator OTUs identified in GN, 226 were significantly related to the low temperature cluster, 22 to medium and 10 to high temperature cluster. Indicator OTUs for warmer temperatures mostly belong to the following phyla: Actinobateria, Chloroflexi, Verrucomicrobia and Acidobacteria, while Betaproteobacteria, Bacteroidets and Gemmatimonadetes contain the highest percentage of OTUs related to the lower temperatures.

d) Comparative analysis based on phylogeny

Comparing the taxa-level response to warming from GO, GN and GN* (see phylogenetic tree in Appendix, Figure A_V) could help us determine which taxa are consistently responsible for the observed shifts in microbial structure. Although it was not possible to directly compare the taxa from the three sites (they contain rather different composition of dominant OTUs), some common selectively-responding OTUs with relatively high abundance for every site (greater than 0.02%) are shown in Table 1 and Table 2.

We observed that common indicators of high temperatures can be found in phyla Acidobacteria Actinobacteria, Chloroflexi and Verrucomicrobia (Table 1). Only two OTUs were found to be indicators of higher temperatures in every of the three sites: OTU_121 for which the phylum is unknown and OTU_74 which belongs to phylum Acidobacteria. This OTU also has a rather high relative abundance in all sites (GO - 0.12%, GN - 0.35%, GN* - 0.4%). As for the indicators of lower temperatures, they come from each of the 7 dominant high-level taxa (the abundance higher than 2.5%), but are most commonly found within Acidobacteria and Actinobacteria lineages (Table 2).

Table 1. Indicator OTUs for high-temperature group (above the shift point), shared by at least two of three sites; only OTUs with the abundance higher than 0.02% for each site were taken into account

OTU #	Rel. abundance (%)			Taxonomy		
	GO	GN	GN*	Phylum	Classis	Ordo
216	NA	0.09	0.14	Acidobacteria	Acidobacteria-6	iii1-15
57	0.28	NA	0.31	Acidobacteria	Solibacteres	Solibacterales
74	0.12	0.35	0.40	Acidobacteria	DA052	Ellin6513
257	NA	0.08	0.04	Actinobacteria	Actinobacteria	Actinomycetales
490	NA	0.05	0.07	Actinobacteria	Actinobacteria	Actinomycetales
513	NA	0.04	0.15	Chloroflexi	Ktedonobacteria	
3310	NA	0.06	0.05	Unknown		
121	0.08	0.16	0.22	Unknown		
427	0.04	0.06	NA	Verrucomicrobia	[Spartobacteria]	[Chthoniobacter.]

Table 2. Indicator OTUs for low-temperature group (below the shift point) shared by all three sites; only 150 top indicator OTUs with the relative abundance higher than 0.02% were taken into account.

OTU #	Rel. abundance (%)			Taxonomy		
	GO	GN	GN*	Phylum	Classis	Ordo
116	0.15	0.21	0.05	Acidobacteria	RB25	
52	0.63	0.05	0.11	Acidobacteria	[Chloracidobacteria]	
525	0.05	0.04	0.08	Acidobacteria	[Chloracidobacteria]	
58	0.43	0.18	0.09	Acidobacteria	Acidobacteria-6	
65	0.27	0.09	0.07	Acidobacteria	Acidobacteriia	Acidobacteriales
77	0.07	0.11	0.14	Acidobacteria	iii1-8	
26	0.23	0.21	0.17	Actinobacteria	Acidimicrobiia	Acidimicrobiales
193	0.06	0.12	0.04	Actinobacteria	Actinobacteria	Actinomycetales
21	0.20	0.53	0.36	Actinobacteria	MB-A2-108	
48	0.15	0.22	0.08	Actinobacteria	Actinobacteria	Actinomycetales
49	0.07	0.21	0.07	Actinobacteria	Acidimicrobiia	Acidimicrobiales
93	0.09	0.24	0.21	Actinobacteria	Actinobacteria	Actinomycetales
45	0.09	0.19	0.06	Bacteroidetes	[Saprospirae]	[Saprospirales]
108	0.15	0.05	0.04	Chloroflexi	Gitt-GS-136	
17	0.21	0.52	0.26	Chloroflexi	Ellin6529	
549	0.16	0.08	0.10	Chloroflexi	Ellin6529	
125	0.20	0.15	0.07	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales
143	0.13	0.05	0.04	Proteobacteria	Gammaproteobacteria	Xanthomonadales
189	0.17	0.09	0.05	Proteobacteria	Betaproteobacteria	
17190	0.06	0.08	0.11	Proteobacteria	Alphaproteobacteria	
174	0.10	0.08	0.08	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]
23	0.43	0.28	0.08	Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]

In order to test if the abundance of different phyla (classes) is significantly different at different temperatures, one-way ANOVA and post hoc Tukey test were applied.

Betaproteobacteria clearly showed a decrease along the temperature gradient for all three sites (Figure 10). For GO, this decrease is gradual and near significant ($p<0.1$). For GN site, the decrease is highly significant ($p<0.001$) and it occurs above the shift point (significant difference between control and samples from +10/+20°C; $p<0.01$). For GN*, the difference is again apparent above the shift point, where the abundance of Betaproteobacteria decreases abruptly ($p<0.001$) and then stays more or less constant (highly significant difference between all the samples above +6°C and the control).

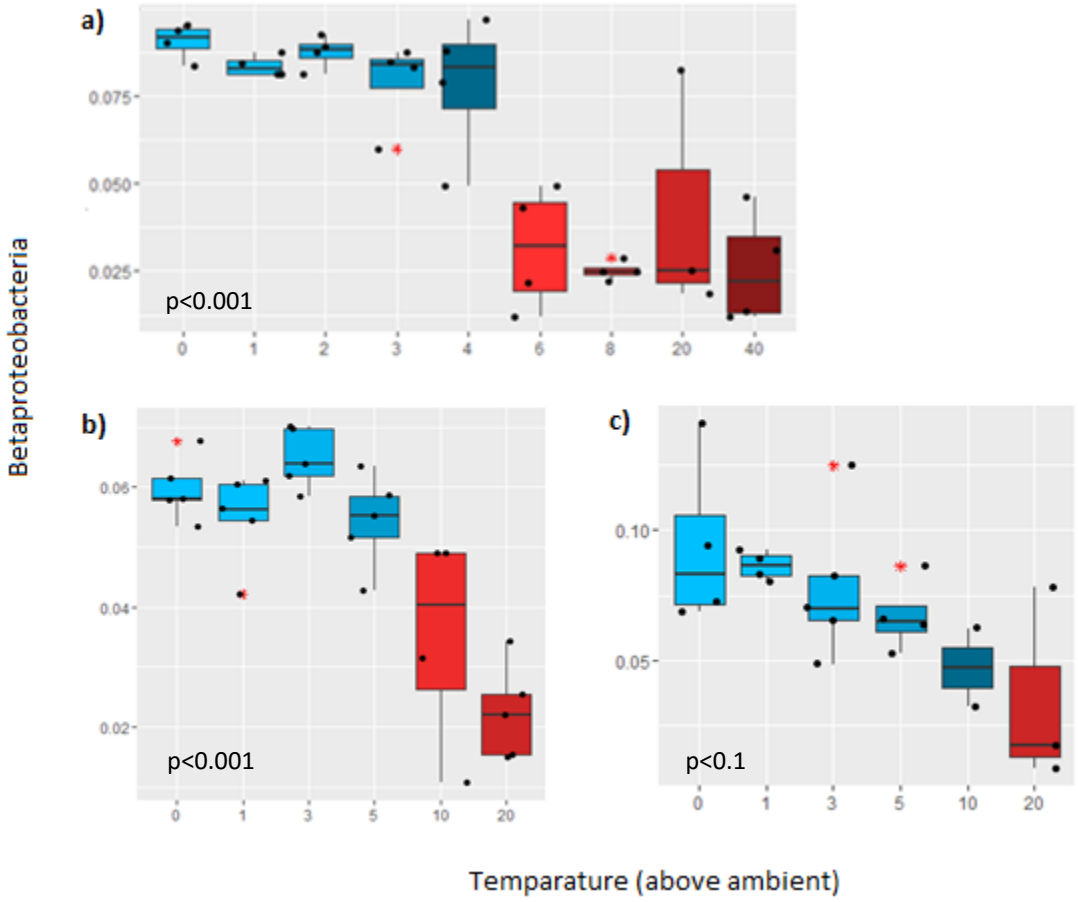

Figure 10: Boxplots showing the change in relative abundance of Betaproteobactera along the temperature gradient for: a) GN*; b) GN and c) GO. Blue boxplots – temperatures below the shift point; red boxplots – temperatures above the shift point

Alphaproteobacteria did not show a particular response to warming in GO and GN, but in GN* they showed an abrupt increase at +6°C after which it remained more or less stable up to +40°C ($p<0.01$). This is exactly the opposite effect of what we have seen for Betaproteobacteria in GN*.

In general, phylum Bacteroidetes showed a decreasing trend with increasing temperatures for all the sites. The difference between the temperatures was near significant for GO ($p<0.1$), highly significant for GN ($p<0.001$) and for GN* ($p<0.01$). In GN, the abundance of Bacteriodetes decreased rather steeply after +5°C and both +10°C and +20°C samples showed a significant difference from the control ($p<0.05$ and $p<0.01$, respectively).

The relative abundance of Chloroflexi showed an increasing trend with temperature elevation, but significant differences from the control were found only for +20°C ($p<0.001$) in GN and for +40°C ($p<0.05$) in GN*.

The abundance of Acidobacteria did not exhibit a significant change along temperature gradient for any of the three sites, except for GN* at temperature +40°C which showed a significant increase compared to the control ($p<0.01$).

3.4 Indirect temperature effects on bacterial communities

a) Bacterial community composition along nutrient gradients

When considering the effect of nitrogen addition on microbial community structure, we observe that although different treatments tend to show some degree of separation, the distinction between them is not clear (Figure 11). Based on both weighted and unweighted UniFrac, a near significant difference ($p<0.1$) was found between different nitrogen treatments, however the percentage of variance explained is relatively low (R^2 = 8% and R^2 = 11.2%, respectively). On the other hand, the samples coming from the highest nutrient concentrations with temperature elevation of +10°C have a distinctive community composition from the others, which is especially apparent when considering unweighted UniFrac. However, this response is driven entirely by temperature elevation since the interaction between temperature and nutrient addition is non-significant ($p>0.05$), while the temperature effect itself is highly significant ($p<0.001$) (Table 3).

Table 3: Results of PERMANOVA analysis for the effect of nitrogen addition and interaction between nitrogen and temperature (below the dashed line) based on weighted and unweighted UniFrac distances

	Weighted UniFrac (R^2)	Unweighted UniFrac (R^2)
N treatment	7.1% .	11.2% .
Temperature alone	16.2% **	10.7% ***
N treatment alone	5.4% .	6.4% .
Interaction N and T	4.9%	6.5% .

. p<0.1; ** p<0.01; *** p<0.001

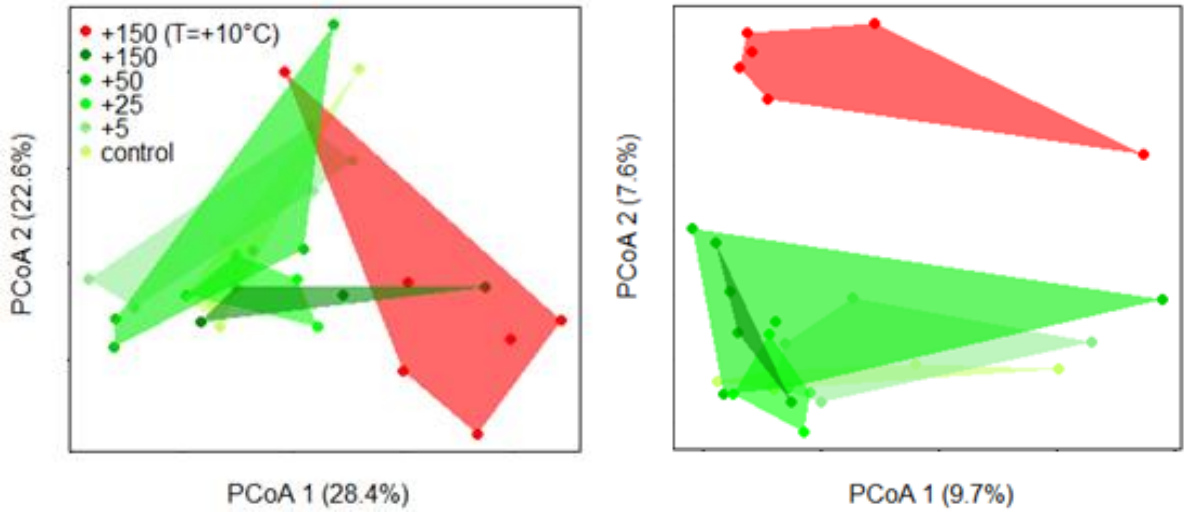

Figure 11: PCoA plots based on: left – weighted and right – unweighted UniFrac distance matrices for N-experiment site; points (samples) and the corresponding polygons are coloured according to N concentrations (green: ambient T; red: +10°C)

b) Influence of other environmental factors

Soil water content (SWC), soil organic carbon stocks (SOC, only available for GO), root biomass (RM) and total aboveground plant biomass (BM) were all significant parameters in explaining the variation between the samples (Table 4, Figure 12). All these factors are significantly negatively correlated to temperature (see Appendix, Figure A_VI). Temperature alone, explained the greatest amount of variation (67% for GN and 81% for GO) followed by SOC and BM for GN (43%), and RM for GO (46%).

The effect of nitrogen concentration in the soil (not shown in the graph), was in both cases uncorrelated to temperature effect and had no significant effect on the separation of samples in the space.

Table 4. The results of envfit analysis with R^2 values and significance for individual effect of every variable on differences between bacterial communities from different samples.

Variable	GO (R^2)	GN (R^2)
T	81% ***	67% ***
SWC	31% *	26% *
SOC		43% ***
RM	46% ***	23% *
BM	37% **	42% ***
N	8%	0.2%

* p <0.05 ** p<0.01 *** p<0.001

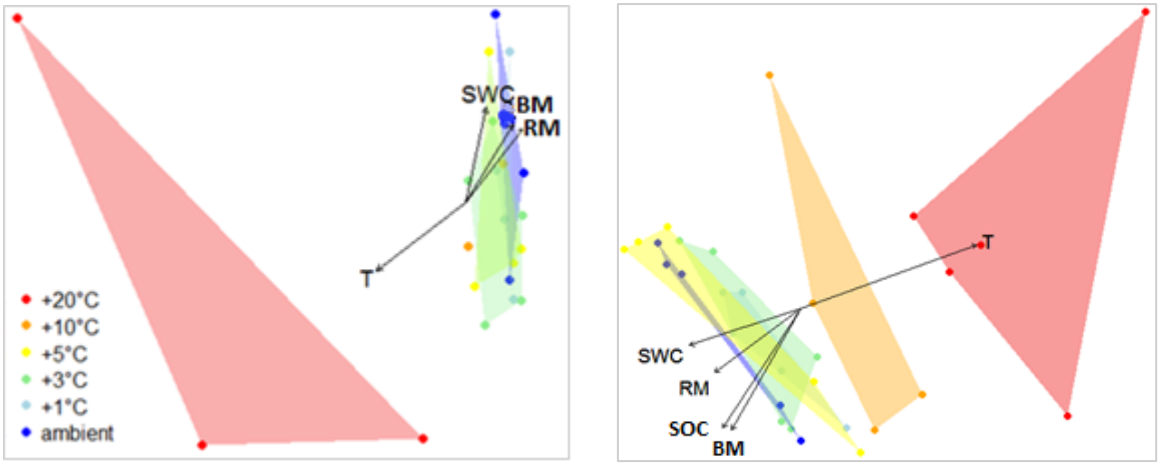

Figure 12. Environmantal vectors fitted onto PCoA ordination (weighted UniFrac) using envfit function; left – GO, right – GN. T: temperature [°C], SWC: soil water content [%], BM: total plant (aboveground) biomass [g/m^2], RM: root biomass [g/m^2], SOC: soil ogranic carbon stocks [t/ha]

The influence of the most important explanatory factors (SOC, BM and RM) on the abundance of the most dominant phyla (classes for Proteobacteria) was tested using simple linear regression. No significant relation was found between these factors and abundances of taxa coming from GO. However, for GN relative abundance of Betaproteobacteria and Bacteroidetes showed a significant increase with the increase of soil organic carbon (R^2=39%, p<0.001; R^2=31%, p<0.01, respectively), while relative abundance of Chloroflexi exhibited a significant decreasing trend (R^2=52%, p<0.001) (Figure 13). Similar,

but weaker responses of these taxa were found for total aboveground plant biomass (BM). The other taxa in GN did not show a significant response to the change in SOC stocks or plant biomass.

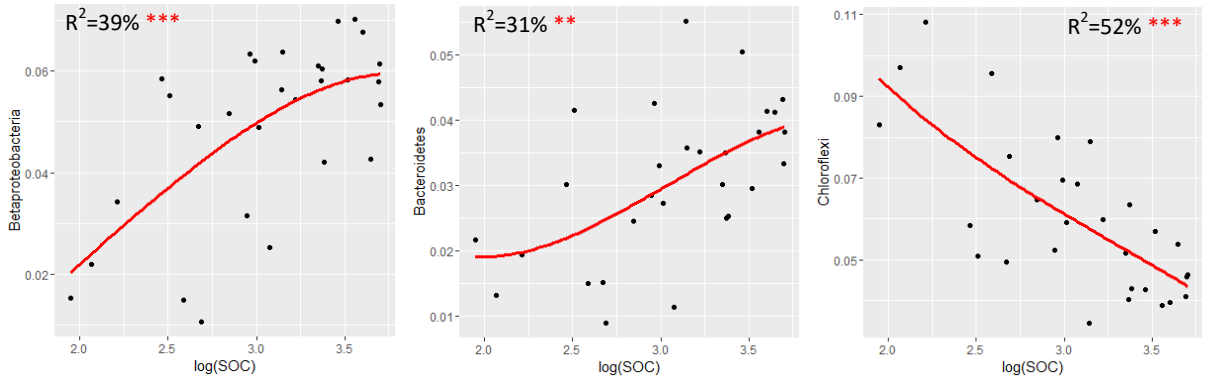

Figure 13: Relationship between relative abundance of: Betaproteobacteria, Bacteroidetes and Chloroflexi and (log) soil organic carbon stocks [t/ha]

4. DISCUSSION

In this study we sought to investigate the responses of bacterial communities to elevated temperatures in the context of predicted global warming. For this purpose, we sampled soils from natural geothermal gradients exhibiting temperature elevations associated with predicted climate change scenarios (up to +5°C), as well as more extreme temperature elevations (up to +20°C). This approach enabled us to investigate temperature thresholds where compositional responses can clearly be detected, thereby allowing us to examine which taxa are particularly sensitive to altered temperature conditions and what are the most important drivers of the shift (temperature itself or combination of factors related to altered temperature conditions).

The study was particularly focused on investigating how the bacterial community responses to elevated temperatures change through time, which was accomplished by comparing the responses from the soils subjected to different exposure periods of continuous natural warming (namely: 4 years, 7 years and more than 50 years).

4.1 Bacterial community composition along temperature gradients

Numerous studies have reported that microbial community structure was sensitive to warming within the predicted range of future temperature increase (Zogg et al, 1997; Rinnan et al, 2007; Frey et al, 2008; Zhang et al, 2005; Sheik et al, 2011; Luo, et al., 2014; DeAngelis et al., 2015). Our study also demonstrated that microbial communities were sensitive to elevated temperatures, but the magnitude of warming was very important since no response (in any site) was found at low temperature elevations (from +1 to +5°C). The lack of response of microbial communities to low-magnitude warming has also been found in several other studies (Schindlbacher et al, 2011; Weedon et al, 2012; Rousk, et al., 2013). This discrepancy between different studies can possibly be explained by the fact that different ecosystems do not always show similar responses to warming (Pold & DeAngelis, 2013; Diepen et al, 2015, Weedon et al, in prep). It has also been suggested that the soil biota responses to disturbance are not uniform since they depend to a large extent on physico-chemical structure of the soil (Griffiths & Philippot, 2013). Moreover, there are some inconsistences in the notion of what is called 'a shift in microbial commity structure' which comes as a consequence of different methodological approaches used for the investigation of microbial responses to warming. For instance, the 'shift' might be refered to as: the shift in the ratio of gram-positive and gram-negative bacteria (Zogg et al., 1997; Frey, et al. 2008), differences in the abundance of particular bacterial phyla (Hayden et al., 2012), differences in abundance of phospholipid fatty acid markers for certain bacterial groups (Rinnan et al., 2007), the differences based on the UniFrac distance matrices (Rui, et al., 2015) etc., which makes it more difficult to compare the results from different studies.

When looking at the effects of warming in GO, the first striking observation is that temperature elevation up to +10°C did not induce a detectable change in the bacterial community composition (Figure 4a). Studies commonly report a shift in microbial community composition due to the warming within the rage of +5°C to +10°C (Zogg et al., 1997; Frey et al., 2008; Wu et al., 2015), so the lack of response in GO to such high temperature elevation is somewhat unexpected. However, it seems that none of the studies conducted so far dealt with such a long warming period as the one found in the old grassland (longer than 50 years).

On the other hand, in the new grassland, temperature elevation of +10°C did produce a distinctive bacterial community composition compared to the control (Figure 4b). The fact that, at +10°C, a difference from the control was detected in the shot-term warmed grassland but not in the long-term

warmed grassland (which are comparable in terms of ecosystem type and climate and generally hold similar bacterial communities (Figure 3), but which differ in terms of exposure period to high temperatures) hint to a conclusion that the observed response in the GN site might be transient.

In this context, it was very interesting to compare observations from 2015 sampling to those obtained in 2012 (Weedon et al, in prep.) in the new grassland, which made it possible to follow how the communities react to warming throughout time. If we assume that the results from 2012 show the earliest (detected) stage of responses to warming, then the results from GO arguably represent the latest "climax" stage in the "succession" of bacterial communities from the period when they are exposed to sudden changes in temperatures to the period when they are entirely adapted to altered conditions. It is not possible to compare the shift points from GN* and GN, due to the lack of data (in 2015 samples were not taken from +6°C, which was a shift point in 2012), but in general it can be concluded that the observed response to warming from 2012 has become less pronounced by 2015 since the communities at +10°C from GO show a much weaker distinction from the control than those in GN* (Figures 5 and 6). So, as the gradient in GN becomes older, the differences between communities at different temperatures seem to become less pronounced and possibly after a certain period they will entirely disappear. These results thus further support the hypothesis that bacterial responses to warming in GN are transient.

However, this kind of dynamics can only be observed until the temperature elevation of +10°C. Above this point (more precisely, somewhere between +10°C and +20°C), the communities don't return to their pre-warming state any more, but they become increasingly divergent through time. This is already apparent from the GN site, where the communities at +20°C did not show signs of recovery towards the original state during 3 years of continuous warming (Figure 6a), but it is especially noticeable when comparing communities from +20°C in GO and GN (Figure 6b).

Based on these results the possibility that bacterial communities in this system are resilient to long-term warming up to +10°C seems plausible. However, better understanding of the potential causes of this kind of response requires consideration of indirect temperature effects (Figure 1).

4.2 Indirect temperature effects

a) Soil microbial responses along nitrogen gradients

It is already well-known from many studies that elevated temperatures tend to enhance respiration rates in microorganisms leading to accelerated decomposition and increased nutrient availability (Peterjohn et al., 1994; Melillo et al., 2011). This might be particularly important for nitrogen limited systems, including subarctic grasslands, where decomposition is normally hampered by low temperatures (Shaw & Harte, 2001; Schmidt et al., 2002). Thus, the effects of warming on microbial communities that we observed might partially be attributed to temperature driven increase of nitrogen availability. In the current study, a nitrogen addition experiment was established to assess the contribution of increased nitrogen availability (arguably the most important indirect temperature effect) to microbial responses to warming.

The lack of clear response to increased nitrogen concentrations (as seen from the analysis of both weighted and weighted UniFrac distance matrices) is not entirely unexpected since the nitrogen addition experiment was implemented only few months before the sampling. For now, the only clear difference from the control can be observed in the plots that are exposed to high concentration of nitrogen at temperatures of +10°C. However, this difference is most likely a pure temperature effect.
It is possible that a longer period of exposure to increased nitrogen conditions is needed to induce a significant change in microbial communities since we see that some degree of separation (albeit non-significant) is already present (Figure 11, Table 3). So, these might be initial community responses to increased nitrogen concentrations after four months of exposure to N addition.

Many studies have detected a shift in microbial community composition as a result of N addition, but those were mostly long-term studies (Frey, et al., 2004; Rinnan et al., 2007; Fierer et al., 2012). Indeed, Rinnan at al. found that 15 years of artificial fertilisation (with 100kg/ha.y of NH_4NO_3) were needed to detect first responses in bacterial community composition. They argue that this delay in microbial response happens because the substrate inhabited by microbes mainly originates from the vegetation present at the site before manipulations. Over time, the substrate quality and quantity are increasingly affected by the treatments and by indirect effects, such as changes in plant biomass and community

composition. Only when the substrate composition is altered, microbial communities will change accordingly.

Interestingly, in the same study by Rinnan et al., it was observed that responses to warming were not in concordance with the responses to fertilisation, even though a positive link is commonly assumed (as discussed above). In addition, Schmidt et al. detected that, while warming induced increase in net mineralization and assimilation of nutrients by plants, nutrient assimilation by microbes was not affected (Schmidt et al. 2002). The responses may also be dependent on the ecosystem in question since the aforementioned studies were focused on (sub)arctic systems specifically.

Given that, in our study, responses to fertilisation were not yet apparent, we are still not able to conclude whether the detected bacterial responses to warming are to some extent induced by higher nutrient availability. Future research regarding bacterial community composition at N experiment site will most likely be able to provide the answer to this question.

b) Additional potential indirect effects of warming

Apart from the effect of nitrogen addition, we also looked at the effect of some other factors potentially influencing bacterial community responses to warming (soil water content, soil organic carbon stocks and plant biomass). These parameters were measured along the warming gradients, which is why the confounding effects of elevated temperatures must be taken into account when explaining their contribution to bacterial community responses. In this analysis, the nitrogen concentrations measured along the warming gradients were also included. That way, we were able to examine to what extent the actual concentration of nitrogen in the soil can be related to the change in bacterial community composition.

Typically, it is expected that changes in soil temperatures may have as a consequence changes in soil water availability (Classen, et al., 2015). In some cases, even the small changes in soil moisture can induce a shift of one dominant member of community to another, while in other cases communities are more adapted to low water availability and exhibit less pronounced compositional shift to changing water regimes (Classen, et al., 2015).

Elevated temperatures can also induce a change in plant productivity (Kirschbaum, 2000). This might have important implications for microbial communities since the interactions between plants and microorganisms are numerous in types and very complex (e.g. many soil microbes rely on the habitat and resources provided by plants' roots) (Classen, et al., 2015). Changes in plant productivity could, thus, alter microbe-plant interactions leading to the change of microbial community composition.
Increase in decomposition rates and net primary production induced by elevated temperatures have contrasting effects on soil organic carbon (Kirschbaum, 2000; Melillo et al., 2011), but it is thought that warming will ultimately reduce SOC by stimulating decomposition more than plant production (Kirschbaum, 2000). Since some bacterial groups seem to be sensitive to the changes in carbon availability in soil (Fierer et al., 2007), the depletion of SOC stocks could have implications for microbial community composition.

We found that all investigated factors (soil water content, soil organic content and plant biomass) except nitrogen concentrations, can be significantly related to the observed pattern in community composition (Figure 11). Since these factors are all negatively correlated to the temperature, they can all possibly be responsible for the observed distinctions.

The fact that we found no higher concentrations of nitrogen at higher temperatures, (although warming typically increases decomposition rates) can probably be explained by accelerated nitrogen turnover rates at elevated temperatures (meaning that it is very quickly used up by the organisms) (Shaver et al., 2000). Therefore, measured concentrations of nitrogen in the soil cannot tell us a lot about the effect of temperature-induced increase in nitrogen availability, which is why the above-described nutrient experiment is a more effective way to examine it.

As for the other factors, soil organic carbon stocks and plant biomass (which are closely correlated) explained the highest amount of variation, meaning that the changes in these two factors with warming could have had an important influence on microbial composition. The study by Rui et al.,2015 also found that plant biomass contributed significantly to determining the bacterial community structure, although, in their case, the effects of temperature and plant biomass were positively correlated. In addition, in the study by Zhang et al., 2005, it has demonstrated that warming-induced alteration of plant growth, rather than the warming itself was responsible for changes in microbial community composition.

These results suggest that indirect temperature effects probably play a significant role in shaping microbial community structure which should be taken into account when explaining the responses (likewise lack of responses) of microbial communities to warming.

4.3 Combining the direct and indirect effects of warming to explain the observed patterns

In previous sections it has been suggested that observed bacterial community responses to warming in the new grassland could be transient. Numerous bacterial traits, such as fast growth rates, physiological flexibility and rapid evolution, suggest that resilience could be a common phenomenon among this group (Allison & Martiny, 2008). However, microbial resilience to perturbations is not often reported in literature and most studies investigating long-term effects of disturbances have shown that clear microbial responses could still be detected after several years of exposure (Allison & Martiny, 2008). Moreover, few studies explicitly focus on the time course of microbial composition (Allison & Martiny, 2008). As for the studies concentrating on the effect of temperatures, they typically show that microbial composition is not resilient within a few years long warming period (Allison & Martiny, 2008; Blankinship et al, 2011). On the contrary, Rinnan et al., 2007 and De Angelis et al., 2015 report that more than 10 years were needed to detect any changes in microbial composition under experimental warming. So, while in this study we argue that during a several decades (possibly centuries) long period of warming, microbial communities adapted to elevated temperatures up to +10°C and went back to their previous state, DeAngelis et al., 2015 showed that 20 years of soil warming were needed to detect differences in community composition between the control and +5°C treatments. However, it must be taken into account that De Angelis et al., 2015 for instance, investigated microbial communities coming from temperate forest soils, while this study investigated soils from subarctic grasslands. It has already been emphasised before that differences in ecosystem types might be the cause of different response patterns within microbial communities. The effects of warming could be particularly important and could act faster in higher latitude ecosystems, (which tend to be both temperature and nutrient-limited) than in other regions (Rustad et al., 2001, Chen et al., 2015). Therefore, the fact that, in the study by De Angelis et al., 2015 considerably longer exposure period to continuous warming (of +5°C) was needed to induce microbial responses than in GN (responses to +6°C were clear after 3 years) illustrates that some systems might react much more slowly to same disturbances than the others, which does not mean that they will not ultimately follow a similar pattern.

It is thought that vegetation plays a particularly important role on how fast the soil can regain its pre-disturbance microbial composition (Zhang et al., 2013) and how fast the microbial communities will react to warming in the first place (Rinnan et al., 2007). The results of our study support the hypothesis that plant productivity may be one of the important factors determining bacterial responses to warming. Therefore, it is indeed possible that differences between microbial community responses to warming are largely a consequence of the fact that some vegetation types react to warming faster than the other.

However, based on the data from our study, it cannot be concluded to what extent temperature-driven change in plant productivity itself affects the observed bacterial responses to warming. Further research should thus focus on disentangling the effects of warming from the effects of warming-induced change in plant productivity. For instance, this could be accomplished by removing the vegetation from the area influenced by soil warming (thereby excluding the effect of vegetation on microbial composition) and comparing the results with those from the areas with intact vegetation cover, as demonstrated in the study by Zhang et al., 2005.

4.4 The effects of warming on different taxa

Overall microbial composition in GN and GO reflects the typical soil microbial composition as found in many other studies with the most dominant phyla being Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Verrucomicrobia, Gemmatimonadetes, Bacteroidetes etc. (He et al., 2012, Janssen, 2006, Koyama et al., 2014, Guan, et al., 2013, Sheik et al., 2011). Proteobacteria was the most abundant phylum both in GO and GN (with the abundance of 29%) which is consistent with the known studies commonly reporting that Proteotobacteria comprise more than 25% of all the sequences extracted from the soils (Spain et al, 2009). Given the large physiological diversity within this phylum, it is difficult to make predictions about their predominant metabolism (Janssen, 2006), which is why it is more practical to focus on the most dominant classes within this phylum (Alpha-, Beta- and Gamma-proteobacteria).
When comparing GO and GN soils, we found very similar abundances of same phyla, except that GO contains considerably more Acidobacteria and less Actinobacteria than GN. This difference can partially be attributed to disproportionally high abundance of Acidobacteria in some +20° GO samples. No apparent reason for the difference in abundance of these two phyla between GO and GN could be found.

One general pattern that arises from our phylogenetic analysis is that phylogenetic relatedness does not necessarily imply common responses to increased temperatures. For instance, many dominant phyla showed no coherent response to increased temperatures, with Actinobacteria showing particular inconsistency in the distribution of 'warm-tolerant' and 'warm-intolerant' OTUs across the phylogenetic tree. This result is not surprising considering that all these phyla are very ubiquitous and include organisms with very different physiological strategies and metabolic properties (Fierer at al., 2007). Despite of this, it has been suggested that high-level taxa can, in some cases, share general physiology and exhibit common ecological responses which might enable us to divide them into ecologically meaningful categories (Fierer at al., 2007; Philippot at al., 2010).

Indeed, our results show that (in spite of the general lack of consistent responses exhibited by most phyla) some of the high-level taxa do show certain coherence in responses to elevated temperatures. So we observed that the abundance of Betaproteobacteria decreases significantly above the shift point (although in GO the decrease is not so abrupt but rather linear). Bacteroidetes exhibited similar responses, albeit less pronounced. It has been shown in some other studies as well that abundance of Betaproteobacteria (Ru et al., 2015) and Bacteroidetes (Wu et al., 2015) decreases with increasing temperatures. Interestingly, exactly these taxa seem to exhibit coherent general behaviour in response to differing C availabilities and are therefore considered to be related to more copiotrophic conditions (Fierer at al., 2007, Aanderud, et al., 2011). Our results also showed that these particular phyla increase in abundance with the increase of soil organic carbon stocks. However, as discussed above, it is not clear whether these responses come as a consequence of warming or temperature-induced decrease in carbon availability.

Furthermore, although we observed that indicators of low temperatures can be found in practically every phylum (Table 2), indicators of higher temperatures tend to appear consistently in certain phyla, mostly: Chloroflexi, Acidobacacteria and Actinobacteria (Figures 8 and 9; Table 1). Chloroflexi are already known to comprise some thermophilic species (Overmann, 2008) and it was found in this study that their abundance tends to increase with warming (significant only for GN), but Acidobacteria did not show a particular response to temperature elevation. Therefore, although Acidobacteria phylum contains many indicators of high temperatures, it does not always show a consistent increase in response to warming because this (highly diverse) phylum also contains whole clades exhibiting a prominent intolerance to elevated temperatures (as seen from phylogenetic trees; Figures 8 and 9). For

instance, it can be observed that even the same class within Acidobacteria phylum (Acidobacteria-6) contains OTUs highly specific for both lower and higher temperatures (Tables 1 and 2).
As for the effects of other factors on this phyla, Acidobactera, which are considered to be oligotrophic taxa (commonly found in high abundances in relatively nutrient poor areas such as bulk soils, unlike in the nutrient rich areas such as rhizosphaere) (Smit et al., 2001; Fierer et al, 2007,) did not show a significant correlation with soil organic carbon stocks, while Chloroflexi (which are not evaluated in the study by Fierer at al.) clearly decrease with the increase in soil organic carbon, indicating that this phylum might also be related to more oligotrophic conditions. It has already been demonstrated that some members of Chloroflexi phylum are linked to oligotrophic conditions (Aanderud, et al., 2011).

Therefore, our findings are consistent with the idea of coherent ecological strategy for members of Betaproteobacteria and Bacteroidetes and the hypothesis that they are related to soils with higher carbon availabilities. If we assume that Chloroflexi exhibit more oligotrophic tendencies (as suggested from our results), we can argue that bacterial community composition from warmer soils tend to switch towards more oligotrophic bacterial taxa compared to soils with lower temperatures.

4.5 Linking structure and functioning

It is clear that warming can affect microbial community composition, but the major question is currently whether these compositional changes are necessarily associated with the changes in ecosystem functioning (Wertz 2006; Reed & Martiny, 2007; Nemergut et al., 2014). Given that many thousands of microorganisms can be found in a single gram of soil and that they are able to rapidly adapt to new environment, the assumption of functional redundancy seems rather plausible (Allison & Martiny, 2008; Strickland, et al., 2009). This practically means that any change in community composition would produce an insignificant change in ecosystem processes regulated by microorganisms. Based on this assumption most global change models marginalise the importance of alterations in microbial community composition (Reed & Martiny, 2007; Strickland et al., 2009).
However, this hypothesis has been challenged by many scientists who were able to demonstrate that changes in microbial composition was in many cases linked to changes in certain ecosystem processes (Pett-Ridge & Firestone, 2005; Balser & Firestone, 2005; Waldrop & Firestone, 2006; Strickland et al., 2009; Reed & Martiny, 2013). These results provide some evidence that responses of microbe-regulated processes to global change are not entirely dependent on changes in environmental conditions, but that

microbial community composition in combination with the environment can ultimately determine these processes (Strickland et al., 2009).

This means that the changes in bacterial community composition that were observed in this study are likely to affect ecosystem functioning. From our data, no firm conclusions as to how bacterial composition translates to functioning can be made. However, this study demonstrated that the abundance of some copiotrophic taxa tend to decline above the shift temperature point which might already broadly point out to some possible consequences to ecosystem functioning. Compared to oligotrophic taxa, copiotrophic bacteria are generally expected to have higher rates of activity per unit biomass, higher turnover rates and higher substrate use efficiencies yielding a smaller standing microbial biomass pool and they are assumed to have a reduced overall rates of heterotrophic respiration (they preferentially consume more labile substrates over the more recalcitrant organic C substrates that make up the bulk of the soil organic C pool) (Fontaine & Barot, 2005; Fierer et al., 2012). This means that the warming-induced decrease in abundance of copiotrophic taxa compared to abundance of oligotrophic taxa could further intensify heterotrophic respiration. However, further research is definitely needed to prove this assumption, and, in general, to clarify how and when exactly the compositional changes will influence particular ecosystem processes.

One way to investigate correlation of compositional changes to functional changes would be to perform measurements of biogeochemical process rates (e.g. denitrification rates) along temperature gradient. However, even if the correlated response between composition and ecosystem functioning would be detected, it would be difficult to disentangle the effects of warming itself from the effects produced by the change in bacterial composition. Better approach would be to establish controlled laboratory experiments which will complement 'natural warming experiment', where the parameters cannot be held constant. One of the possible ways to do it would be to regrow microbial communities sampled at different soil temperatures (in this case, above and below shift point) under controlled conditions and measure the rates of certain processes (Langenheder et al., 2006). In this case, any differences in process rates between soil samples could be attributed to the difference in their bacterial community compositions (Reed & Martiny, 2007).

4.6 Ramifications for climate change

Given that we found no sign of shift in microbial community composition at temperature elevations relevant for the predicted climate scenarios, the conclusion that immediately arises is that microbial communities in this system will not be affected by future global warming. However, Thopmson et al, 2013 indicted that future climates are likely to include extreme events which might have greater impacts on ecological systems than changes in mean temperatures alone. This was supported by Kreyling et al., 2015 who stated that going 'beyond the realism' is essential in identifying thresholds and tipping points that will allow us to evaluate possible effects of extreme events. The effect of extreme events is, however, very rarely incorporated in current studies which are often focused only on the temperatures associated with mean predicted climate warming and typically apply static warming treatments (Thompson et al., 2013; Kayler, et al., 2015).

Despite of the lack of responses to low magnitude warming found for this system, two important temperature thresholds were detected: one where bacterial communities are no longer resilient (around +6°C) (Weedon et al., in prep.) and the other where they stop showing resilience (between +10°C and +20°C) after a very long period of continuous warming. This means that, for this particular system, if the temperatures increase locally more than 6°C, significant changes in bacterial community composition can be expected. Also, if the temperature elevation exceeds the threshold point above +10°C, bacterial communities might permanently retain their new compositions, which would most likely have functional implications, as discussed above. These results thus provide a better mechanistic understanding on what can be expected under different temperature conditions, even if such high temperature elevations are not anticipated by the current climate models.

4.7 Methodological limitations

Although molecular methods have added much to our knowledge about incredibly diverse soil microbial communities, they also have some limitations that have to be taken into account.

Firstly, the entire analysis of microbial community composition is based on rather small soil samples that should be representative for a particular area. However, soil is a very heterogeneous system containing many microhabitats that might be more or less suitable for different organisms (soil bacteria tend to be highly aggregated in certain "hotspots") which makes it very difficult to include the entire diversity

present within that part of the soil (Kirk, et al., 2004). Secondly, some controversies exist about the biases related to the DNA extraction procedures since lysis efficiency can sometimes vary between and within microbial groups (de Lipthay et al., 2004; Feinstein et al., 2009, Kirk et al., 2004). Also, biases related to PCR amplification (with GC priming regions amplifying better than AT rich sites) are often emphasised in the literature (Macrae, 2000). Thirdly, 16s rRNA based analysis can be hindered by PCR artefacts and sequencing errors (which might not be entirely eliminated by quality filtering) possibly resulting in overestimation of species diversity (Puente-Sánchez et al., 2015) .
Finally, since the analysis of bacterial communities is performed based on a single marker (V3 region of 16s rRNA gene), the differentiation between closely related species is limited compared to the analysis based on larger regions (Poretsky et al., 2014).

However, it should be emphasised that all the samples were subjected to exactly the same sampling and extraction procedures. This means that eventual biases and errors that might appear should be more or less equally distributed between the samples and they should not have considerable effect on between-sample composition (β diversity). On the other side, these errors would significantly affect the within-sample composition (α diversity), which is why it was not examined in this study.

5. CONCLUSION

The results of our study support the hypothesis that, in this study system, bacterial communities are ultimately resilient to long-term warming. However, above a certain temperature elevation (between +10°C and +20°C) bacterial communities lose the ability to return to their pre-warming state and become increasingly different from those at lower temperatures. Furthermore, it has been shown that temperature-driven changes in plant productivity and soil organic carbon stocks were correlated to the observed changes in bacterial community composition, while the effect of increase of nitrogen availability remains unknown since the responses to nitrogen addition were not yet detected. Finally, our results support the idea that some high-level taxa (Betaproteobacteria, Bacteroidetes and Chloroflexi) can exhibit coherent ecological responses. So, it has been shown that, with increasing temperatures, Betaproteobacteria and Bacteroidetes (which are thought to be copiotrophic taxa) decrease in abundance, while the abundance of Chloroflexi (possibly related to more oligotrophic conditions) shows increasing tendencies.

Better understanding of causes and consequences of these patterns requires further research which should primarily be focused on difficulties in disentangling the direct and indirect effects of warming and examining how the changes in bacterial community composition translate to changes in functioning.

REFERENCES

Aanderud, Z. T., & Lennon, J. T. (2011). Validation of heavy-water stable isotope probing for the characterization of rapidly responding soil bacteria. Applied and environmental microbiology, 77(13), 4589-4596.

Aerts, R. (2010). Nitrogen-dependent recovery of subarctic tundra vegetation after simulation of extreme winter warming damage to Empetrum hermaphroditum. *Global Change Biology*, 16(3), 1071-1081.

Allison, S., & Martiny, J. (2008). Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences 105.Supplement 1*, 11512-11519.

Anderson, M. (2001). A new method for non-parametric multivariate analysis of variance. *Austral ecology*, 26(1), 32-46.

Arnórsson, S. (1995). Geothermal systems in Iceland: structure and conceptual models - I. High-temperature areas. *Geothermics*, 24 (5/6): 561 - 602.

Balser, T., & Firestone, M. (2005). Linking microbial community composition and soil processes in a California annual grassland and mixed-conifer forest. *Biogeochemistry*, 73: 395–415.

Bardgett, R. (2011b). Plant-soil interactions in a changing world. . *F1000 Biol Rep*, 3(16), 569.

Bardgett, R., Freeman, C., & Ostle, N. (2008). Microbial contributions to climate change through carbon cycle feedbacks. . *The ISME Journal*, 2(8), 805-814.

Bartram, A., Lynch, M., Stearns, J., Moreno-Hagelsieb, G., & Neufeld, J. (2011). Generation of Multimillion-Sequence 16S rRNA Gene Libraries from Complex Microbial Communities by Assembling Paired-End Illumina Reads. *Applied and Environmental Microbiology*, 3846-3852.

Blankinship, J., Niklaus, P., & Hungate, B. (2011). A meta-analysis of responses of soil biota to global change. *Oecologia*, 165(3), 553-565.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*, 6(8):1621-1624.

Caporaso, J., Bittinger, K., Bushman, F., DeSantis, T., Andersen, G., & Knight, R. (2010b). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, 26(2): 266-267.

Caporaso, J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F., Costello, E., et al. (2010c). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* , 7, 335–336.

Caporaso, J., Lauberb, C., Waltersc, W., Berg-Lyonsb, D., Lozuponea, C., Turnbaughd, P., et al. (2010). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Washington University School of Medicine*, 4516-4522.

Castro, H., Classen, A., Austin, E., Norby, R., & Schadt, C. (2010). Soil microbial community responses to multiple experimental climate change drivers. *Applied and environmental microbiology*, 76(4), 999-1007.

Chen, J., Luo, Y., Xia, J., Jiang, L., Zhou, X., Lu, M., Liang, J., Shi, Z., Shelton, S. & Cao, J., (2015). Stronger warming effects on microbial abundances in colder regions. *Scientific reports,5*.

Classen, A., Sundqvist, M., Henning, J., Newman, G., Moore, J., Cregger, M., et al. (2015). Direct and indirect effects of climate change on soil microbial and soil microbial-plant interactions: What lies ahead? *Ecosphere* , 6(8):130.

Daniel, R. (2005). The metagenomics of soil. *Nat Rev Microbiol 3*, 470-478.

Davidson, E., & Janssens, I. (2006). Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. *Nature*, 440(7081), 165-173.

De Deyn, G., Cornelissen, J., & Bardgett, R. (2008). Plant functional traits and soil carbon sequestration in contrasting biomes. *Ecology letters*, 11(5), 516-531.

de Lipthay, J., Enzinger, C., Johnsen, K., Aamand, J., & Sørensen, S. (2004). Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. *Soil Biology and Biochemistry*, 36(10), 1607-1614.

DeAngelis, K., & al, e. (2015). Long-term forest soil warming alters microbial communities in temperate forest soils. *Frontiers in microbiology*, 6: 104.

DeSantis, T., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology*, 72(7), 5069-5072.

Diepen, L., Varney, R. M., & Blanchard, J. (2015). Long-term forest soil warming alters microbial communities in temperate forest soils. *Frontiers in microbiology*, 6.

Dufrêne, M., & Legendre, P. (1997). Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecol. Monogr.*, 67, 345–366.

Duhamel, M., & Peay, K. (2015). Does Microbial Diversity Confound General Predictions? *Trends in Plant Science*, 20(11): 695-697.

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26 (19): 2460-2461.

Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods*, (10) :996-8.

Falkowski, P., Fenchel, T., & Delong, E. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science*, 320, 1034–1039.

Feinstein, L., Sul, W., & Blackwood, C. (2009). Assessment of bias associated with incomplete extraction of microbial DNA from soil. *Applied and environmental microbiology*, 75(16), 5428-5433.

Fierer, N., & Jackson, R. (2006). The diversity and biogeography of soil bacterial communities . *Proceedings of the National Academy of Sciences of the United States of America*, 103(3), 626-631.

Fierer, N., Bradford, M., & Jackson, R. (2007). Toward an ecological classification of soil bacteria. *Ecology*, 88(6), 1354-1364.

Fierer, N., Lauber, C., Ramirez, K., Zaneveld, J., Bradford, M., & Knight, R. (2012). Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *The ISME journal*, 6(5), 1007-1017.

Fontaine, S., & Barot, S. (2005). Size and functional diversity of microbe populations control plant persistence and long-term soil carbon accumulation. *Ecology Letters*, 8(10), 1075-1087.

Frey, S., Drijber, R., Smith, H., & Melillo, J. (2008). Microbial biomass, functional capacity, and community structure after 12 years of soil warming. *Soil Biology and Biochemistry,* , 40(11), 2904-2907.

Frey, S., Knorr, M., Parrent, J., & Simpson, R. (2004). Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management*, 196(1), 159-171.

Gans, J., Wolinsky, M., & Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science 309*, 1387-1390.

Geirsson, K., & Arnórsson, S. (1995). Conceptual model of the Hveragerdi geothermal reservoir based on geochemical data. *In World Geothermal Congress, , International Geothermal Association*, (2): 1251-1256.

O'Gorman, E.J., Benstead, J.P., Cross, W.F., Friberg, N., Hood, J.M., Johnson, P.W., Sigurdsson, B.D., & Woodward, G. (2014). Climate change and geothermal ecosystems: natural laboratories, sentinel systems, and future refugia. *Global Change Biology*, 20: 3291-3299.

Griffiths, B., & Philippot, L. (2013). Insights into the resistance and resilience of the soil microbial community. *FEMS microbiology reviews*, 37(2), 112-129.

Griffiths, R., Whiteley, A., O'Donnell, A., & Bailey, M. (2003). Physiological and community responses of established grassland bacterial populations to water stress. *Applied and Environmental Microbiology*, 69(12), 6961-6968.

Guan, X., Wang, J., Zhao, H., Wang, J., Luo, X., Liu, F., et al. (2013). Soil bacterial communities shaped by geochemical factors and land use in a less-explored area, Tibetan Plateau. *BMC genomics*, 14(1), 820.

Hayden, H., & al, e. (2012). Changes in the microbial community structure of bacteria, archaea and fungi in response to elevated CO2 and warming in an Australian native grassland soil. *Environmental microbiology*, 14 (2) 3081-3096.

He, Z., Piceno, Y., Deng, Y., Xu, M., Lu, Z., DeSantis, T., Andersen, G., Hobbie, S.E., Reich, P.B., & Zhou, J. (2012). The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide. *The ISME journal* , 6.2: 259-272.

Hobbie, S. (2008). Nitrogen effects on decomposition: a five-year experiment in eight temperate sites. *Ecology*, 89(9), 2633-2644.

Janda, J., & Abbott, S. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *J Clin Microbiol. 2007* , 45(9): 2761–2764.

Janssen, P. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and environmental microbiology*, 72(3), 1719-1728.

Karhu, K., Auffret, M.D., Dungait, J.A., Hopkins, D.W., Prosser, J.I., Singh, B.K., Subke, J.A., Wookey, P.A., Ågren, G.I., Sebastià, M.T., & Gouriveau, F., (2014). Temperature sensitivity of soil respiration rates enhanced by microbial community response. *Nature*, 513(7516), 81-84.

Kayler, Z., De Boeck, H., Fatichi, S., Grünzweig, J., Merbold, L., Beier, C., et al. (2015). Experiments to confront the environmental extremes of climate change. *Frontiers in Ecology and the Environment*, 13(4), 219-225.

Kirk, J., Beaudette, L., Hart, M., Moutoglis, P., Klironomos, J., Lee, H., et al. (2004). Methods of studying soil microbial diversity. *Journal of microbiological methods*, 58(2): 169-188.

Kirschbaum, M. (2000). Will changes in soil organic carbon act as a positive or negative feedback on global warming? *Biogeochemistry*, 48(1), 21-51.

Koyama, A., Wallenstein, M., Simpson, R., & Moore, J. (2014). Soil bacterial community composition altered by increased nutrient availability in Arctic tundra soils. *Frontiers in microbiology*, 5, 516.

Kreyling, J., Jentsch, A., & Beier, C. (2014). Beyond realism in climate change experiments: gradient approaches identify thresholds and tipping points. *Ecology letters*, 17(1), 125.

Kuczynski, J., Stombaugh, J., Walters, W., González, A., Caporaso, J., & Knight, R. (2011). Using QIIME to analyze 16S rRNA gene sequences from Microbial Communities. *Curr Protoc Bioinformatics*, 1-28.

Langenheder, S., Lindström, E., & Tranvik, L. (2006). Structure and function of bacterial communities emerging from different sources under identical conditions. *Applied and environmental microbiology*, 72(1), 212-220.

Letunic, I., & Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic acids research*, gkr201.

Loreau, M., Downing, A., Emmerson, M., Gonzalez, A., Hughes, J., Inchausti, P., et al. (2002). A new look at the relationship between diversity and stability. In M. Loreau, S. Naeem, & P. Inchausti, *Biodiversity and ecosystem functioning: synthesis and perspectives* (pp. 77-91). New York: Oxford University Press.

Lozupone, C., & Knight, R. (2005). UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Applied and Environmental Microbiology*, 71(12):8228-8235.

Lozupone, C., Hamady, M., Kelley, S., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and environmental microbiology*, 73(5), 1576-1585.

Luo, C. R.-R., Johnston, E., Wu, L., Cheng, L., Xue, K., Tu, Q., et al. (2014). Soil microbial community responses to a decade of warming as revealed by comparative metagenomics. *Applied and environmental microbiology*, 80(5), 1777-1786.

Lynch, J., Benedetti, A., Insam, H., Nuti, M., Smalla, K., Torsvik, V., et al. (2004). Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. *Biol Fertil Soils*, 40: 363–385.

Macrae, A. (2000). The use of 16S rDNA methods in soil microbial ecology. *Brazilian Journal of Microbiology*, 31(2), 77-82.

Magnússon, Á., & Vídalín, P. (1918-1921). Jarðabók. Annað bindi [The farm register of Iceland. 2nd Volume]. *Copenhagen* , (Originally published in 1708) (In Icelandic).

McDonald, D., Price, M., Goodrich, J., Nawrocki, E., DeSantis, T., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal*, 6(3), 610-618.

Melillo, J.M., Butler, S., Johnson, J., Mohan, J., Steudler, P., Lux, H., Burrows, E., Bowles, F., Smith, R., Scott, L., & Vario, C., (2011). Soil warming, carbon–nitrogen interactions, and forest carbon budgets. *Proceedings of the National Academy of Sciences* , 108(23): 9508-9512.

Nazaries, L., Tottey, W., Robinson, L., Khachane, A., Al-Soud, W. A., Sørenson, S., et al. (2015). Shifts in the microbial community structure explain the response of soil respiration to land-use change but not to climate warming. *Soil Biology and Biochemistry*, 89, 123-134.

Nemergut, D., Shade, A., & Violle, C. (2014). When, where and how does microbial community composition matter? *Frontiers in microbiology*, 5.

Nemergut, D., Townsend, A., Sattin, S., Freeman, K., Fierer, N., Neff, J., et al. (2008). The effects of chronic nitrogen fertilization on alpine tundra soil microbial communities: implications for carbon and nitrogen cycling. *Environmental Microbiology*, 10, 3093-3105.

Ogram, A., Sayler, G., & Barkay, T. (1987). The extraction and purificationof microbial DNA from sediments. *Journal of Microbiological Methods*, 7, 57–66.

Oksanen, J., & al. (2015). Vegan: Community Ecology Package. R package version 2.4-0. https://github.com/vegandevs/vegan.

Overmann, J. (2008). Green Nonsulfur Bacteria. *In: eLS. John Wiley*.

Peterjohn, W., Melillo, J., Steudler, P., Newkirk, K. B., & Aber, J. (1994). Responses of trace gas fluxes and N availability to experimentally elevated soil temperatures. *Ecological Applications*, 4(3), 617-625.

Pett-Ridge, J., & Firestone, M. (2005). Redox fluctuation structures microbial communities in a wet tropical soil. *Appl Environ Microbiol*, 71: 6998–7007.

Pold, G., & DeAngelis, K. (2013). Up against the wall: the effects of climate warming on soil microbial diversity and the potential for feedbacks to the carbon cycle. *Diversity*, 5(2), 409-425.

Poretsky, R., Rodriguez-R, L. L., Luo, C., Tsementzi, D., & Konstantinidis, K. (2014). Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PloS one*, 9(4), e93827.

Price, M., Dehal, P., & Arkin, A. (2009). FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular biology and evolution*, 26(7), 1641-1650.

Prosser, J., Bohannan, B.J., Curtis, T.P., Ellis, R.J., Firestone, M.K., Freckleton, R.P., Green, J.L., Green, L.E., Killham, K., Lennon, J.J., & Osborn, A.M. (2007). The role of ecological theory in microbial ecology. *Nature Reviews Microbiology 5*, 384-392.

Puente-Sánchez, F., Aguirre, J., & Parro, V. (2015). A novel conceptual approach to read-filtering in high-throughput amplicon sequencing studies. *Nucleic Acids Res.* , gkv1113v1-gkv1113. .

R Core Team (2015). R: A language and environment for statistical computing. *R Foundation for Statistical Computing, Vienna, Austria.*, URL https://www.R-project.org/ .

Ramirez, K., Lauber, C., Knight, R., Bradford, M., & Fierer, N. (2010). Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems . *Ecology*, 91(12)3463-3470.

Reed, H., & Martiny, J. (2007). Testing the functional significance of microbial composition in natural communities. *FEMS microbiology ecology*, 62(2), 161-170.

Reed, H., & Martiny, J. (2013). Microbial composition affects the functioning of estuarine sediments. *The ISME journal*, 7(4), 868-879.

Rinnan, R., Michelsen, A., Baath, E., & Jonasson, S. (2007). Fifteen years of climate change manipulations alter soil microbial communities in a subarctic heath ecosystem. *Global Change Biology*, 13:28–39.

Roberts, D. (2016). Labdsv: Ordination and Multivariate Analysis for Ecology. R package version 1.8-0. http://CRAN.R-project.org/package=labdsv.

Roselló-Mor, R., & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev*, 25:39-67.

Rousk, J., Smith, A. R., & Jones, D. L. (2013). Investigating the long-term legacy of drought and warming on the soil microbial community across five European shrubland ecosystems. *Global Change Biology*, 19(12), 3872-3884.

Rui, J., Li, J., Wang, S., An, J., Liu, W., Lin, Q., et al. (2015). Responses of bacterial communities to simulated climate changes in alpine meadow soil of the Qinghai-Tibet Plateau. *Applied and environmental microbiology*, 81(17), 6070-6077.

Rustad, L.E.J.L., Campbell, J., Marion, G., Norby, R., Mitchell, M., Hartley, A., Cornelissen, J., & Gurevitch, J. (2001). A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia*, 126(4), 543-562.

Schindlbacher, A., Rodler, A., Kuffner, M., Kitzler, B., Sessitsch, A., & Zechmeister-Boltenstern, S. (2011). Experimental warming effects on the microbial community of a temperate mountain forest soil. *Soil Biology and Biochemistry*, 43(7), 1417-1425.

Schmidt, I., Jonasson, S., Shaver, G., Michelsen, A., & Nordin, A. (2002). Mineralization and distribution of nutrients in plants and microbes in four arctic ecosystems: responses to warming. *Plant and Soil*, 242(1), 93-106.

Shaver, G. R., Canadell, J., Chapin, F. S., Gurevitch, J., Harte, J., Henry, G., & Rustad, L. (2000). Global Warming and Terrestrial Ecosystems: A Conceptual Framework for Analysis. *BioScience*, 50(10), 871-882.

Shaw, M., & Harte, J. (2001). Response of nitrogen cycling to simulated climate change: differential responses along a subalpine ecotone. *Global Change Biology*, 7(2), 193-210.

Sheik, C., Beasley, W., Elshahed, M., Zhou, X., Luo, Y., & Krumholz, L. (2011). Effect of warming and drought on grassland microbial communities. *The ISME journal*, 5(10), 1692-1700.

Sinclair, L., Osman, O., Bertilsson, S., & Eiler, A. (2015). Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the Illumina platform. *PloS one*, 10(2), e0116955.

Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S., & Wernars, K. (2001). Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Applied and Environmental Microbiology* , 67.5: 2284-2291.

Spain, A., Krumholz, L., & Elshahed, M. (2009). Abundance, composition, diversity and novelty of soil Proteobacteria. . *The ISME journal*, 3(8), 992-1000.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, btu033.

Stocker, T., & al, e. (2013). *Technical Summary. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.* Cambridge: Cambridge University Press.

Strickland, M., Lauber, C., Fierer, N., & Bradford, M. (2009). Testing the functional significance of microbial community composition. *Ecology*, 90(2), 441-451.

Tharanga, M., & Munasinghe, N. (2014). *Geothermal exploration in Gufudalur, Hveragerdi, SW-Iceland.* Reykjavik, Iceland : United Nations University.

Thompson, R., Beardall, J., Beringer, J., Grace, M., & Sardina, P. (2013). Means and extremes: building variability into community-level climate change experiments. *Ecology Letters*, 16(6), 799-806.

Thorbjörnsson, P., Saemundsson, K., Kristinsson, S., Kristjansson, B., & Ágústsson, K. (2009). Southern lowland earthquakes 29th May 2008, effect of groundwater level, geothermal activity and fractures. (In Icelandic). *In: ORKURANNSÓKNIR*, Í. (ed.).

Tiedje, J., Cho, J., Murray, A., Treves, D., Xia, B., & Zhou, J. (2001). Soil teeming with life: new frontiers for soil science. In Sustainable Management of Soil Organic Matter. *Edited by Rees RM, Ball BC, Campbell CD, Watson CA. CAB Internationa*, 393-412.

Torsvik, V., & Ovreas, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr Opin Microbiol*, 5: 240-245.

Waldrop, M., & Firestone, M. (2006). Response of microbial community composition and function to soil climate change. *Microb Ecol*, 52: 716–724.

Wang, Q., Garrity, G., Tiedje, J., & Cole, J. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology*, 73(16), 5261-5267.

Weedon, T., Kowalchuk, G., Aerts, R., van Hal, J., van Logtestijn, R., Taş, N., Röling, W., & Bodegom, P. (2012). Summer warming accelerates sub-arctic peatland nitrogen cycling without changing enzyme pools or microbial community structure. *Global Change Biology*, 18(1), 138-150.

Wertz, S., Degrange, V., Prosser, J., Poly, F., Commeaux, C., Freitag, T., et al. (2006). Maintenance of soil functioning following erosion of microbial diversity. *Environmental Microbiology*, 8(12), 2162-2169.

Whitman, W., Coleman, D., & Wiebe, W. (1998). Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences*, 95(12), 6578-6583.

Winsley, T., Van Dorst, J., Brown, M., & Ferrari, B. (2012). Capturing greater 16S rRNA gene sequence diversity within the domain Bacteria. *Applied and environmental microbiology*, 78(16), 5938-5941.

Woodward, G., Dybkjær, J., Ólafsson, J., Gíslason, G., Hannesdóttir, E., & Friberg, H. (2009). Sentinel systems on the razor's edge: effects of warming on Arctic geothermal stream ecosystems . *Global Change Biology*, 16: 1979-1991.

Wu, J., Xiong, J., Hu, C., Shi, Y., Wang, K., & Zhang, D. (2015). Temperature sensitivity of soil bacterial community along contrasting warming gradient. *Applied Soil Ecology*, 94, 40-48.

Xu, G., Chen, J., Berninger, F., Pumpanen, J., Bai, J., Yu, L., et al. (2015). Labile, recalcitrant, microbial carbon and nitrogen and the microbial community composition at two Abies faxoniana forest elevations under elevated temperatures. *Soil Biology and Biochemistry*, 91, 1-13.

Zhang, B., Wang, H., Yao, S., & Bi, L. (2013). Litter quantity confers soil functional resilience through mediating soil biophysical habitat and microbial community structure on an eroded bare land restored with mono Pinus massoniana. *Soil Biology and Biochemistry*, 57, 556-567.

Zhang, W., Parker, K. M., Luo, Y., Wan, S., Wallace, L. L., & Hu, S. (2005). Soil microbial responses to experimental warming and clipping in a tallgrass prairie. *Global Change Biology*, 11(2), 266-277.

Zhao, C., & al. (2016). Soil microbial community composition and respiration along an experimental precipitation gradient in a semiarid steppe. *Scientific Reports*, 6, Article number: 24317.

Zogg, G., Zak, D., Ringelberg, D., MacDonald, N., Pregitzer, K., & White, D. (1997). Compositional and functional shifts in microbial communities due to soil warming. *Soil Science Society of America Journal* , 61:475–481.

APPENDICES

Appendix I: Nominal and measured soil temperatures

Table A_I: Nominal and real (measured) temperatures in GN and GO

Plot	Nominal T(°C)	T (°C) in GN	T (°C) in GO
1_A	+0	5.6	5.1
1_B	+1	6.0	6.0
1_C	+3	6.6	6.9
1_D	+5	10.3	8.7
1_E	+10	13.0	14.3
1_F	+20	22.7	15.9
2_A	+0	5.5	5.4
2_B	+1	6.2	5.5
2_C	+3	6.5	7.4
2_D	+5	9.0	8.6
2_E	+10	13.8	10.1
2_F	+20	20.8	17.6
3_A	+0	6.0	5.7
3_B	+1	6.4	6.2
3_C	+3	8.6	7.9
3_D	+5	10.0	9.0
3_E	+10	17.4	14.1
3_F	+20	24.6	22.4
4_A	+0	5.8	5.9
4_B	+1	6.4	5.6
4_C	+3	7.5	6.8
4_D	+5	9.2	8.9
4_E	+10	16.9	14.4
4_F	+20	21.2	20.0
5_A	+0	5.9	6.8
5_B	+1	6.7	7.0
5_C	+3	9.1	7.6
5_D	+5	9.7	8.1
5_E	+10	17.5	12.8
5_F	+20	21.2	25.9

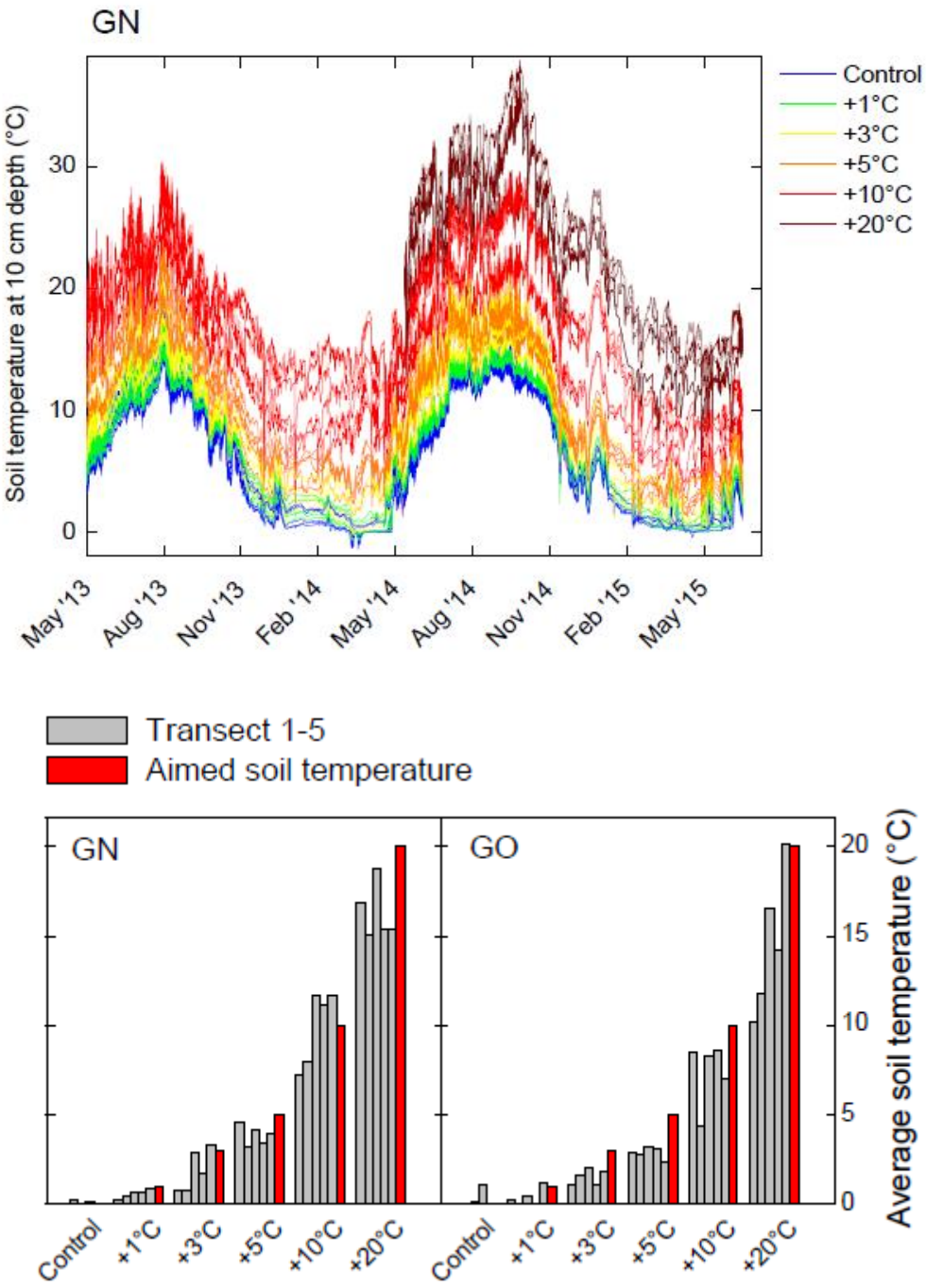

Figure A_I: Top: temperature changes measured from 10cm depth for the period between May 2013 and May 2015 ; bottom: real and aimed temperature differential for GN and GO; from Niki Leblans.

Appendix II: Primers used in the library construction for Illumina sequencing

Table A_II: Primers used in library preparation for sequencing. Lowercase letters – adapter sequences necessary for binding to the flow cell, italic lowercase – binding sites for the Illumina sequencing primers, bold uppercase – index sequences , regular uppercase – the V3 region primers (341F on for the forward primers and 518R for the reverse primers). For reverse primers starting from 2R only the index sequences are shown, the rest of the sequence remains the same as in 1R. From Bartram et al., 2011.

V3_F	aatgatacggcgaccaccgagatct*acactctttccctacacgacgctcttccgatct*CCTACGGGAGGCAGCAG
V3_1R	caagcagaagacggcatacgagat**CGTGAT***gtgactggagttcagacgtgtgctcttccgatct*ATTACCGCGG CTGCTGG
V3_2R-96R	CGTGA, ACATCG, GCCTAA, TGGTCA, CACTGT, ATTGGC, GATCTG, TCAAGT, CTGATC, AAGCTA, GTAGCC, TACAAG, CGTACT, GACTGA, GCTCAA, TCGCTT, TGAGGA, ACAACC, ACCTCA, ACGGTA, AGTTGG, CTCTCT, CAAGTG, CCTTGA, ACCACT, AGTGTC, AGAAGG, TTATCC, TTAAGG, TTCTTG, TTCAAC, TTGTGA, TTGACT, TATTCG, TATAGC, TAACTC, TACCAA, TACGTT, TAGTAC, TAGATG, TCTACA, TCTGAT, TCATGT, TGTCTA, ATTCTC, ATACCT, ATGCAA, AATCCA, AATGGT, AACTAG, AACACT, AAGAGA, ACTTAC, ACATTG, ACGAAT, AGTCAT, AGAAGT, CTTATG, CTAGAA, CATCTT, CACATA, CCAATT, CGATTA, GTTAGT, GTAACA, GTGTAT, GATAAG, GAATCT, TTCCGT, TTCGCA, TTGGTC, TGACAG, ATCTGC, ACACGA, AGGTTC, CATGAC, GCTATC, GGACTT, GGCAAT, TCTCGG, TCAGCG, TGTGCC, TGCACG, AAGGCC, ACCAGG, AGCCTG, AGCGAC, CTACGC, CTCCAG, CCGTAG, CGGTGT, CGGAAC, GTGCTG, GAACGG, GGATGC, GGCGTA

Appendix III: Technical replicates

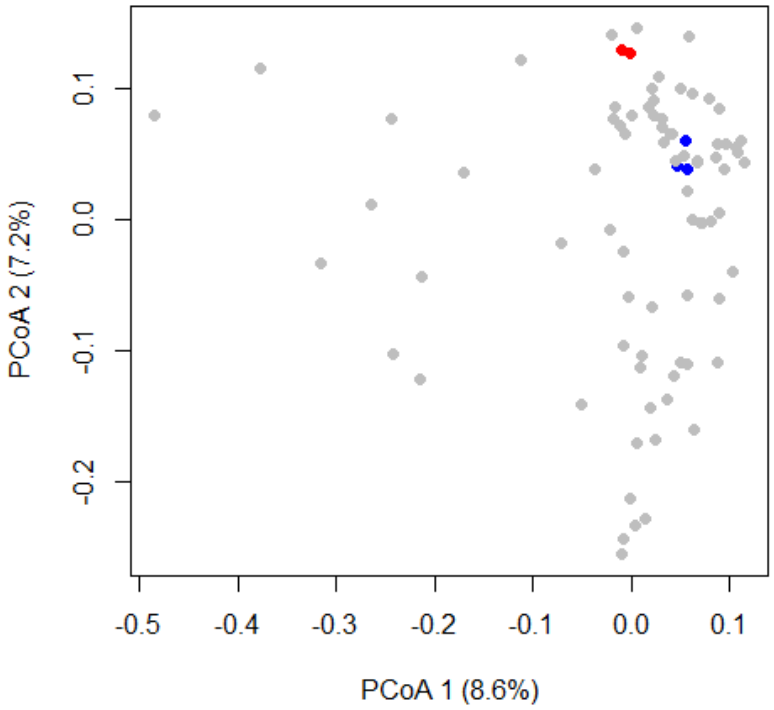

Figure A_III: PCoA ordination based on weighted UniFrac distances for all samples in the study. Red and blue points indicate technical replicates (same sample, separate PCR and sequencing procedure). Relatively close clustering of technical replicates show the reproducibility of the sample preparation and sequencing procedure.

Appendix IV: Relative abundances of phyla

Table A_IV. The percentage of sequences belonging to different phyla from GN and GO

Phylum	GO (%)	GN (%)
Proteobacteria	29	29
Acidobacteria	25	18
Actinobacteria	11	21
Chloroflexi	7	6
Verrucomicrobia	5.99	4.87
Gemmatimonadetes	3.57	2.90
Bacteriodetes	2.57	3.25
Firmicutes	2.44	3.52
Nitrospirae	2.41	1.74
WS3	1.54	1.32
Planktomycetes	0.85	1.19
TM6	0.56	0.51
TM7	0.47	0.51
Chlamydiae	0.30	0.47
Elusimicrobia	0.30	0.17
OD1	0.29	0.23
Armatimonadetes	0.22	0.16
Cyanobacteria	0.18	0.14
Chlorobi	0.16	0.18
AD3	0.90	0.21
Fibrobacteres	0.08	0.07
Tenericutes	0.06	0.03
Spirochaetes	0.04	0.02
Undefined	4.85	3.94

Appendix V: GN* phylogenetic tree

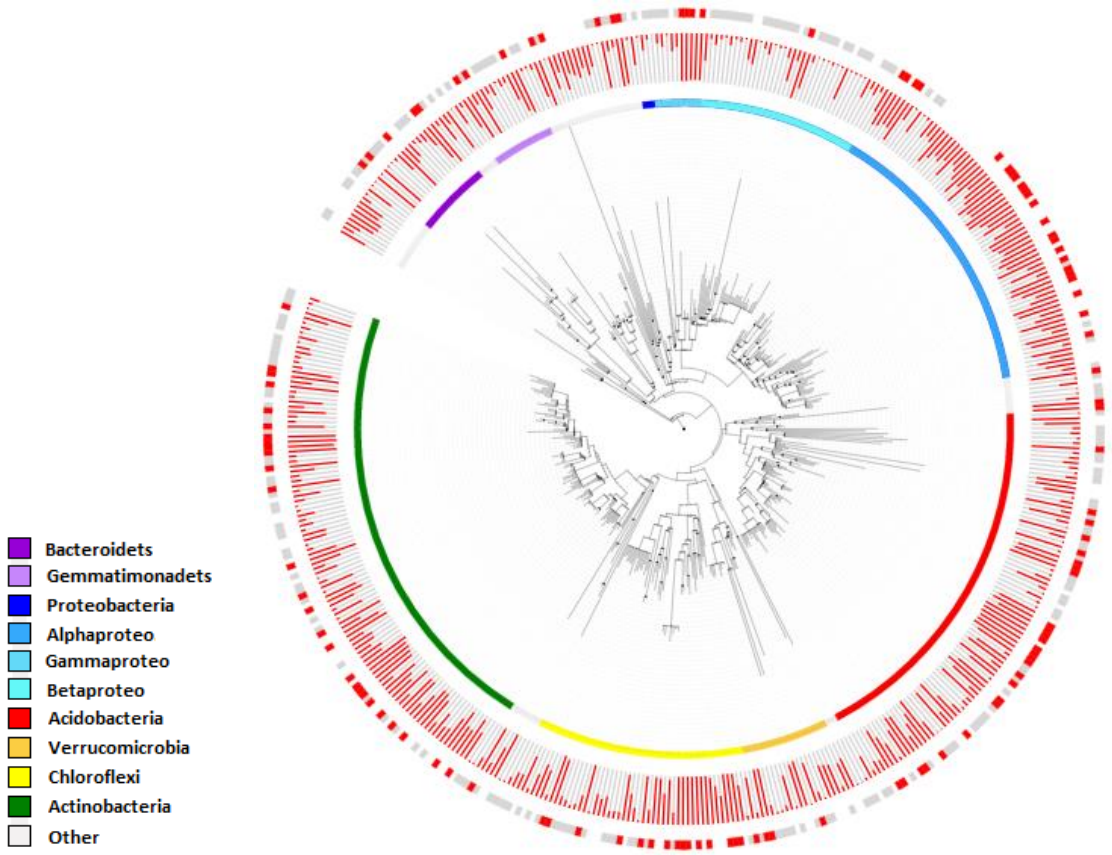

Figure A_V: Phylogenetic tree based on 500 most abundant OUTs from the new grassland (study in 2012, by Weedon et al.). First inner circle – phylum-level assignment for each OTU; second inner circle – relative abundance of each OTU in: grey – the low (from +0°C to +4°C); red – high (from +6°C to +40°C) temperature clusters; outer circle – indicator OTUs for the low temperature (grey) and high temperature (red) clusters.

Appendix VI: Relation between soil temperatures and other environmental factors

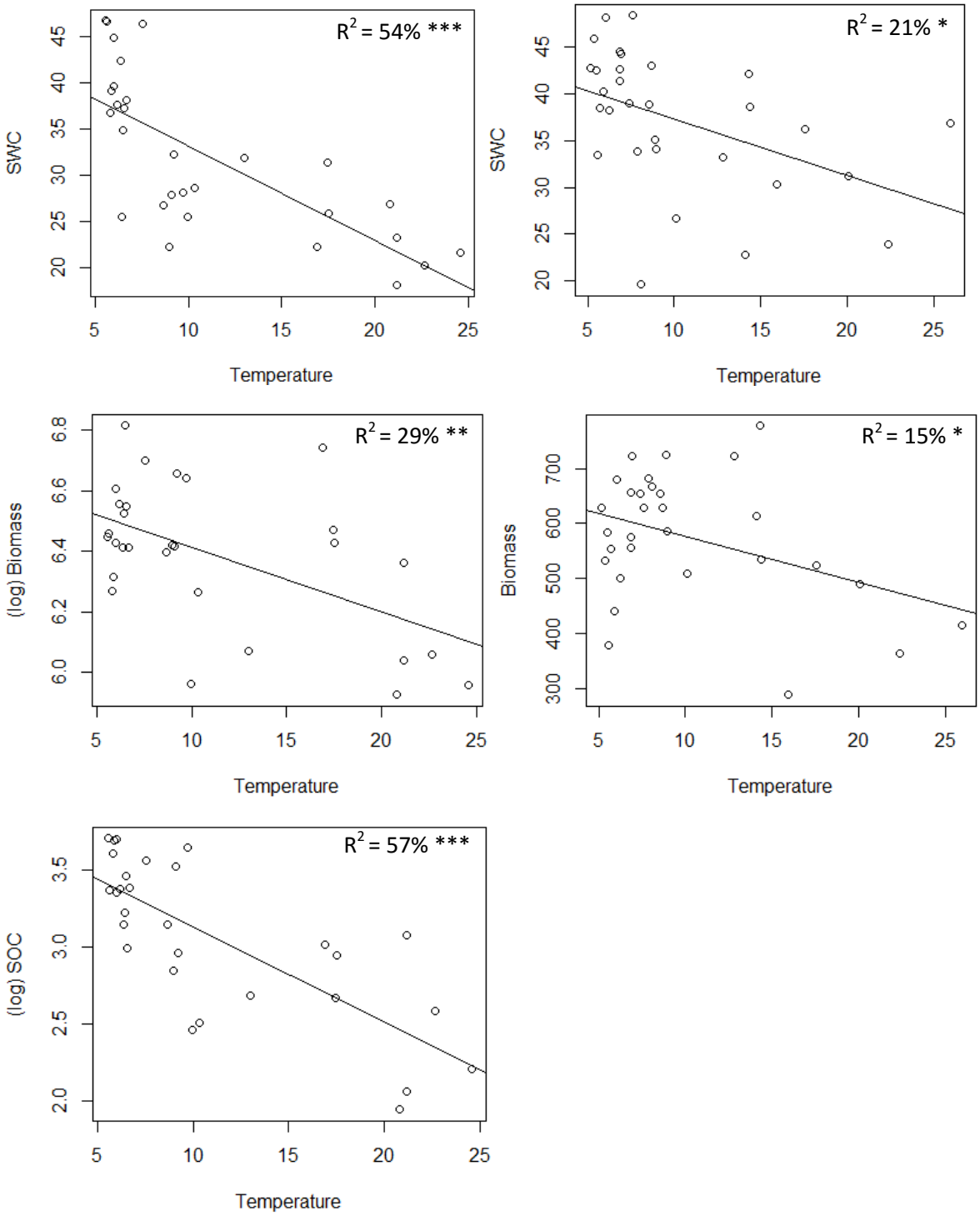

Figure A_VI: The change of SWC - soil water content [%], BM - plant biomass [g/m^2] and SOC - soil organic carbon stocks [t/ha] with temperature increase; right: GN, left: GO.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my supervisor James Weedon who has patiently led me through every step in creation of this thesis. His devotion and enthusiasm continuously encouraged me to do my best while working on the thesis. The knowledge I have acquired while working with him, starting from lab techniques, bioinformatic and statistical analysis of data, to the methods of scientific writing, is invaluable to me.

In addition, I would like to thank all the people who helped me in organising and conducting my fieldwork in Iceland. Special thanks to Bjarni Sigurdsson and the entire ForHot team for providing all the necessary conditions for our study at the ForHot site.

I am particularly grateful to Niki Leblans, Steven Dauwe, Stephanie Van Loock and Wendelien Meynzer for providing me extra information and data from their own studies.

I wish to thank Johan De Gruyter and Cindy De Jonge for their help during the lab work.

I also want to thank Lisa Bloss for reviewing the text and for her useful comments regarding the layout (not to forget encouraging stickers).

I would like to thank my family and friends for their encouragement, optimism and belief in me.

Finally, I would like to mention that the JoinEU-SEE scholarship programme provided the funding for my masters studies at the University of Antwerp.