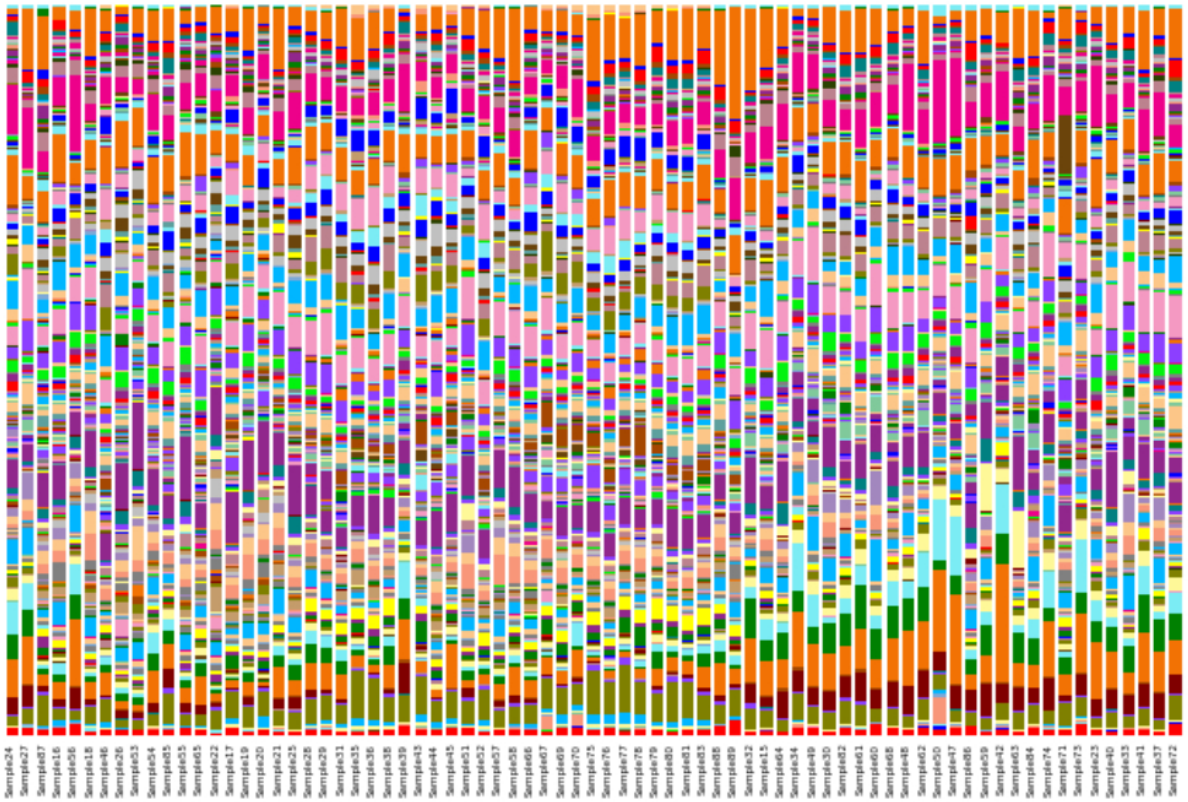
Shifts in soil prokaryotic communities of boreal and alpine semi-natural grasslands along temperature and precipitation gradients:
An investigation into the effects of graminoid removal



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Cover image: Stacked bars representing compositions (at genus level) of prokaryotic communities in 75 soil samples from semi-natural grasslands of western Norway.

ABSTRACT

Biotic interactions are important drivers of ecological responses to climate change. By studying the effects of graminoid removal on forb populations along temperature and precipitation gradients, Olsen *et al.* (2016) concluded that temperature increase shifted the net outcome of plant interactions from facilitation to competition in the boreal and alpine semi-natural grassland ecosystems. The study thus raised the concern that graminoids, the dominant plant functional group in these ecosystems, would further outcompete the subordinate groups such as the forbs as the climate continues to warm up. This Master´s thesis project was an extension to the study, digging deeper into the experimental set-up to investigate how soil prokaryotes had responded to five years of the graminoid removal. The results showed no significant change to the overall community composition, nor the diversity (number of observed OTUs, Chao1, Shannon *H'* and Simpson's *E*), of the soil prokaryotes due to the removal. This could be interpreted as a positive outcome, as the results casted doubt over the likelihood of impact of the reverse scenario – increased graminoid dominance – on soil prokaryotic communities under a warmer climate. While graminoid removal yielded no detectable response, the overall community composition was found to vary significantly along both the temperature and precipitation gradients. Redundancy analysis on the community composition data at phylum level further revealed that the two climate variables combined explained 20.5% of the variation in the data, with 11.1% and 9.34% of the variation attributed to precipitation and temperature, respectively. Relative abundance of the *K*-selected acidobacteria increased with increasing temperature and precipitation, while the *r*-selected proteobacteria decreased in relative abundance as the temperature increased. This change in the ratio of the two dominant soil bacterial phyla was indicative of a shift towards a more competitive environment for the prokaryotes as temperature rose – just as it was for the plants. All in all, the soil prokaryotic community in these boreal and alpine semi-natural grasslands demonstrated a considerable level of ecological resilience to natural and anthropogenic environmental changes. It is hoped that such resilience would help impede further climate change impacts on the semi-natural grassland ecosystem.

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Acknowledgments

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List of abbreviations

ALR	Ålrust (sampling site)
ANOVA	Analysis of variance
ARH	Arhelleren (sampling site)
bp	(Nucleotide) base pair
BSA	Bovine serum albumin
DCA	Detrended correspondence analysis (unconstrained ordination)
ddNTPs	Dideoxynucleotides
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United nations
FAU	Fauske (sampling site)
FEMS	Federation of European Microbiological Societies
GB	Gigabytes
GUD	Gudmedalen (sampling site)
Gt	Gigatonne
HOG	Høgsete (sampling site)
ISME	International Society for Microbial Ecology
LAV	Låvisdalen (sampling site)
mRNA	Messenger ribonucleic acid
NINA	Norsk institutt for naturforskning
NSC	Norwegian Sequencing Centre
OTU	Operational taxonomic unit
OVS	Øvstedal (sampling site)
PCR	Polymerase chain reaction
PLoS	Public Library of Science
QIIME	Quantitative Insights Into Microbial Ecology
RAM	Rambera (sampling site)
RAM	Random access memory
RDA	Redundancy analysis (constrained ordination)
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SKJ	Skjellingahaugen (sampling site)
ULV	Ulvehaugen (sampling site)
VES	Veskre (sampling site)
VIK	Vikesland (sampling site)

INTRODUCTION

I. Soil microorganism – plant interactions in the context of climate change

Virtually all terrestrial ecosystems consist of above-ground and below-ground communities that interact to drive ecosystem-level processes. In a typical scheme, plants act as the primary producers and capture light energy to convert atmospheric carbon into organic carbon, leading to biomass growth and provision of habitat and nutrient resources for other organisms such as the obligate root symbionts and pathogens. Below-ground microorganisms, in turn, act as decomposers to break down dead plant biomass and release nutrients into soil that are then taken up by plants (Wardle *et al.,* 2004). Such plant-microbe interactions operate through a web of feedback systems. Positive feedback increases the magnitude of a process and results in a directional change, whereas a negative feedback decreases the magnitude of the process until its effect is stabilized (Ehrenfeld *et al.*, 2005). Plant-microbe interactions also operate through a myriad of pathways involving physical, biogeochemical, and biological properties and processes. This includes the community compositions of the plant-microbe biota (Van Der Heijden *et al.,* 2008; Eskelinen *et al.,* 2009; Waldrop *et al.,* 2017). Shifts in the composition of a plant community, for example, may alter the chemical composition of the organic litter it produces, causing the soil microbial community that mediates access to the nutrient pools to respond concordantly. This response may then escalate (positive feedback) into a lasting change to the microbial community, or diminish (negative feedback). Either way, it depends on further responses from the plant community, and vice versa (Reynolds *et al.,* 2003). Plant-microbe interactions have been recognized as a major factor in the formation of soil (Jenny, 1941; Ehrenfeld *et al.*, 2005, Lambers *et al.*, 2009), in the evolution of terrestrial flora (Selosse and Le Tacon, 1998; Lau and Lennon, 2011), and in shaping community structures (Van Der Putten *et al.,* 2013; Classen *et al.,* 2015). Because they are fundamental to ecological processes, they are also important drivers of ecological responses to global environmental changes.

Anthropogenic activities since the advent of industrialization have been implicated as a primary force behind the current acceleration of global warming (Karl and Trenberth, 2003).

At the present rate, the average global surface temperature is estimated to keep rising by 4°C over the course of the 21st century, with extreme temperature and precipitation events projected to become more frequent in many regions (Kharin *et al.,* 2007; IPCC, 2014). An ever-increasing number of studies have presented evidence that the climate change has left ecological impacts ranging from latitudinal and altitudinal range shifts to coral bleaching (Walther *et al.,* 2002; Van der Putten, 2012). Climate modulates these impacts directly through physiological responses to factors such as temperature and precipitation and indirectly through biotic interactions (Classen *et al.,* 2015; Klanderud *et al.,* 2015; Waldrop *et al.,* 2017).

As the driver of the approximately 120 Gt annual flux of carbon in and out of terrestrial ecosystems – an amount much greater than that released through the combustion of fossil fuels – soil microorganisms have the potential to significantly alter the concentration of atmospheric carbon and thus the climate (Classen *et al.,* 2015). Soil microorganisms and the ecological processes they mediate are sensitive to temperature. Warming has been demonstrated to directly increase soil microbial respiration that channels carbon from soils to the atmosphere, but it remains unclear and highly complex how global warming would affect soil microorganisms and, ultimately, the global carbon stock in the long term (Bradford *et al.,* 2008; Hagerty *et al.,* 2014; Karhu *et al.,* 2014).

Numerous studies have investigated and argued for the critical role precipitation plays in regulating soil microbial responses to climate change (Castro *et al.,* 2010; Zhang *et al.,* 2013). Liu *et al.* (2009) reported their findings that experimental warming in a semi-arid environment, on the contrary, suppressed soil microbial activities and reduced respiration rates by inducing water stress. Increased precipitation, on the other hand, consistently exhibited positive impacts on respiration rates and other microbial parameters such as growth. The results of their study thus suggest that soil water availability may be a more influential factor than temperature. This is corroborated by another study looking into the single and combined effects of precipitation, warming, and elevated carbon dioxide concentration on the plant and soil communities of an old-field in Tennessee (Kardol *et al.,* 2010). The study found that precipitation had the largest impact compared to the other two environmental factors, and that interactions between the factors in mediating impacts were also largely

controlled by changes in water availability. It is also to be taken into consideration that high soil water availability may contribute to waterlogged, anoxic conditions such as in peat soils (Inisheva, 2006). The lack or absence of oxygen due to poor aeration in such conditions would cause the microbial decomposition of organic matter to occur anaerobically and potentially produce methane, a greenhouse gas estimated to be 25 times more potent than carbon dioxide (Tveit *et al.,* 2013; Tveit *et al.,* 2015).

Another important mechanism behind climate change impacts is the biotic interactions between plant and soil microbial communities. Given the influence they mutually have on each other, plant-microbe interactions may facilitate or hinder community transitions in response to the changes in climate. Classen *et al.* (2015) presented six different scenarios on this *(fig. 1)* and even proposed that the indirect effects of climate change through such biotic interactions may dominate over its direct effects for the soil microbial community. In the case of the old-field study mentioned earlier (Kardol *et al.,* 2010), for example, the effect of precipitation on the soil community was found to vary depending on the plant the soil was associated with. The relative abilities and rates at which plants and soil microorganisms respond to the climate change may also come into play. The lack of presence of a root symbiont due to differing migration responses, for example, may hinder plant growth and invasion into a new area (Nuñez *et al.,* 2009). Yet, much still remains to be understood about how climate change affects soil microorganisms and their biotic interactions with plants. There is an urgency to fill such knowledge gaps to allow more accurate prediction and better mitigation strategies of the imminent global climate change (IPCC, 2014; Classen *et al.,* 2015).

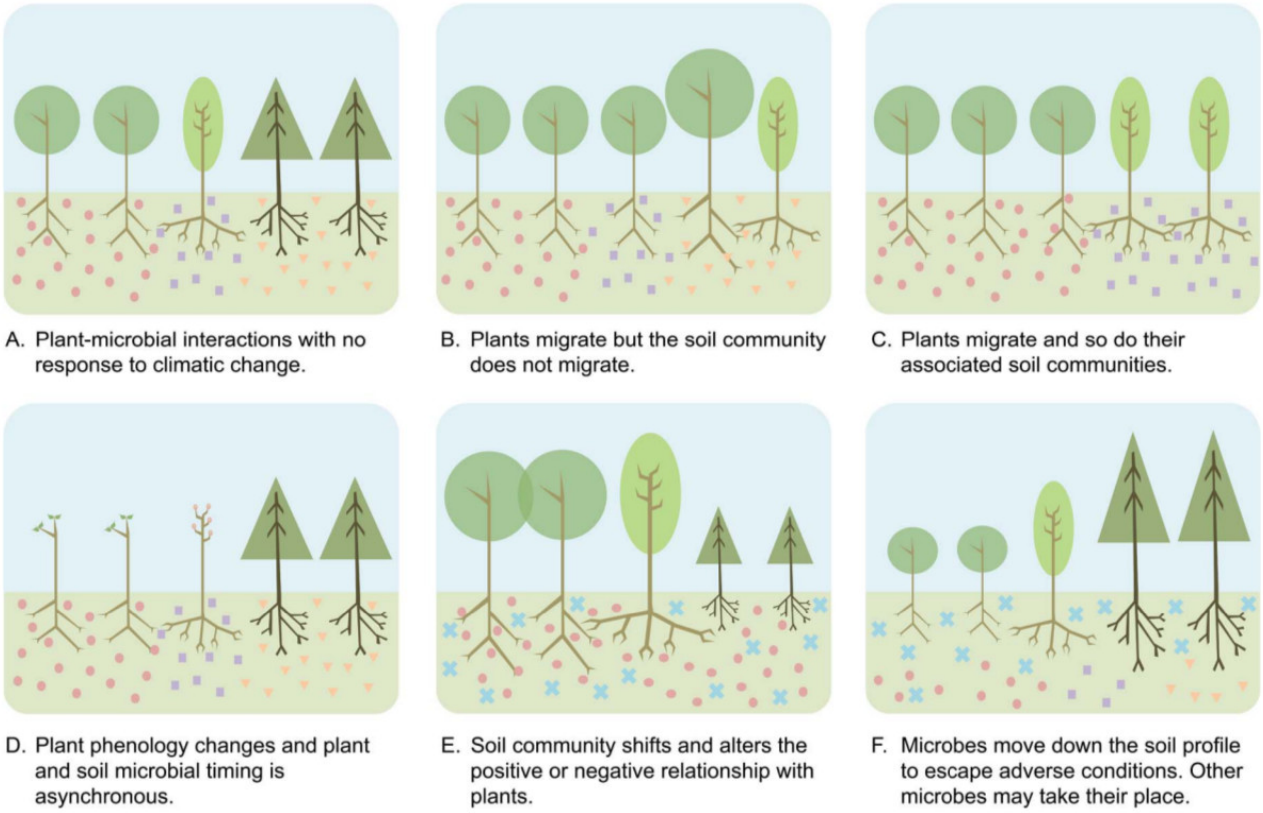


Figure 1. Six potential scenarios of responses of plant and soil microbial communities to changes in climate. Source: Classen *et al.,* 2015.

II. Background study

After the arctic regions, ecosystems of the alpine regions are amongst those most vulnerable to global warming. There is increasing evidence that the altitudinal limits of alpine and sub-alpine vegetation have risen within the last 100 years (Kullman, 2004). Klanderud and Birks (2003), for example, reported that more than half of the vascular plants growing on the alpine mountains of Jotunheimen, central Norway, were observed at higher altitudes in 1998 than in 1930-31, indicating a mean elevational advance of 1.2 m per year over the 68-year period. Global warming has also been associated with observed changes in the phenology of alpine species (Gallagher *et al.,* 2009; Mohandass *et al.,* 2015). It is feared that such responses may lead to the loss – or at its worst, extinction – of species that do not benefit from the climate change. Global warming thus pose a threat to biodiversity (Thuiller, 2007; Bellard *et al.*, 2012).

A study by Olsen *et al.* (2016) explored how the removal of graminoids, the dominant plant functional group in the semi-natural grassland ecosystem, affected the population dynamics of four subordinate forb species. The study was conducted at 12 field sites in western Norway that collectively formed a climate grid with natural temperature (alpine to boreal) and precipitation (continental to oceanic) gradients. This allowed for assessment of how climate would influence the plant interactions. The study found that the growth rates of three out of the four forb populations were lower in the removal plots than in the control plots at the colder alpine sites, whereas the opposite outcome (i.e. higher in the removal plots) was consistently observed at the warmer boreal sites. This implied that at low temperatures, graminoids facilitated the growth of these subordinate species, but this interaction turned into competition at higher temperatures. No systematic variation in the effect of graminoid removal on population growth was found along the precipitation gradient. It was thus concluded that temperature, rather than precipitation, was the primary determinant in the net outcome of interactions between dominant and subordinate plant groups in the semi-natural grasslands. The study also raised the concern that the dominant groups would further outcompete the subordinate ones under global warming, resulting in increased dependence on disturbance (i.e. grazing) to maintain biodiversity in the alpine, semi-natural grasslands (Olsen *et al*., 2016).

The above-ground removal of graminoids in the treatment plots since 2011 had also led to a natural decline of the graminoid populations over the years *(fig. 2),* prompting the interest to probe further into the experimental study and investigate how the below-ground microbial communities had responded to the removal. This may further unravel the role soil microorganisms play in the network of biotic interactions within the semi-grassland ecosystem. An investigation into how these biotic interactions vary with climate factors may also provide insights useful for predicting outcomes of the possible scenario of increased graminoid dominance under global warming.

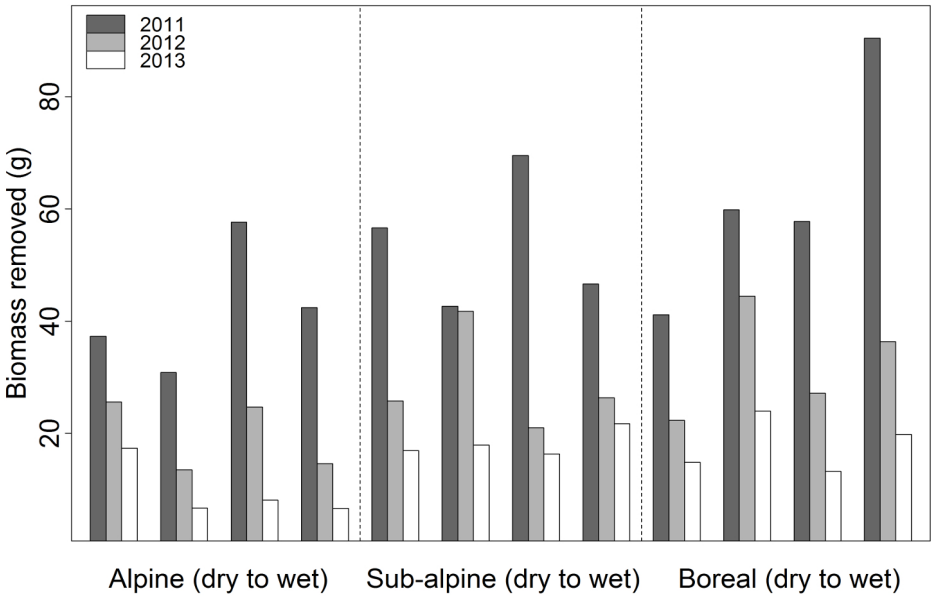


Figure 2. Total above-ground biomass (g) of graminoids removed from the treatment plots at twelve semi-natural grassland sites varying in temperature (alpine, sub-alpine, boreal) and precipitation (four levels from dry to wet) over the period of 2011-2013. Location: western Norway. Source: Siri L. Olsen, Vigdis Vandvik.

III. Norwegian semi-natural grasslands: soil conditions and microbial biota

Semi-natural grasslands cover an extensive part of Norway and are a major contributor to the cultural landscapes of the country. The unsown, wild vegetation of these grasslands is shaped and maintained through centuries of low-intensity (i.e. no ploughing) land use and agricultural activity. Traditionally, these grasslands are grazed by livestock of most commonly sheep and dairy cow. It is also common to cut and harvest the vegetation for fodder, sometimes in combination with spring and autumn grazing. Despite the dominance of members of the Poaceae (grasses) family, the plant communities of semi-natural grasslands are generally considered to be highly diverse and the conservation value of these landscapes has long been recognized (Austrheim, 2002; Vandvik, 2002; Helgadòttir *et al.,* 2014).

Large parts of Norway sit on a relatively thin layer of glacial till varying in texture and nutrient value. A considerable portion of this is peat soils, which are acidic, poor in nutrients and require drainage for cultivation (Helgadòttir *et al.,* 2014). As mentioned earlier, anoxic

conditions are common in such soils, potentially harbouring a broad diversity of microorganisms ranging from the facultative aerobe *Escherichia coli* to the strictly anaerobic archaeal group of methanogens (Madigan and Martinko, 2006). On the other end of the spectrum is calcareous soils, which contain substantial amounts of calcium carbonate and are alkaline. The organic matter content of such soils is generally low, and so are the availabilities of mineral nutrients such as phosphorus, zinc and iron (FAO, 2018). Plants growing in calcareous soils may therefore rely on symbiotic microorganisms to assist in nutrient acquisition (Lambers *et al.,* 2009).

Regardless of the soil type, it is important to consider that microbial communities operate at very small spatial and time scales due to their minuscule sizes and high turnover rates (Sessitsch *et al.,* 2001; Or *et al.,* 2012). One tiny soil aggregate can accommodate numerous microenvironments that differ physically and/or chemically, each hosting a microbial community that may be distinct from the other. A soil aggregate may also provide shelter from a more hostile condition outside. Even the oxygen level within a single soil particle can vary dramatically (Madigan and Martinko, 2006). Sampling microbial community at a scale that would adequately capture its diversity and function in an ecosystem thus presents a practical challenge.

As shown in *fig. 3*, the soil matrix is a highly heterogeneous and complex environment. A typical, mature soil consists of a number of layers or horizons differing in substrate, physiochemical conditions and biota. Microbial growth and activity, as well as those of other soil organisms, is usually highest at the A (surface) horizon of the soil profile due to the accummulation of dark organic matter there and the presence of living roots. Plants secrete sugars, amino acids, hormones and vitamins into the soil through these roots. The region immediately surrounding these roots, also known as the rhizosphere, is therefore particularly dense with microorganisms that feed on these nutrients and form symbiotic or parasitic relationships with the plants (Madigan and Martinko, 2006; Lambers *et al.,* 2009; De Vrieze, 2015).

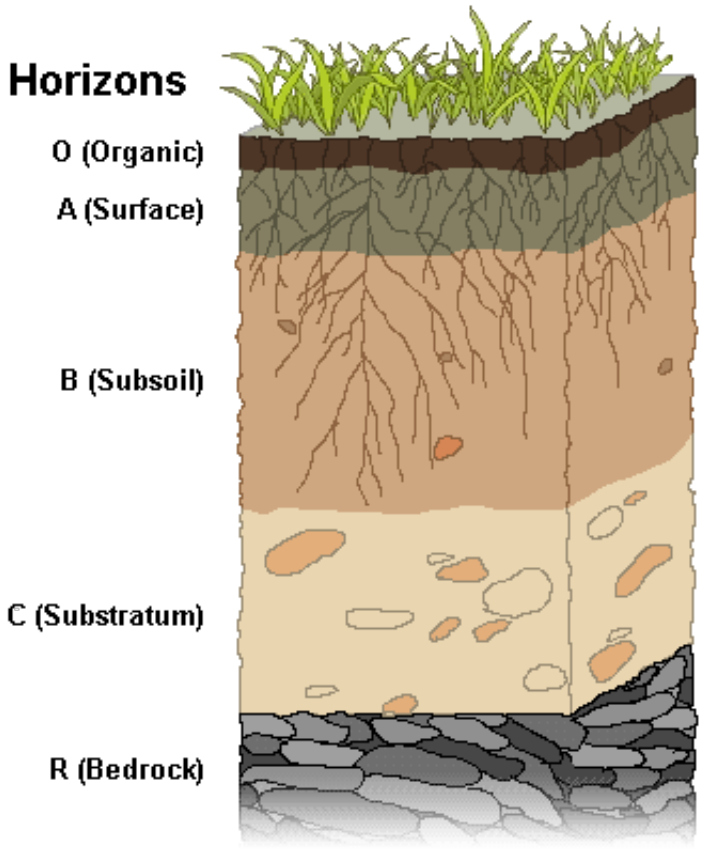


Figure 3. Profile of mature soil. Source: Wilson Biggs, distributed under a CC BY-SA 4.0 license.

Soil microorganisms can be classified into a number of taxonomic groups, namely bacteria, archaea, viruses, fungi, algae, and protozoa. Bacteria usually form the most abundant group, with a single gram of soil estimated to contain up to 10 billion counts of cells belonging to thousands of different species (Whitman *et al.,* 1998; Torsvik and Øvreås, 2002; De Vrieze, 2015). Two of the most common bacterial divisions or phyla in soil are proteobacteria and acidobacteria. Proteobacteria is the largest and most diverse phylum of bacteria, all of which are gram negative. Many bacteria of medical, industrial, and agricultural significance belong to this phylum, including the pathogen *Escherichia* and the nitrogen fixing rhizobia (Madigan and Martinko, 2006). Acidobacteria is also a diverse phylum of bacteria, found in a myriad of environments but is particularly abundant in soils. As hinted by its name, many acidobacteria are known to be acidophilic. Despite of their abundance and ubiquitous distribution in soils, relatively little is known about their physiologies and ecological roles, which is in part due to the difficulties in culturing a majority of the phylum members (Quaiser *et al.,* 2003; Kielak *et*

al., 2016). Much of what is currently known about the phylum has therefore been deduced through studies on their genetic materials. Smit *et al.* (2001), for example, analysed available 16S rRNA sequences to reveal any pattern of abundance of five bacterial divisions, including acidobacteria, in relation to the soil nutrient profile. The study found higher abundances of acidobacteria in soils with low nutrient input or high content of recalcitrant substrates, whereas α- and γ-proteobacteria appeared to thrive better in soils that were rich in readily available nutrients. These results thus prompted Torsvik and Øvreås (2002) to comment that α- and γ-proteobacteria may be *r*-selected, which is selection for bacteria with potentially high growth rates, whereas acidobacteria may be *K*-selected, which is selection for bacteria with lower growth rates but are typically strong competitors for limited resources.

IV. Studying microbial diversity

As Torsvik and Øvreås (2002) put it, "microbial diversity describes complexity and variability at different levels of biological organization. It encompasses genetic variability within taxons (species), and the number (richness) and relative abundance (evenness) of taxons and functional groups (guilds) in communities." Three levels of assessment of microbial – or any ecological – diversity have also been defined: alpha (α) diversity is the mean diversity within a site or habitat, beta (β) diversity refers to the difference in diversity between two or more sites/habitats, and gamma (γ) diversity is a combination of the two to represent the diversity of the region (Whittaker, 1960).

The assessment of microbial diversity, however, has always been a challenging task in microbiology. At the root of this is the inscrutable nature of microbial communities from natural environments (Øvreås and Curtis, 2011). It is notoriously difficult, if at all possible, to culture a vast majority of microorganisms, rendering culture-based methods highly inadequate and biased for the assessment of diversity. The incapability to isolate microorganisms into pure cultures also means that there is a lack or absence of morphological and physiological data necessary for traditional classification of a taxon into a species (Rosselló-Mora and Amann, 2001; Handelsman, 2004). The study of microbial diversity and ecology have thus largely relied on methods of molecular biology in these last few decades.

The extraction of genetic materials directly from environmental samples, by-passing the need to first cultivate the microorganisms in the samples, further revolutionized the field and paved the way for environmental genomics (Amann *et al.,* 1995; Handelsman, 2004). Operational taxonomic unit (OTU) has become a practical alternative to the concept of species, using mathematical algorithms to calculate the similarities of microbial DNA sequences and 'cluster' together those that meet a pre-defined threshold (i.e. commonly 97% similarity) (Schmidt *et al.,* 2014). In this way, an OTU serves as a proxy for a microbial species when describing and comparing microbial communities (Øvreås and Curtis, 2011).

Usually, OTUs are defined from sequences of the prokaryotic 16S ribosomal RNA (rRNA) gene or the eukaryotic 18S rRNA gene (Schmidt *et al.,* 2014). rRNA is very useful for phylogenetic and taxonomic studies, because it is found across virtually all cells and living organisms and contains regions that evolve very slowly and are therefore highly conserved (Woese *et al.,* 1990; Madigan and Martinko, 2006). Since the focus of this thesis is on prokaryotic communities, there will be no further discussion of the 18S rRNA gene. 16S rRNA is one of the components of the prokaryotic ribosome that has an essential function in initiating protein synthesis. The gene that encodes 16S rRNA is approximately 1500 nucleotide base pairs (bp) long and contains nine hypervariable regions in addition to the conserved regions *(fig. 4)*. These hypervariable regions (V1-9) are the key to the differentiating and identification of the prokaryotic taxa. The conserved regions, on the other hand, allow for the design of primers that reliably amplify target sections of the gene across different taxa. While it is ideal to obtain and analyse the entire length of the gene, it is often not feasible to do so for varying reasons including the constraint of cost. For this reason, many studies have chosen the V4 region for analysis, as it is only around 100 bp long, is semi-conserved (i.e. less instraspecies diversity), and has been demonstrated to provide resolution at the phylum level comparable to that of the full gene (Schmalenberger *et al.,* 2001; Walters *et al.,* 2015; Yang *et al.,* 2016).

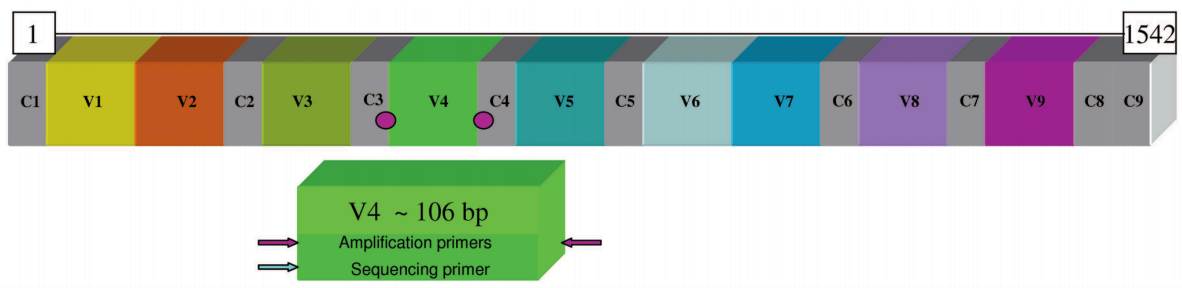


Figure 4. Schematic diagram of the 16S rRNA gene, showing its conserved (grey colour) and hypervariable (other colours) regions. The pink circles and arrows represent the sites for primer binding for DNA amplificiation of the hypervariable V4 region. Source: Petrosino *et al.,* 2009 (reproduced with permission from the American Association for Clinical Chemistry).

Progress in microbial ecology goes in tandem with developments in the techniques of molecular biology. Earlier studies in microbial diversity used the approach of community fingerprinting such as denaturant gradient gel electrophoresis (DGGE; Muyzer *et al.,* 1993) and terminal restriction fragment length polymorphism (T-RFLP; Liu *et al.,* 1997), both of which provided a quick and affordable way to characterize microbial communities (Øvreås and Curtis, 2011). DNA sequencing has also played an important role and has come a long way since the earliest method of sequencing through two-dimensional gel electrophoresis (Min Jou *et al.,* 1972). Soon after, Sanger *et al.* (1977), with contributions from others in the scientific community, pioneered the method of DNA sequencing through primer elongation with chain-terminating dideoxynucleotides (ddNTPs). The demand for lower cost and faster sequencing since then has led to the current generation of high-throughput sequencing methods, which parallelize the sequencing process to achieve an output of thousands to millions of sequences (reads) per run (Hutchison, 2007). These high-throughput methods vary in the number of reads per run, the average length of reads, accuracy, and cost (Lanzén, 2013). One such high-throughput method is Illumina, previously knowned as Solexa.

Illumina uses the approach of "sequencing-by-synthesis", which builds on the process of Sanger sequencing by overriding or reversing the chain termination step and allowing the chain to continue to elongate. In addition, Illumina massively parallelizes this process by running the sequencing of many different, barcoded template strands all at once. Altogether,

the modifications to the classical Sanger sequencing have enabled Illumina to generate an enormous amount of reads in a single run and at a significantly lower cost (Shokralla *et al.,* 2012). Illumina, however, produces shorter reads compared to other high-throughput methods such as pyrosequencing, although recent protocols involving paired-end reads allow lengths up to 600 bp (2 x 300 bp) (Lanzén, 2013; Illumina, 2014). An overview of the steps involved in Illumina sequencing is presented below in *figure 5*.

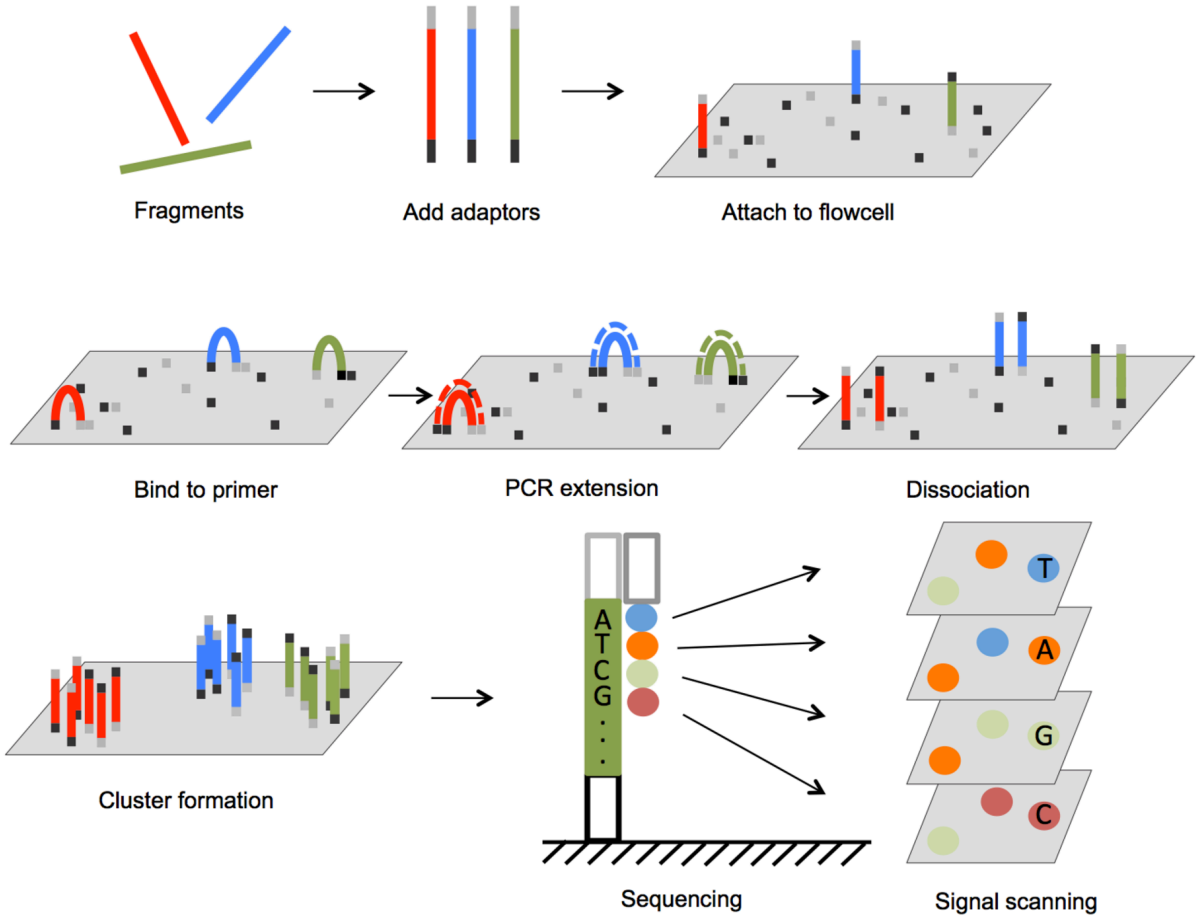


Figure 5. A summary of the workflow in Illumina sequencing. Firstly, barcoded adaptors are added onto both ends of the sequence fragments. Fragments then bind to primers attached onto a flowcell, and bridge polymerase chain reactions (PCR) are initiated to amplify each fragment into a cluster of fragments. Each fragment in the cluster serves as a template strand for sequencing, using ddNTPs that release fluorescent signals. At every sequencing cycle, one complementary ddNTP binds to the template strand and the chain is terminated. The released signal is then scanned to identify the nucleotide. The chain termination is then reversed, allowing the next cycle to start and another ddNTP to be added onto the chain.
Source: <<http://www.3402bioinformaticsgroup.com/service/>>.

Regardless of the choice between community fingerprinting or high-throughput sequencing, polymerase chain reaction (PCR) is required for the analysis of 16S rRNA gene in this context of diversity assessment. PCR is needed to produce multifold copies of only the desired section (e.g. 16S rRNA gene) of the extracted environmental DNA. The method involves numerous cycles of repeated heating and cooling to drive the reactions that exponentially amplify the target section (Madigan and Martinko, 2006). While PCR is a powerful tool, it is not flawless. It is therefore important to consider the errors that PCR may introduce, as they may have a significant impact on the profiling of the community (Lanzén, 2013).

V. Project objectives and approach

The objective of this Master´s project was two-fold. The first was to determine the effects of five years of graminoid removal on the composition and diversity of below-ground prokaryotic communities in boreal and alpine semi-natural grasslands. The second was to investigate how these effects vary along the climate gradients of temperature (boreal to alpine) and precipitation (coastal to continental). The rationale was that findings from this study may provide insights into how below-ground prokaryotic communities would respond to increased graminoid dominance at semi-grasslands, as prospected to happen under a warmer climate by Olsen *et al.,* 2016. In other words, it was hoped that this study would shed light on the role of soil prokaryotes in mediating this graminoid dominance, and further unravel the network of biotic interactions within the semi-grassland ecosystem.

A number of research questions were therefore specified as follows:

- Did the removal of graminoids lead to significant difference in the overall soil prokaryotic community composition?
- Did the impact of graminoid removal on community composition vary along the climate gradients of temperature and precipitation?
- Did the soil prokaryotic community vary along the climate gradients of temperature and precipitation?

- Did graminoid removal have significant impacts on the OTU richness and evenness of the soil prokaryotic community?
- Did the impacts of graminoid removal on soil prokaryotic diversity vary along the climate gradients of temperature and precipitation?
- Did the OTU richness and evenness of the soil prokaryotic community vary along the climate gradients of temperature and precipitation?

In order to answer the questions above, soil samples were collected in the summer of 2016 from control and graminoid removal plots across twelve semi-grassland sites that formed a climate grid with natural temperature and precipitation gradients. The experimental set up was established in 2011 by Olsen *et al.* (2016) and had been maintained since then. The samples were frozen immediately after collection to prevent further changes to the microbial community contained in the soil. DNA was extracted directly from the soil samples, thus capturing a snapshot of the microbial community at sampling time. PCR then amplified a target section covering the V4 region of the 16S rRNA gene from the environmental DNA, generating barcoded PCR amplicons that were sent to the Norwegian Sequencing Centre for Illumina sequencing. Bioinformatics tools were used to process the sequences and 'cluster' them into OTUs based on the threshold of 97% sequence similarity. The OTUs were identified and classified into prokaryotic taxa by matching their representative sequences to the Greengenes 16S rRNA gene database. In this way, the OTU composition of the prokaryotic community in each soil sample was determined. Bioinformatics tools were also used to calculate estimations of prokaryotic diversity for each community or sample, with Chao1 and Shannon Diversity Index selected as measures of OTU richness and Simpson´s Evenness Index selected as a measure of OTU evenness. Differences in OTU composition between communities were quantified by their Bray-Curtis dissimmilarity. Data and statistical analyses were then performed on these data. This outline of the methodology is described in more details in the next section.

MATERIALS AND METHODS

I. Sampling sites and experimental design

Twelve semi-natural grassland sites scattered across the fjord landscapes of western Norway *(fig. 6)* were sampled in this study. They were carefully selected to form a climate grid, combining three levels of summer temperature (mean of the four warmest months; approximately 6.5, 8.5, and 10.5°C) with four levels of annual precipitation (approximately 0.6, 1.2, 2.0, and 2.7 m), using interpolated climate data from the normal period 1961-1990 (Skarpaas *et al.,* 2016). The two climate variables were not correlated to each other and other variables were kept as constant as possible. The sites were located on south-facing, shallow slopes with calcareous bedrock. The plant communities belonged under the plant-sociological association Potentillo-Festucetum ovinae (Fremstad, 1997), and could be classified into three functional groups: graminoids, forbs, and bryophytes. Common species included the graminoids *Agrostis capillaris, Anthoxanthum odoratum, Deschampsia cespitosa, Nardus stricta*, and the forbs *Achillea millefolium, Bistorta vivipara, Potentilla erecta* (Gya, 2017). Specific details regarding the locations, geophysical and climate characteristics of the sites are gathered together in *table 1*. Collectively known as the "SeedClim" sites, they were established as research sites in 2009 and had been maintained and subjected to numerous studies since then (Meineri *et al.,* 2013; Meineri *et al.*, 2014; Klanderud *et al.,* 2015; Olsen *et al.*, 2016; Skarpaas, *et al.,* 2016; Gya, 2017; Klanderud, *et al.,* 2017; Althuizen *et al.,* 2018).

There were five separate blocks at every site, and within each block, a pair of control and graminoid removal (treatment) plots measuring 25 cm x 25 cm each were set up. Whilst the vegetation in the control plot was left undisturbed, any above-ground growth of graminoid species was cut and collected twice – in the beginning and at the end of the growing season. This experimental set-up had been maintained every year since 2011.

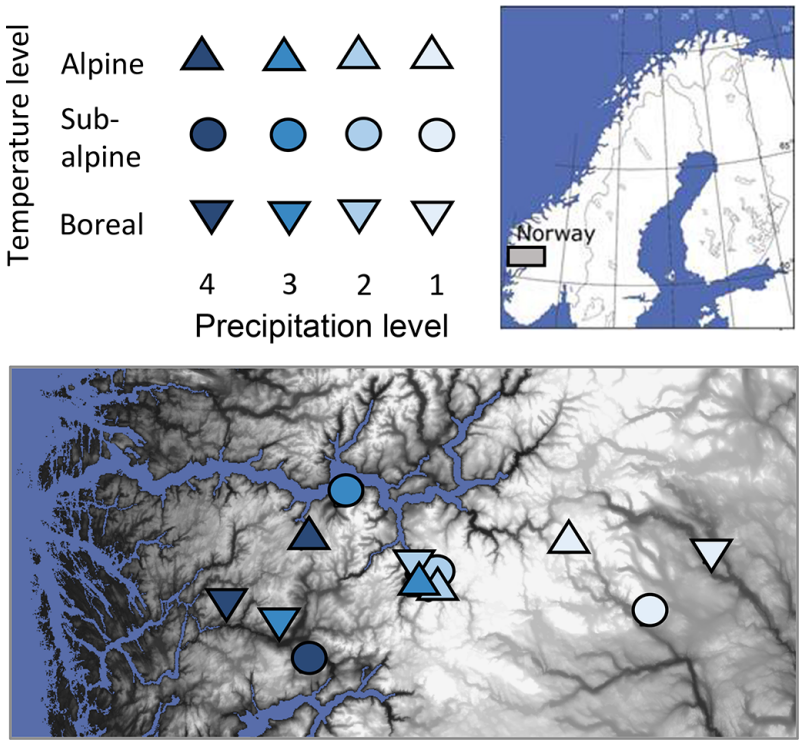


Figure 6. Locations of the twelve "SeedClim" semi-grassland study sites, each having a unique combination of mean summer temperature (alpine, sub-alpine, boreal; approximately 6.5, 8.5, and 10.5°C respectively) and annual precipitation level (approximately 0.6, 1.2, 2.0, and 2.7 m). Source: Klanderud *et al.,* 2015.

Table 1. The SeedClim sites, locations (World Geodetic System of 1984 datum), climate and geophysical characteristics: altitude (m above sea level), mean summer temperature (°C), mean annual precipitation level (m) and types of underlying bedrock.

Site	**Coordinate x**	**Coordinate y**	**Altitude (m)**	**Temperature (°C)**	**Precipitation (m)**	**Bedrock**
Ulvehaugen (ULV)	61°1'27.40" N	8°7'24.30" E	1208	6.17	0.596	Rhyolite, ryodacite, dacite
Låvisdalen (LAV)	60°49'23.10" N	7°16'33.50" E	1097	6.45	1.321	Phyllite, mica schist
Gudmedalen (GUD)	60°49'57.90" N	7°10'32.20" E	1213	5.87	1.925	Phyllite, mica schist
Skjellingahaugen (SKJ)	60°56'0.50" N	6°24'54.10" E	1088	6.58	2.725	Marble
Ålrust (ALR)	60°49'13.00" N	8°42'16.80" E	815	9.14	0.789	(Meta)sandstone, shale
Høgsete (HOG)	60°52'33.70" N	7°10'36.00" E	700	9.17	1.356	Phyllite, mica schist

Rambera (RAM)	61°5'11.80" N	6°37'48.90" E	769	8.77	1.848	Phyllite, mica schist
Veskre (VES)	60°32'40.10" N	6°30'52.80" E	797	8.67	3.029	(Meta)sandstone, shale
Fauske (FAU)	61°2'7.50" N	9°4'43.60" E	589	10.3	0.600	Phyllite, mica schist
Vikesland (VIK)	60°52'49.20" N	7°10'11.30" E	474	10.6	1.161	Phyllite, mica schist
Arhelleren (ARH)	60°39'54.60" N	6°20'14.60" E	439	10.6	2.044	Phyllite, mica schist
Øvstedal (OVS)	60°41'24.20" N	5°57'53.50" E	346	10.8	2.923	Ryolite, ryodacite, dacite

II. Sample collection

Soil samples were collected from all, but one, sites during the period of July 4th to 8th, 2016. Samples from the site GUD were collected on August 16th, 2016. Gloves were worn throughout the entire procedure, and contact with other surfaces was avoided as much as possible. Tools were cleaned and then sterilized prior to every sampling (of a different plot) by dousing the surfaces with absolute ethanol and igniting them on fire on a safety plate. Tools were then left briefly to cool before a ca. 5 cm slit was cut into the ground within the plot and sample from the surface or top layer (O and A) of the soil horizon was scooped to fill one sterile MO BIO PowerBead Tube and two sterile, empty cryotubes. A total of three, full tubes of soil samples were collected from every plot and kept cold in ice until sampling was completed for the site. The samples were then immediately frozen inside a portable tank containing liquid nitrogen for a couple days of temporary storage. When the tank was full, samples were kept as cold as possible for a few hours until all the samples were transported to the research facility. Samples were then transferred to a -80°C freezer for long term storage.

III. DNA extraction, quantitation and quality control

Samples from three out of the five blocks at every site were selected for DNA extraction and any downstream procedure and analysis. The 72 selected samples, plus one redundant sample, are listed in *appendix A*. Two sets of pseudoreplicates were made to allow for detection of

significant inconsistencies in techniques (see *appendix A)*.

Extraction of DNA directly from the soil samples was carried out in separate batches using the PowerSoil® DNA Isolation Kit manufactured by MO BIO Laboratories, Inc., following the protocol described in the instruction manual (version 02232016). Prior to the start of the procedure, frozen samples were left to thaw at room temperature for a few minutes. 0.25–1.22 g of soil sample was added into a PowerBead Tube using a pre-sterilized spatula (see *appendix A* for exact amounts). Some of the samples were already pre-loaded into the PowerBead Tubes, which contained beads and buffer to assist in homogenizing the mixture and protect DNA from degradation. Microbial cells in the mixture were then lysed, releasing DNA collected in the supernatant. A series of precipitation reactions and centrifugation followed, in order to remove non-DNA organic and inorganic materials from the supernatant. DNA was then extracted by filtering the supernatant through a silica membrane, which trapped only DNA under high salt concentration. The silica-bound DNA was cleaned with ethanol solution before it was eluted from the membrane into the provided elution buffer twice. DNA extracts were then kept at 4°C for downstream procedures on the same or next day, or stored at -20°C until the next procedure.

The concentration of DNA in the solution was quantified through spectrophotometry using the Qubit® dsDNA High Sensitivity Assay Kit, following the protocol described in its User Guide (Thermo Fisher Scientific Inc., 2015). If the concentration was too high for the high-sensitivity assay (>600 ng/mL after dilution in the assay tube), the procedure was then repeated for that particular DNA extract using the Qubit® dsDNA Broad Range Assay Kit (Thermo Fisher Scientific Inc., 2015).

5 μL of the DNA extract solution was then electrophoresed on 1% agarose gel to allow for visual evaluation of the quality of the DNA extract. Images of the UV light-illuminated gels are presented in *appendix B*.

IV. Polymerase Chain Reaction of 16S ribosomal RNA gene

There were two stages in the preparation of the 16S rRNA gene amplicons for the Illumina MiSeq sequencing system. In the first stage, the target V4 region of the gene was amplified such to include an overhang of Illumina adapter oligonucleotide. To minimize PCR drift, PCR mixtures were first prepared in triplicates for every sample and later pooled together after the PCR. Each 20 µL PCR mixture consisted of 10 µL Qiagen HotStarTaq Master Mix, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.5 µL 100% BSA, 5–10 ng of DNA extract as PCR template, and nuclease-free water.
The nucleotide sequence of the forward primer ('adapter-N5-519F') was
5'CTACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-CAGCMGCCGCGGTAA,
whereby M=A/C.
The sequence of the reverse primer ('adapter-806R') was
5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-GGACTACHVGGGTWTCTAAT,
whereby H=A/C/T, V=A/C/G, and W=A/T.

Positive and blank controls were included in every PCR run. For the positive control, 5 ng of *Escherichia coli* genomic DNA was used as the PCR template, whereas nuclease-free water substituted for PCR template in the blank control. The PCR was initiated at 95°C for 15 min (denaturation), followed by 25 cycles of thermal conditions at 95°C for 20 sec (denaturation) – 55°C for 30 sec (primer annealing) – 72°C for 30 sec (elongation), and then finalized at 72°C for 7 min (elongation) before termination and cooling at 4°C.

After pooling together the PCR mixtures that corresponded to the same samples, 2 µL of each of the mixtures was electrophoresed on 1.5% agarose gel to check for the presence and size of the amplicons. The PCR amplicons were then purified through the Zymo Research DNA Clean & Concentrator™-5 columns following the manufacturer's protocol (version 1.2.0), prior to having their DNA concentrations measured using the Qubit® dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific Inc., 2015).

The second stage in the preparation of the final amplicons targeted the overhanging Illumina adapter oligonucleotides and added 'barcode' nucleotide sequences into the amplicons. A

combination of 8 versions of the forward primer and 12 versions of the reverse primer allowed for identification of up to 96 uniquely barcoded amplicons such that they could all be sequenced in parallel. Unlike in the previous stage, the PCR was not divided into triplicates for every sample. Each 50 µL PCR mixture consisted of 25 µL Qiagen HotStarTaq Master Mix, 23 µL of the solution containing the purified amplicons from the first stage, and a specific combination of 1 µL barcoded forward primer (10 µM) and 1 µL barcoded reverse primer (µM).

The nucleotide sequence of the barcoded forward primer ('adapter-barcode-linker') was 5'AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-ACACTCTTTCCCTACACGACG, and the sequence of the barcoded reverse primer ('adapter-barcode-linker') was 5'CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT. The different versions of the barcode region of the forward and reverse primers are described below in *table 2.*

Table 2. Different versions of the barcode region of forward and reverse primers for the 2nd stage of PCR.

Forward ID	Sequence	Reverse ID	Sequence
F1	TAGATCGC	R1	TCGCCTTA
F2	CTCTCTAT	R2	CTAGTACG
F3	TATCCTCT	R3	TTCTGCCT
F4	AGAGTAGA	R4	GCTCAGGA
F5	GTAAGGAG	R5	AGGAGTCC
F6	ACTGCATA	R6	CATGCCTA
F7	AAGGAGTA	R7	GTAGAGAG
F8	CTAAGCCT	R8	CCTCTCTG
		R9	AGCGTAGC
		R10	CAGCCTCG
		R11	TGCCTCTT
		R12	TCCTCTAC

Positive and blank controls were also included in every PCR run, using the same controls as in the first stage but with their amounts adjusted accordingly. The PCR was initiated at 95°C for 15 min (denaturation), followed by 15 cycles of thermal conditions at 95°C for 20 sec (denaturation) – 62°C for 30 sec (primer annealing) – 72°C for 30 sec (elongation), and then finalized at 72°C for 7 min (elongation) before termination and cooling at 4°C.

The presence and size of the amplicons were verified by running 2 μL of each of the PCR mixtures through electrophoresis on 1.5% agarose gel and then visualizing the gel under UV light. The amplicons were purified using the Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc., 2013) and eluted into 10 mM Tris buffer.

As the final steps before pooling together the 16S rRNA gene amplicons, the DNA concentrations of the purified amplicons in solutions were determined using the Qubit® dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific Inc., 2015). The purity of the amplicons were also measured using the nanoDrop1000 spectrophotometer calibrated with 10 mM Tris buffer ($A_{260/280}$ = 1.8–2.1, $A_{260/230}$ = 1.5–2.9).

V. High-Throughput Sequencing: Illumina MiSeq System

45 ng of each sample solution of 16S rRNA gene amplicon was pooled together and shipped to the Norwegian Sequencing Centre (NSC) in Oslo, Norway. Prior to sequencing using the Illumina MiSeq V2 system, a well-characterized PhiX library was added as a means for quality control.

VI. Post-sequencing processing

Sequences received from the NSC were processed and analysed on the Bio-Linux 8 bioinformatics workstation platform (Field *et al.*, 2006), using the tools BBDuk of BBTools (Bushnell, 2014) and QIIME (Caporaso *et al.,* 2010). Pipeline containing the series of commands used, along with detailed annotations, is provided in *appendix C*. The steps involved in the processing of the raw sequence reads into community compositions are briefly described as follows.

Firstly, the raw sequences were demultiplexed using the QIIME script split_libraries.py, based on data provided in a mapping file. Demultiplexing identified the sequence reads based

on their barcodes, divided them into separate files for every sample, and subsequently removed from the reads the bases that corresponded to the forward and reverse adapters, their adjacent barcodes, and any linker sequence. Any control PhiX sequence was then filtered away using the multi-functional BBTools script bbduk.sh. Complementary forward and reverse ("paired-end") reads were merged into single consensus sequences using the script bbmerge.sh, and then the remaining portions of the forward and reverse primer sequences ('N5-519F' and '806R', respectively) were trimmed off. The sequence reads were quality trimmed and following this, reads shorter than 200 bases were discarded. As the final step in the processing of the reads, the FASTQ sequence files were converted into FASTA files using the script reformat.sh.

QIIME-compatible labels were individually added to the FASTA files before all the FASTA files were concatenated into one, such that sequences from all the samples were merged together for the downstream processes. This was particularly important when picking the OTUs, which was the next step, as doing this separately would lead to different IDs being assigned to the same OTUs and thus overestimate the total number of the OTUs. The QIIME script pick_de_novo_otus.py ran a series of processes that led to the construction of a phylogenetic tree and an OTU table. In OTU picking, sequences that were at least 97% similar were assigned to an OTU *de novo* using the uclust clustering algorithm (Edgar, 2010). A representative sequence was then picked for each OTU, and the representative sequences were aligned to the template sequences from the Greengenes Database (DeSantis *et al.,* 2006) using the PynaST method. This allowed each representative sequence – or OTU – to be assigned to a taxon. The number of times an OTU/taxon appeared in each sample was then counted and tabulated into an OTU table. The OTU table was later translated into community compositions, specifically the relative abundances of OTUs or taxa, at genus, class and phylum taxonomic levels for further analyses. In addition to generating an OTU table, the QIIME script also filtered away highly variable and therefore uninformative regions of the 16S rRNA amplicon sequences, and constructed a phylogenetic tree based on these filtered sequences using the FastTree method (Price *et al.,* 2009).

It is to be noted that singletons, which are OTUs formed by single sequence reads, were not filtered away from the dataset so as to include as much of the available data as deemed fit for

the downstream data analyses, particularly for rarefaction analysis. Theoretically, the choice of keeping or removing singletons should not matter for the alpha and beta diversity analyses in this study. This is because many of the singletons would have been removed through rarefaction (sub-sampling) of the OTU table prior to calculation of alpha diversity estimates for the samples. As for the beta diversity analysis, singletons were not included as only the common OTUs or taxa (i.e. mean relative abundance of phylum >2%) would be analysed.

VII. Data and statistical analyses

Statistical analyses of the microbial community compositions were conducted using R, with the packages 'phyloseq' (McMurdie and Holmes, 2013) , 'vegan' (Oksanen *et al.,* 2018) and 'tidyverse' (Wickham, 2017) added in. 'phyloseq' was chosen specifically for handling and analyzing high-throughput microbial community data. Multivariate analysis of the community data required the package 'vegan'. 'tidyverse' was a set of compatible packages with powerful tools for data manipulation and visualization. Selected sections of the pipeline are provided in *appendix C*. Prior to any analysis, the data was processed such to, among others, remove samples 79 ('VES II R2'), 83 ('VES II C2'), and 89 ('ULV III C1'). The redundant sample 89 was removed to maintain equal sample size for the analyses, whilst samples 79 and 83 were both pseudoreplicates of samples 38 and 31 respectively. The pseudoreplicates were removed after a preliminary analysis found no difference to indicate any significant technical inconsistency. Removal of the pseudoreplicates was necessary to avoid violating the assumption of independence underlying the statistical analyses.

Beta diversity analysis

Since the data had more than one outcome variable (i.e. the multitude of OTUs/taxa that comprised a community), multivariate methods and statistics were employed for analysis of the data. Only OTUs belonging to phyla present at relative abundances greater than 2% were included in the analyses. Overall difference between community compositions was quantified by calculating their Bray-Curtis dissimilarity (d_{BC}; Bray and Curtis, 1957), based on the

following formula:

$$d_{BC} = \frac{\Sigma_{i=1}^{R} |p_i - q_i|}{\Sigma_{i=1}^{R} (p_i + q_i)}$$

where p and q are the OTU/taxa abundance of the first and second community or dataset respectively, and R is their combined abundance (Lanzén, 2013).

Firstly, detrended correspondence analysis (DCA), an unconstrained ordination method (Hill and Gauch, 1980), was applied on the dataset to uncover the inherent gradients (i.e. directions of changes) in the community compositions. The first ordination axis (i.e. the main gradient) was found to be less than 3 SD units in length (see Results section). The linear method redundancy analysis (RDA; Van Den Wollenberg, 1977) was therefore chosen over unimodal methods for constraining the ordination axes to the environmental variables, namely plot type (graminoid removal or control), mean summer temperature, and annual precipitation level. RDA is the constrained version of the ordination method principal components analysis (PCA; ter Braak, 1994). Constrained ordination allowed for statistical testing (i.e. Monte Carlo permutation test) of formulated hypotheses. For more details, please refer to *appendix C.*

Alpha diversity analysis

QIIME, through the script alpha_diversity.py, could also provide estimates of alpha, or within-sample, diversity by calculating a wide selection of metrics on the OTU table. Prior to doing this, the OTU table had to be rarefied to an even sub-sampling depth (i.e. minimum no. of sequence reads) to avoid bias in the estimations, considering that there was a considerable range in the numbers of sequence reads across the samples (21 772 – 155 222 reads; see Result section I). For this study on microbial communities, the metrics Chao1 and Shannon-Wiener diversity index *(H')* were chosen as estimators of richness, whilst the evenness of the communities was estimated by Simpson's evenness *(E)* index. The numbers of OTUs and singletons observed in every sample were also computed through the same script. Linear mixed-effects models were then built to assess whether graminoid removal, or the mean summer temperature and annual precipitation level, had any significant relationship with these measures of alpha diversity. For more details, please refer to *appendix C.*

RESULTS

I. Sequence reads

A sum of 20 million raw reads were produced from the Illumina sequencing run of 16S rRNA gene amplicons from 75 samples (including 1 redundant sample and 2 pseudoreplicates). Out of this sum, only 5 409 021 reads remained after processing to obtain high-quality (Phred score > 27) paired-end reads more than 200 bases long. An example set of reports from the quality control tool FastQC (Babraham Bioinformatics) before and after processing of sequence reads are available in *appendix D*. The processed reads ranged from 21 772 to 155 222, averaging at 72 120 reads per sample. Clustering of these reads based on 97% sequence similarity resulted in a total of 336 493 unique OTUs. Excluding the redundant sample and pseudoreplicates, the lowest number of OTUs observed in a sample was 4 867 and the highest observed was 28 787. The number of OTUs across the samples averaged at 11 813 (SD = 3 886). 6% to 24% (mean = 12%, SD = 3.5%) of the OTUs in the samples were singletons. Individual data for all the samples are provided in *appendix E*.

II. Microbial community composition

Out of a total of 53 bacterial and archaeal phyla detected in the soil samples (as listed in *table 4)*, seven were identified to be present at the mean relative abundance of at least 2% in all of the soil samples. All of these seven phyla belonged to the bacterial domain. The most abundant group of bacteria across all samples belonged to the phylum proteobacteria, followed by the phyla acidobacteria, actinobacteria, bacteroidetes, verrucomicrobia, planctomycetes, and chloroflexi. In addition, four phyla were detected to be occasionally present at the relative abundance of at least 2%. These were nitrospirae, AD3, gemmatimonadetes, and firmicutes.

Table 4. List of bacterial and archael phyla detected in the soil samples.

Count	Kingdom	Phylum	Count	Kingdom	Phylum
1	Bacteria	Proteobacteria	29	Bacteria	Spirochaetes
2	Bacteria	Acidobacteria	30	Bacteria	BRC1
3	Bacteria	Actinobacteria	31	Archaea	[Parvarchaeota]
4	Bacteria	Bacteroidetes	32	Bacteria	OD1
5	Bacteria	Verrucomicrobia	33	Bacteria	NKB19
6	Bacteria	Planctomycetes	34	Bacteria	Chlamydiae
7	Bacteria	Chloroflexi	35	Bacteria	FBP
8	Bacteria	Nitrospirae	36	Bacteria	MVP – 21
9	Bacteria	AD3	37	Bacteria	[Thermi]
10	Bacteria	Gemmatimonadetes	38	Bacteria	OP11
11	Bacteria	Firmicutes	39	Bacteria	GOUTA4
12	Bacteria	WS3	40	Bacteria	Lentisphaerae
13	Bacteria	WPS – 2	41	Archaea	Euryarchaeota
14	Bacteria	Armatimonadetes	42	Bacteria	GAL15
15	Bacteria	Cyanobacteria	43	Bacteria	SR1
16	Bacteria	Elusimicrobia	44	Bacteria	PAUC34f
17	Archaea	Crenarchaeota	45	Bacteria	ZB3
18	Bacteria	TM6	46	Bacteria	WS5
19	Bacteria	TM7	47	Bacteria	OC31
20	Bacteria	Fibrobacteres	48	Bacteria	SBR1093
21	Bacteria	Chlorobi	49	Bacteria	NC10
22	Bacteria	OP3	50	Bacteria	SAR406
23	Bacteria	FCPU426	51	Bacteria	WS4
24	Bacteria	GN02	52	Unassigned,Other	
25	Bacteria	WS2	53	Bacteria	
26	Bacteria	Tenericutes	54	Bacteria	Other
27	Bacteria	GN04			
28	Bacteria	BHI80 – 139			

Fig. 7 presents a visual comparison of the averaged bacterial phyla compositions in soil samples from the graminoid removal (treatment) and control plots, across the different mean summer temperatures and annual precipitation levels that characterize the sites. Details regarding these averaged phyla compositions, such as the mean and standard deviation values, are provided in *appendix F*. Looking at *fig. 7*, the stacked bars for the treatment and control

plots look rather similar to one another at every site, taking into account natural variations in the data. Differences in the stacked bars are more apparent when comparing the different temperature and precipitation levels. While it is difficult to visually discern any difference in mean relative abundance for many of the phyla, there are a couple clear trends in the figure. Actinobacteria appeared to decrease in abundance with increasing precipitation. Acidobacteria, on the other hand, appeared to increase in abundance with increasing precipitation, particularly at the warmest sites. In regards to comparison between the control and treatment plots, only the driest sub-alpine site Ålrust (ALR; precipitation: 0.6 m, temperature: 8.5°C) showed a more obvious difference. The phyla appeared to be more evenly distributed in the treatment plots than in the control plots. There was also a marked increase in the relative abundance of bacteroidetes, and a decrease in the relative abundance of acidobacteria, with graminoid removal. More figures showing the microbial compositions at class and genus levels for every individual soil sample analysed in this study are provided in *appendix G*. Similar findings can be drawn from studying these figures. With the exception of Ålrust, there is hardly any noticeable difference in the stacked bars amongst the replicates and between plot types.

To better visualize any correlation between the phyla and mean summer temperature or annual precipitation level, the two variables were plotted against each other in *fig. 8*. The scatter plot shows that the following phyla may positively correlate with mean summer temperature: acidobacteria, verrucomicrobia, bacteroidetes, planctomycetes, firmicutes, and AD3, while proteobacteria and chloroflexi appeared to become less abundant as it got warmer. As for the annual precipitation level, the following phyla appeared to correlate positively: proteobacteria, acidobacteria, planctomycetes, chloroflexi, and nitrospirae. Actinobacteria and bacteroidetes, on the other hand, appeared to negatively correlate with precipitation.

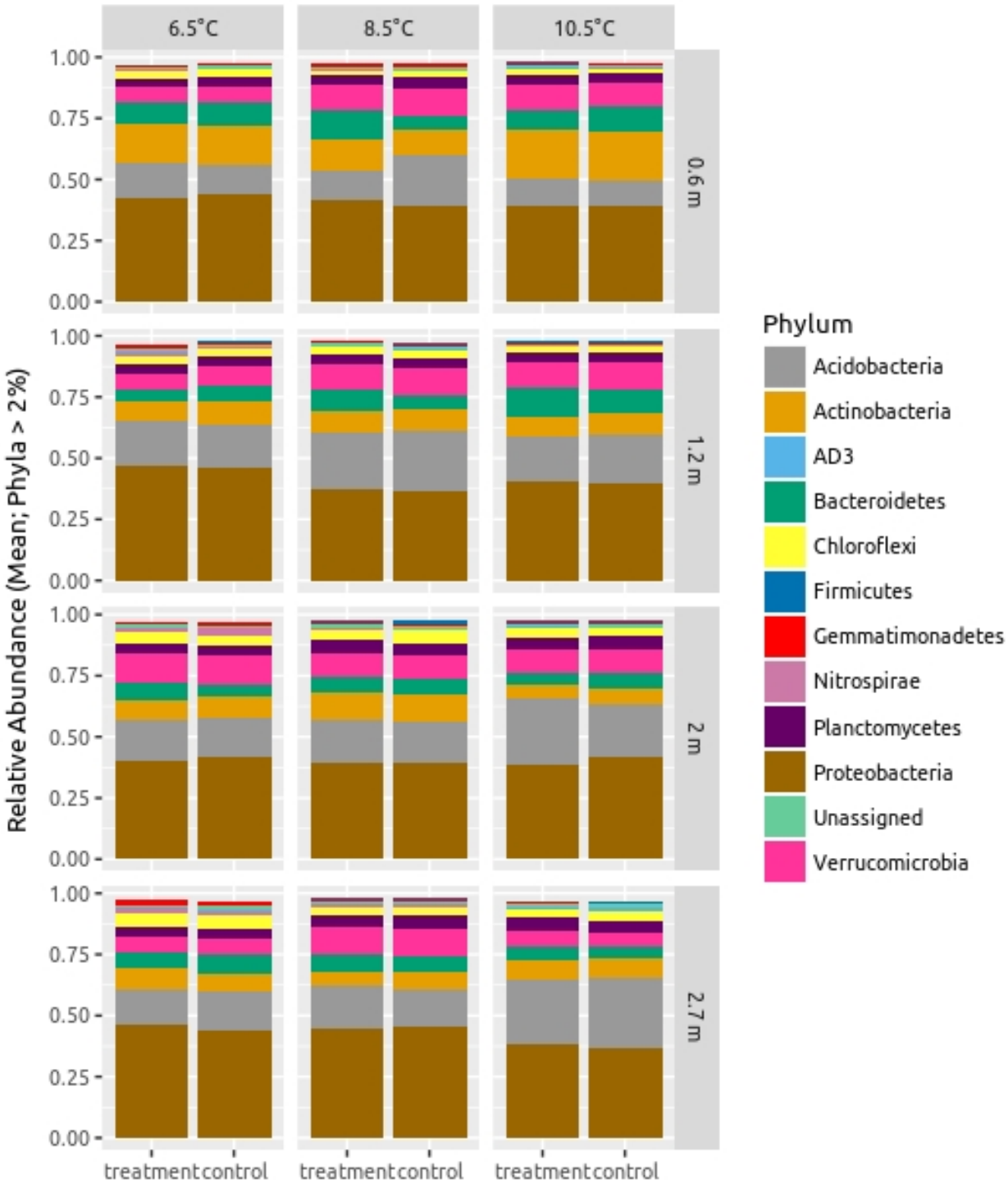


Figure 7. Stacked bar charts showing the compositions, in terms of mean relative abundances, of common (>0.02) bacterial phyla found in the control and graminoid removal (treatment) soil samples (n=3) at twelve sites, each having a unique set of mean summer temperature (6.5, 8.5, 10.5°C) and annual precipitation level (0.6, 1.2, 2, 2.7 m).

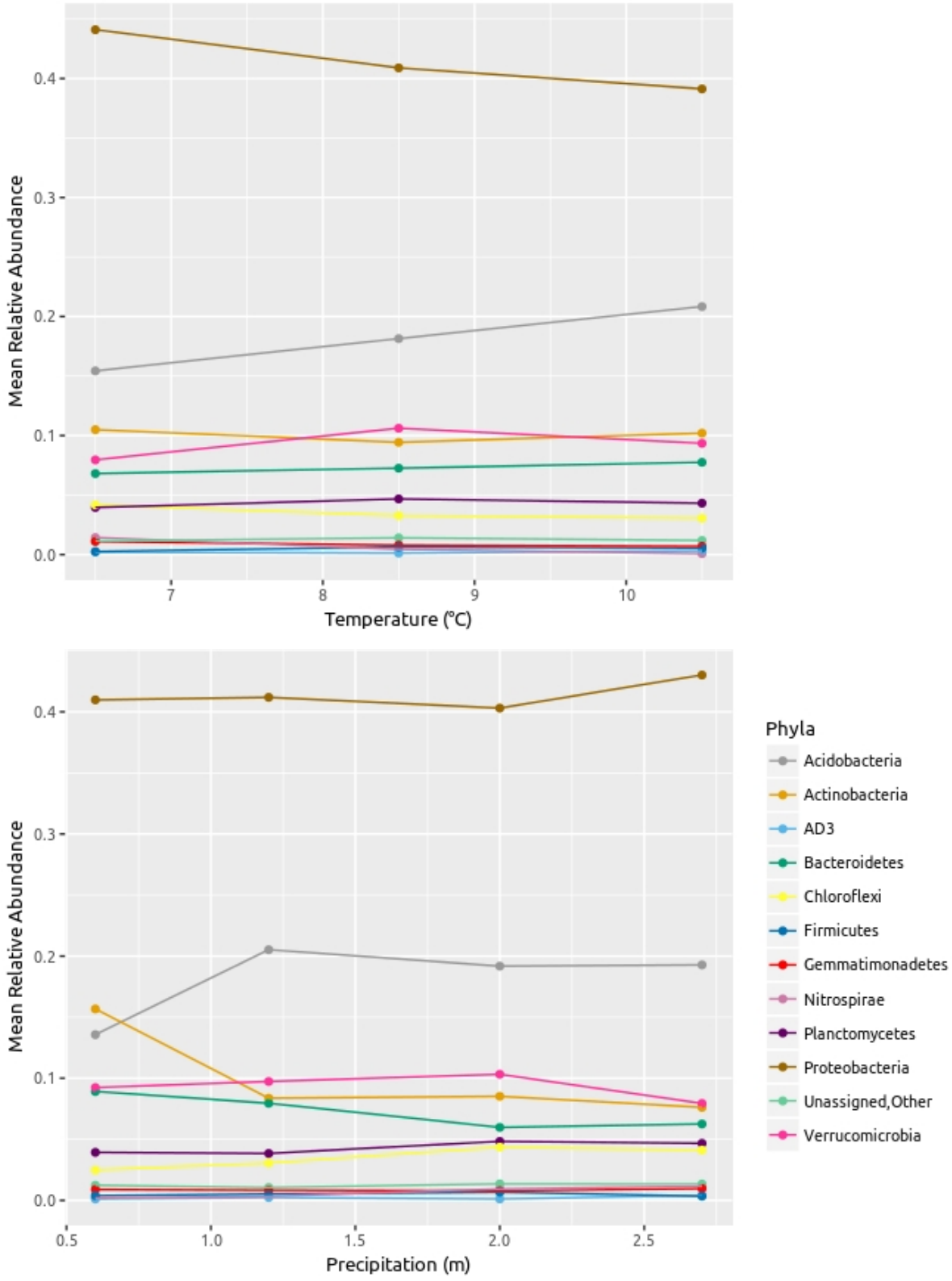


Figure 8. Mean relative abundances of common (>0.02) bacterial phyla in soil samples at different levels of mean summer temperature (top, n=24) and annual precipitation (bottom, n=18).

III. Beta diversity analysis: Constrained ordination analysis

Redundancy analysis (RDA) was chosen as the suitable constrained ordination method after the community data were found to have short gradients under the method detrended correspondence analysis (e.g. length of first axis DCA1 = 0.99691). The RDA found no statistically significant difference in the overall composition of the microbial community, specifically the common phyla that made up >95% of the community, between the treatment and control plots across the sites ($F = 0.1847$, $df = 1$, $p = 0.945$). This result was consistent with the visual assessment of the microbial communities between the two types of plot in *fig. 7* (and *appendix G*). The RDA did, however, reveal significant variation in the microbial communities along the gradients of temperature and precipitation ($F = 8.7832$, $df = 2$, $p = 0.005$). With the plot type partialled out as a covariable, the two constraining variables (temperature and precipitation) combined explained 20.5% of the variance. Individually, temperature accounted for 9.34% of the variance and precipitation accounted for 11.1% of the variance.

The constrained ordination diagrams are presented in *fig. 9(a)*, with the plot for the taxa magnified in *fig. 9(b)*. The diagrams show that the first ordination axis (12.1%) was related to precipitation, meaning that precipitation was the more dominant factor out of the two climate variables in influencing the soil prokaryotic community composition. The ordination of the samples in the diagram was deemed to be more or less consistent with what was already known about the sites. Samples belonging to the driest sites FAU, ULV and ALR were separated out on the left side of the diagram, while samples belonging to the wetter sites ARH and OVS were on the right. The second ordination axis (8.5%) was related to temperature, with samples from the alpine sites SKJ and LAV separated out on the bottom side of the diagram and samples from the boreal sites ARH and OVS separated out on the top. The ordination of phyla in *fig. 9(b)* also revealed results that confirmed some of the observations made from *fig. 8*. For example, acidobacteria were more abundant in soils from warmer and wetter sites. The abundance of actinobacteria, on the other hand, was lower at the wetter sites. Proteobacteria were found to negatively correlate with temperature. The plot also shows that the phyla AD3, firmicutes, planctomycetes and gemmatimonadetes had similar occurrence pattern in the soil samples.

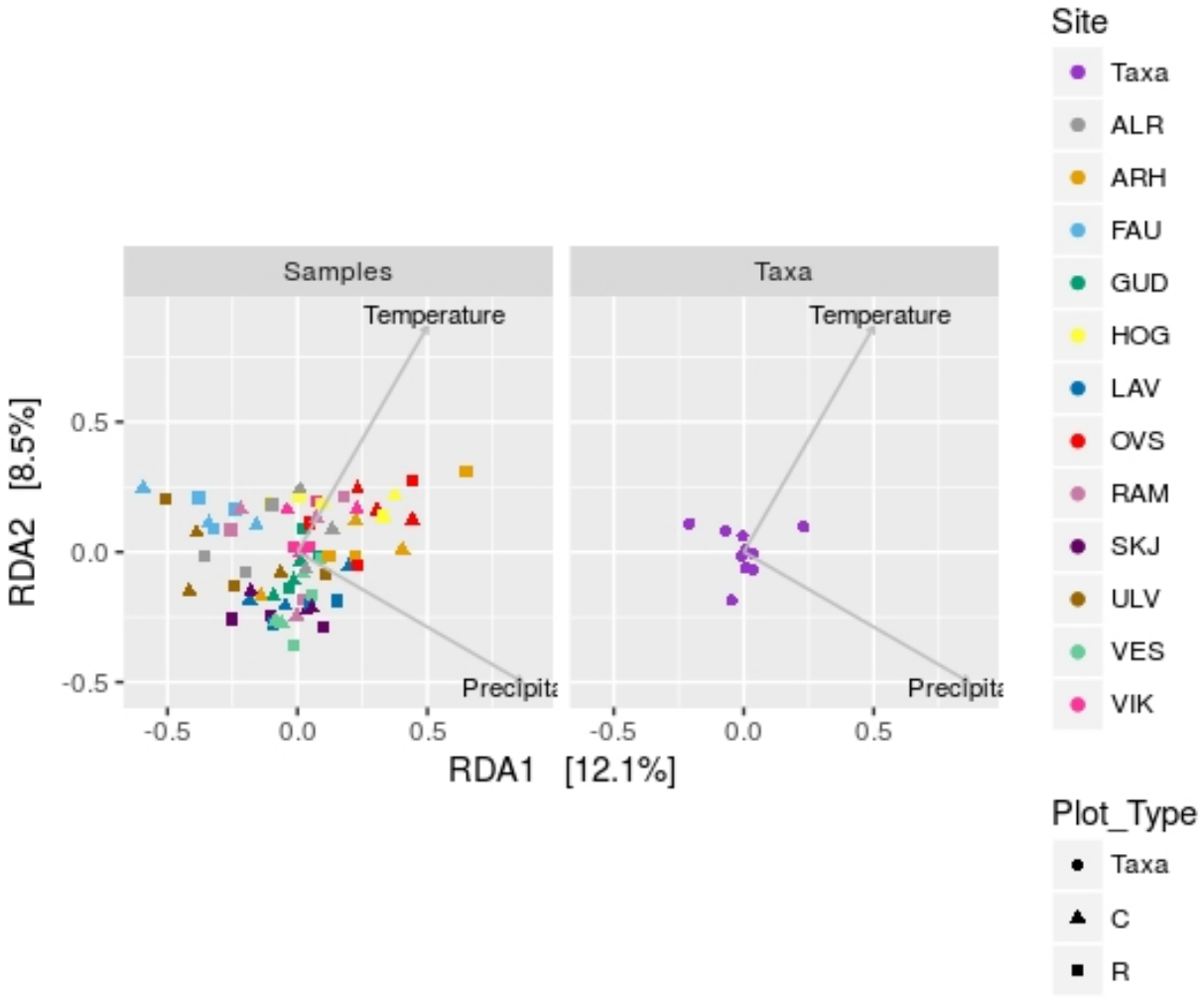


Fig. 9(a): Ordination diagram based on redundancy analysis (RDA) of 12 bacterial phyla (including 1 unassigned group) in 72 soil samples, constrained to the environmental variables mean summer temperature and annual precipitation level (arrows). The plot is split between samples (colour for site, shape for plot type, C = control, R = graminoid removal) and taxa (phyla), and both share the same axis scales.

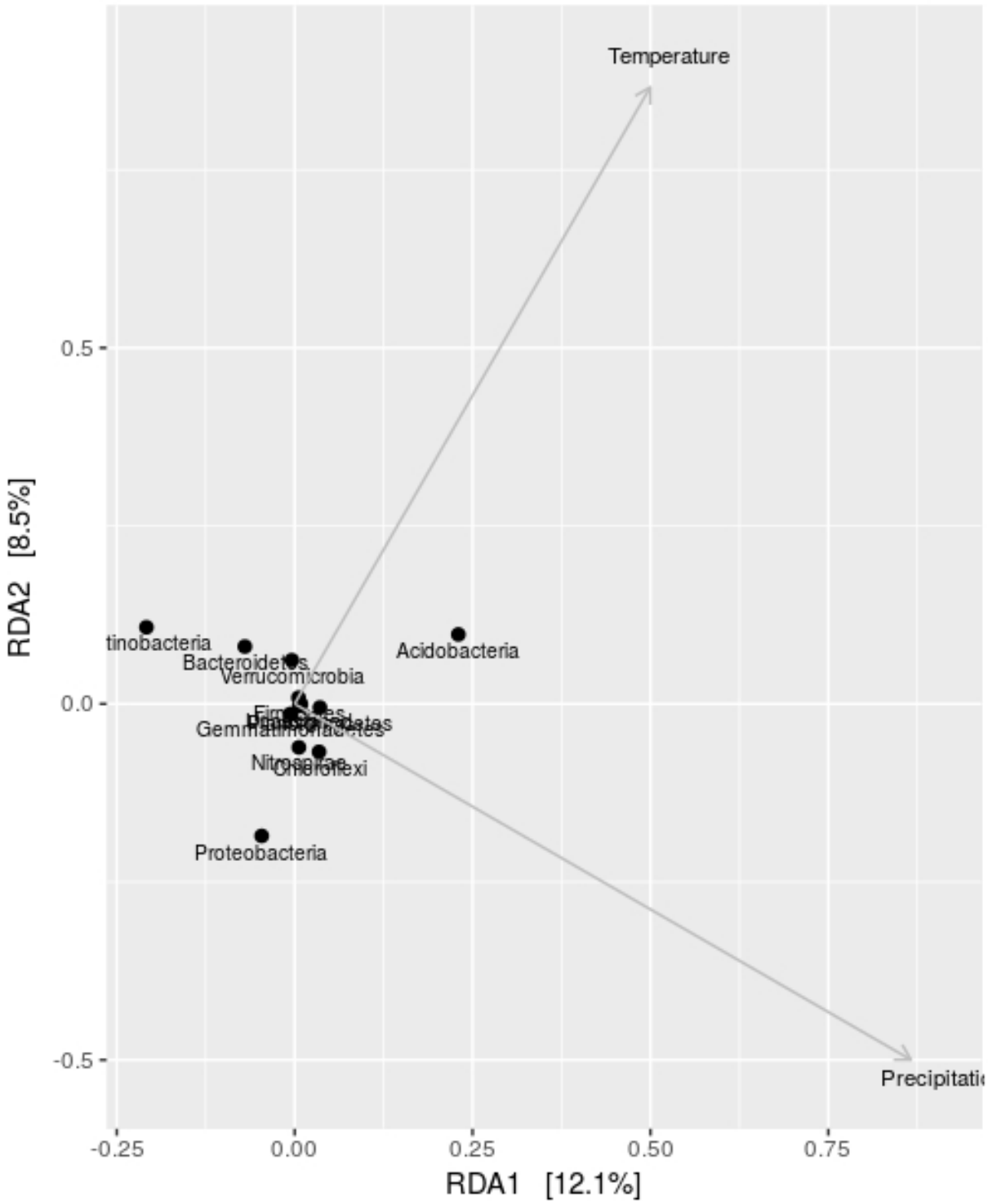


Fig. 9(b): Ordination plot based on redundancy analysis (RDA) of 12 bacterial phyla, including one unassigned group, in 72 soil samples, with the constraining variables mean summer temperature and annual precipitation levels represented as arrows.

IV. Alpha diversity analysis

A number of alpha diversity metrics were calculated on the rarefied (sub-sampled) OTU table through the QIIME pipeline. The metrics Chao1, Shannon *H'* Index and the number of observed OTUs provided estimations of richness, while Simpson´s *E* provided a measure of evenness. Box plots showing how these estimates compare between the two plot types and across the different levels of mean summer temperature and annual precipitation are presented in *fig. 10 – 13.* There were a few notable observations that could be derived from these figures. Firstly, all of the diversity metrics (richness and evenness) clearly dropped as the annual precipitation level increased from 0.6 m (driest) to 1.2 m. Both Shannon diversity and Simpson´s evenness also appeared to decrease with increasing temperature. Graminoid removal, however, hardly appeared to have any effect on the community richness and evenness.

ANOVA testing of the linear mixed-effects models built on these metrics, however, did not reveal significant outcome to indicate that graminoid removal, temperature and precipitation affected the richness of the microbial community (no. of observed OTUs, Chao1 and Shannon diversity). The only significant result from this analysis is the relationship between the (Simpson's) evenness of the microbial community and temperature ($F = 6.5241$, $df = 10$, $p = 0.0287$; *fig. 13b*).

(a)

(b)

(c)

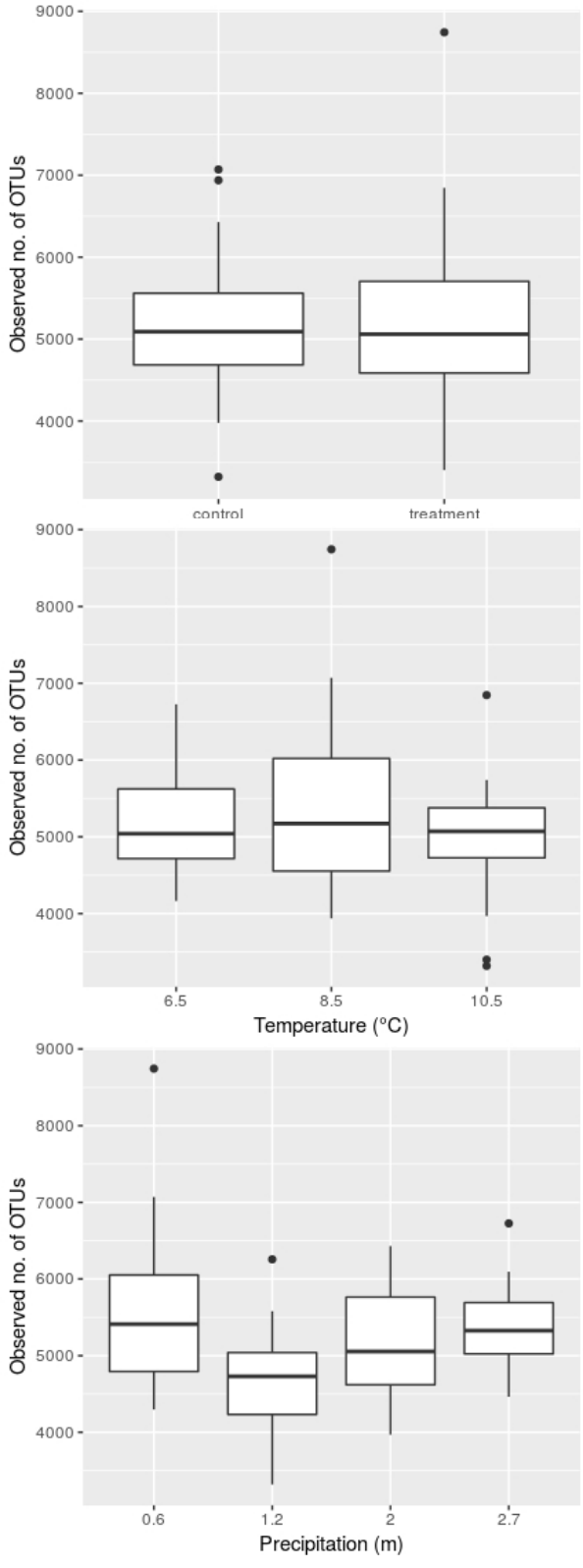


Figure 10. Box plots showing the number of OTUs observed in soil samples from (a) graminoid removal and control plots (n=36), (b) sites with three levels of mean summer temperature (n=24), and (c) sites with four levels of annual precipitation (n=18).

(a)

(b)

(c)

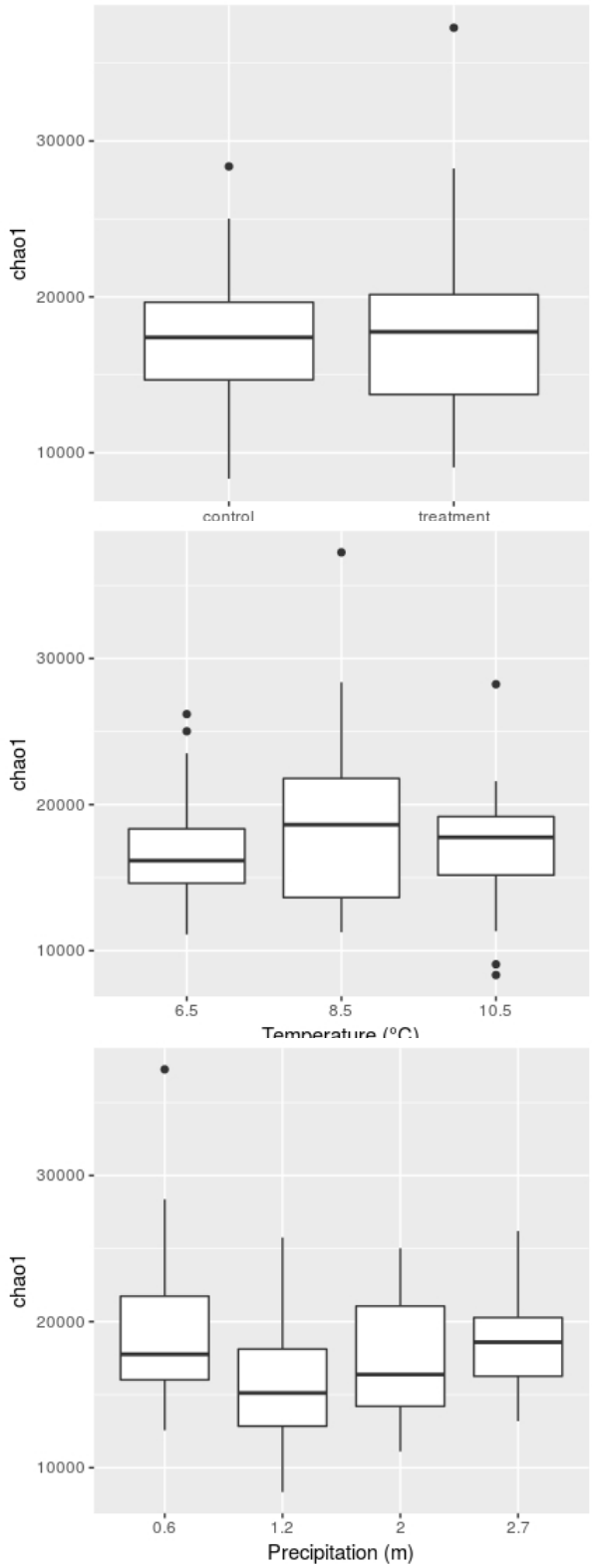
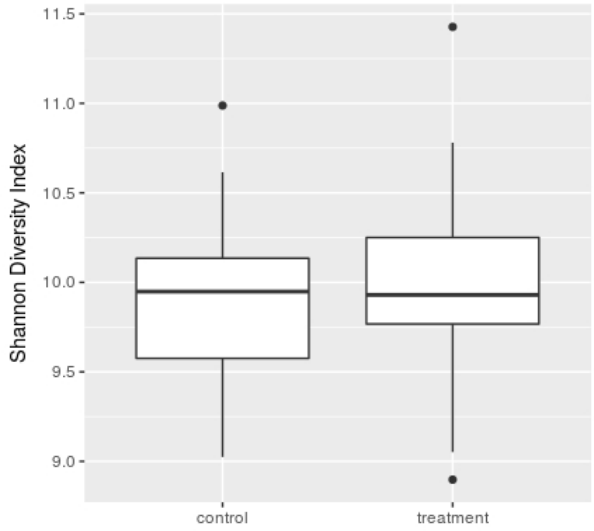
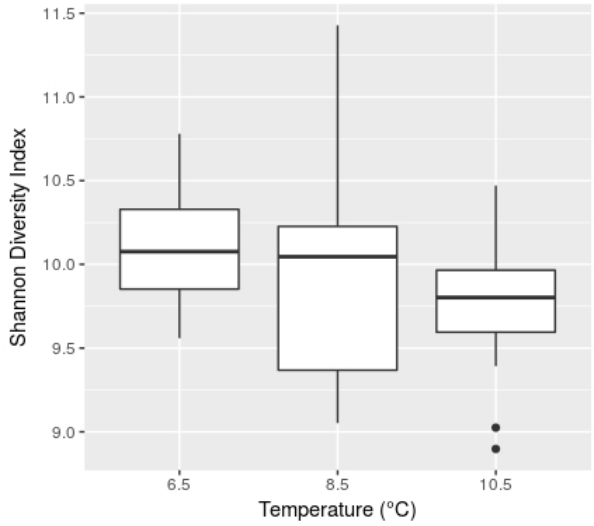


Figure 11. Box plots of Chao1 richness estimate of soil prokaryotic communities in samples from (a) graminoid removal and control plots (n=36), (b) sites with three levels of mean summer temperature (n=24), and (c) sites with four levels of annual precipitation (n=18).

(a)



(b)



(c)

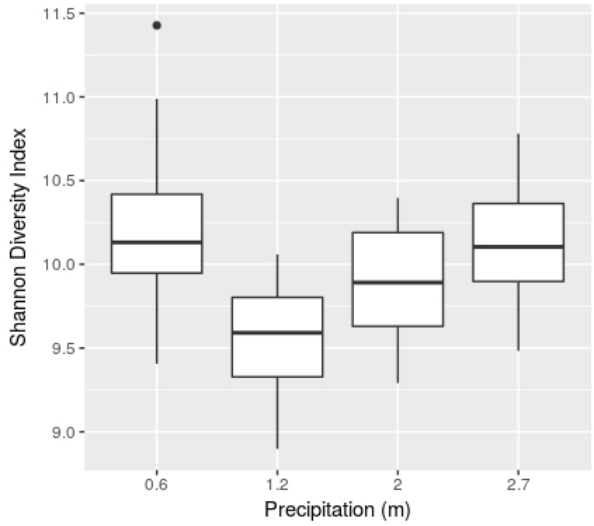
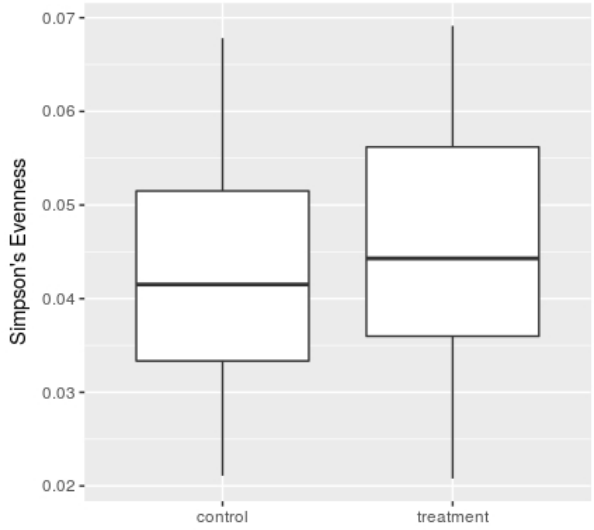
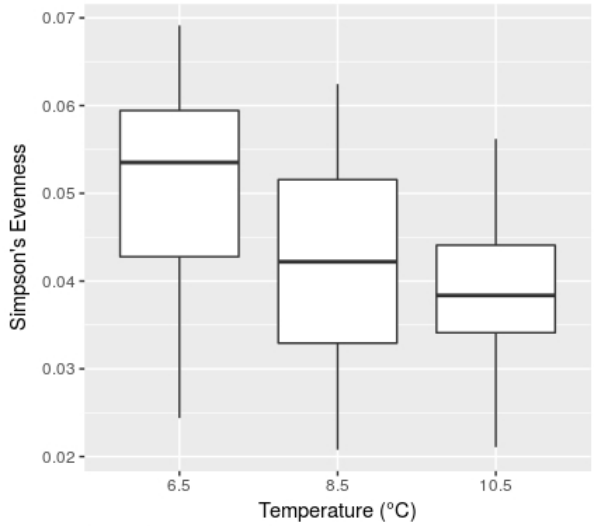


Figure 12. Box plots of Shannon diversity index of soil prokaryotic communities in samples from (a) graminoid removal and control plots (n=36), (b) sites with three levels of mean summer temperature (n=24), and (c) sites with four levels of annual precipitation (n=18).

(a)



(b)



(c)

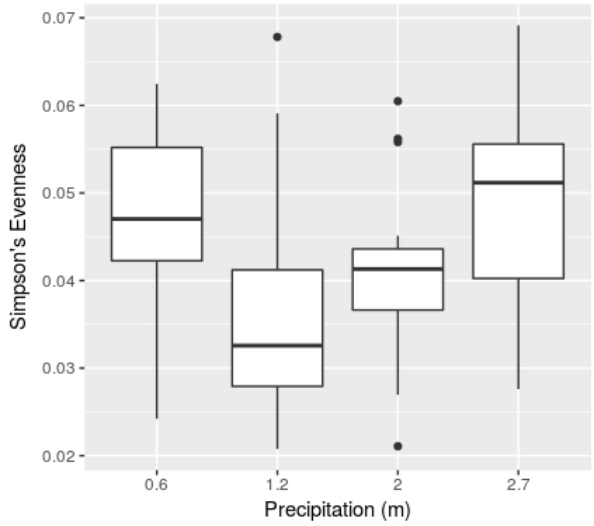


Figure 13. Box plots of Simpson's evenness of soil prokaryotic communities in samples from (a) graminoid removal and control plots (n=36), (b)* sites with three levels of mean summer temperature (n=24), and (c) sites with four levels of annual precipitation (n=18).

*statistically significant

DISCUSSION

I. Response of soil prokaryotic community to graminoid removal

The results showed that five years of above-ground removal of graminoids had no effect on the overall (>95%) composition of the soil prokaryotic community in the boreal and alpine semi-natural grasslands. There were a few possible scenarios behind this. Firstly, graminoid removal may not have changed the overall nutrient content of the litter the plot produced, such that the group of decomposers that broke down this litter remained largely the same. This could be ascertained by gathering and analyzing more data on relevant variables, such as the carbon and nitrogen content in the undecomposed plant litter and in the soil. Secondly, graminoid removal may have initially prompted a more drastic response from the prokaryotic community, but the community may have eventually recovered or re-stabilized itself over the years. This may be an indication of a negative feedback at work. A comparable study by Urcelay *et al.* (2009) lent support to this recovery hypothesis, as the study found that graminoid removal in a woody ecosytem significantly reduced the total colonization of plant roots by arbuscular mycorrhizal fungi after five months, but this initial response eventually disappeared after 17 and 29 months. Thirdly, no effect of graminoid removal was detected on the common taxa that made up the majority of the community and on phylum level. A further probe into the rare taxa and individual genuses in these communities may have revealed interesting findings to add to this study.

In any case, the results provided evidence to suggest that soil prokaryotic community in the semi-natural grassland ecosystem possessed a considerable level of ecological resilience to natural or anthropogenic environmental changes. Ecological resilience in this context could be interpreted as the ability of the community to resist or recover from the disturbance of graminoid removal so as to retain the same structure and functions (Holling, 1973; Hodgson *et al.*, 2015). This finding thus concurred with the conclusion drawn from another similar study (Marshall *et al.,* 2011) set in a northern Canadian grassland, which also found almost no response from the soil microbial community – as measured through their substrate-induced respiration (i.e. a proxy for metabolic diversity) and phospholipid fatty acid analysis

(i.e. a proxy for community composition) – after five years of graminoid removal. This may be a positive news, in light of the prospect of increased graminoid dominance under a warmer climate (Olsen *et al.,* 2016). If the removal of graminoids did not yield any significant impact on the community structure and functions of soil prokaryotes, then the reverse scenario – increased graminoid dominance – would likely not either. Such stability may help impede further climate change impacts on the semi-natural grassland ecosystems.

II. Shifts in soil prokaryotic community along temperature and precipitation gradients

While no response to graminoid removal was detected from the results, this was not the case for the climate variables of temperature and precipitation. The prokaryotic community composition was found to vary significantly with the two variables. Ordination analysis of the data further determined that precipitation predominated slightly over temperature in shaping the community composition of soil prokaryotes in the semi-natural grasslands, with 11.1% of the observed variance in data attributed to precipitation while temperature explained 9.34% of the variance. This left approximately 80% of the variance to be explained by other factors and random variation. It was perhaps not a surprise to find that the prokaryotic community shifted along the gradients of temperature and precipitation, as prokaryotes – or any living organism – were known to vary in the ranges of environmental conditions they could survive or grow optimally in (Madigan and Martinko, 2006). In addition to the direct effects, temperature and precipitation (or water availability) could also have exerted further effects indirectly, through other biotic components of the ecosystem (e.g. plants) that interacted with the soil prokaryotic community.

Water availability has been recognized as one of the primary determinants of soil microbial community composition and activities (Drenovsky *et al.,* 2004; Madigan and Martinko, 2006). The latter, which includes microbial respiration and growth, has been discussed briefly in the introduction to this thesis. Water is an essential component of physiological structures and processes. Extreme lack of water, such as in dessication experiments, had been demonstrated to cause damage to microbial cells, leading to a decline in activity (Potts, 1994). Drought conditions may thus select for microorganisms that are able to tolerate the extreme dryness by

producing protective molecules such as osmolytes (Warren, 2014). A recent study by Meisner *et al.* (2018) also showed how drought and re-wetting events left impacts on the microbiome composition that persisted long after the events. For example, the study found that the archaeal phylum Thaumarchaeota, known to thrive in dessert soils and other extreme environments, was particularly abundant in soil with a history of drought.

Water also mediates impacts by regulating the oxygen and nutrient availabilities in soils (Drenovsky *et al.,* 2004). Water is necessary for microorganisms and plants to access and absorb nutrients (Viets, 1972). Adequate amount of water in well drained soil can also sustain high level of oxygen for microorganisms. In contrast, the oxygen concentration of soil solution in waterlogged condition is usually very low due to the relatively quick consumption of dissolved oxygen without sufficient replenishment (Madigan and Martinko, 2006). The latter would have been compounded by abundant presence of organic matter or nutrition in the soil solution, which further increases the biological demand for oxygen and eventually selects for facultative and obligate anaerobic microorganisms (Drenovsky *et al.,* 2004).

Temperature is another important environmental factor affecting microorganisms, particularly on microbial growth. Although growth and community assemblage are two separate processes, they are ultimately and intimately related. Every microorganism has a minimum temperature below which growth cannot occur, an optimum temperature at which enzyme-mediated physiological processes occur at maximal rates, and a maximum temperature above which proteins denature and the cell lyses (Madigan and Martinko, 2006). Although these three cardinal temperatures could differ greatly for different microorganisms, it was speculated that many of the soil prokaryotes in this study shared similar or overlapping ranges of temperature for growth, such to allow precipitation – or more precisely, water availability – to be the more important factor in affecting the prokaryotic communities, at least on the phylum level and within the temperature range studied here (mean summer temperature = 5.87–10.8°C).

The results revealed that the relative abundance of acidobacteria increased with increasing temperature and precipitation. Proteobacteria, on the other hand, were observed to become less abundant as the temperature rose. Considering that proteobacteria may be *r*-selected and

acidobacteria may be *K*-selected (Torsvik and Øvreås, 2002), a parallel could be drawn between this result and that of the study by Olsen *et al.* (2016). Studies have proposed that (α-, β-, γ-) proteobacteria are *r*-strategists and are adapted to thrive in less-crowded environments with plentiful resources by growing and reproducing rapidly. Acidobacteria, meanwhile, are *K*-strategists in that they are adapted to compete successfully for limited resources at the expense of growth (Smit *et al.,* 2001; Fierer *et al.,* 2007). The observed increase in dominance of acidobacteria, and decrease in proteobacteria, could thus be indicative of a more competitive environment for the prokaryotes at the warmer sites. This would be congruent with the finding by Olsen *et al.* (2016) that warming brought about a shift in the net outcome of interactions amongst the plants from facilitation to competition.

The only statistically significant result from the alpha diversity analysis was the decrease in evenness (Simpson's *E*) of the community with increasing temperature. This could perhaps be linked to the observed increase in dominance of competitive bacteria, particularly acidobacteria, and the probable shift towards a more competitive environment in general, as the temperature rose. There was also a notable trend, consistent across the calculated estimates of alpha diversity, that richness and evenness dropped considerably as the annual precipitation level rose from 0.6 m (driest) to 1.2 m. While the statistical test did not find this trend to be significant, it was nonetheless an interesting observation worth looking further into. It was to be noted that all three of the driest sites (ULV, ALR, FAU) were located at the eastern end of the site range, roughly 100 km away from the next driest sites (LAV, HOG, VIK) *(fig. 6)*. Considering that these sites were relatively farther away from all of the other sites, other factors that differentiated these sites may have a stronger role in influencing the diversity of their prokaryotic communities.

III. Discussion on uncertainties in data

In any case, it is important to take into account the (un)reliability of the diversity estimates when drawing conclusions from the results. Firstly, the diversity estimates analysed in this study were calculated from an OTU table that was rarefied only once (i.e. one random sub-sample of the entire data), which meant that a portion of the available data was not included

in calculating the estimates. Repeating the rarefaction multiple times (e.g. 100 times) would have improved the reliability of the data and confidence in the results. The choice of alpha diversity metrics could also have influence on the findings of this study, as different metrics may not be equally sensitive to the underlying distributions of the taxa nor produce the same outcome. The non-parametric estimator Chao1 and Shannon *H'* and Simpson's *E* indices were chosen for this study based on recommendation from other studies on bacterial communities (Hill *et al.,* 2003; Lanzén, 2013). However, the only and closest possible way to verify that these chosen metrics reflected true diversity of the data, or at least capture the true differences or trends within the dataset, would be to run the post-sequencing processing and analyses on a similar but artificial (mock) community with pre-defined diversity.

As discussed briefly in the introduction to this thesis, the assessment of diversity is particularly challenging in microbiology. One aspect that lends to this challenge is in obtaining adequate sample size and/or sequencing depth, especially for samples from highly complex environments such as the soil. The loss of a substantial portion of the raw sequence reads during processing (mean recovery rate = 52.3%) first raised the concern of insufficient sequencing depth. This was confirmed for many of the samples, as their rarefaction curves *(appendix H)* had not even begun to flatten out. This meant that the data would highly underestimate the true diversity of the prokaryotic community in the soil samples. Fortunately, this may not as severely affect the analysis of trends in community composition and diversity, as a study by Lundin *et al.* (2012) had demonstrated that a relatively small number of sequence reads could be enough to uncover the majority of trends when comparing compositions (i.e. 1000 denoised sequences per sample to explain 90% of trends in β-diversity) and diversities (i.e. 5000 denoised sequences per sample to explain 80% of trends in α-diversity).

Singletons were found to contribute 6–24% (mean = 12%, SD = 3.5%) of OTUs in the analysed samples. Singletons are a subject of contention amongst microbial ecologists, as their presence could arise from either genuine rare taxa or errors introduced during PCR or sequencing. The latter would have erroneously increased OTU richness of the community. As explained earlier in the methodology section of this thesis, the presence of singletons should not matter much for most of the analyses in this study, since many of the singletons would

have been excluded from the rarefied (sub-sampled) OTU table or filtered out (i.e. mean relative abundance of phylum <2%) from the compositional data.

IV. The next steps

Time constraint limited the depth of analyses that could be done for this thesis. Much yet remains to be uncovered from the millions of sequences obtained from the high-throughput sequencing of the 16S rRNA genes. The next step would be to further analyse the community data, down to the class and genus levels. This would, for example, reveal which classes of proteobacteria dominated in the soil samples and which had responded significantly to the climate variables. Analyses at such higher resolutions are likely to unveil valuable or interesting findings to add to this study. Another step would be to probe into the rare taxa, particularly those that are highly relevant to the carbon flux of the terrestrial ecosystem. One such example is the archaeal class methanobacteria, which are known to produce methane as an essential part of their metabolism (Madigan and Martinko, 2006). A quick look into the data revealed presence of these methanogens in soil samples from the site VES, which received on average the highest level of precipitation amongst all of the sites *(table 1)*.

To improve the accuracy of the diversity estimations, the alpha diversity metrics should be re-calculated on multiple (e.g. 100) sub-samples of the OTU table. A number of bioinformatics tools or methods have also been developed to minimise bias and noise in the sequence data (Lanzén, 2013). Examples include AmpliconNoise (Quince *et al.,* 2011) and amplicon sequence variants (ASVs) methods (Callahan *et al.,* 2017). It might be worthy to explore the usage of such tools or methods on the sequence reads.

Althuizen *et al.* (2018) had gathered more data characterizing the soils at the study sites, such as the soil pH and the carbon and nitrogen content. The addition of such relevant environmental variables into the analyses may lead to further insights into the network of abiotic interactions in the ecosystem. A number of future studies can also be suggested, considering that there are remaining soil samples stored frozen. Analysis of the fungal community in the soils, such through amplification and sequencing of the 18S rRNA genes

from the environmental DNA, would be a suitable complement to this study. The shift of focus onto the active fraction of the microbial communities, such by extracting mRNA from the samples, or onto a specific ecological function of interest, such through real-time PCR of a specific target (e.g. methanogens), may also pave the way for valuable, new findings.

CONCLUSIONS

The lack of response to five years of removal of graminoids in the semi-natural grasslands signified some level of resilience of the soil prokaryotic communities to major environmental disturbances. In contrast, the soil prokaryotic communities varied significantly along the natural gradients of temperature and precipitation across the western Norwegian fjord landscapes. Such conflicting responses perhaps give a glimpse into the complexity of biotic and abiotic interactions that run the terrestrial ecosystem.

To the best my knowledge, this study is the first to apply the approach of high-throughput sequencing of the 16S rRNA gene to examine the composition and diversity of soil prokaryotic communities in a plant removal experiment.

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Appendix A

Table listing all the soil samples analysed in this study. The columns correspond to (*l* to *r*): sample no. and sequencing ID (No.), sample label, plot ID, plot type, person handling the sample (I.I.; JT = Jesslyn Tjendra, WG = William Garcia), soil mass (S.M.; g), concentration of 1st elution of DNA extract ([D1]; ng/µL), concentration of 2nd elution of DNA extract ([D2]; ng/µL), concentration of 1st PCR amplicon ([P1]; ng/µL), concentration of 2nd PCR amplicon ([P2]; ng/µL), sample purity $A_{260/280}$, sample purity $A_{260/230}$. NA = not available.

No.	Label	Plot ID	Plot Type	I.I.	S.M.	[D1]	[D2]	[P1]	[P2]	260/280	260/230
15	ALR II C1	TTC31	Control	JT	NA	21,2	4,64	0,88	49,3	1,88	2,17
25	ALR II R1	RTC	Removal	JT	NA	1,35	0,72	1,39	45,5	1,96	2,4
56	ALR III C2 (48)	TTC37	Control	WG	0,25	16,4	5,68	2,46	28,2	1,83	2,22
55	ALR III R2 (45)	RTC	Removal	WG	0,24	7,48	5,88	3,73	30,1	1,83	2,17
88	ALR IV C2 (54)	TTC45	Control	WG	NA	NA	9,04	6,08	16,7	NA	NA
87	ALR IV R2 (51)	RTC	Removal	WG	NA	NA	8,2	1,6	24,8	1,93	2,22
86	ARH I C1 (278)	TTC211	Control	WG	0,6	23,2	5,4	1,94	22,8	1,93	1,72
85	ARH I R1 (275)	RTC	Removal	WG	0,6	22,4	4,28	2,51	22	1,99	2,29
72	ARH II C2 (285)	TTC216 (Cc:216)	Control	WG	0,27	23,6	6,92	2,37	19,6	1,8	2,17
68	ARH II R2 (282)	RTC	Removal	WG	0,26	28,4	4,64	1,45	14,5	1,88	2,31
47	ARH III C1 (293)	TTC222 (Cc:222)	Control	JT	0,72	24	21,2	1,03	49,9	1,87	2,23
42	ARH III R1 (290)	RTC	Removal	JT	0,74	95,2	16	1,92	44,6	1,9	2,21
19	FAU I C1	TTC51	Control	JT	0,74	18,6	3,86	0,67	38,9	1,85	2,05
18	FAU I R1	RTC	Removal	JT	0,96	19,7	3,16	0,8	38,8	1,93	2,02
22	FAU II C2	TTC57	Control	JT	0,24	1,39	0,89	2,28	40,7	1,94	2,32
21	FAU II R2	RTC	Removal	JT	0,25	1,54	1,42	5,8	41,6	1,87	2,26
58	FAU III C1 (77)	TTC61	Control	WG	0,84	8,44	8,36	11	23,6	1,87	2,22
57	FAU III R1 (74)	RTC	Removal	WG	1	5,08	1,72	1,51	27,7	1,88	2,21
76	GUD II C2 (351)	TTC156 (Cd:156)	Control	WG	0,51	19,8	4,4	3,78	20,3	1,8	1,82
75	GUD II R2 (348)	RTC	Removal	WG	0,54	22	5,08	2,35	19,4	1,82	2,11
35	GUD III C1 (356)	TTC165 (Cd:165)	Control	JT	0,85	112	10,8	2,26	43,5	1,81	2,12
36	GUD III R1 (353)	RTC	Removal	JT	0,83	98	11,4	1,8	44,4	1,84	2,12
78	GUD IV C1 (362)	TTC167	Control	WG	0,53	23,2	8,88	6,86	17,5	1,77	2,13
77	GUD IV R1 (359)	RTC NewSiri	Removal	WG	0,52	19,8	6,56	2,42	12,3	2,07	1,54
60	HOG I C2 (96)	TTC101	Control	WG	0,25	9,72	2,88	2,53	20,4	1,89	2,06
59	HOG I R2 (93)	RTC	Removal	WG	0,24	9,2	3,22	1,77	24,9	1,9	2,24
62	HOG II C1 (101)	TTC110	Control	WG	0,8	42,8	9,28	1,99	31,9	1,83	2,15
61	HOG II R1 (98)	RTC	Removal	WG	0,84	42	12,2	1,54	13,1	1,94	2,2
41	HOG III C1 (107)	TTC115	Control	JT	0,94	27,5	13,2	2,15	45,4	1,9	2,23
27	HOG III R1 (104)	RTC	Removal	JT	0,89	19	9,36	1,58	40,3	1,88	2,08
66	LAV I C1 (155)	TTC78	Control	WG	0,82	101	15,4	0,77	14	1,85	1,89

65	LAV I R1 (152)	RTC	Removal	WG	0,69	19,8	18	0,98	16,8	1,93	2,12
71	LAV II C2 (162)	TTC85	Control	WG	0,52	22	12,2	5,27	21,5	1,83	2,2
67	LAV II R2 (159)	RTC	Removal	WG	0,53	22	6,84	1,31	16,4	1,92	2,28
48	LAV III C1	TTC87	Control	JT	0,81	91,6	22,4	1,34	41,9	1,89	2,11
23	LAV III R1	RTC	Removal	JT	0,75	0,5	1,89	0,53	43,9	1,97	2,33
34	OVS I C1 (314)	TTC286	Control	JT	1,02	18,2	11,1	1,8	43,1	1,85	2,26
39	OVS I R1 (311)	RTC	Removal	JT	1	9,48	5,52	1,46	43,1	1,84	2,21
50	OVS II C2 (321)	TTC291	Control	JT	0,66	98	25	2,4	34,1	1,86	2,06
49	OVS II R2 (318)	RTC	Removal	JT	0,64	129	21,2	1,83	37,6	1,82	2,11
74	OVS III C1 (326)	TTC 297	Control	WG	0,55	88,8	21,4	3,51	19,7	1,88	2,01
73	OVS III R1 (323)	RTC	Removal	WG	0,51	63,6	22	2,38	16,1	1,83	2,27
33	RAM IV C1 (215)	TTC194	Control	JT	0,72	56,4	10,1	1,26	48,1	1,8	2,18
51	RAM IV R1 (212)	RTC	Removal	JT	0,77	121	19,5	2,02	38,6	1,84	2,15
84	RAM V C1 (221)	TTC198	Control	WG	0,6	22	NA	8,86	18	2,03	2,24
82	RAM V R1 (218)	RTC	Removal	WG	0,6	24	20,4	1,75	18	1,98	2,25
29	RAM VI C2 (228)	TTC203 (Cc:203)	Control	JT	0,3	43,2	8,2	1,56	48	1,86	2,22
28	RAM VI R2 (225)	RTC newSiri	Removal	JT	0,24	57,6	9	1,52	43,1	1,84	2,15
45	SKJ I C1	TTC236	Control	JT	0,86	142	21,6	1,14	38,7	1,88	2,06
43	SKJ I R1	RTC NewSiri	Removal	JT	0,81	123	15,8	1,8	39,9	1,88	2,24
17	SKJ II C2	TTC243	Control	JT	0,63	23,6	3,47	0,85	38,9	1,9	2
44	SKJ II R2	RTC NewSiri	Removal	JT	0,6	123	15,6	1,52	43,8	1,84	2,18
70	SKJ III C1 (260)	TTC246 (Cd:246)	Control	WG	0,51	16,8	12,6	11,9	16,5	1,76	2,88
69	SKJ III R1 (257)	RTC	Removal	WG	0,53	69,2	10,6	4,5	20,3	1,99	2,29
26	ULV I C1	TTC5	Control	JT	0,91	0,49	1	0,62	46,8	1,93	2,41
24	ULV I R1	RTC	Removal	JT	0,61	0,61	1,87	0,53	48,1	1,95	2,21
20	ULV II C2	TTC6	Control	JT	0,81	2,78	1,21	0,63	44,8	1,85	2,09
16	ULV II R2	RTC	Removal	JT	0,76	23,4	5,2	0,55	33,7	1,9	2,03
89*	ULV III C1 (16)	TTC11	Control	WG	0,41	22,4	11,7	12	13,2	1,84	2,07
53	ULV V R2 (2)	RTC	Removal	WG	0,54	21,6	10,5	4,63	30,7	1,88	2,15
54	ULV V T232 (5)	TTC23	Control	WG	0,53	21,6	10,9	5,65	28,8	1,87	1,92
81	VES I C1 (185)	TTC263	Control	WG	1,12	18	8,8	3,35	19,6	2,03	2,21
80	VES I R1 (182)	RTC	Removal	WG	0,81	19,9	11	1,27	17,8	2,16	2,56
31	VES II C2 (192)	TTC270	Control	JT	0,32	101	8,68	1,12	42,5	1,85	2,1
83*	VES II C2 (192)	TTC270	Control	WG	0,32	22	16,7	0,87	18,3	1,92	2,18
38	VES II R2 (189)	RTC	Removal	JT	0,3	22,4	6,16	1,35	31,7	1,96	2,19
79*	VES II R2 (189)	RTC	Removal	WG	0,3	21,2	11,7	1,02	14,1	1,98	2,48
52	VES III C1 (197)	TTC271	Control	JT	0,83	24	18,8	1,16	29	1,87	2,11
46	VES III R1 (194)	RTC	Removal	JT	1,22	139	17	1,19	38,3	1,82	2,11
32	VIK I C1	TTC126	Control	JT	0,84	29,1	10,6	1,94	46,1	1,85	2,13

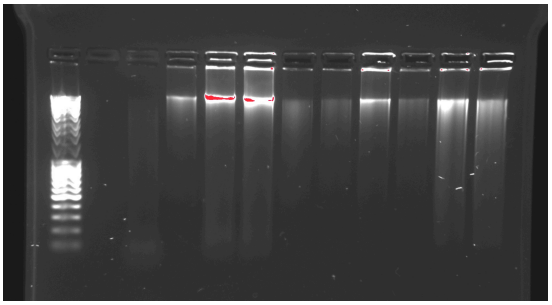
40	VIK I R1	RTC	Removal	JT	0,75	19,8	5,96	2,38	44,8	1,87	2,1
30	VIK II C2	TTC134	Control	JT	0,25	9,68	3,77	0,76	41,3	1,84	2,17
37	VIK II R2	RTC	Removal	JT	0,24	7,6	2,42	1,08	44,2	1,84	2,14
64	VIK III C1 (137)	TTC140	Control	WG	0,35	23,7	7,48	0,56	13,9	1,84	2
63	VIK III R1 (134)	RTC	Removal	WG	0,8	17,1	5,8	0,99	9,88	1,79	1,59

* Sample 89 was a redundant sample.

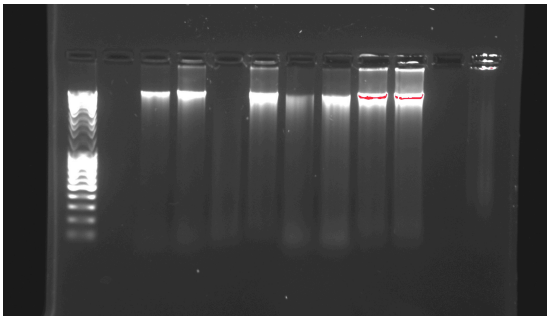
** Samples 79 and 83 were both pseudoreplicates of samples 38 and 31 respectively.

Appendix B

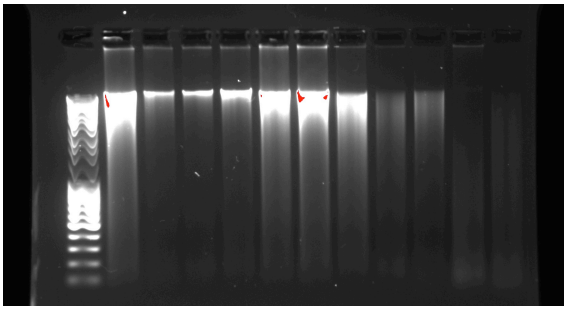
Images of UV light-illuminated gels of DNA extracts.
DNA ladder: Thermo Scientific MassRuler DNA Ladder Mix
(https://www.thermofisher.com/order/catalog/product/SM0403)



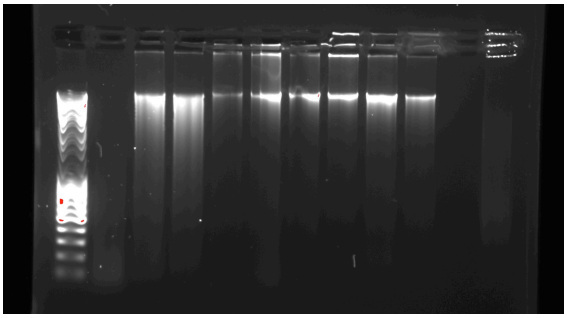
Lane 1: DNA ladder
Lane 2: —
Lane 3: Sample 25 (ALR II R1)
Lane 4: Sample 15 (ALR II C1)
Lane 5: Sample 42 (ARH III R1)
Lane 6: Sample 47 (ARH III C1)
Lane 7: Sample 18 (FAU I R1)
Lane 8: Sample 19 (FAU I C1)
Lane 9: Sample 21 (FAU II R2)
Lane 10: Sample 22 (FAU II C2)
Lane 11: Sample 36 (GUD III R1)
Lane 12: Sample 35 (GUD III C1)



Lane 1: DNA ladder
Lane 2: —
Lane 3: Sample 27 (HOG III R1)
Lane 4: Sample 41 (HOG III C1)
Lane 5: Sample 23 (LAV III R1)
Lane 6: Sample 48 (LAV III C1)
Lane 7: Sample 39 (OVS I R1)
Lane 8: Sample 34 (OVS I C1)
Lane 9: Sample 49 (OVS II R2)
Lane 10: Sample 50 (OVS II C2)
Lane 11: Nuclease-free water
Lane 12: *Escherichia coli* genomic DNA (50 ng)



Lane 1: DNA ladder
Lane 2: Sample 51 (RAM IV R1)
Lane 3: Sample 33 (RAM IV C1)
Lane 4: Sample 28 (RAM VI R2)
Lane 5: Sample 29 (RAM VI C2)
Lane 6: Sample 43 (SKJ I R1)
Lane 7: Sample 45 (SKJ I C1)
Lane 8: Sample 44 (SKJ II R2)
Lane 9: Sample 17 (SKJ II C2)
Lane 10: Sample 16 (ULV II R2)
Lane 11: Sample 20 (ULV II C2)
Lane 12: Sample 24 (ULV I R1)



Lane 1: DNA ladder
Lane 2: Sample 26 (ULV I C1)
Lane 3: Sample 46 (VES III R1)
Lane 4: Sample 52 (VES III C1)
Lane 5: Sample 40 (VIK I R1)
Lane 6: Sample 32 (VIK I C1)
Lane 7: Sample 37 (VIK II R2)
Lane 8: Sample 30 (VIK II C2)
Lane 9: Sample 38 (VES II R2)
Lane 10: Sample 31 (VES II C2)
Lane 11: Nuclease-free water
Lane 12: *Escherichia coli* genomic DNA (50 ng)

Appendix C

BIOINFORMATICS PIPELINE

The following pipeline was run on the Bio-Linux 8 bioinformatics workstation platform (Field *et al.*, 2006), using the tools BBDuk of BBTools (Bushnell, 2014) and QIIME 1 (Caporaso *et al.,* 2010).

Processing of sequences

```
# Demultiplexing

# Repeat the following pipeline until ##### for every sample

# Access the directory containing the raw reads and unzipped all the files.
gunzip *.gz

# Scan the reads for k-mers of at least 31 bases that match the referred sequence (phiX),
# allowing 1 base mismatch, and filter those reads away.
# Override the default setting and specify 2 GB of RAM for this procedure.
bbduk.sh -Xmx2g in1=R1.fastq in2=R2.fastq out1=unmatched1.fastq out2=unmatched2.fastq
outm1=matched1.fastq outm2=matched2.fastq ref=/usr/share/bowtie2/indexes/phiX.fasta
k=31 hdist=1 stats=stats.txt

# Merge together the pairs of complementary or overlapping forward and reverse reads.
bbmerge.sh in1=unmatched1.fastq in2=unmatched2.fastq out=R1R2merged.fastq

# Scan the merged reads for 12-20 base k-mers that match the referred 'N5-519F' sequence,
# allowing 1 base mismatch and every possibility of 5 degenerate bases (5 N's).
# Override the default setting and specify 2 GB of RAM for this procedure.
bbduk.sh -Xmx2g in=R1R2merged.fastq out=RmergedFA.fastq
literal=NNNNNCAGCMGCCGCGGTAA ktrim=n k=20 mink=12 hdist=1 copyundefined

# Scan the merged reads for 12-20 base k-mers that match the referred '806R' sequence,
# allowing 3 base mismatches.
bbduk.sh -Xmx2g in=RmergedFA.fastq out=RmergedRA.fastq
literal=GGACTACHVGGGTWTCTAAT ktrim=n k=20 mink=12 hdist=3

# Scan the right and left ends of the merged reads and trim away
# regions that are scored below 27 by the Phred algorithm.
# Discard reads that are shorter than 200 bases.
bbduk.sh -Xmx2g in=RmergedRA.fastq out=Rtrimmed.fastq qtrim=rl minlen=200 trimq=27

# Reformat the FASTQ sequence files into FASTA files.
reformat.sh in=Rtrimmed.fastq out=Rtrimmed.fasta
```

Constructing an OTU table and a phylogenetic tree

```
# Add a copy of the metadata mapping file into the relevant directory.
```

```
# An example of the mapping file is available at
# http://qiime.org/_static/Examples/File_Formats/Example_Mapping_File.txt
# Manually edit the SampleID column of the file.
# Activate the QIIME environment.
# Add QIIME-compatible label to the FASTA file
add_qiime_labels.py -i Sequence_Data -m Sequence_Data/Mapping_File.txt -c SampleID -n
0 -o Qiime_Formatted
# Exit Qiime

#####

# Access the directory containing all the FASTA files.
# Concatenate all the sequences together into one FASTA file using the cat command.
# Add a copy of the metadata mapping file into the same directory.
# Manually edit the SampleID column of the file.
# Activate the QIIME environment.

# Run a workflow script for de novo OTU picking, taxonomy assignment, phylogenetic tree
construction, and
# OTU table construction, and store all outputs in a directory (e.g. RockyData)
# Use the 'nohup &' command to keep the workflow running even after logging out, etc.
nohup pick_de_novo_otus.py -i RockyData.fasta -o RockyData &

# Access the directory containing the OTU table,
# and convert the .biom format of the OTU table into a tab-delimited .txt format.
biom convert -i otu_table.biom -o otu_table.txt --to-tsv
# Translate the OTU table into tables and stacked bar charts of community compositions.
# Community composition at genus level
summarize_taxa.py -L 6 -i otu_table.biom -o otu_summarised_genus
biom summarize-table -i otu_table.biom -o otu_table_summary_genus.txt
plot_taxa_summary.py -i otu_summarised_genus/otu_table_L6.txt -l Genus -o
bar_charts_genus -c bar
zip -r bar_charts_genus.zip bar_charts_genus
# Community composition at class level
summarize_taxa.py -L 3 -i otu_table.biom -o otu_summarised_class
biom summarize-table -i otu_table.biom -o otu_table_summary_class.txt
plot_taxa_summary.py -i otu_summarised_class/otu_table_L3.txt -l Class -o bar_charts_class
-c bar
zip -r bar_charts_class.zip bar_charts_class
# Community composition at phylum level
summarize_taxa.py -L 2 -i otu_table.biom -o otu_summarised_phylum
biom summarize-table -i otu_table.biom -o otu_table_summary_phylum.txt
plot_taxa_summary.py -i otu_summarised_phylum/otu_table_L2.txt -l Phylum -o
bar_charts_phylum -c bar
zip -r bar_charts_phylum.zip bar_charts_phylum
```

Alpha diversity metrics and rarefaction curves

```
# Add a copy of the metadata mapping file into the relevant directory.
# Activate the QIIME environment.
# Run a workflow script for performing alpha rarefaction and generating rarefaction curves,
# storing the outputs in a directory (e.g. arare).
alpha_rarefaction.py -i otu_table.biom -m Mapping_File.txt -o arare -t rep_set.tre

# Rarefy the OTU table to even sampling depth (i.e. minimum sequence reads = 21 772)
single_rarefaction.py -i otu_table.biom -o otu_table_even21772.biom -d 21772
# Compute alpha diversity metrics on the rarefied OTU table.
# The chosen metrics are observed no. of OTUs, chao1 richness estimator, no. of singletons,
# shannon diversity index, and simpson's evenness index
alpha_diversity.py -i otu_table_even21772.biom -m observed_otus,chao1,singles,shannon,
simpson_e  -o adiv.txt -t rep_set.tre
```

DATA ANALYSIS PIPELINE

The following pipeline was run using R version 3.2.0 via the platform RStudio version 1.1.414. The packages 'phyloseq' (version 1.22.3), 'vegan' (version 2.4-6) and 'tidyverse' (version 1.2.1) were installed prior to running the pipeline.

Data processing

```
# Load the relevant packages
library("tidyverse")
library("phyloseq")
library("vegan")

# Set the working directory
setwd("/home/jesslyn/BIO_399/Analysis/RockyData/")
# Load the sample ID, OTU composition, and alpha diversity metrics files
SampleID.df <- read.csv(file = "SampleID.csv")
OTU_phylum.df <- read.csv(file = "NO_singleton_removal/OTU_Phylum.csv")
alphadiversity.df <- read.csv(file = "NO_singleton_removal/alphadiversity.csv")
# Remove Sample89 from the dataframes above
SampleID.df <- filter(SampleID.df, X!="Sample89")
alphadiversity.df <- filter(alphadiversity.df, X!="Sample89")
OTU_phylum.df$Sample89 <- NULL

# Combine the dataframes into a phyloseq object

# Combine the first two taxonomic columns of OTU dataframe into one column
OTU_phylum.df <- unite(OTU_phylum.df, OTU_phylum, Kingdom:Phylum, sep = ",",
remove = TRUE)
# Convert the first column into row names
OTU_phylum.df <- OTU_phylum.df %>% remove_rownames %>%
column_to_rownames(var = "OTU_phylum")
```

```
# Assign the OTU table as a phyloseq object
OTU_phylum = otu_table(OTU_phylum.df, taxa_are_rows = TRUE)

# And then the sample ID
# Add two columns of data (mean summer temperature and annual precipitation) into the dataframe
SampleID.df <- SampleID.df %>%
  mutate(Temperature = as.numeric(if_else(Site %in% c('ULV', 'GUD', 'LAV', 'SKJ'), '6.5', if_else(Site %in% c('OVS', 'FAU', 'VIK', 'ARH'), '10.5', '8.5')))) %>%
  mutate(Precipitation = as.numeric(if_else(Site %in% c('ULV', 'ALR', 'FAU'), '0.6', if_else(Site %in% c('LAV', 'HOG', 'VIK'), '1.2', if_else(Site %in% c('GUD', 'RAM', 'ARH'), '2', '2.7')))))
# Convert the first column into row names
SampleID.df <- SampleID.df %>% remove_rownames %>% column_to_rownames(var = "X")
# Assign the Sample ID as a phyloseq object
SampleID = sample_data(SampleID.df)

# And then the alpha diversity metrics
# Convert the first column into row names
alphadiversity.df <- alphadiversity.df %>% remove_rownames %>% column_to_rownames(var = "X")
# Assign the alpha diversity metrics as a phyloseq object
alphadiversity = sample_data(alphadiversity.df)

# Merge all three together
dataframe <- merge_phyloseq(OTU_phylum, SampleID, alphadiversity)

# Remove pseudoreplicates (samples 79 and 83) from the data
dataframe <- prune_samples(rownames(sample_data(dataframe)) != "Sample83", dataframe)
dataframe <- prune_samples(rownames(sample_data(dataframe)) != "Sample79", dataframe)

# For each sample, remove phyla that have relative abundance less than 0.02
df_ordination = dataframe
wh0 = genefilter_sample(df_ordination, filterfun_sample(function(x) x > 0.02))
dford = prune_taxa(wh0, df_ordination)
```

Unconstrained ordination – Detrended Correspondence Analysis (DCA)

```
# DCA on phylum-level data object, using bray-curtis dissimilarity
df_phylum_dca <- ordinate(dford, "DCA", "bray")
df_phylum_dca
```

Beta diversity analysis: Constrained ordination – Redundancy Analysis (RDA)

```
# Compute the bray-curtis disimilarities for phylum-level data object
brayphylum <- phyloseq::distance(physeq = dford, method = "bray")
```

```
# Constrain the ordination to the variable 'Plot_Type' (graminoid removal vs. control),
# partialling out ‘Temperature’ and ‘Precipitation’ as covariables.
# Ordinate: ~ Plot_Type + Condition(Temperature,Precipitation)
df_phylum_rda_2 <- ordinate(
  physeq = dford,
  method = "RDA",
  distance = brayphylum,
  formula = ~ Plot_Type + Condition(Temperature,Precipitation)
)
# Do a permutation test on the ordination
set.seed(1)
anova.cca(df_phylum_rda_2, permutations = how(Plots(strata = sample_data(dford)$Site,
type = "free")))
# Summary of the model
summary(df_phylum_rda_2)

# Constrain the ordination to the environmental variables 'Temperature' and 'Precipitation',
# partialling out ‘Plot_Type’ as a  covariable.
# Ordinate: ~ Temperature + Precipitation + Condition(Plot_Type)
df_phylum_rda <- ordinate(
  physeq = dford,
  method = "RDA",
  distance = brayphylum,
  formula = ~ Temperature + Precipitation + Condition(Plot_Type)
)
# Do a permutation test on the ordination
set.seed(1)
anova.cca(df_phylum_rda, permutations = how(Plots(strata = sample_data(dford)$Site, type
= "free")))
# Summary of the model
summary(df_phylum_rda)

# Constrain the ordination to the environmental variable 'Temperature',
# partialling out ‘Plot_Type’ and ‘Precipitation’ as covariables.
# Ordinate: ~ Temperature + Precipitation + Condition(Plot_Type,Precipitation)
df_phylum_rda_3 <- ordinate(
  physeq = dford,
  method = "RDA",
  distance = brayphylum,
  formula = ~ Temperature + Condition(Plot_Type,Precipitation)
)
# Do a permutation test on the ordination
set.seed(1)
anova.cca(df_phylum_rda_3, permutations = how(Plots(strata = sample_data(dford)$Site,
type = "free")))
# Summary of the model
summary(df_phylum_rda_3)
```

```
# Constrain the ordination to the environmental variable 'Precipitation',
# partialling out ‘Plot_Type’ and ‘Temperature’ as covariables.
# Ordinate: ~ Precipitation + Condition(Plot_Type,Temperature)
df_phylum_rda_4 <- ordinate(
  physeq = dford,
  method = "RDA",
  distance = brayphylum,
  formula = ~ Temperature + Condition(Plot_Type,Precipitation)
)
# Do a permutation test on the ordination
set.seed(1)
anova.cca(df_phylum_rda_4, permutations = how(Plots(strata = sample_data(dford)$Site,
type = "free")))
# Summary of the model
summary(df_phylum_rda_4)
```

Alpha diversity analysis

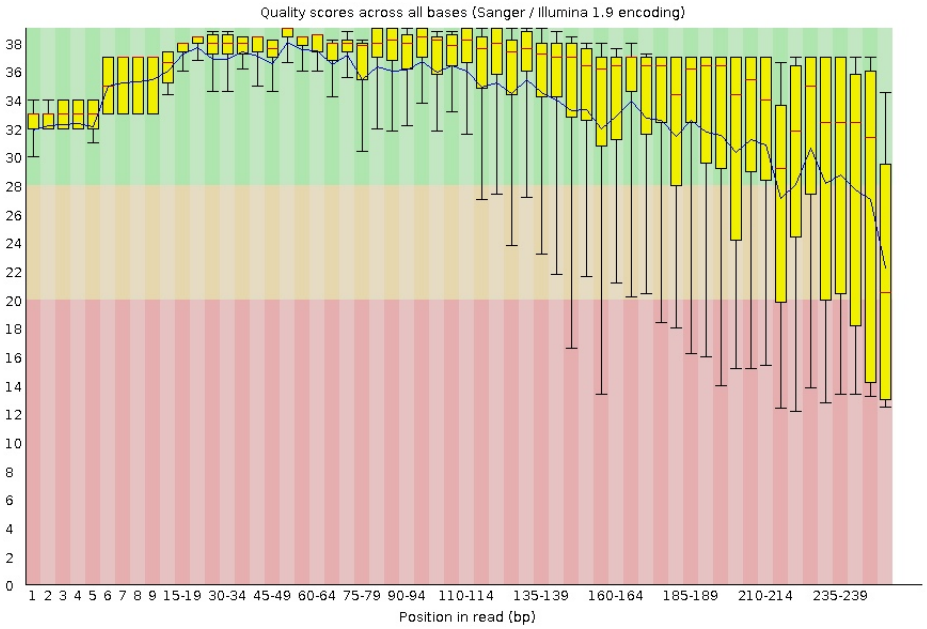
```
# For the following analysis, it does not matter which taxonomic level data is used,
# since the alpha diversity estimates correspond to the 72 samples instead of the taxa
dfphylum = dataframe
df2 <- data.frame(sample_data(dfphylum))

# Linear mixed-effects model:
# Observed OTUs ~ temperature + precipitation + plot type
lme_otu <- lme(observed_otus ~ Temperature + Precipitation + Plot_Type, data = df2,
random = ~+1|Site)
# Analysis of variance (statistical testing)
anova(lme_otu)

# Linear mixed-effects model:
# Chao1 ~ temperature + precipitation + plot type
lme_chao1 <- lme(chao1 ~ Temperature + Precipitation + Plot_Type, data = df2, random =
~+1|Site)
# Analysis of variance (statistical testing)
anova(lme_chao1)

# Linear mixed-effects model:
# Shannon diversity ~ temperature + precipitation + plot type
lme_shannon <- lme(shannon ~ Temperature + Precipitation + Plot_Type, data = df2, random
= ~+1|Site)
# Analysis of variance (statistical testing)
anova(lme_shannon)

# Linear mixed-effects model:
# Simpson evenness ~  temperature + precipitation + plot type
lme_simpsone <- lme(simpson_e ~ Temperature + Precipitation + Plot_Type, data = df2,
random = ~+1|Site)
```

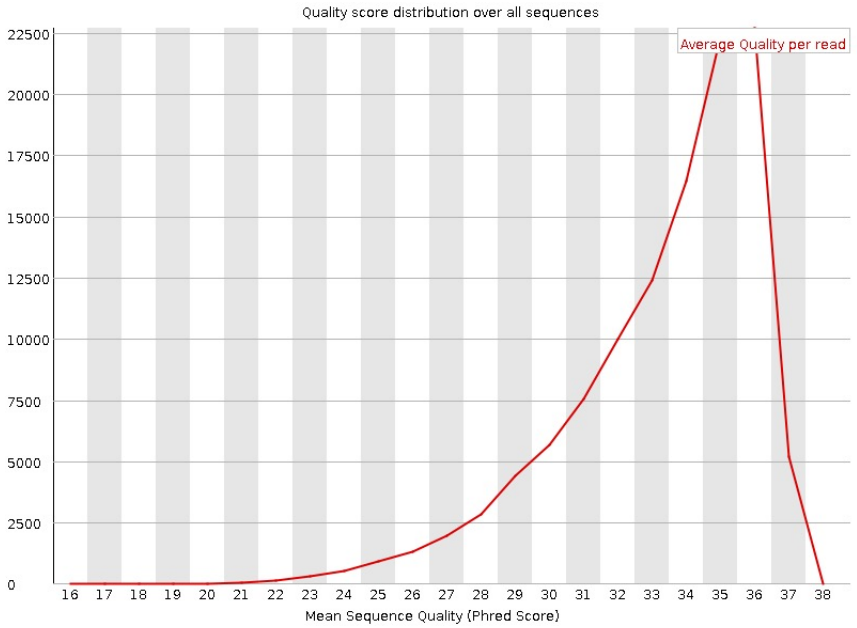
```
# Analysis of variance (statistical testing)
anova(lme_simpsone)
# Summary of the model
summary(lme_simpsone)
```

Appendix D

Select sections of an example FastQC report on raw sequence reads.
Sample 43, forward reads (chosen at random):

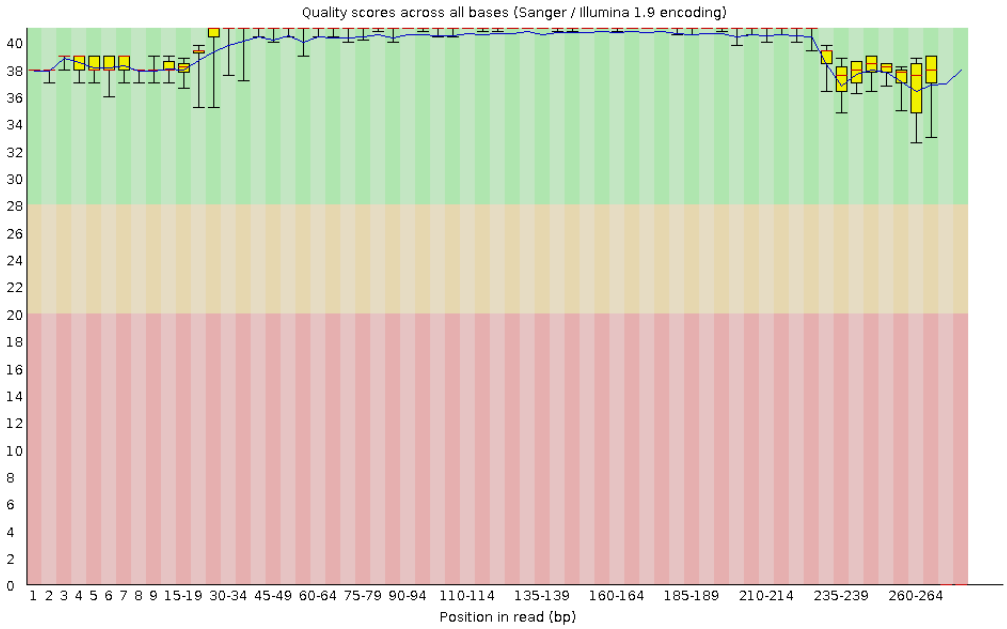
Filename	43R1.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	115097
Sequences flagged as poor quality	0
Sequence length	251
%GC	57

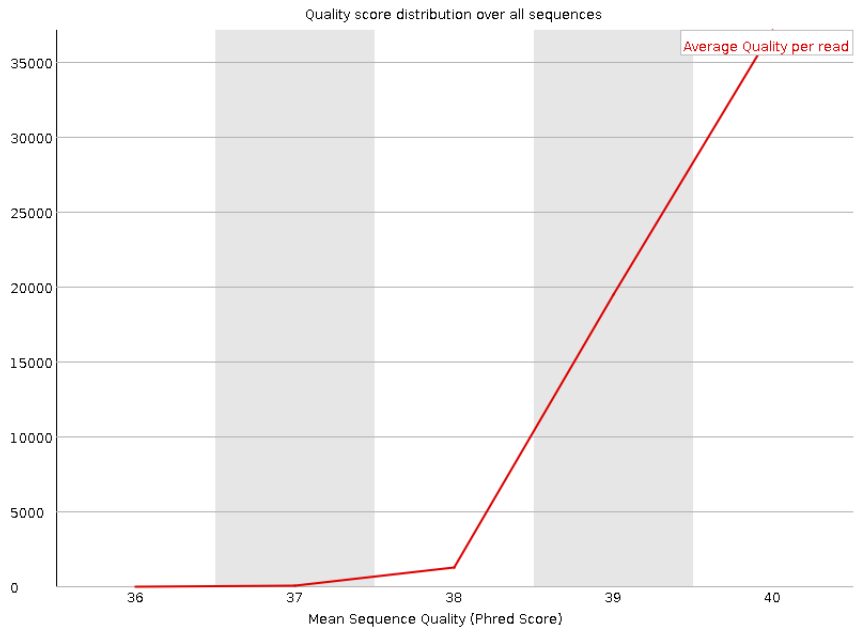




Select sections of an example FastQC report on the sequence reads after processing. Sample 43 (chosen at random), merged reads:

Filename	Rtrimmed_43.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total sequences	57938
Sequences flagged as poor quality	0
Sequence length	200-275
%GC	56





Appendix E

Table listing the number of raw forward and reverse sequence reads in the sample, the percentages of outcomes (joined, ambiguous, no solution) from merging of the forward and reverse reads, the final numbers of processed reads, OTUs and singletons in the 75 samples.

				Merging of Sequences						
No.	Label	Raw Input (reads)	Raw Input (pairs)	Joined (%)	Ambiguous (%)	No solution (%)	Reads	OTUs	Singleton	Singleton (%)
15	ALR II C1	238916	119458	37,16	15,75	47,09	41628	11201	7787	18,71
25	ALR II R1	242486	121241	54,07	9,60	36,33	60334	11323	8171	13,54
56	ALR III C2 (48)	362660	181328	60,28	7,76	31,97	100407	13591	9537	9,50
55	ALR III R2 (45)	444114	222055	64,63	6,78	28,59	132411	15122	9801	7,40
88	ALR IV C2 (54)	490704	245351	63,96	7,19	28,86	144831	28787	21387	14,77
87	ALR IV R2 (51)	399656	199825	34,88	17,50	47,63	64580	20399	15701	24,31
86	ARH I C1 (278)	322020	161008	55,19	9,15	35,66	81551	12179	8626	10,58
85	ARH I R1 (275)	358634	179315	51,75	10,26	38,00	85293	11598	7905	9,27
72	ARH II C2 (285)	291288	145643	57,98	8,12	33,90	77514	11661	8246	10,64
68	ARH II R2 (282)	311594	155796	60,78	7,61	31,61	87023	10014	6620	7,61
47	ARH III C1 (293)	214582	107291	49,89	10,42	39,68	49420	9217	6556	13,27
42	ARH III R1 (290)	194556	97277	47,23	11,37	41,40	42418	8521	6172	14,55
19	FAU I C1	231022	115510	61,13	7,86	31,01	65047	10691	7423	11,41
18	FAU I R1	207300	103650	58,68	8,52	32,80	55903	10955	7555	13,51
22	FAU II C2	219030	109511	61,20	7,73	31,08	61765	11959	8518	13,79
21	FAU II R2	284726	142363	65,47	6,61	27,92	86136	19431	14587	16,93
58	FAU III C1 (77)	563948	281970	59,78	7,80	32,42	155222	17508	11644	7,50
57	FAU III R1 (74)	411022	205509	64,52	6,42	29,06	122379	17956	12391	10,13
76	GUD II C2 (351)	260080	130040	64,03	6,59	29,38	76930	11516	7716	10,03
75	GUD II R2 (348)	263408	131703	35,25	16,32	48,43	43009	7681	5101	11,86
35	GUD III C1 (356)	219462	109731	62,35	7,18	30,47	62938	13866	10426	16,57
36	GUD III R1 (353)	193590	96795	59,52	7,86	32,62	52958	11693	8665	16,36
78	GUD IV C1 (362)	284144	142072	57,34	8,35	34,32	75008	12327	8447	11,26
77	GUD IV R1 (359)	264180	132090	65,92	6,01	28,07	80527	10019	6261	7,78
60	HOG I C2 (96)	290828	145414	56,86	8,24	34,90	76210	9598	6731	8,83
59	HOG I R2 (93)	308778	154388	61,58	7,00	31,42	87084	10224	7094	8,15
62	HOG II C1 (101)	337750	168875	58,43	7,77	33,80	90881	12289	8710	9,58
61	HOG II R1 (98)	244710	122353	54,32	9,22	36,46	61086	13937	10678	17,48
41	HOG III C1 (107)	220722	110359	59,32	7,23	33,45	60498	10475	7556	12,49
27	HOG III R1 (104)	199004	99501	38,59	14,84	46,57	35517	6963	5012	14,11
66	LAV I C1 (155)	275110	137555	58,39	8,62	32,99	73692	9496	5728	7,77
65	LAV I R1 (152)	308364	154179	66,86	6,12	27,02	95027	10719	6873	7,23
71	LAV II C2 (162)	257484	128741	60,94	7,64	31,43	72187	10559	7245	10,04
67	LAV II R2 (159)	303354	151677	64,15	7,23	28,62	89595	11664	7649	8,54
48	LAV III C1	240622	120311	47,20	11,41	41,39	52494	9428	6770	12,90
23	LAV III R1	204346	102172	62,55	7,14	30,31	58716	9817	6767	11,52
34	OVS I C1 (314)	208010	104003	62,41	7,33	30,27	59588	12126	8944	15,01
39	OVS I R1 (311)	212814	106407	22,03	26,84	51,12	21772	5300	3859	17,72
50	OVS II C2 (321)	190226	95113	56,05	8,86	35,09	49201	9675	7021	14,27
49	OVS II R2 (318)	220064	110032	50,12	10,90	38,99	50434	8924	6469	12,83
74	OVS III C1 (326)	232282	116140	56,30	8,58	35,12	60211	9049	6328	10,51
73	OVS III R1 (323)	311748	155874	51,11	10,22	38,68	73322	12191	8668	11,82
33	RAM IV C1 (215)	236468	118232	66,68	6,03	27,29	72638	12551	9164	12,62
51	RAM IV R1 (212)	210646	105323	34,90	16,84	48,26	33519	8279	6133	18,30
84	RAM V C1 (221)	299076	149537	56,55	8,38	35,08	77662	9452	6503	8,37
82	RAM V R1 (218)	279600	139799	58,79	8,07	33,14	75504	9293	6355	8,42

29	RAM VI C2 (228)	289192	144596	67,36	6,02	26,63	89264	18493	13786	15,44
28	RAM VI R2 (225)	216812	108405	65,77	6,50	27,74	65410	13463	9919	15,16
45	SKJ I C1	501964	250980	53,47	9,75	36,79	124221	21607	15584	12,55
43	SKJ I R1	230194	115097	54,46	9,45	36,09	57938	13972	10311	17,80
17	SKJ II C2	174950	87475	67,51	6,26	26,23	54476	10148	7019	12,88
44	SKJ II R2	190956	95478	48,01	11,59	40,40	42470	9443	6694	15,76
70	SKJ III C1 (260)	271324	135661	59,79	8,58	31,63	74458	11568	7857	10,55
69	SKJ III R1 (257)	310726	155363	64,06	6,91	29,03	91978	14336	9817	10,67
26	ULV I C1	265038	132518	58,24	8,58	33,18	71186	11900	8255	11,60
24	ULV I R1	295024	147512	60,96	7,61	31,43	82908	11826	8143	9,82
20	ULV II C2	233658	116828	59,49	8,47	32,05	64145	11511	7792	12,15
16	ULV II R2	203668	101833	65,13	6,87	28,00	61187	8362	5336	8,72
89*	ULV III C1 (16)	300214	149994	66,42	8,58	25,00	81491	48183	41972	51,51
53	ULV V R2 (2)	450424	225212	65,12	6,59	28,29	135575	17996	12596	9,29
54	ULV V T232 (5)	384404	192201	57,80	8,43	33,77	102070	18690	13580	13,30
81	VES I C1 (185)	282950	141473	63,87	6,61	29,52	83340	11201	7388	8,86
80	VES I R1 (182)	283374	141687	59,97	7,81	32,22	78376	10548	6942	8,86
31	VES II C2 (192)	239408	119703	66,32	6,38	27,30	73001	13808	9995	13,69
83**	VES II C2 (192)	292956	146478	58,67	8,02	33,31	79275	10208	6522	8,23
38	VES II R2 (189)	177244	88622	44,65	12,61	42,75	36623	8261	5999	16,38
79**	VES II R2 (189)	322278	161138	63,64	6,76	29,60	94578	13506	9388	9,93
52	VES III C1 (197)	229176	114588	63,64	6,80	29,56	67031	11179	7765	11,58
46	VES III R1 (194)	200642	100321	49,58	10,86	39,56	45901	10374	7378	16,07
32	VIK I C1	206242	103121	63,05	6,86	30,10	59843	10766	7877	13,16
40	VIK I R1	199698	99848	56,96	8,31	34,73	52482	10525	7648	14,57
30	VIK II C2	186834	93416	62,04	7,09	30,87	53245	9600	6889	12,94
37	VIK II R2	202198	101099	39,39	14,43	46,18	36884	7395	5379	14,58
64	VIK III C1 (137)	255412	127704	66,17	6,16	27,68	77810	7447	4710	6,05
63	VIK III R1 (134)	225604	112802	38,12	15,35	46,53	39786	4867	2977	7,48

* Data pertaining to sample 89 were excluded from post-analyses.

** Samples 79 and 83 were both pseudoreplicates of samples 38 and 31 respectively and were therefore excluded from post-analyses.

Appendix F

Table: Mean (with standard deviation, S.D.) relative abundances (%) of common (>2%) bacterial phyla found in control and graminoid removal soil samples at the twelve sites. (n=3)

Site	Temperature (°C)	Precipitation (m)	Phylum	Removal		Control	
				Mean	S.D.	Mean	S.D.
ULV	6.5	0.6	Proteobacteria	42.74	4.42	44.03	3.48
			Acidobacteria	13.97	7.12	12.04	4.53
			Actinobacteria	15.88	7.54	16.26	4.63
			Bacteroidetes	8.63	4.35	9.28	2.62
			Verrucomicrobia	6.43	0.57	6.51	1.57
			Planctomycetes	3.62	0.72	4.02	0.54
			Chloroflexi	3.26	0.82	2.82	1.38
			Nitrospirae	0.22	0.16	0.33	0.26
			AD3	0.09	0.07	0.08	0.13
			Unassigned	1.19	0.07	1.21	0.26
			Gemmatimonadetes	0.77	0.18	0.74	0.09
			Firmicutes	0.23	0.20	0.24	0.19
ALR	8.5	0.6	Proteobacteria	41.70	4.99	39.58	5.54
			Acidobacteria	11.90	2.51	20.60	4.07
			Actinobacteria	12.72	1.91	10.35	0.86
			Bacteroidetes	12.05	3.56	5.15	0.99
			Verrucomicrobia	10.33	4.44	11.29	4.87
			Planctomycetes	3.94	1.10	5.02	2.41
			Chloroflexi	2.08	0.80	2.07	0.69
			Nitrospirae	0.05	0.06	0.16	0.14
			AD3	0.06	0.03	0.19	0.11
			Unassigned	1.48	0.70	1.73	0.59
			Gemmatimonadetes	1.16	0.30	1.01	0.29
			Firmicutes	0.38	0.32	0.39	0.33
FAU	10.5	0.6	Proteobacteria	38.85	1.93	38.99	0.98
			Acidobacteria	11.91	2.05	11.00	2.79
			Actinobacteria	19.43	2.33	19.41	7.60
			Bacteroidetes	8.13	0.66	10.28	1.57
			Verrucomicrobia	10.74	0.46	10.12	3.62
			Planctomycetes	3.59	1.17	3.42	1.24
			Chloroflexi	2.52	0.72	2.14	0.51
			Nitrospirae	0.22	0.14	0.19	0.25
			AD3	0.16	0.15	0.13	0.19
			Unassigned	1.02	0.14	0.83	0.18
			Gemmatimonadetes	0.89	0.23	0.80	0.14
			Firmicutes	0.51	0.53	0.58	0.52
LAV	6.5	1.2	Proteobacteria	46.92	3.41	45.88	0.90
			Acidobacteria	18.08	4.48	18.04	7.02

			Actinobacteria	8.16	1.22	9.40	2.15
			Bacteroidetes	4.96	1.43	6.31	0.92
			Verrucomicrobia	6.43	0.73	7.77	1.65
			Planctomycetes	3.84	0.69	4.05	0.68
			Chloroflexi	3.65	1.90	3.57	0.85
			Nitrospirae	1.52	2.39	0.45	0.44
			AD3	0.75	1.16	0.11	0.10
			Unassigned	0.86	0.17	0.96	0.15
			Gemmatimonadetes	1.22	0.79	0.75	0.23
			Firmicutes	0.20	0.12	0.49	0.53
HOG	8.5	1.2	Proteobacteria	37.71	0.72	36.75	1.40
			Acidobacteria	22.95	3.48	24.77	5.86
			Actinobacteria	8.55	4.58	8.19	7.32
			Bacteroidetes	8.75	6.18	6.03	0.49
			Verrucomicrobia	10.85	2.23	10.95	1.22
			Planctomycetes	3.99	0.77	4.07	0.41
			Chloroflexi	2.57	0.69	3.31	0.44
			Nitrospirae	0.01	0.01	0.01	0.00
			AD3	0.15	0.11	0.37	0.17
			Unassigned	1.47	0.51	1.52	0.42
			Gemmatimonadetes	0.70	0.15	0.82	0.18
			Firmicutes	0.35	0.20	0.46	0.42
VIK	10.5	1.2	Proteobacteria	40.41	3.29	39.48	2.86
			Acidobacteria	18.73	0.80	20.59	2.73
			Actinobacteria	7.70	4.70	8.22	4.74
			Bacteroidetes	12.09	6.59	9.57	1.18
			Verrucomicrobia	10.66	2.46	11.78	2.92
			Planctomycetes	3.64	0.44	3.48	0.78
			Chloroflexi	2.50	0.27	2.73	0.60
			Nitrospirae	0.00	0.00	0.03	0.04
			AD3	0.03	0.01	0.02	0.01
			Unassigned	0.83	0.26	0.74	0.21
			Gemmatimonadetes	0.74	0.19	0.77	0.16
			Firmicutes	0.78	0.62	0.87	0.52
GUD	6.5	2.0	Proteobacteria	40.35	4.55	42.08	2.63
			Acidobacteria	16.81	1.38	15.46	2.57
			Actinobacteria	8.09	0.44	9.05	0.58
			Bacteroidetes	6.85	1.27	5.04	0.85
			Verrucomicrobia	11.95	2.73	11.34	1.46
			Planctomycetes	4.31	0.94	4.40	1.05
			Chloroflexi	4.35	0.77	4.38	0.48
			Nitrospirae	2.06	0.73	2.79	1.28
			AD3	0.01	0.01	0.05	0.06
			Unassigned	1.02	0.20	1.17	0.12
			Gemmatimonadetes	0.99	0.25	1.00	0.17

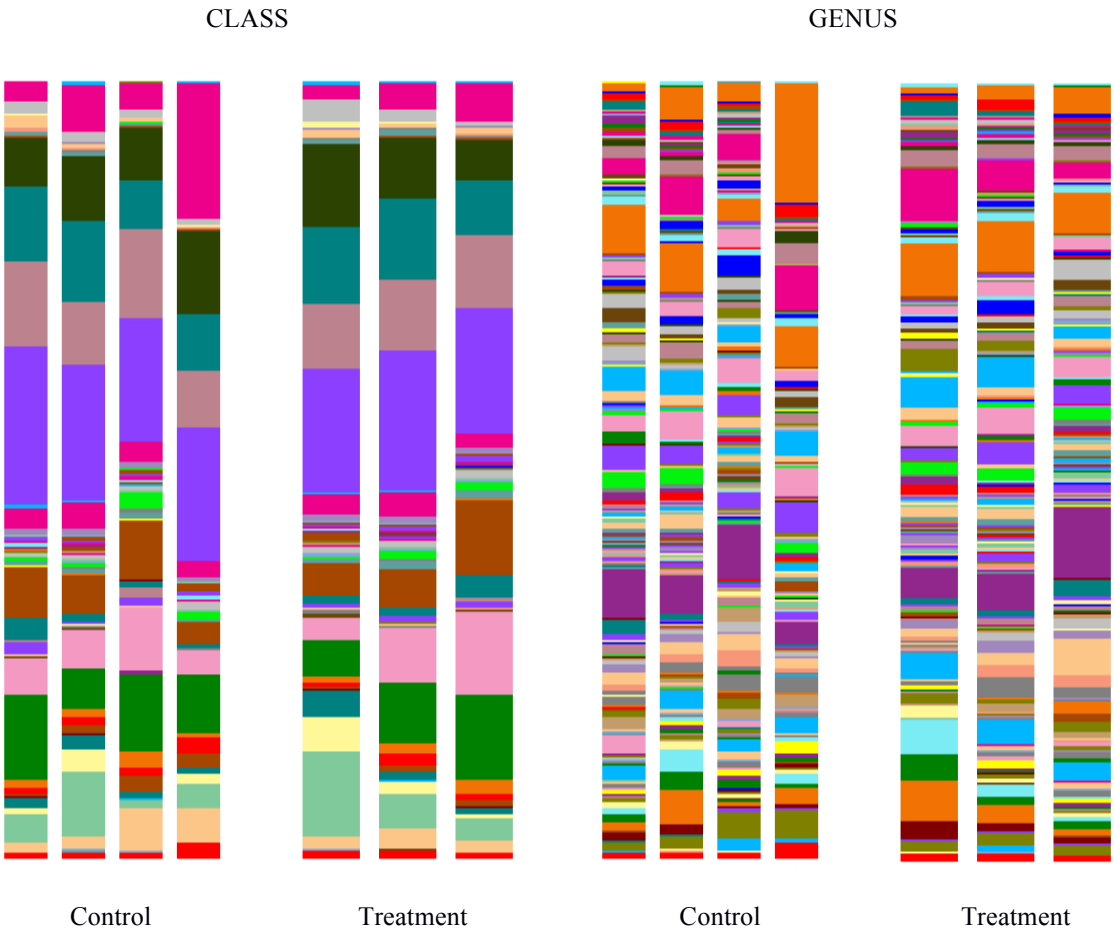
			Firmicutes	0.17	0.02	0.21	0.08
RAM	8.5	2.0	Proteobacteria	39.69	6.37	39.48	5.59
			Acidobacteria	17.07	5.60	16.49	2.27
			Actinobacteria	11.13	3.74	11.69	5.52
			Bacteroidetes	6.73	3.02	5.88	1.11
			Verrucomicrobia	9.76	2.62	9.61	2.01
			Planctomycetes	5.01	1.22	4.77	0.99
			Chloroflexi	4.61	0.75	5.63	0.87
			Nitrospirae	0.26	0.29	0.34	0.57
			AD3	0.09	0.07	0.16	0.14
			Unassigned	1.71	0.76	1.39	0.17
			Gemmatimonadetes	0.74	0.18	0.67	0.19
			Firmicutes	1.02	0.73	1.40	0.93
ARH	10.5	2.0	Proteobacteria	38.75	4.87	41.49	4.52
			Acidobacteria	27.25	10.28	22.02	9.70
			Actinobacteria	5.05	1.28	6.14	2.31
			Bacteroidetes	5.26	1.91	6.13	2.68
			Verrucomicrobia	9.40	3.17	9.91	4.63
			Planctomycetes	5.03	1.45	5.47	1.36
			Chloroflexi	3.97	0.79	3.21	0.53
			Nitrospirae	0.12	0.12	0.06	0.03
			AD3	0.25	0.32	0.16	0.21
			Unassigned	1.20	0.14	1.65	0.33
			Gemmatimonadetes	0.68	0.03	0.64	0.255
			Firmicutes	0.48	0.22	0.78	0.48
SKJ	6.5	2.7	Proteobacteria	46.59	1.36	44.22	0.64
			Acidobacteria	13.75	4.13	15.33	3.29
			Actinobacteria	9.45	3.75	7.71	2.29
			Bacteroidetes	5.96	1.29	7.47	2.56
			Verrucomicrobia	6.72	1.43	6.55	1.62
			Planctomycetes	3.45	0.77	4.00	0.55
			Chloroflexi	5.62	1.15	5.73	0.23
			Nitrospirae	2.34	1.80	1.77	0.88
			AD3	0.06	0.06	0.55	0.85
			Unassigned	1.34	0.16	1.41	0.08
			Gemmatimonadetes	1.91	0.54	1.51	0.25
			Firmicutes	0.14	0.10	0.42	0.36
VES	8.5	2.7	Proteobacteria	45.93	4.66	46.31	3.62
			Acidobacteria	16.70	1.90	14.70	1.23
			Actinobacteria	5.62	1.14	7.24	1.01
			Bacteroidetes	7.35	1.47	6.17	1.34
			Verrucomicrobia	10.49	3.47	11.67	1.98
			Planctomycetes	5.29	1.15	5.33	1.37
			Chloroflexi	3.11	0.75	2.87	0.41
			Nitrospirae	1.31	0.44	1.45	0.19

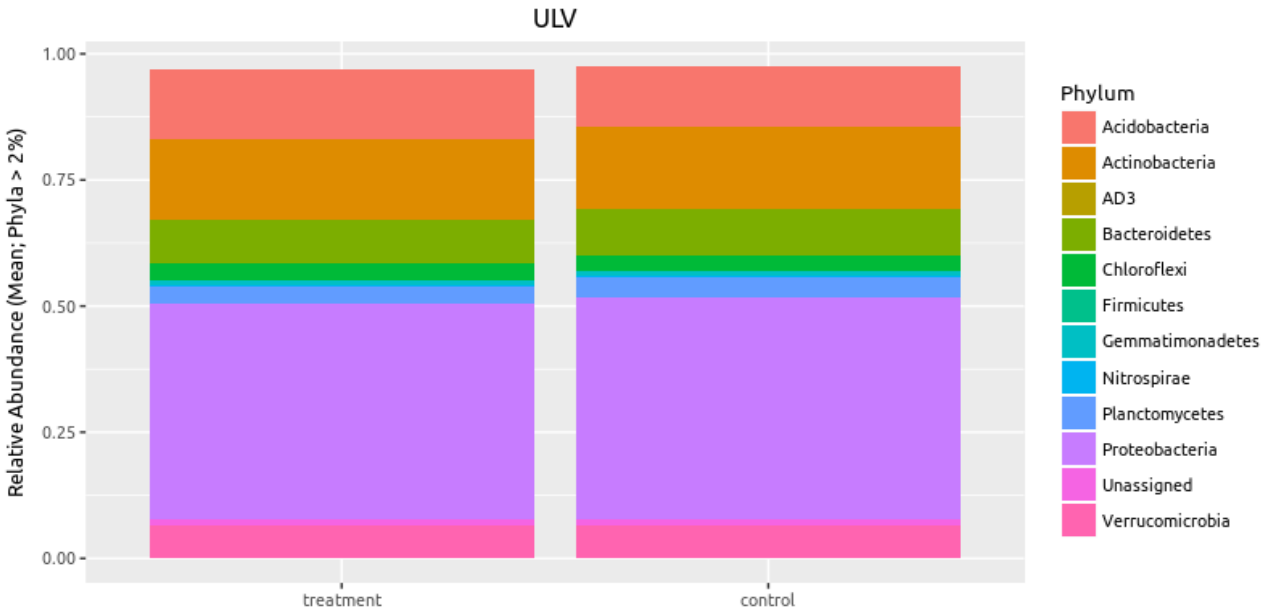
			AD3	0.01	0.01	0.02	0.02
			Unassigned	1.05	0.22	0.96	0.06
			Gemmatimonadetes	0.65	0.03	0.64	0.19
			Firmicutes	0.34	0.31	0.82	1.00
OVS	10.5	2.7	Proteobacteria	38.49	5.57	36.56	1.54
			Acidobacteria	25.95	4.38	29.28	2.55
			Actinobacteria	8.40	5.21	7.31	3.13
			Bacteroidetes	5.38	1.26	5.24	0.74
			Verrucomicrobia	6.43	1.73	5.76	1.71
			Planctomycetes	5.28	0.89	4.68	1.60
			Chloroflexi	3.74	0.88	3.66	0.83
			Nitrospirae	0.01	0.01	0.05	0.04
			AD3	0.29	0.13	1.58	2.14
			Unassigned	1.79	0.10	1.51	0.27
			Gemmatimonadetes	0.54	0.12	0.57	0.12
			Firmicutes	0.20	0.07	0.17	0.12

Appendix G

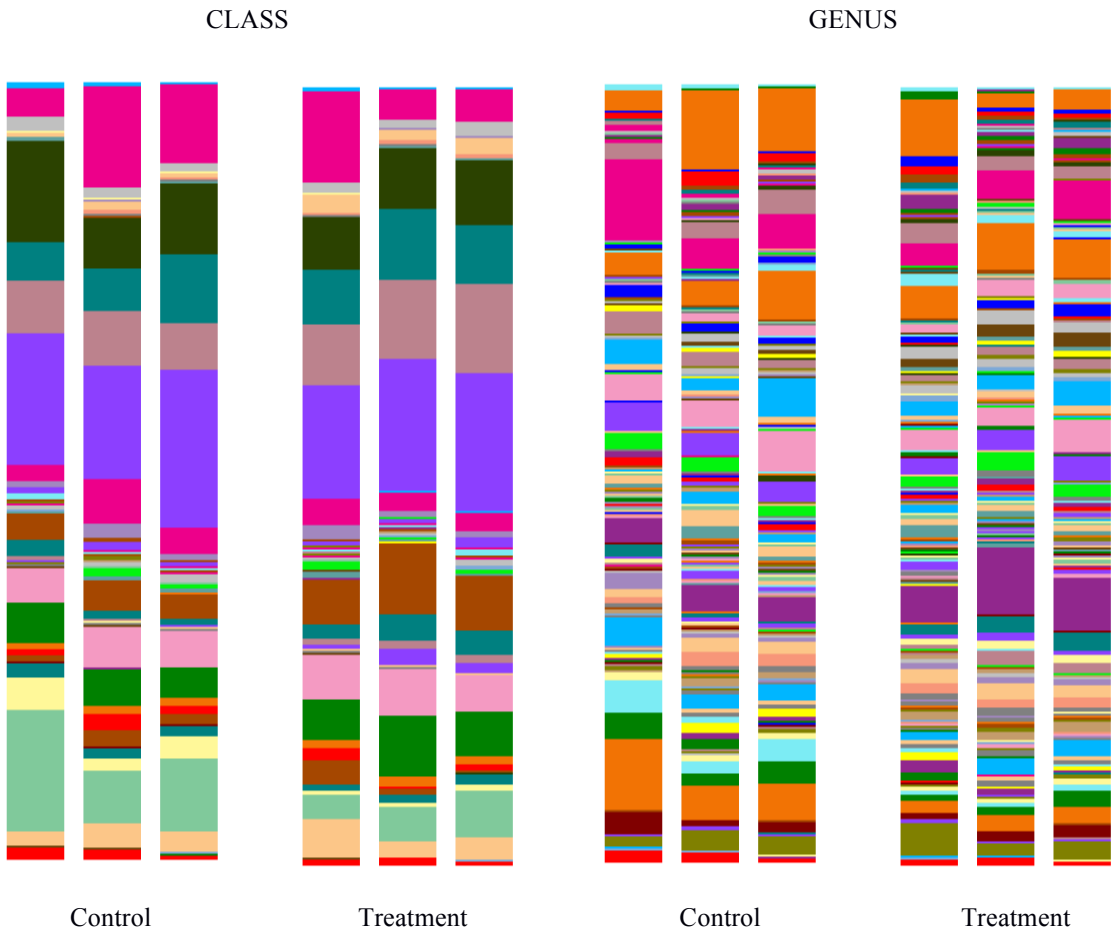
Stacked bar charts showing the compositions of prokaryotic communities in 75 soil samples (including 1 redundant sample and 2 pseudoreplicates) at genus, class, and phylum taxonomic levels. For the phylum level, only the averaged compositions of common taxa (mean relative abundance > 2%) are shown.

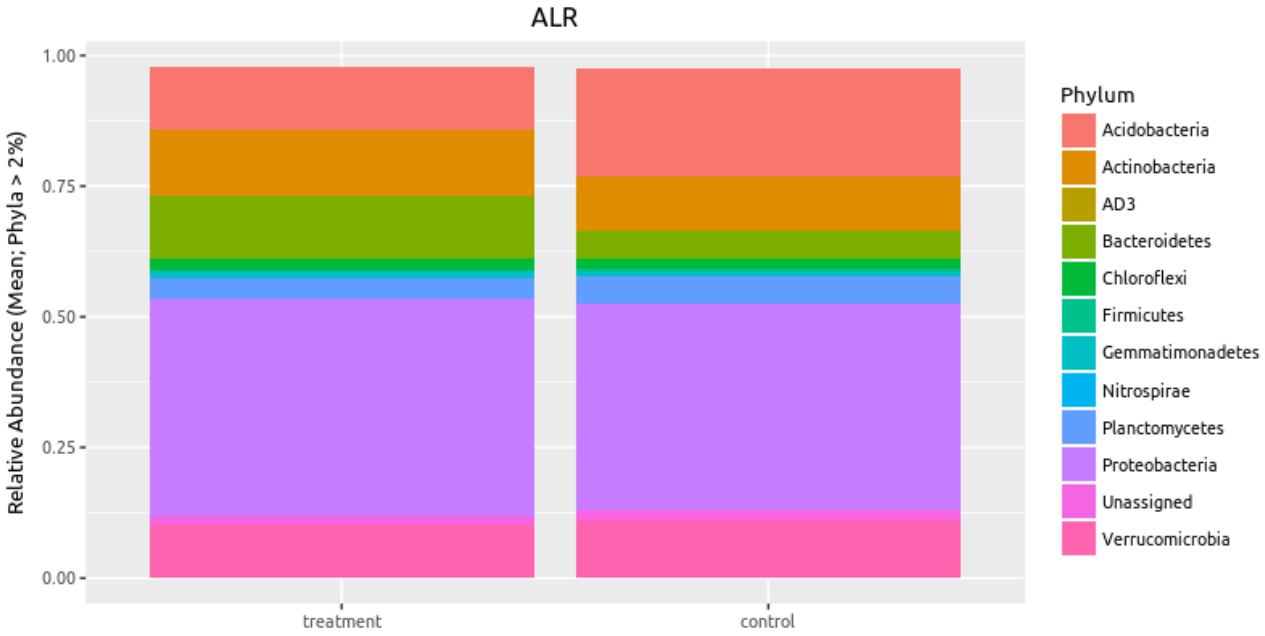
Ulvhaugen (ULV): 6.5°C, 0.6 m



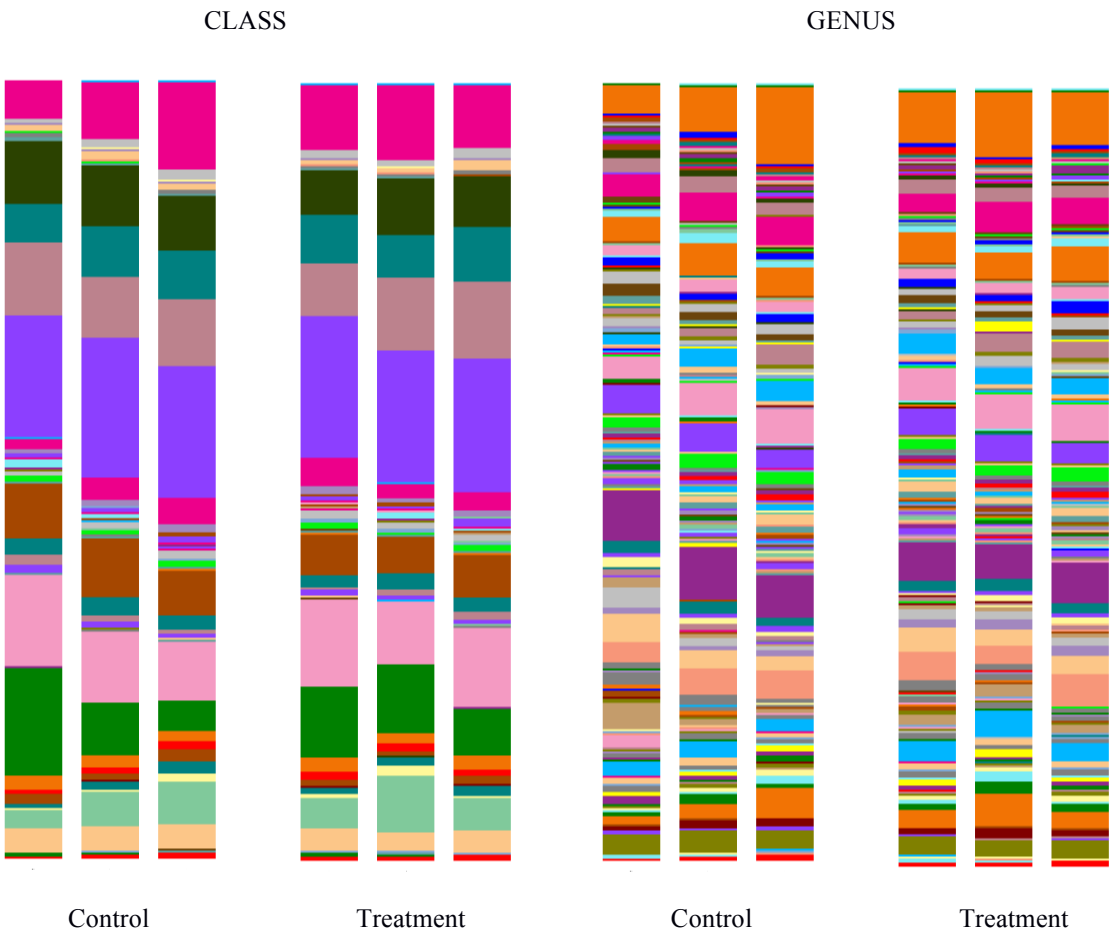

ULV
Relative Abundance (Mean; Phyla > 2%)
1.00
0.75
0.50
0.25
0.00
treatment
control
Phylum
Acidobacteria
Actinobacteria
AD3
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
Proteobacteria
Unassigned
Verrucomicrobia

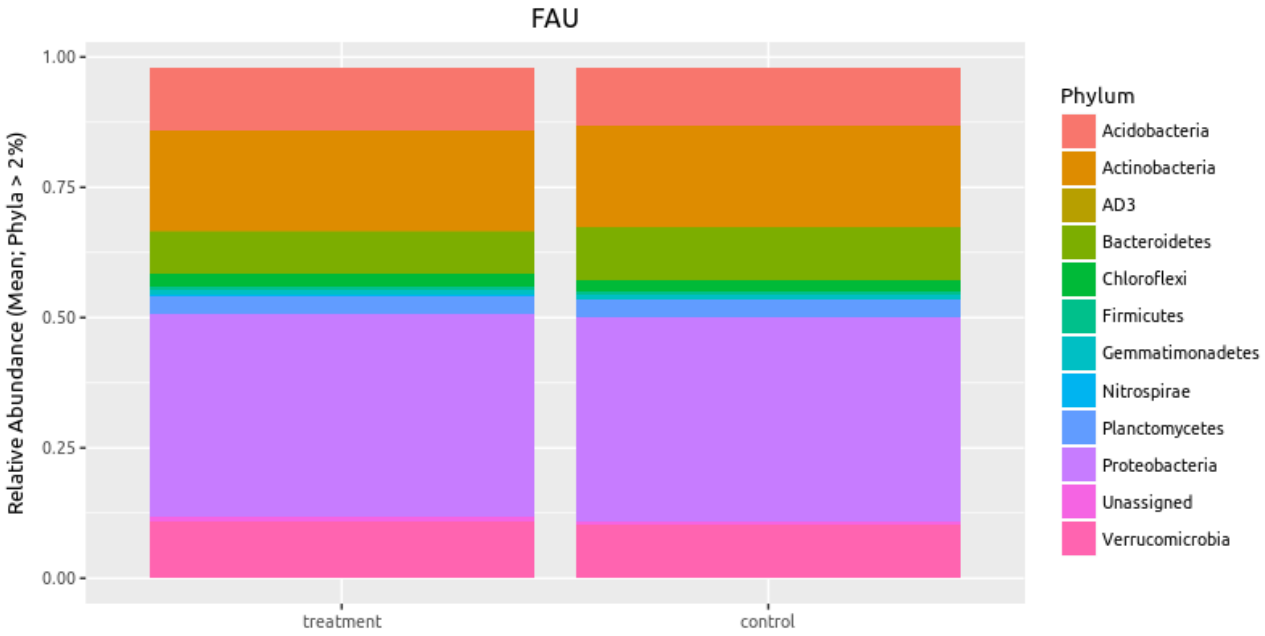
Ålrust (ALR): 8.5°C, 0.6 m



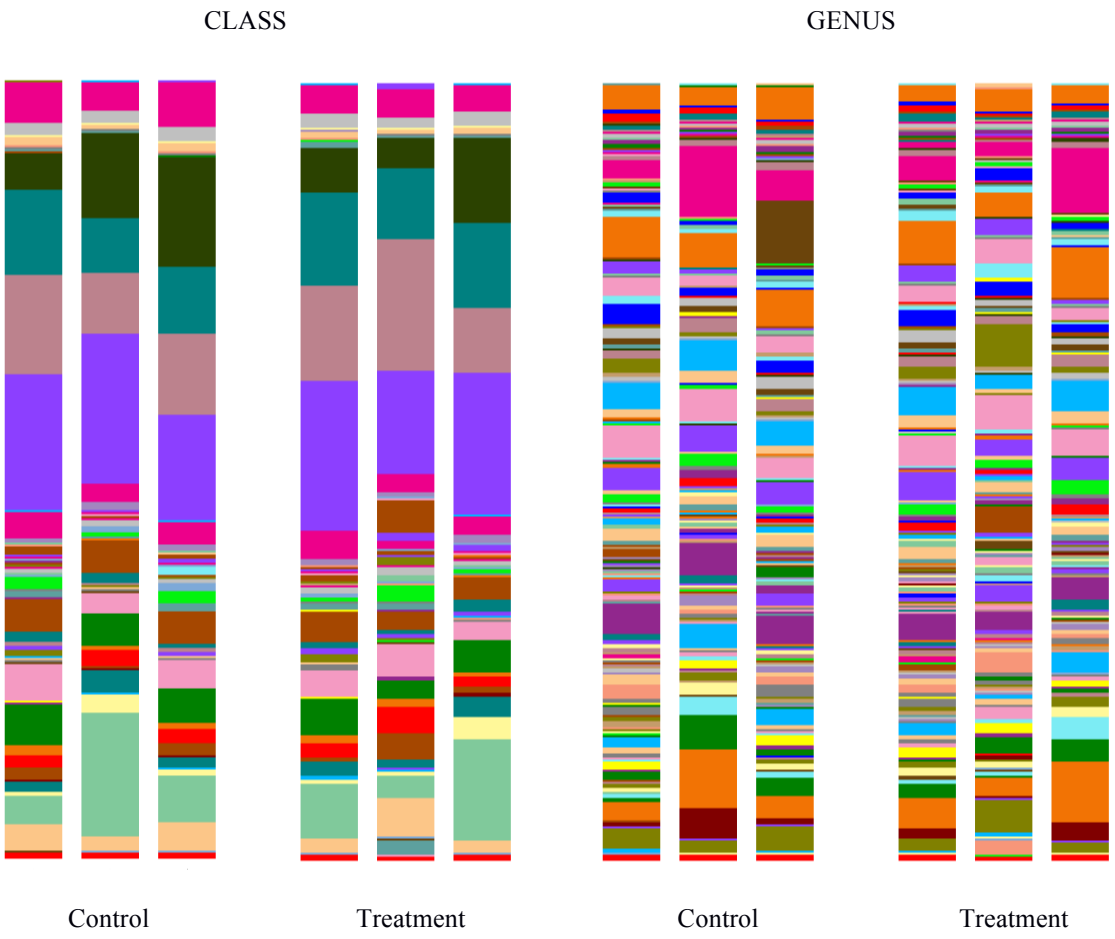


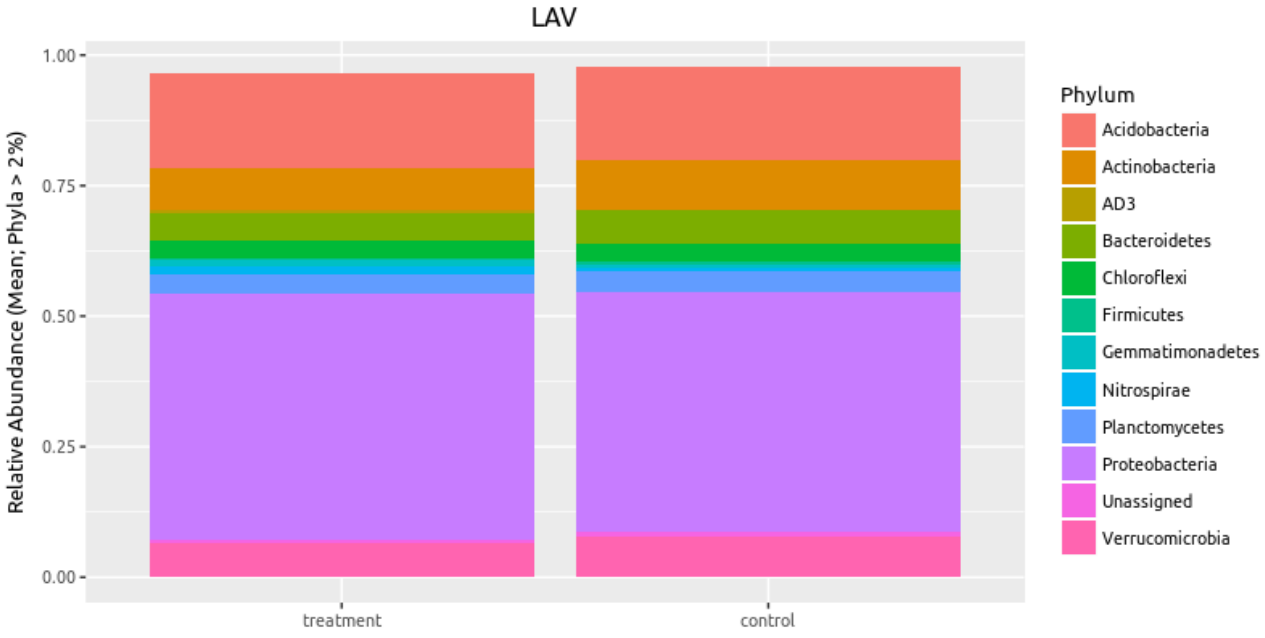
Fauske (FAU): 10.5°C, 0.6 m



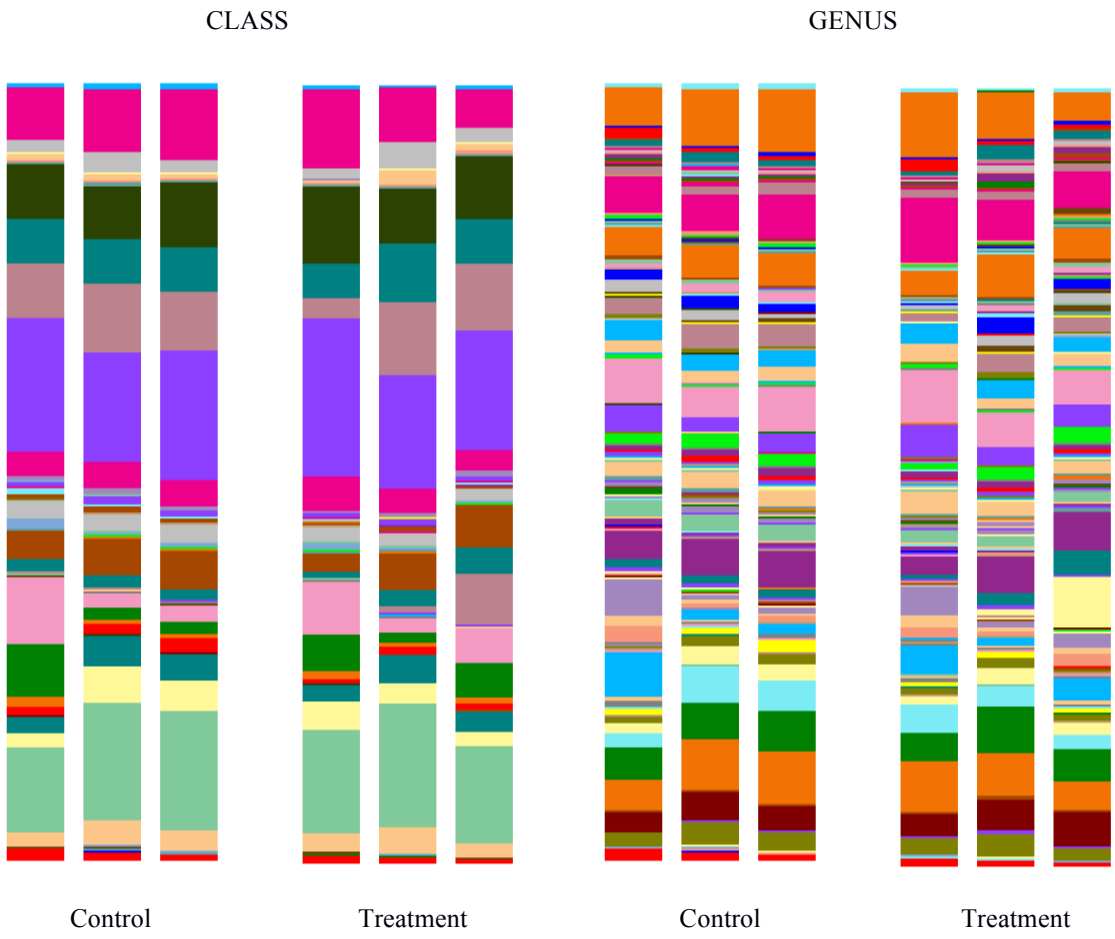


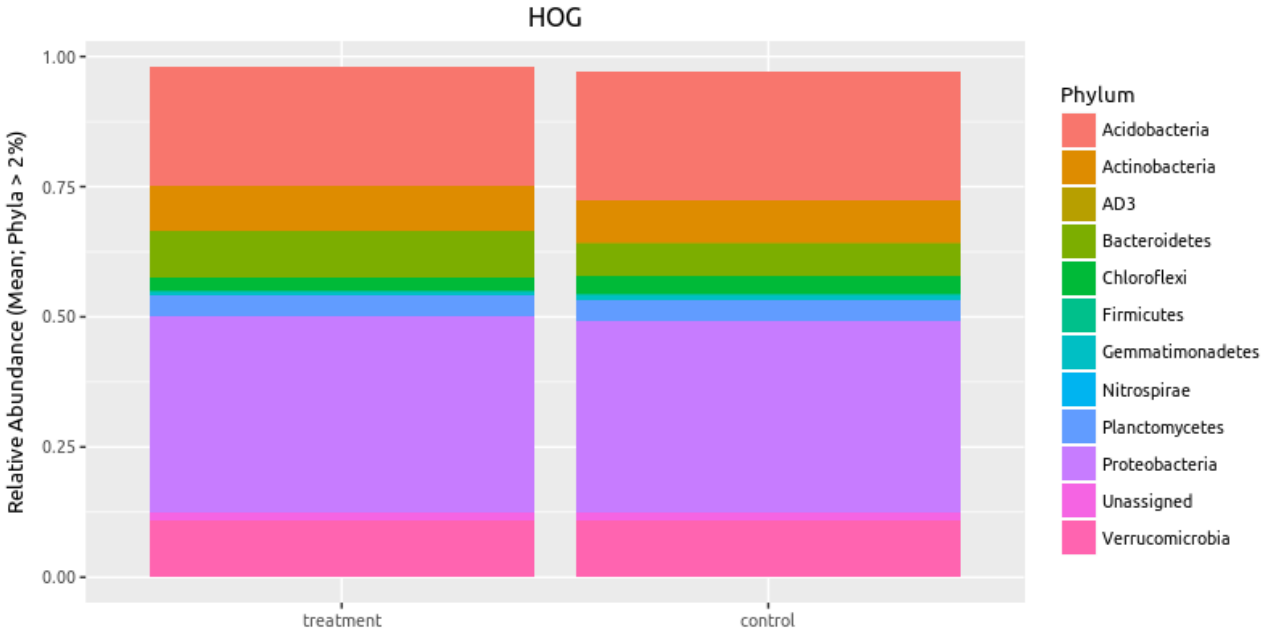
Låvisdalen (LAV): 6.5°C, 1.2 m



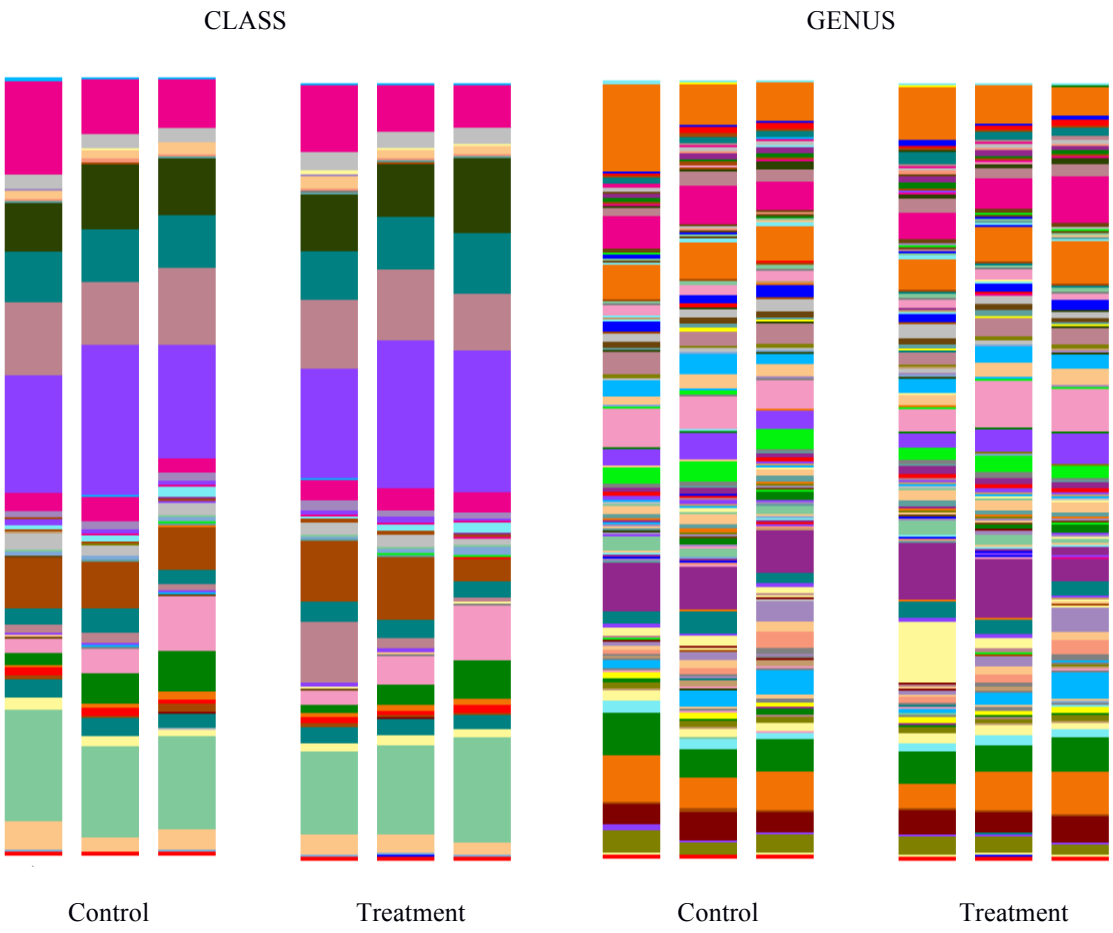


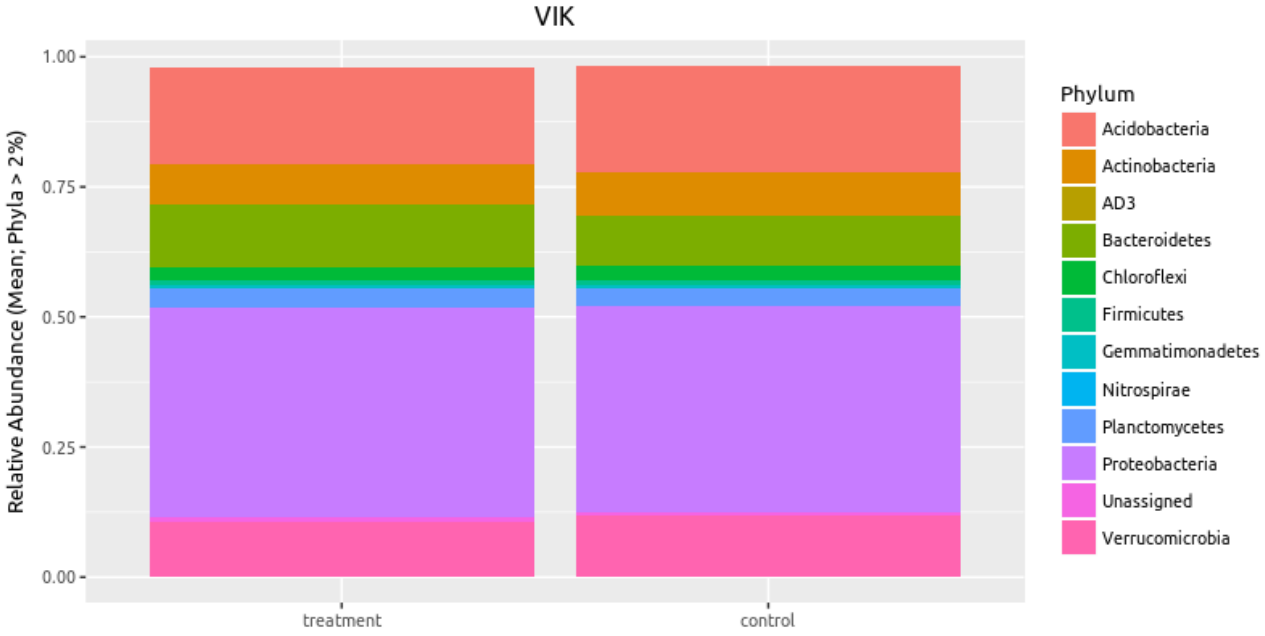
Høgsete (HOG): 8.5°C, 1.2 m





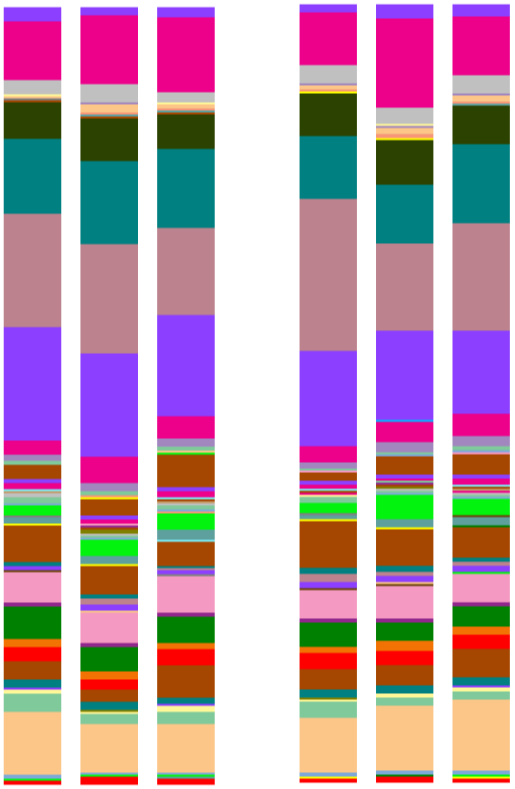
Vikesland (VIK): 10.5°C, 1.2 m





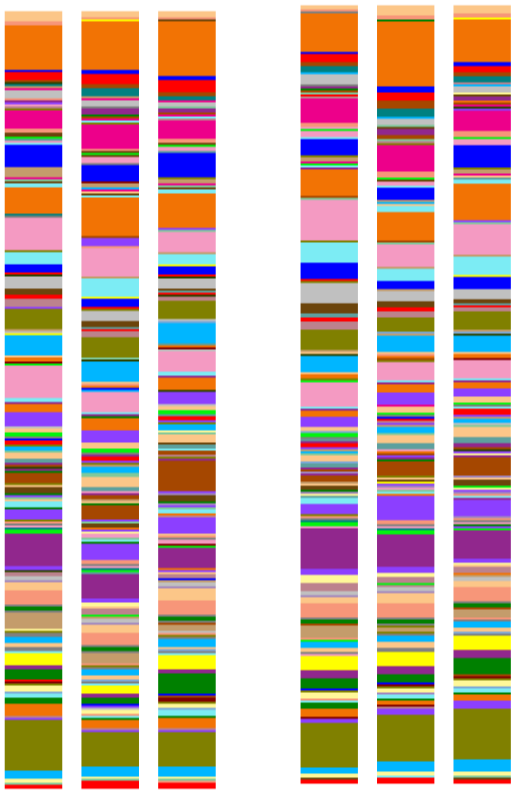
Gudmedalen (GUD): 6.5°C, 2 m

CLASS

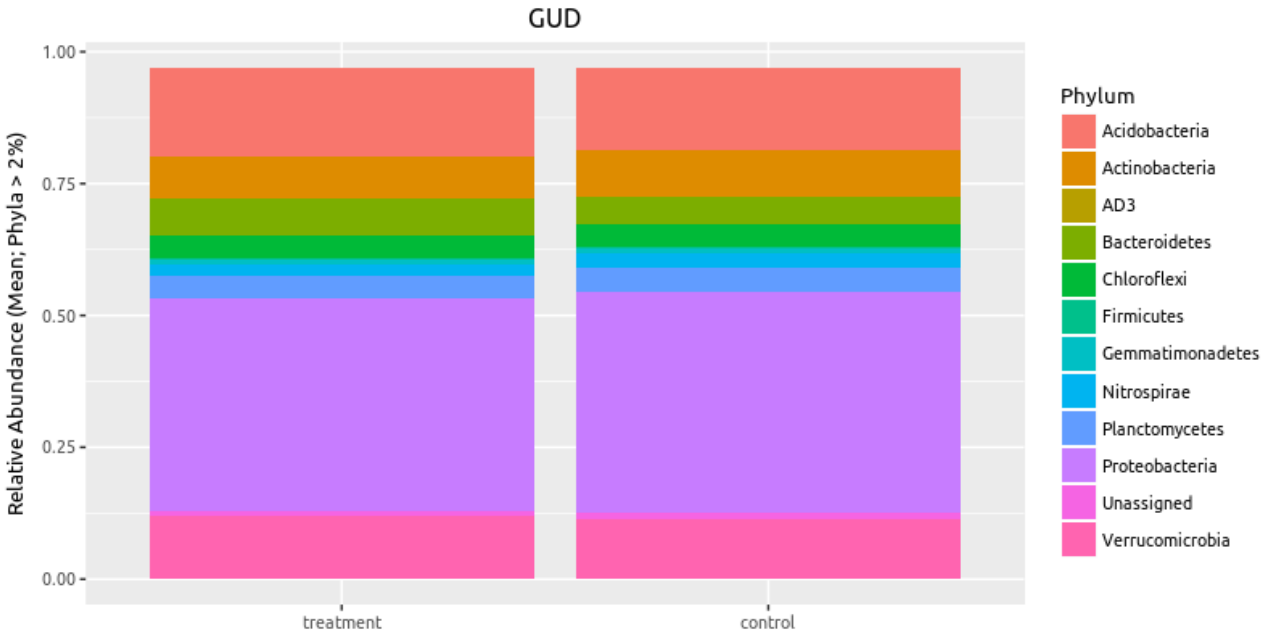


Control Treatment

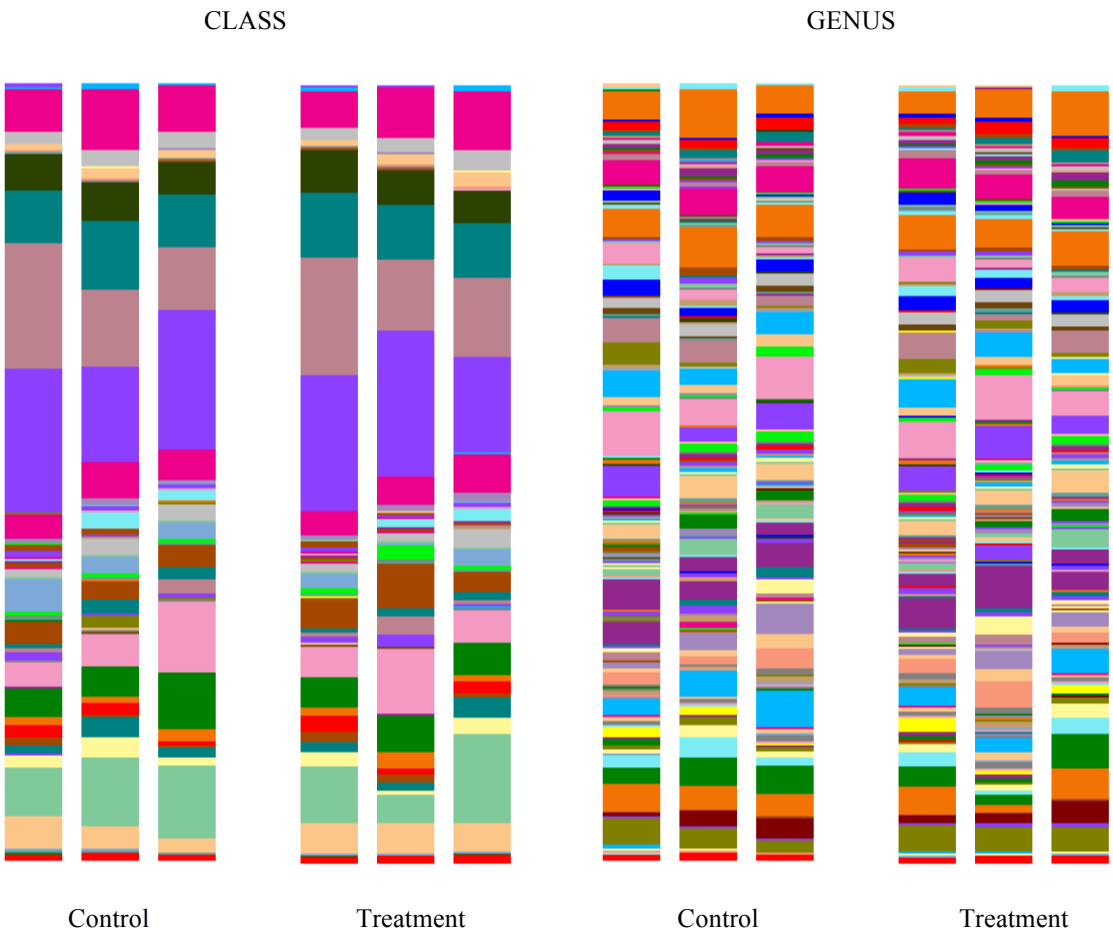
GENUS

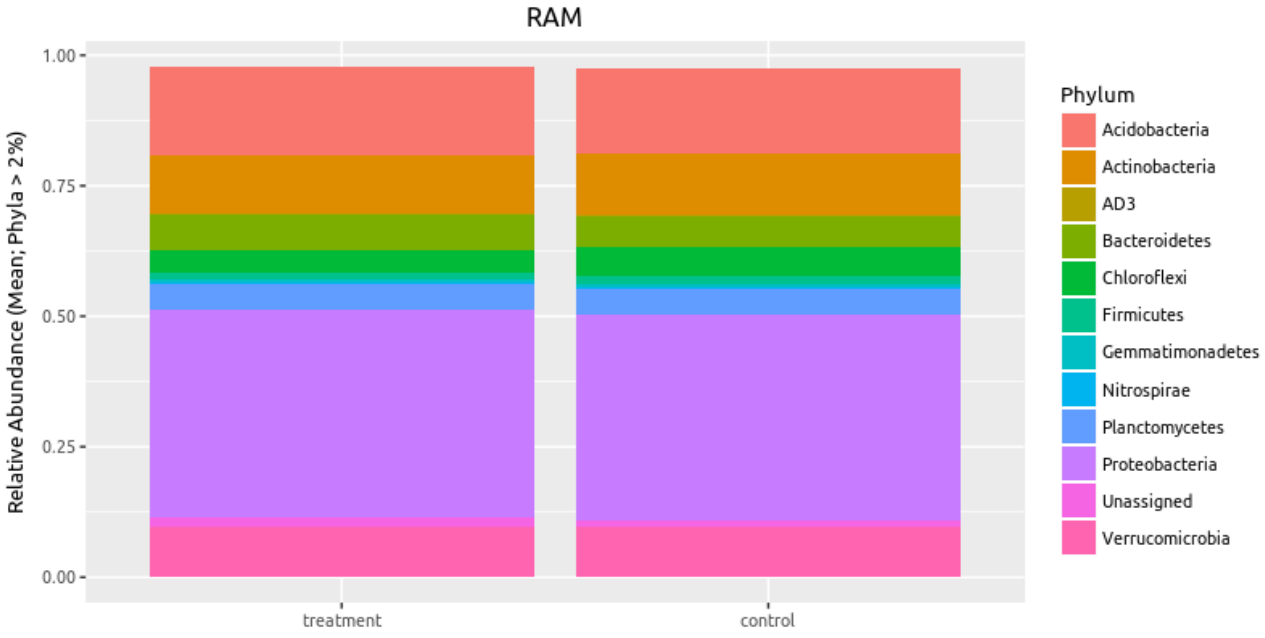


Control Treatment

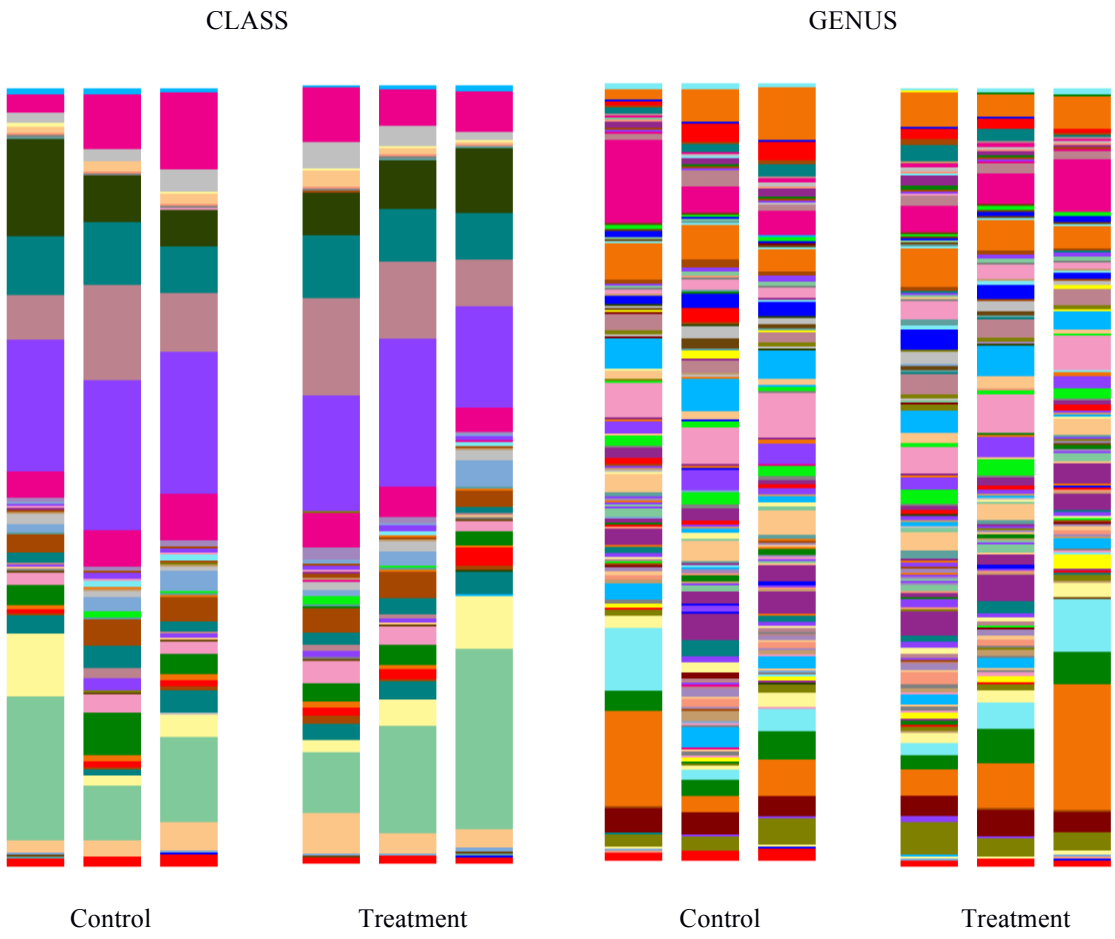


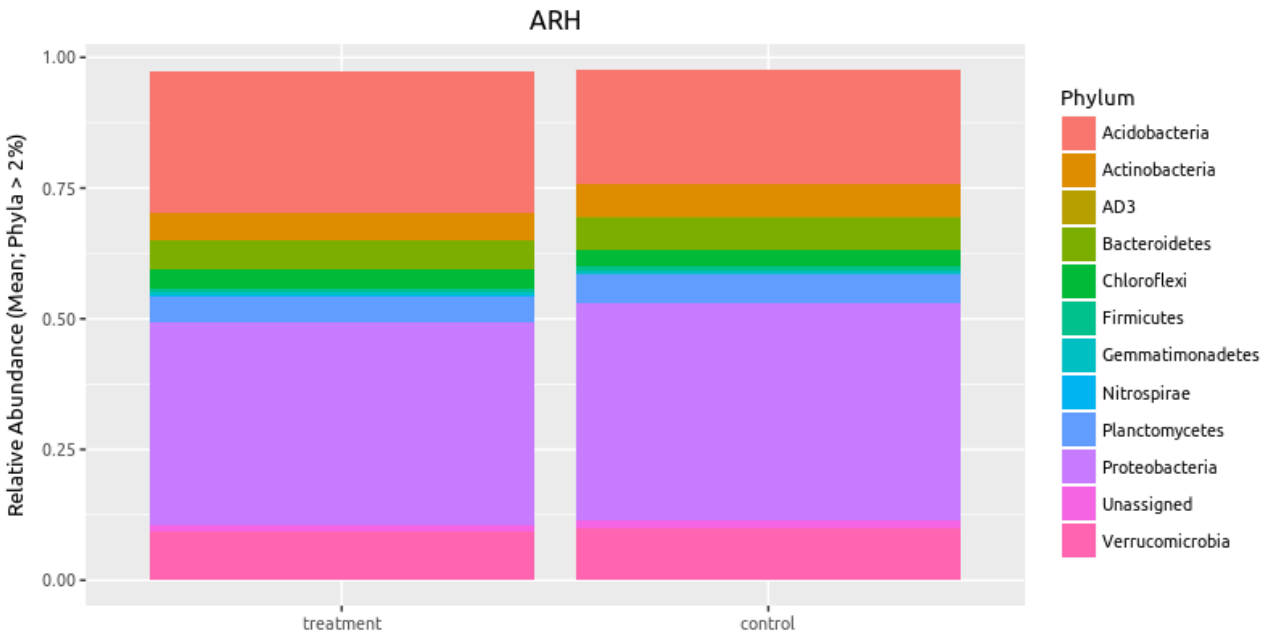
Rambære (RAM): 8.5°C, 2 m





Arhelleren (ARH): 10.5°C, 2 m

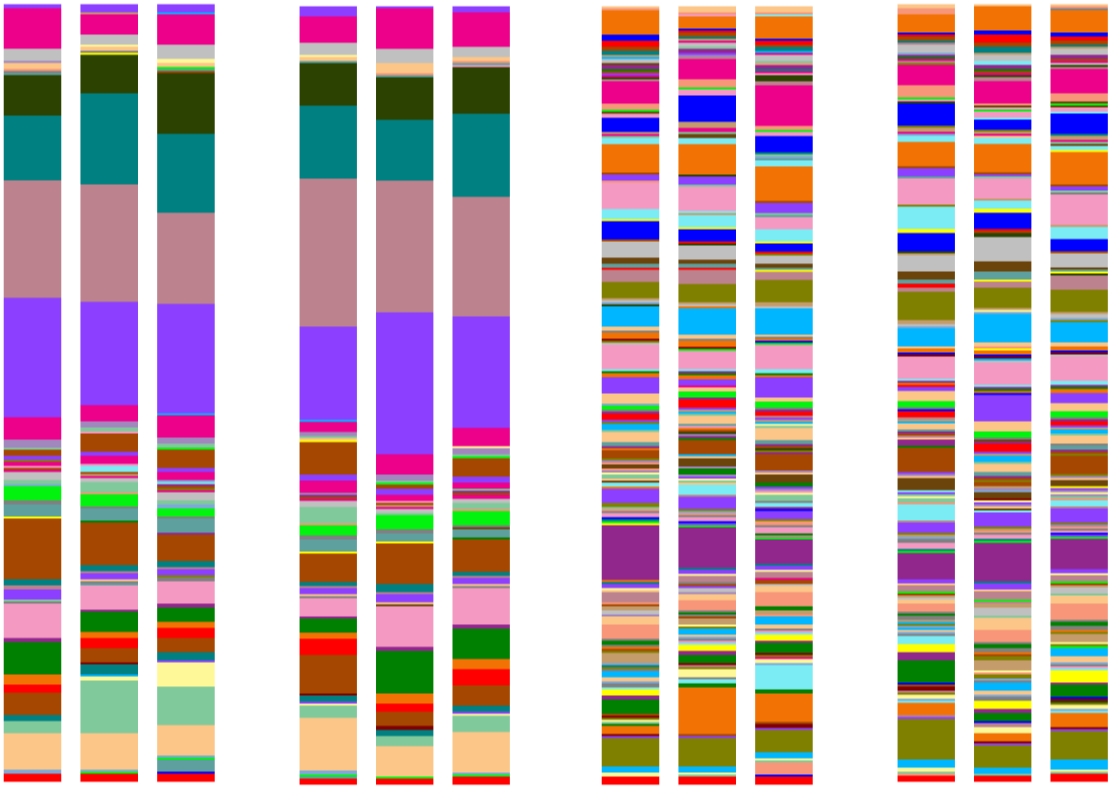




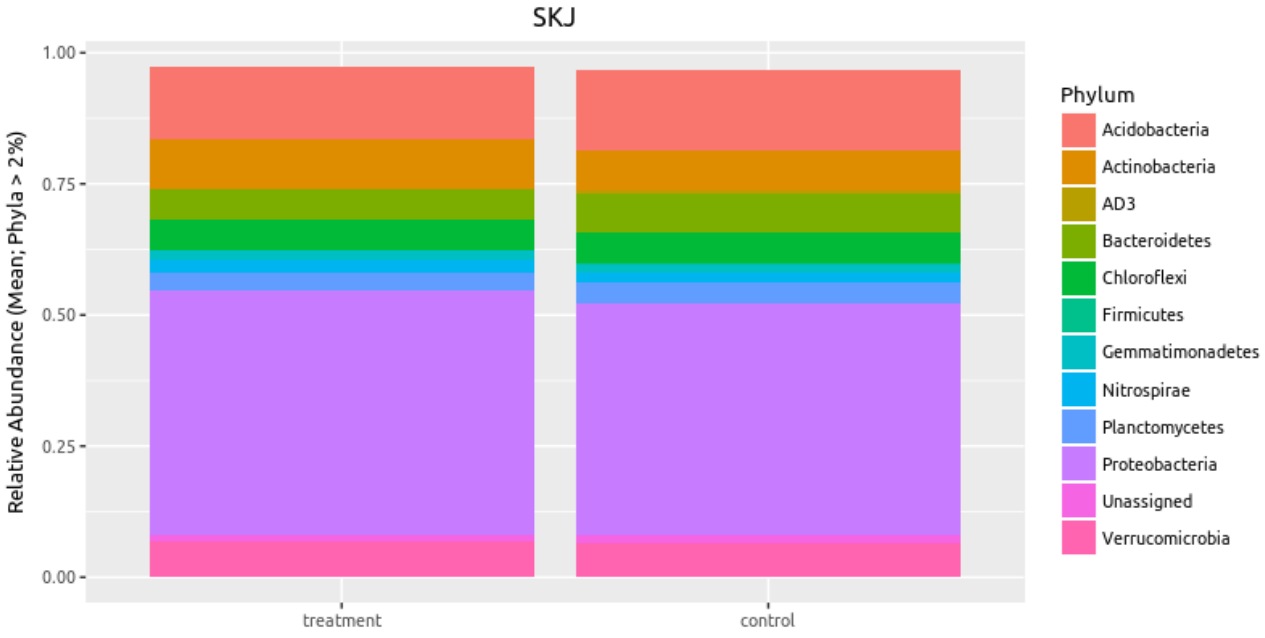
Skjellingahaugen (SKJ): 6.5°C, 2.7 m

CLASS

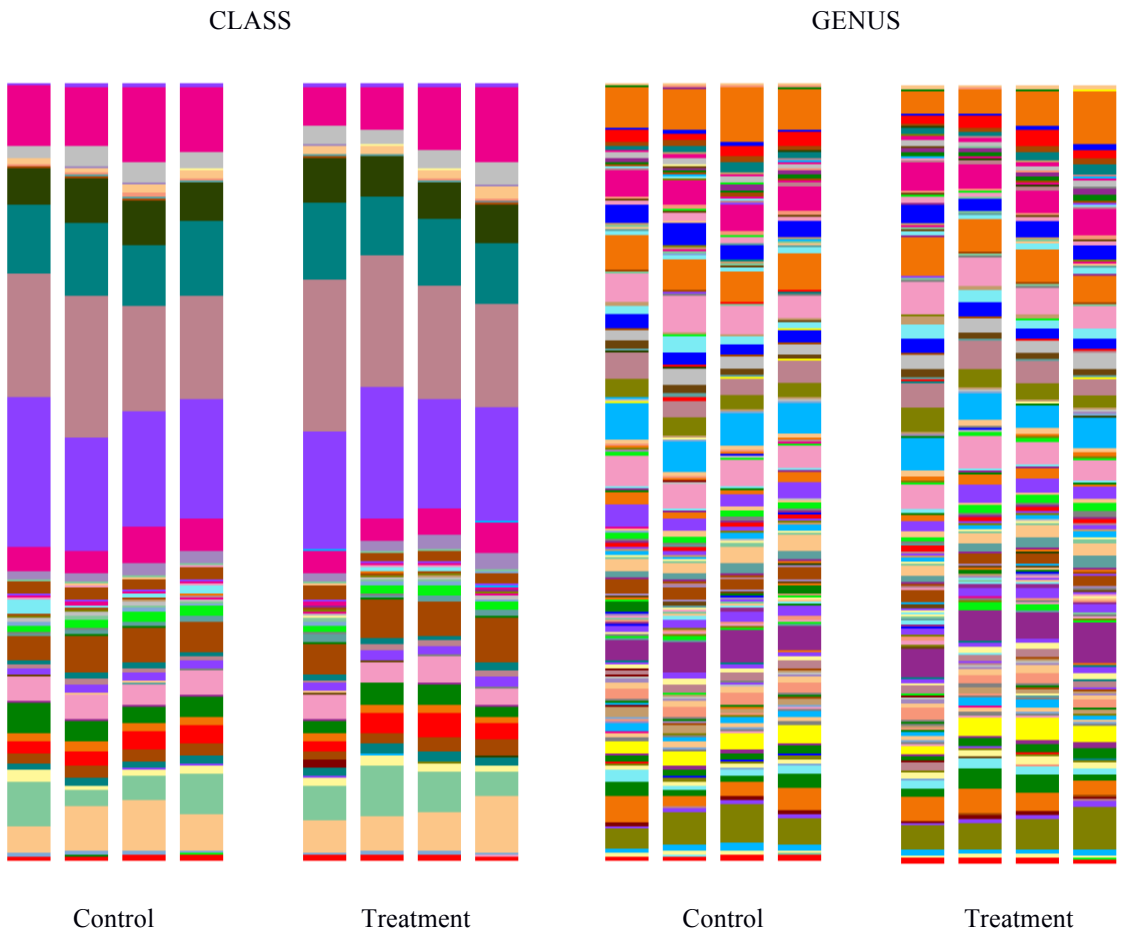
GENUS

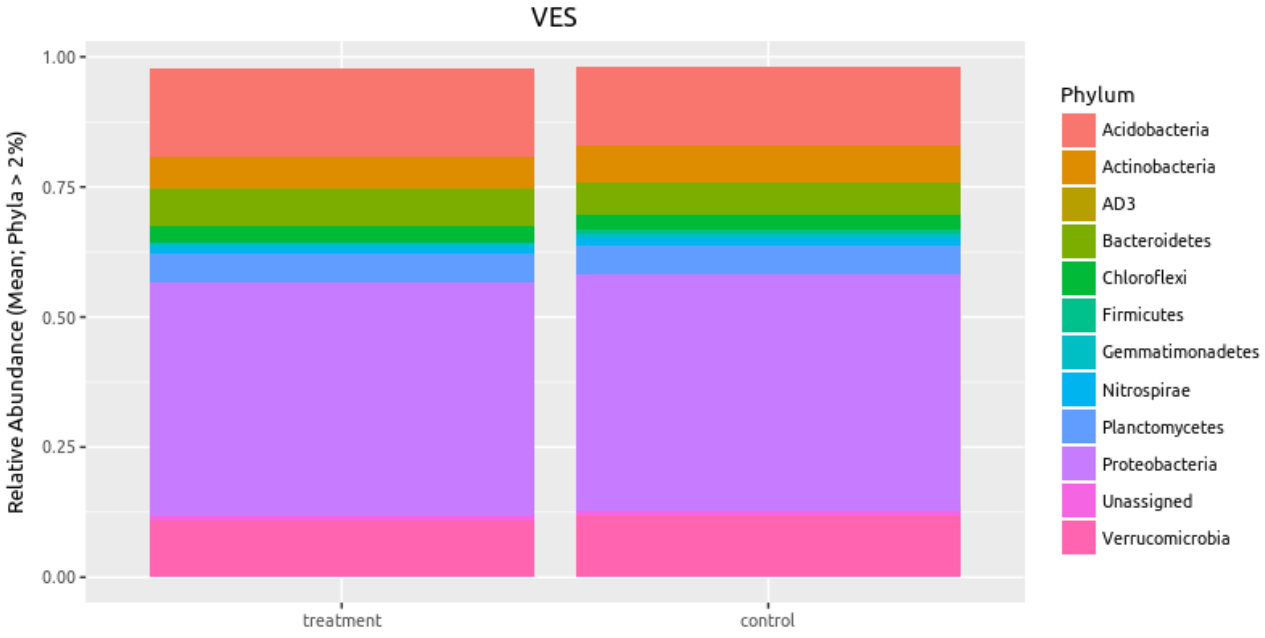


Control Treatment Control Treatment

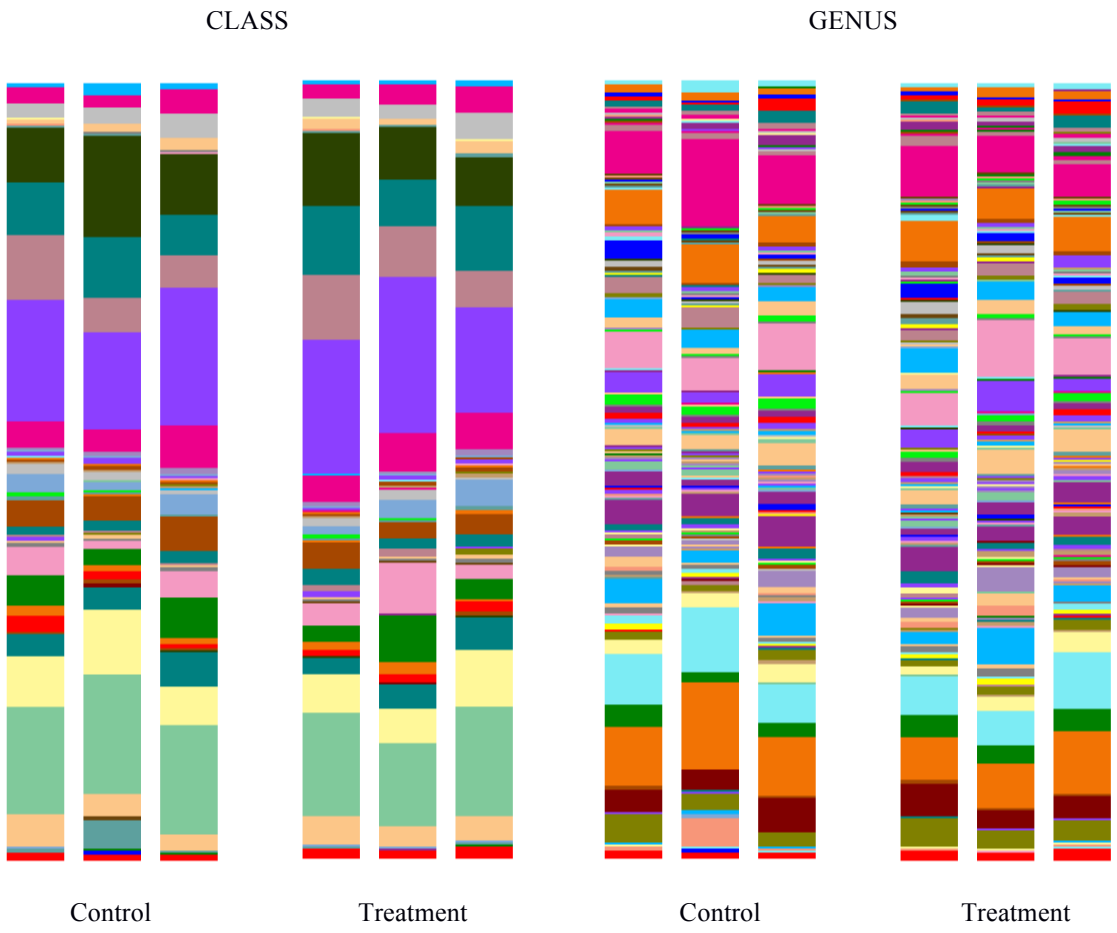


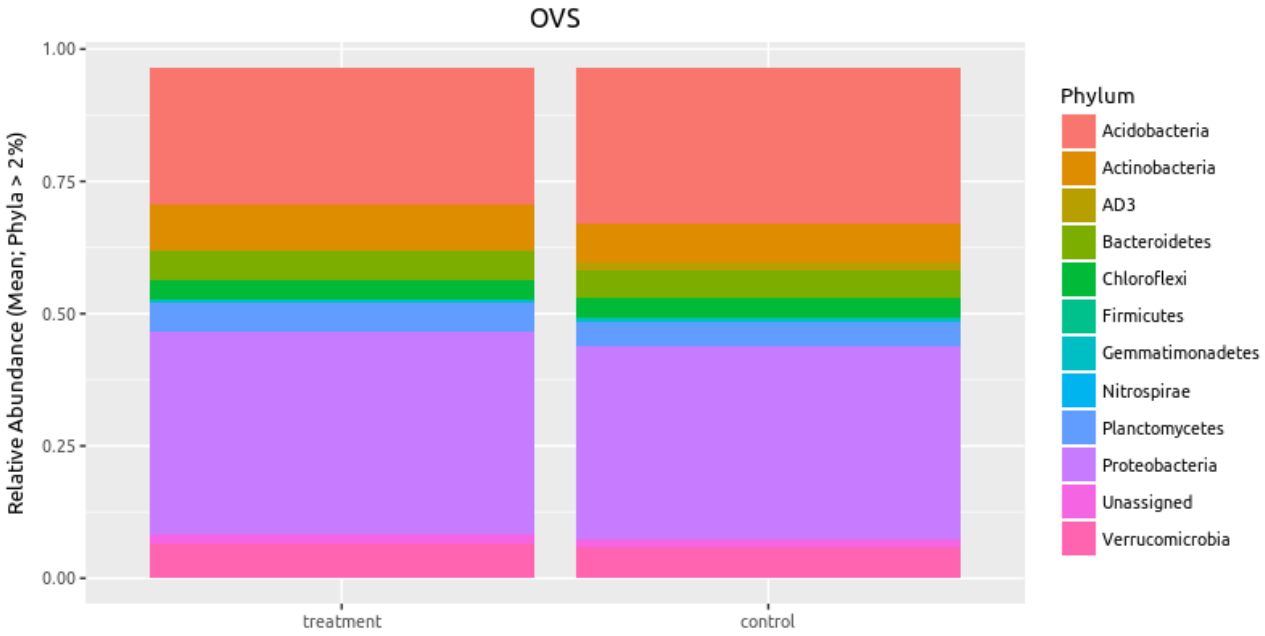
Veskre (VES): 8.5°C, 2.7 m



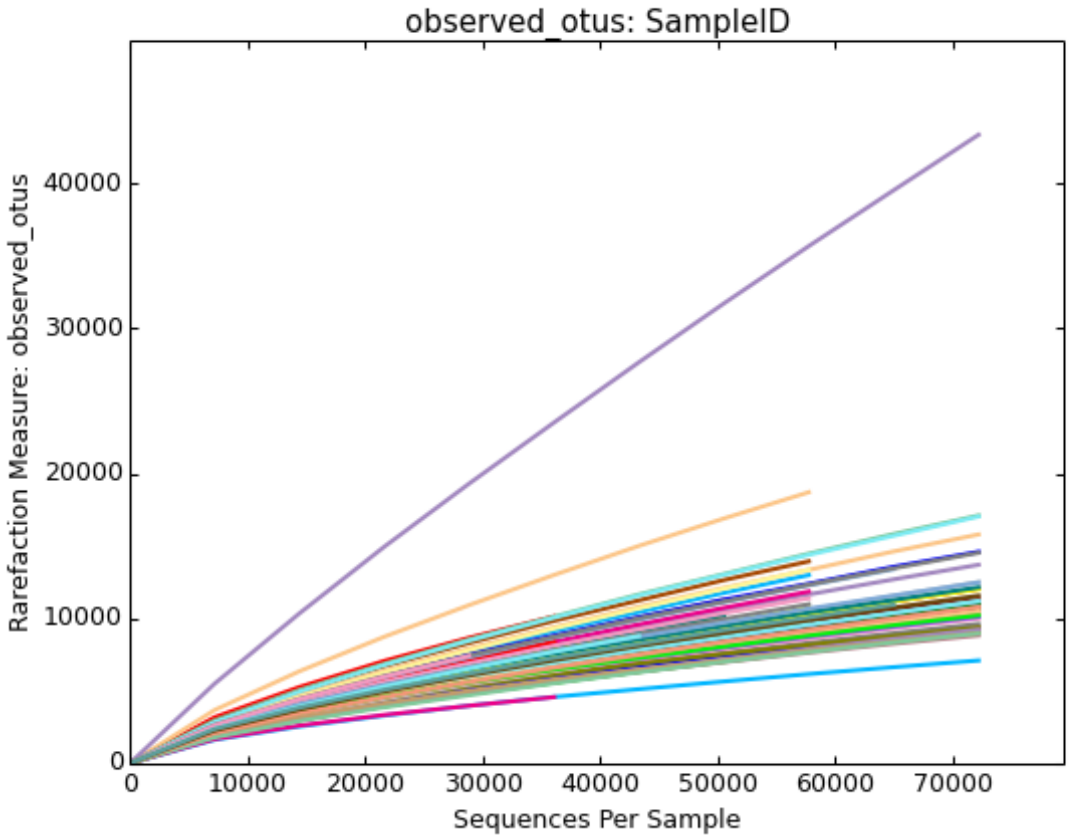


Øvstedalen (OVS): 10.5°C, 2.7 m





Appendix H



Rarefaction curves for the observed number of OTUs in 75 soil samples. The topmost curve belonged to Sample 89.