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Microbial responses to warming and seasonal temperature changes in sub-Arctic forest and grassland soils

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Abbreviations

A_T	Ambient soil temperature, non-warmed soil
C	Carbon
CO_2	Carbon dioxide
CUE	Carbon use efficiency
CH_4	Methane
DNA	Deoxyribonucleic acid
DW	Dry weight (e.g., of soil)
E_T	Elevated soil temperature, warmed soil
EtOH	Ethanol
FN	“Forest New”, medium-term warming forest site
GN	“Grassland New”, medium-term warming grassland site
GO	“Grassland Old”, long-term warming grassland site
GC	Gas chromatography
GWC	Gravimetric water content
GWP	Global warming potential
IPCC	Intergovernmental Panel on Climate Change, United Nations
KCl	Potassium chloride
mRNA	Messenger RNA
N	Nitrogen
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SOC	Soil organic carbon
TNA	Total nucleic acids
WW	Wet weight (e. g., of soil)
y	Years

Abstract

Atmospheric carbon dioxide (CO_2) levels and global temperatures have increased steadily over the past 100 years (IPCC, 2018). Greenhouse gases, such as CO_2, and their emissions from soils play an important role in shaping future climate scenarios. Soil microorganisms are responsible for the turnover of soil organic matter and the release of CO_2 to the atmosphere (Hartley et al., 2008), thereby influencing whether soils act as carbon (C) sinks or sources (Jansson & Hofmockel, 2020). How soil microorganisms respond to warming is therefore a key question for understanding how climate change affects the global terrestrial C cycle and CO_2 emissions from soil. The ForHot research site in Iceland allows *in situ* long-term warming studies on natural soil warming gradients, enabling research that can answer this question (Sigurdsson et al., 2016). In this master thesis, ForHot forest soils were analyzed focusing on changes in RNA:DNA ratios and CO_2 production rates between long-term warmed (~15 y; +3 °C) and non-warmed soils as indicator for changes in microbial physiologies that can affect ecosystem-scale processes. Based on the observation of a downregulation of the microbial protein biosynthesis machinery (i.e., reduced ribosome contents) and increased microbial metabolic activities in long-term warmed grassland soils (Söllinger et al., 2022; Walker et al., 2018), a seasonal survey and a short-term warming experiment were conducted to investigate these physiological and metabolic warming responses in forest soil. Obtained RNA:DNA ratios, used as proxy for cellular ribosome contents of the entire microbial population, indicated a reduction of the average cellular ribosome content in warmed forest soils throughout the year, except in winter, indicating a temperature threshold for ribosomal adjustments. Short-term warming incubations of forest soils further demonstrated a reduction in the average cellular ribosome content after six weeks, but only at the highest warming extent (+9 °C; non-warmed *in situ* temperature: 2 °C), supporting the idea of a temperature threshold for microbial ribosomal adjustments. Finally, a metatranscriptomics analysis of long-term warmed (>50 y) grassland soils, confirmed the above-mentioned downregulation of the protein biosynthesis machinery in all seasons except winter. In conclusion, the reduction in cellular ribosome contents seems to be a common microbial physiological response to warming and seasonal temperature changes that occurs already after a few weeks at a higher temperature, and still occurs after years and decades of warming. The response appears to be controlled by a temperature threshold, below which the effect of warming is absent or even reversed.

1 Introduction

1.1 A warming world

Greenhouse gases, such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and fluorinated gases, are responsible for trapping heat inside the atmosphere and consequently warming the Earth's surface. The impact of greenhouse gases on global warming is measured as global warming potential (GWP), which estimates the energy that one metric ton of emitted greenhouse gas absorbs relative to the energy absorbed by the same mass of CO_2 (EPA, 2022b). While CH_4 has a shorter lifespan than CO_2, it has a 27 – 30 times higher GWP over a 100-year period. N_2O, in contrast, has a GWP 273 times that of CO_2 over a 100-year period, and has a half-life in the atmosphere of more than 100 years. Fluorinated gases, such as chlorofluorocarbons, hydrofluorocarbons, hydrochlorofluorocarbons, perfluorocarbons, and sulfur hexafluoride, are estimated to trap thousands to tens of thousands times more heat in the atmosphere than CO_2. (EPA, 2022b)

Earth's carbon (C) and nitrogen (N) cycles are the natural sources of CO_2, CH_4, and N_2O. CO_2 circulates between soils, oceans, plants, animals, and the atmosphere as a part of the C cycle. N_2O remains in the atmosphere for decades before being removed by microbial denitrification or photochemical reactions. CH_4 is emitted from natural sources such as wetlands and removed by methanotrophic microorganisms or photochemical reactions in the atmosphere (IPCC, 2007, 2013a). Fluorinated gases originate mainly from human-related activities and have no significant natural source (IPCC, 2019).

The levels of atmospheric CO_2 and global temperatures have been steadily increasing since the industrial revolution (IPCC, 2018). Over the last 200 years, the levels of CO_2 have increased by 50% due to human activities (*Carbon Dioxide*, 2022), while global temperatures have also risen rapidly over the last century (IPCC, 2018). According to the Intergovernmental Panel on Climate Change (IPCC), human activities, chief among them being land use, are the main drivers of global greenhouse gas emissions. CO_2 is the primary greenhouse gas emitted to the atmosphere as a result of human activities, mainly through the burning of fossil fuels, waste, and biological materials, and by reducing the C uptake capacity of natural sinks, such as forests and soils (EPA, 2022a). CH_4 and N_2O are also emitted as a result of agricultural activities and land use change, while fluorinated gases are emitted from commercial, and industrial processes, but typically in smaller quantities (EPA, 2022a).

1.2 The terrestrial C cycle

Soil microorganisms such as bacteria, archaea, fungi, and protists are key players in the turnover of soil organic matter and play a significant role in the terrestrial C cycle (Hartley et al., 2008). Plant roots or decaying biomass provide C input that is metabolized by soil microorganisms. Stabilization of atmospheric C in the form of soil organic matter after C-fixation by plants and microorganisms serves as a major C storage mechanism, while microbial decomposition of soil organic matter releases CO_2 and CH_4 to the atmosphere (Canadell et al., 2007). The ratio between C incorporated into the cellular biomass to the total organic C taken up by the cell, defined as microbial C use efficiency (CUE), indicates how much C is directed to anabolic reactions and thus remains in the soil. Thus, CUE demonstrates the microbial metabolic influence on whether soils act as C sinks or sources (Jansson & Hofmockel, 2020; Manzoni et al., 2012).

Due to slow decomposition in cold environments, northern high latitude soils store almost 30% of global soil C, making these environments particularly important in the context of global warming (CAFF, 2013). The IPCC (2013a) estimates that average temperatures in Arctic and sub-arctic regions will have increased by +6 °C by 2099. Soils are also abundant in other climate zones, and microbial activities, including decomposition, are expected to increase globally as a result of warming (Megonigal et al., 2003). However, plant growth and CO_2 fixation are also expected to increase, and the resulting equilibrium between these processes under warming, which remains elusive, will determine if warming leads to net emissions (Jansson & Hofmockel, 2020).

Natural emissions of greenhouse gases are balanced by the sink function of atmospheric chemical reactions, plants, soils and oceans, but anthropogenic emissions are disturbing this balance (IPCC, 2021). Soils, especially high-latitude forests, grasslands, peatlands, and permafrost regions, serve as C storages that are on the verge of releasing large amounts of C into the atmosphere (EPA, 2022a). This could potentially trigger a feedback loop that accelerates climate change by further warming the atmosphere and increasing the C loss from soils (Cavicchioli et al., 2019). In oxic soils, such as the sub-arctic forest and grassland soils under investigation in this master thesis, CO_2 is the most relevant microbial-derived greenhouse gas (Schlesinger & Andrews, 2000). In addition to amplifying radiative forcing and therefore natural greenhouse gas emissions via warming, CO_2 can also amplify emissions of other greenhouse gases via other feedback loops. For example, a meta-analysis of 49 published

studies on greenhouse gas emissions from soils found that elevated CO_2 levels stimulated CH_4 emissions form wetlands and N_2O emissions from upland soils (van Groenigen et al., 2011).

Thus, greenhouse gas emissions from soils play a crucial role in shaping future climate scenarios and the soil microbial response to global warming will ultimately determine how the terrestrial C cycle will change in the future.

1.3 Microbial responses to warming

1.3.1 Temperature effects on metabolic activities

Heat is characterized by higher motion in atoms, resulting in an increased kinetic energy. The relevance of temperature for biological processes lies in the principle that higher energy of a substrate means higher reactivity of the same (*Effect of Temperature on Enzymatic Reaction*, 2022). High temperatures accelerate enzymatic reaction rates on a molecular level and thus can lead to higher microbial activities and growth rates. Additionally, the increased molecular movement that follows higher temperatures means more motion within molecular structures, including membranes and proteins. Microorganisms can counteract this by synthesizing more rigid structures that offset the sometimes-damaging effects of such increased fluidity. Any microorganism is adapted to a certain temperature range where its performance of important reactions is most efficient, but if temperatures exceed this range, adjusting to the new temperature conditions becomes necessary. For microorganisms undergoing physiological adjustments to cope with increased temperatures, an adjustment time is required (Bárria et al., 2013). The acceleration of reaction rates at warmer temperatures suggests that higher microbial activities in warmed soils can lead to more respiration by soil microorganisms, linking microbial temperature responses directly to global warming, and highlighting that microbial adjustment to warming matters.

1.3.2 Warming effects on soil

Natural soil warming caused by geothermal activity allows studies on the effects of elevated soil temperatures and comparison with non-warmed soil temperatures in areas not affected by geothermal warming (Sigurdsson et al., 2016). At the ForHot *in situ* warming site close to Hveragerði, Iceland, studies on soil warming have been carried out for 15 years. One grassland at the site has been warmed for more than 50 years (possibly since 1708), while after an

earthquake in 2008, warming began at a second grassland and a forest site, the latter being the focus of this thesis. The natural warming gradients at the ForHot sites are seasonally consistent and stable since measurements began in 2013 and include non-warmed control plots as well as plots with a gradually increasing degree of warming. All sites are unmanaged and have been intensively studied regarding gas fluxes, soil biogeochemistry, plant growth and diversity, microbial diversity and community dynamics in response to soil warming (Sigurdsson et al., 2016).

In the longest warmed ForHot grassland soils, Walker et al. (2018) reported reduction of soil C and N in topsoil, and nutrient depletion of 11% per 1 °C increase co-occurring with a reduced microbial biomass. A systemic overreaction was observed after 5 – 8 years of warming, while after >50 years of warming the system had stabilized and was not altered by further warming (Walker et al., 2020). The overreaction was characterized by an increase in biotic activity due to accessible soil organic carbon (SOC) and nutrients, resulting in a rapid decline in C pools after 5 – 8 years of warming. Other studies conducted on different soil ecosystems reported a similar overreaction and subsequent return to pre-warming respiration rates within the first years of warming, possibly due to the depletion of easily degradable substrates (Kirschbaum, 2004; Knorr et al., 2005). Large and proportional C and N losses in warmed ForHot grassland soils were also reported by Marañón-Jiménez et al. (2019). Furthermore, a persistent increase in microbial respiration per unit of microbial biomass was found with warming (Marañón-Jiménez et al., 2018; Walker et al., 2018), showing a mechanism at ecosystem level where a microbial physiological adjustment is apparently lacking. The authors suggested that C depletion as a consequence of soil warming leads to increased energy costs for microorganisms to sustain the same metabolic rates and resource acquisition, ultimately resulting in a weaker C storage capacity in warmed soils (Marañón-Jiménez et al., 2018).

A decrease in microbial biomass in warmed soils was found in several studies but it is still uncertain whether this is linked to a reduction in respiration rates (Marañón-Jiménez et al., 2018; Melillo et al., 2017; Walker et al., 2018). For example, after the onset of warming, an initial increase in soil respiration was followed by a subsequent decrease over time and a return to pre-warming respiration rates (Kirschbaum, 2004; Knorr et al., 2005).. It has been suggested that this could mean that the long-term warming response of soil respiration is less strong than the short-term response (Romero-Olivares et al., 2017).

1.3.3 Microbial responses to long-term warming

Long-term warming experiments are important as it is not possible to infer long-term responses from the extrapolation of short-term responses (Romero-Olivares et al., 2017; Torn et al., 2015). However, difficulties lie in the availability and maintenance of long-term study sites, plus the source of warming. Artificial warming is cost intensive and prolongs the required timeframe as installing heating cables disturbs the soil environment (Melillo et al., 2017). Thus, the natural warming gradients powered by geothermal activity at the ForHot research site are unique and with evidence linking back to the 18th century ForHot forms the world's longest lasting *in situ* warming experiment (Sigurdsson et al., 2016).

In another truly long-term warming study, the Harvard Forest experiment, 26-year soil warming of a mid-latitude hardwood forest was used to investigate soil warming effects during the growing season (April – November) (Melillo et al., 2017). They found a four-phase pattern in soil organic matter decay and CO_2 fluxes over two decades of warming. Phases of substantial C loss and no detectable loss seem to be related to depletion of microbially accessible C pools and their subsequential regeneration. A decrease in fungal abundance, a community shift towards gram-positive bacteria, an increase in bacterial evenness, an increase in abundance of bacteria with low ribosomal RNA (rRNA) operon copy numbers, and a decrease in microbial biomass in warmed soil was also found (Melillo et al., 2017). In line with the large shifts in soil composition occurring alongside microbial shifts in the Harvard Forest, it has been proposed that a combination of temperature adjustment and substrate limitation form the so-called warming effect (Crowther & Bradford, 2013; Hartley et al., 2008; Kirschbaum, 2004). Domeignoz-Horta et al. (2023) suggested that long-term warming affects microbial physiology indirectly via reduced C availability, and that this has a larger impact on soil C pools than the changes in microbial physiology caused by the direct effect of temperature. However, studies on microorganisms in pure cultures exposed to substrate saturation and temperature change demonstrate large direct effects of temperature change on microbial physiology (e.g., Tveit et al. (2023)). This might imply that the interwoven direct and indirect effects of temperature cannot easily be disentangled.

1.3.4 Downregulation of the protein biosynthesis machinery

Possible microbial responses of the combined effects of warming are changes in microbial communities and their interactions, physiological adjustments, and adaptation on a genetic

level. While microbial communities may shift in quantity and composition, physiological adjustments occur on an individual scale and may include transcriptional and translational regulations of growth and metabolic pathways (Söllinger et al., 2022). In a recent study by Söllinger et al. (2022) conducted on the ForHot grasslands, a downregulation of the bacterial protein biosynthesis machinery in warmed soil was observed, coinciding with a lower microbial biomass, RNA, and soil substrate content. In addition, gene expression connected to replication and central metabolic pathways were up-regulated. The taxon-independent downregulation of the protein biosynthesis machinery in warmed soils was also reflected by a reduction in cellular RNA/ribosome content and suggested a common microbial physiological response to warming (Söllinger et al., 2022).

Ribosomes are the macromolecules responsible for protein biosynthesis. These molecules consist of two subunits, a large and a small one, and differ in size between prokaryotes (30S, 50S) and eukaryotes (40S, 60S). The subunits consist of rRNAs and proteins (ribosomal proteins). Due to the high number of ribosomes in both prokaryotic and eukaryotic cells, around 95% of the total RNA in cells are rRNAs (Lafontaine & Tollervey, 2001). Messenger RNA (mRNA) is transcribed from the template-DNA by an enzyme, the RNA polymerase, and by ribosomes translated into polypeptide chains, which will turn into the mature proteins. The translation process is an expensive cell process, and thus the reduction of the cellular ribosome content in response to warming as described by Söllinger et al. (2022), could liberate energy and matter that can be used to accelerate other processes such as microbial respiration.

1.3.5 Seasonal dynamics of soil microorganisms

Seasonality in ecosystems is defined by periodic changes of environmental conditions in an annual cycle (Williams et al., 2017) and is characterized by periods of cold and heat, aridity and humidity. Influenced by the accessibility of resources essential to their survival, such as nutrients, water, and energy from of heat, populations might grow and decline due to seasonal changes (Fretwell, 1972).

In soil, the seasonality of microorganisms and plants plays a crucial role for nutrient retention and C and N cycling. Gündler et al. (2021) reported that microbial biomass peaks in winter and thereafter declines in spring and summer. In summer, high growth rates, respiration and turnover rates were observed and with soil warming, these high rates were prolonged into

autumn and winter. Furthermore, Gündler et al. (2021) reported a decrease in microbial biomass caused by soil warming, possibly leading to high C and N losses in winter. In addition, substrate availability was lower in summer, challenging the soil microorganisms with nutrient limitation and possibly leading to thermal adjustment of microbial respiration, while in autumn, no temperature sensitivity in C cycling could be detected (Gündler et al., 2021). The lack of a temperature response in autumn could be due to the autumn litter input by plants, that provides substrates and possibly negates an effect of temperature (Domeignoz-Horta et al., 2023). Based on these observations of large seasonal shifts in temperature and substrate availability, microbial physiological response to seasonal change is likely to be a common phenomenon. Understanding seasonal shifts will therefore be important for understanding responses to global warming.

1.4 Objectives and research questions

The main objective of this master project is to investigate the effects of short- and long-term warming and seasonal temperature changes on microorganisms in the naturally warmed sub-arctic ForHot forest soils and compare these to warming effects on grassland soil communities. To achieve this the project includes three parts:

1. Seasonal survey on long-term warmed (14 y) forest soils
2. Short-term warming experiment (6 weeks) with forest soils
3. Metatranscriptome analysis of long-term warmed (>50 y) grassland soils

In a study conducted on grassland soil from the ForHot research site, it was proposed that microorganisms exposed to warming reduce their ribosome content and subsequently reallocate energy and matter previously used for ribosomes and ribosomal protein synthesis to other processes (Söllinger et al., 2022). This leads to highly active microbial cells in warmed soil and may have large potential consequences for the soil ecosystem, the terrestrial C cycle, and the climate system.

To investigate if the same effects of warming occur in the long-term warmed ForHot forest soils, a seasonal survey of forest soils as well as an incubation experiment were conducted. The first part of this project is a seasonal comparison and assessment of whether the expected physiological effect of temperature, namely the reduction of ribosome content, occurs throughout all seasons. RNA per unit of soil was used to indicate the ribosome content per unit

of soil and DNA per unit of soil served as an indicator for the approximate number of microbial cells, while the resulting RNA:DNA ratio gave a proxy for the average cellular ribosome contents of the microbial populations. Additionally, a short-term warming experiment with different incubation temperatures (chosen according to the *in situ* temperatures in the non-warmed and warmed forest plots) was used to investigate a potential temperature threshold for cellular ribosome reduction and test the hypothesis that higher temperatures lead to higher microbial activities, resulting in increased greenhouse gas production at higher temperatures. The focus was set on CO_2 emission rates (i.e., soil microbial respiration), since CO_2 is the most abundant greenhouse gas and has a long half-live in the atmosphere, implying that today's C emissions will have consequences for hundreds of years.

In addition to the two main parts of this thesis, the seasonal ForHot forest survey and the incubation experiments using ForHot forest soils, a metatranscriptomics analysis of long-term warmed ForHot grassland soils was conducted to study adjustments of the microbial protein biosynthesis machinery throughout seasonal temperature changes.

The following research questions are aimed to be answered in this thesis:

1. Is the reduction of the cellular ribosome content that has been shown in warmed grassland soils also triggered in warmed forest soils? *Total nucleic acid (TNA) extractions were used to determine the RNA and DNA contents in non-warmed and long-term warmed soils with the RNA:DNA ratio giving a proxy for average cellular ribosome contents in the microbial population.*
2. Are the same physiological adjustments occurring throughout the seasons? *Also here, TNA extractions were used to obtain RNA:DNA ratios of non-warmed and long-term warmed soils and compare them during four seasons.*
3. What feedback do these microbial responses have on soil processes such as CO_2 emissions? *A short-term warming experiment was conducted to measure CO_2 emission rates from short-term warmed forest soils using gas chromatography and RNA:DNA ratios were determined using TNA extractions to investigate the relationship between cellular ribosome content and metabolic activity (i.e., CO_2 emission).*
4. Can the seasonal pattern of cellular ribosome content reduction in forest soils also be found on transcriptional level in seasonal grassland soils? *A metatranscriptomics analysis of long-term warmed grassland soil was conducted to compare transcripts in non-warmed and warmed soils across seasons.*

1.5 Hypothesis

The underlying hypothesis for a common microbial warming response is that cells exposed to warming reduce their ribosome content. Due to higher enzymatic reaction rates at increased temperatures, the protein biosynthesis machinery works more efficiently and allows soil microorganisms to produce the required number of proteins with a reduced number of ribosomes. Thereby, energy and matter previously used for ribosome production is liberated and can be re-allocated to other metabolic pathways, permitting the microorganisms to maintain a high metabolic activity.

For this project it is hypothesized:

#1 The downregulation of the protein biosynthesis machinery (i.e., the reduction of cellular ribosome contents) occurs after long-term warming of forest soils from the natural ForHot warming experiment.

#2 The adjustments (i.e., of ribosome contents) occur throughout the year and across different soils (forest and grassland), in a varying degree depending on the season and the effective temperatures.

#3 Cellular ribosome content reduction occurs after three to six weeks of warming with a stronger effect at higher warming extends, accompanied by higher CO_2 production rates at higher temperatures.

The rationale for the formulation of hypotheses #1 and #3 are the observations by Söllinger et al. (2022) of a reduction in average cellular ribosome content of the microbial populations after both long- and short-term warming and increased CO_2 emission rates in warmed soil. The rationale for hypothesis #2 is the described seasonality of soil microbial communities and functioning (see chapter 1.3.5), and the general relationship between temperatures and reaction rates suggesting that temperature can drop to a certain threshold, below which a temperature increase from one cold temperature to another does not trigger ribosome reduction.

2 Material and Methods

In order to reveal changes in average cellular ribosome contents of the microbial population and CO_2 production rates between non-warmed (A_T) and warmed (E_T) forest soils as indicators for changes in microbial physiology and metabolism, the following methods were used:

- Seasonal forest soil sampling and microcosm soil incubations to measure *in situ* properties and conduct short-term warming experiments
- Determining physiochemical soil properties to obtain relevant context data
- Total nucleic acid (TNA) extractions to measure total RNA and DNA and estimate average cellular ribosome contents using RNA:DNA ratios as proxy
- KCl extraction and chloroform fumigation of soil to estimate microbial C and N
- Gas chromatography (GC) to measure CO_2 emissions from soils

Additionally, in a small side project I tried to improve the established TNA extraction protocol towards a more sustainable protocol. For that the use of glass ware was tested as alternative to single-use plastic tubes in the final TNA quantification step.

Furthermore, seasonal metatranscriptome data from long-term warmed ForHot grassland soil that was obtained in parallel to the seasonal forest soil survey was analyzed, looking for transcriptional patterns indicating ribosome reduction in warmed soils.

2.1 Sampling sites and soil sampling

The sampling sites are part of the longest lasting *in situ* soil warming experiment worldwide, the ForHot project in Iceland (forhot.is). The research sites are located on the former grounds of the Agricultural University of Iceland, close to the village of Hveragerði (48°00'28.8'' N, 21°10'40.8'' E, **Figure 1 A**). ForHot includes two subarctic grassland sites and a forest site, where the focus of this thesis is the forest (**Figure 1 B**). Replicated soil temperature gradients (n = 5 at each side) caused by natural geothermal activity allow to study ambient (non-warmed) soil temperature (A) as well as elevated soil temperatures of +3 °C (D) and +6 °C I above non-warmed. The grasslands have been warmed by geothermal activity for years (GN, "grassland new", 14 y) and decades (GO, "grassland old", >50 y), while the forest site, same as GN, is exposed to warming since an earthquake on the 29th of May 2008 (FN, "forest new", 14 y). The soil is classified as Silandic Andosol in all sites, but the plant coverage differs between forest

and grasslands. The Sitka spruce forest was planted by the Agricultural University in the 1970s and the dominating vascular plant species is *Picea sitchensis* with *Equisetum arvense* and *Geranium sylvaticum* in the understory, while the grasslands are dominated by *Agrostis capillaris* with varying vascular plant and moss cover (Sigurdsson et al., 2016).

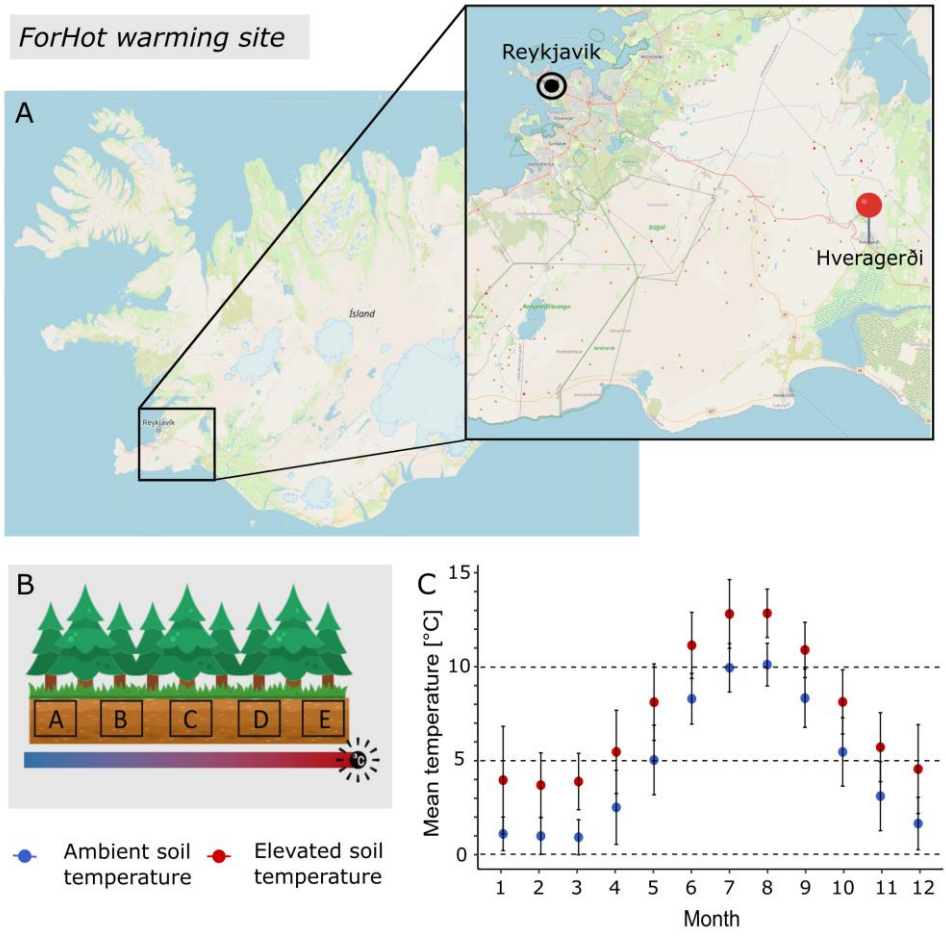

Figure 1. ForHot sampling site in Iceland. Map of Iceland showing the location of Hveragerði (**A**). Schematic overview of one out of five replicated soil temperature transects in the forest site with letters (A – E) marking the permanent plots along the temperature gradient with an increasing degree of warming (**B**). In this study forest A (non-warmed, +0°C) and D (elevated temperature, +3°C) plots were investigated. Annual temperature profile of forest A and D plots measured at 10 cm depth every 30 minutes from 2013 – 2022 (mean ± standard deviation of each month) (**C**).

Topsoil (0 – 10 cm depth) was sampled with a metal corer (3 cm diameter) under sterile conditions. Samples for TNA extractions were immediately frozen in liquid N or kept on dry ice. For biomass extractions samples were cooled with frozen cool packs and stored in a fridge at +4°C. Fresh samples used for the incubation experiment were transported on cool packs to simulate *in situ* temperature during transport. Samples in GN and GO were taken from A (non-warmed) and E (+6 °C) plots, while in FN soil was sampled from A and D (+3 °C) plots (**Figure 1 BC**). The elevated temperatures in E plots were selected for comparison to non-warmed plots because a warming of 6 °C equals the predicted range of warming within the next

60 – 100 years (IPCC, 2018). Massive dying of trees in the forest site (at +6 °C and higher) led to the exclusion of the E (+6 °C) plots and instead, the less warmed D (+3 °C) plots were used in this study, because the change in vegetation might additionally alter the soil microbiome and microbial activities.

2.2 Seasonal survey of forest soil

2.2.1 Soil preparation

For the seasonal survey soil cores were sampled as described in 2.1 (Table 1). Parts of the cores were flash frozen while fresh soil aliquots were used to determine pH and gravimetric water content (GWC). pH was determined with a 0.05 M $CaCl_2$ solution; for that 2 g soil and 5 mL 0.05 M $CaCl_2$ solution were mixed and the pH was measured using a portable pH meter (Multi 350i, WTW, Weilheim, Germany). GWC was determined by drying soil subsamples (~2 g) at 100 °C for 24 h and subtracting dry weight (DW) from fresh wet weight (WW). The flash frozen soil was ground by hand using liquid N to prevent RNA degradation and frozen at -80 °C for further analysis. Several seasons were sampled by collaborators and those cores were shipped on dry ice to Tromsø. While pH was not measured from those cores, GWC was determined using the ground soil.

All equipment was sterilized by washing, autoclaving, or baking at 200 °C for 2 h and wiping with 70% ethanol (EtOH) before use. Disposable equipment was ordered sterile.

Table 1. Sampling conditions for the seasonal survey of forest soil (FN). Soil at 0 – 10 cm depth was samples from non-warmed (A) and warmed (D, average +3°C) plots (Figure 1). In winter, the ForHot grassland sites GN (~15 years of warming) and GO (>50 years of warming) were added to the survey with average warming of +9°C and +6°C, respectively. Soil temperature (mean ± standard deviation), actual warming and weather conditions as measured during the sampling.

Sampling date	Site	Plot	Soil temperature [°C]	Actual warming [°C]	Weather condition
October 25, 2021	FN	A	5.8 ± 0.3	+4.9	Cloudy/windy
		D	10.7 ± 1.8		
February 16, 2022	FN	A	0.9 ± 0.7	+4.16	Cloudy/sunny
		D	5.06 ± 1.7		
	GN	A	0.3 ± 0.2	+10.2	Cloudy/sunny
		E	10.5 ± 4.5		
February 15, 2022	GO	A	0.1 ± 0.2	+5	Cloudy/sunny
		E	5.1 ± 2.7		
May 11, 2022	FN	A	5.3 ± 0.3	+4.1	Cloudy
		D	9.4 ± 0.7		
June 26, 2022	FN	A	10.4 ± 0.6	+5.1	Cloudy
		D	15.5 ± 2.3		
February 22, 2023	FN	A	0.7 ± 0.2	+4.7	Snowing
		D	5.4 ± 1.1		
	GN	A	0.5 ± 0.3	+10.8	Light rain/snow
		E	11.3 ± 5.1		
	GO	A	0.8 ± 0.9	+5.1	Light rain/snow
		E	5.9 ± 2.9		

2.2.2 TNA extraction and quantification

Total nucleic acids (TNA), RNA and DNA, were extracted and quantified to investigate changes in average cellular ribosome contents, RNA per unit of soil was used to indicate the ribosome content, while DNA per unit of soil served as an indicator for the approximate number of microbial cells and the resulting RNA:DNA ratios gave a proxy for the average cellular ribosome contents of the microbial population.

TNAs were extracted with triple-bead beating phenol-chloroform-extractions following the protocol by Angel et al. (2012). To minimize bias, the extractions were conducted in triplicates (technical replicates) and randomized using atmospheric noise (random.org). Approximately 0.3 g soil was weight into lysis matrix E tubes containing silica beads (MP Biomedicals, CA, USA). A phosphate buffer, a detergent solution containing 10% cetyltrimethylammonium bromide (CTAB) and phenol:chloroform:isoamylalcohol (PCI, 25:24:1) was added to the tubes.

In a FastPrep machine (MP Biomedicals, CA, USA) the samples were shaken for 30 sec at 6.5 m s^{-1} to physically lyse the cells. After centrifugation the aqueous phase was retained, and the process was repeated with fresh reagents twice (i.e., triple bead-beating). The supernatants were pooled and PCI, as well chloroform:isoamylalcohol (24:1) extractions were performed. The nucleic acids were precipitated in PEG 8000 (polyethylene glycol, molecular weight 8000) and the pellet was dissolved in 100 µL Rnase-free water. The eluate was treated with RiboLock Rnase Inhibitor (ThermoFisher, MA, USA) to avoid RNA degradation. The detailed protocol for TNA extractions can be found in Appendix I.

To quantify nucleic acids, a Qubit 2.0 Fluorometer with Qubit™ dsDNA HS Assay and Qubit™ RNA HS Assay Kit (ThermoFisher, MA, USA) was used, following the manufacturer's instruction. To determine the RNA content in the samples 2 µL of the TNA extracts were used, while 0.5 – 1 µL of the TNA extracts were used for determination of the DNA content. Agarose gel electrophoresis (1% agarose gel with PAGE GelRed® Nucleic Acid Gel Stain (Biotium, CA, USA) was conducted to inspect the quality of the extracts. The detailed protocol and a representative gel picture can be found in Appendix I.

2.2.2.1 Sustainable Qubit glass test

To reduce plastic waste in environmental research laboratories, glass ware was tested as an alternative for preparing buffer solutions used in Qubit™ measurements. Usually a 15 mL sterile single-use plastic tube was used for the preparation of these buffer solutions. Four different treatments of glass ware represent different levels of sterilization to avoid nucleic acid contamination or degradation and were compared to the standard plastic tubes:

- 15 mL sterile single-use plastic centrifugation tubes (as standard)
- 50 mL glass bottle, untreated (straight from shelf)
- 50 mL glass bottle, dry-autoclaved
- 50 mL glass bottle, wet-autoclaved (dH_20)
- 50 mL glass bottle, wet- and dry-autoclaved (dH_20)

The buffer-reagent-mix was prepared in the respective glass bottle and used for RNA and DNA concentration measurements following the manufacturer's instructions (see also 2.2.2). For all treatments, three samples were used and measured in triplicates. The mean relative differences between the treatments and the standard single-use plastic tubes were calculated and visualized using R (R Core Team, 2021) (**Figure 2)**.

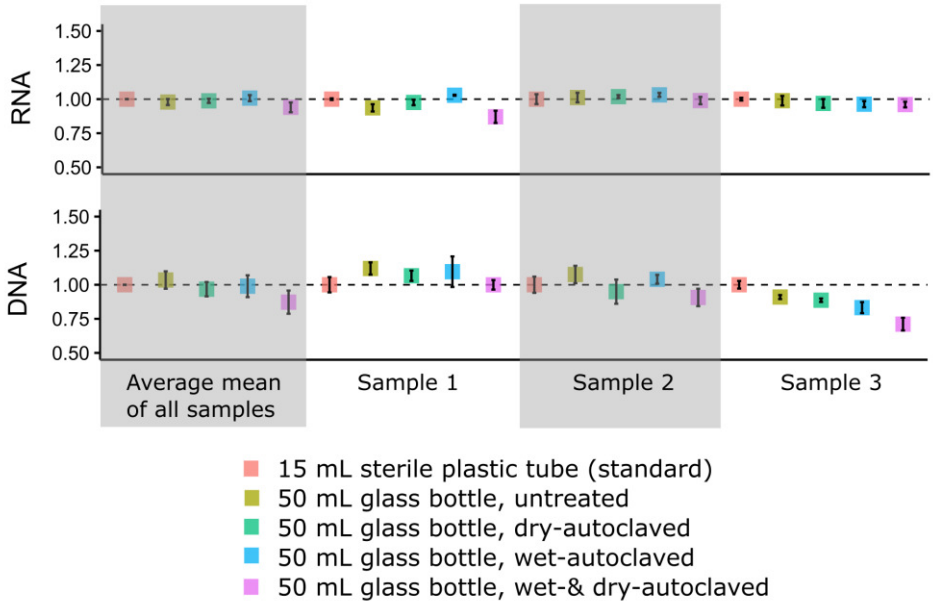

Figure 2. Mean (± standard deviation) of relative differences in RNA and DNA concentrations measured in soil extracts using different treated glass ware and the standard 15 mL single-use plastic tube to prepare the buffer solutions for Qubit™ measurements.

For further measurements untreated glass bottles were used and recommended as new lab standard as they do not result in any considerable differences in RNA and DNA concentration, are easily available and might be used more frequently than glass ware with more preparation intensive treatments. However, it should be noted that the "untreated glass bottles" are washed with our standard lab dishwasher program that includes rinsing with distilled water at the end of the washing cycle. If the glass bottles would have been used right from the start more than 100 single-use 15 mL plastic tubes could have been saved in the course of my work.

2.2.3 Microbial biomass

To estimate microbial biomass in warmed and non-warmed soils, dissolved C and N contents in the soil samples were determined using chloroform fumigation and subsequently KCl (potassium chloride) extractions. Via the comparison of dissolved C and N contents in fumigated and non-fumigated samples microbial biomass in soils can be estimated (Hood-Nowotny et al., 2010).

2.2.3.1 KCl extraction

Prior to KCl extractions, samples were fumigated in chloroform to break open cell walls and release C and N from within the cells. For that, 2 g soil were weighed into aluminum cups and fumigated in a desiccator with 70 ml chloroform for 48 h. Non-fumigated samples were used

as controls and were processed immediately. Between weighing steps, sub-samples were stored at the respective temperature to minimize alteration of the microbial biomass after sampling. Blanks were added to each filtration round as purity controls for the KCl solution. The filtration manifold with stainless steel chimneys (10 x 20 mL; DHI LAB Products, Hørsholm, Denmark) was washed with MiliQ-water and 70% EtOH before use.

KCl extractions were conducted by adding 15 mL of 1 M KCl to 2 g of soil (either fresh control soil or fumigated soil). The samples were shaken horizontally for 30 min at 125 rpm and filtered using the filtration unit with quantitative ashless filter paper (grade 40; Wathman®, Little Chalfont, UK) and a vacuum pump. The extract was stored at -20 °C for further analysis.

2.2.3.2 C and N analysis

The KCl extracts were sent to the Centre for Microbiology and Environmental Systems Science at the University of Vienna, Austria, for further analysis. Due to delays in the measurements results could not be obtained.

2.3 Short-term warming experiment

Samples from the non-warmed plots of the five forest transects (biological replicates) were taken by Páll Sigurðsson, Agricultural University of Iceland, and shipped to Tromsø (Table 2). For the short-term warming experiment the soil was sieved, and physiochemical soil properties were determined (see 2.3.1). Before soil warming, the samples were pre-incubated at *in situ* temperature (2 °C) for three weeks, to allow the microcosms to stabilize after the soil processing. Warming at three different temperatures was conducted for six weeks (see **Figure 3** for the detailed experimental setup). Eight weeks of cooling were added after the termination of the warming incubation period. Controls at 2 °C were incubated in parallel. CO_2 measurements, using GC, and TNA extractions were conducted at the starting point and after week one, two, three and six of warming, as well as after eight weeks of cooling. In addition, microbial biomass extractions were performed at the starting point, after week one, three and six of warming and week eight of cooling.

Table 2. Sampling conditions for the short-term warming experiment. Soil at 0 – 10 cm depth was sampled from non-warmed ForHot forest plots (A, n = 5). Soil temperature (mean ± standard deviation), actual warming and weather conditions as measured during the sampling.

Sampling date	Site	Plot	Soil temperature [°C]	Weather condition
April 6, 2022	FN	A	2.0 ± 0.4	Cloudy

2.3.1 Soil preparation

For the short-term warming experiment the soil was sieved (2 mm mash size) on ice to prevent a rise in temperature. pH and gravimetric water content were determined as described in 2.2.1. To determine the bulk density (necessary for GC calculations), a glass veil was weighed empty and after being filled with MiliQ water (the fill line was marked). After drying the glass veil, 4 g of soil were weighed into the glass veil. The veil was then filled to the line with MiliQ water again and the soil bulk density was calculated using displacement of the soil and the water density at processing temperature (water density at 21 °C = 0.9979955).

The sieved soil was weighed into 100 mL serum flasks (glass) and covered with aluminum foil to allow air exchange but prevent contamination. Each bottle contained approximately 40 g of soil and was placed in an incubator at 2 °C (*in situ* temperature at the sampling time, Table 2). All equipment was sterilized as described in 2.2.1. In addition, incubation flasks and rubber stoppers (used to seal incubation flasks for GC measurements) were wet- and subsequently dry-autoclaved.

2.3.2 Soil incubation and sampling

Before starting the soil warming incubations, the sample flasks were pre-incubated at *in situ* temperature (approximate April mean temperature of 2 °C) for three weeks (**Figure 3**). The main incubation was conducted for six weeks at four different temperatures: *In situ* temperature of 2 °C (+0 °C, non-warmed control), 5 °C (+3 °C compared to non-warmed), 8 °C (+5 °C), and 11 °C (+9 °C). After six weeks of soil warming all sample flasks were incubated for eight weeks at 2°C (i.e., cooled back to the *in situ* temperature).

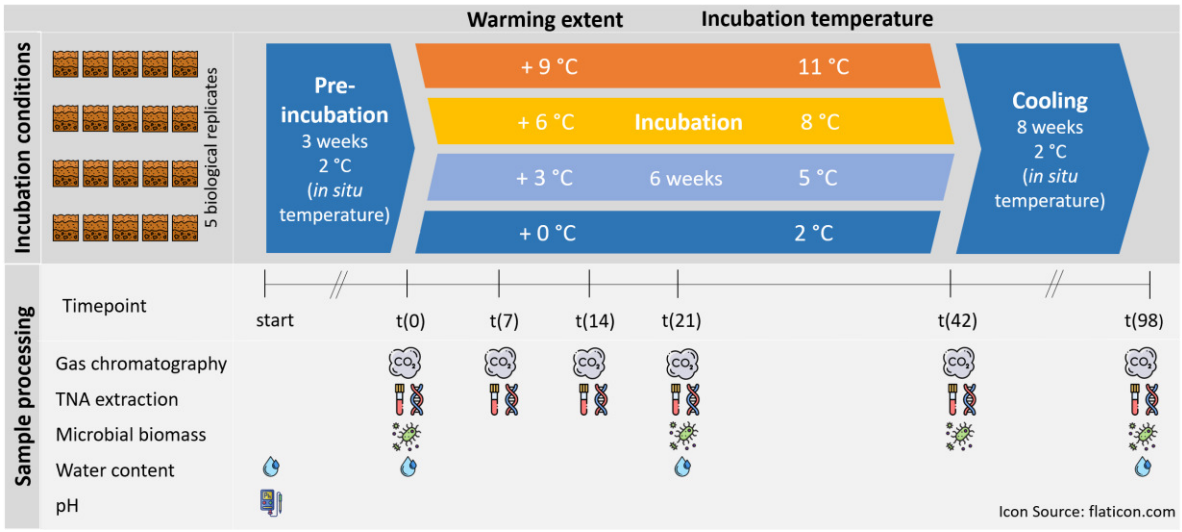

Figure 3. Overview of the forest soil short-term warming experiment with incubation conditions and sample processing. Soil was sampled from non-warmed control plots of all 5 forest transects in April 2022 and pre-incubated at *in situ* temperature for three weeks (start – t0). Short-term warming of six weeks was started at t0 and terminated at t42 with subsequent cooling until t98. Treatments of the main warming incubation were +3 °C warming to 5 °C, +6 °C warming to 8 °C and +9 °C warming to 11 °C, plus a non-warmed control at 2 °C. Soil properties such as pH and gravimetric water content were determined before pre-incubation. During the experiment CO_2 emission rates were determined using gas chromatography (GC). Total nucleic acid (TNA) extractions were used for approximation of the average cellular ribosome contents of the microbial population, microbial biomass was extracted, and water content was measured to take drying into account. Sub-samples were taken at the starting point of the warming treatments t0 and during incubation after 1 week (t7), 2 weeks (t14), three weeks (t21) and six weeks (t42). For the cooling treatment, sub-samples were collected at the start of the cooling incubation (t42) and after eight weeks (t98).

When sampling was conducted during the incubation period, sub-samples were collected as quickly as possible and under sterile conditions. The incubation flasks were placed on ice during sampling for TNA and microbial biomass extractions to prevent a rise in temperature and thus possible changes in TNA and biomass contents. Sub-samples for TNA extractions (see 2.3.3) were taken from the incubated sampling flasks at the starting point (t_0), and after day 7 (t_7), 14 (t_{14}), 21 (t_{21}), 42 (t_{42}) and 98 (t_{98}). Measurements of 24 h CO_2 accumulation (see 2.3.5) were conducted at the same days. Samples for microbial biomass were collected at the starting point (t_0), at day 21 (t_{21}), 42 (t_{42}) and 98 (t_{98}).

2.3.3 TNA extraction

Sub-samples (~2 g) for TNA extractions were sampled as quickly as possible, immediately placed on dry ice and frozen at -80 °C for further processing. These soil samples were ground on liquid N and TNAs were extracted from ~0.3 g soil as described in 2.2.2 with the following adjustment in the extraction protocol: In the last bead-beating step, 150 µL PCI were added

instead of 200 µL to avoid overflowing tubes during shaking. This did not change the final volume of the precipitate.

2.3.4 Microbial biomass

Sub-samples for KCl extractions were sampled as quickly as possible; ~2 g of soil were placed in the desiccator with chloroform immediately and controls were returned to the respective incubator until further processing. Non-fumigated control samples were processed immediately after sampling was finished. Fumigations and KCl extractions were done as described in 2.2.3.

2.3.5 Gas chromatography (GC)

CO_2 emission rates were obtained using a gas chromatograph (SRI 8610C gas chromatograph, SRI Instruments, CA, USA with 8600-PKDC 3m 9`Haysep D Column 80/100 mesh, Samsi) equipped with a flame ionizing detector (FID). H_2 was used as carrier gas and the oven temperature was set to 40 °C. An airtight gas syringe (Pressure-Lok® Precision Analytical Syringe, A-2 series, VICI Precision Sampling, Schenkon, Switzerland) with a needle (Luer Needles A-2, VICI Precision Sampling, Schenkon, Switzerland) was used to inject standards and samples. For integration of peak areas, the software PeakSimple version 4.88 was used.

Several CO_2 standards of different concentrations (400 ppm, 2500 ppm, 5000 ppm, 10 000 ppm) were measured to create a standard curve (**Figure 4**). One mL head space gas was collected from the sample flasks and injected in the GC port. The syringe was flushed with air and the needle wiped with optical paper in between injections. Samples were measured grouped by temperature treatment and with rising incubation temperature (2 → 11 °C). At each timepoint the starting CO_2 concentration was measured immediately after the flasks were capped using air-tight rubber stoppers and metal crimps. After the sampling the flasks were returned to the respective temperature. After 24 h, the cumulative CO_2 accumulation in the flasks were measured again. Afterwards flasks were de-capped, covered with aluminum foil instead and placed back in the respective incubator.

To generate the standard curve, the amount of substance of gas (n) in the standards was calculated with the ideal gas law (general gas equation).

Air pressure (**p**) and room temperature (**T**) were measured before the GC was conducted. The injected gas volume (**V**) was 1 mL for all standard gases and the ideal gas constant (**R**) of 8.31446261815324 $m^3 \cdot Pa \cdot K^{-1} \cdot mol^{-1}$ was used. All values were transferred to SI units.

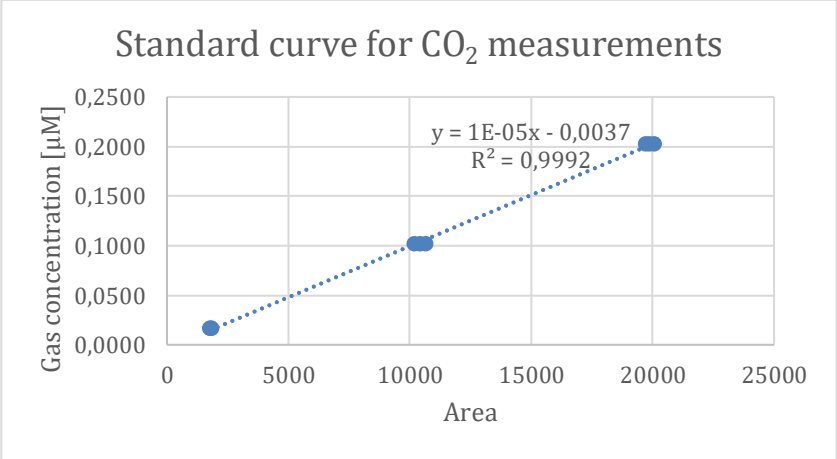

Figure 4. Standard curve for CO_2 measurements with GC: Several standard gases with known concentrations were used to generate a standard curve. The slope of the curve is required to calculate CO_2 concentrations in the incubation flasks with different warming treatments.

CO_2 emission rates in nM h^{-1} g^{-1} DW soil were calculated from the 24 h cumulative CO_2 accumulation. The area under the gas peak, integrated by PeakSimple, multiplied with the slope of the standard curve allowed to calculate CO_2 concentrations in µM mL^{-1} and nM mL^{-1}. The CO_2 increase per mL was calculated by subtracting the start CO_2 concentration [nM ml^{-1}] from the 24 h concentration [nM mL^{-1}], where the start concentration was measured with GC at the beginning of the 24 h accumulation. To determine the CO_2 increase per h [nM mL^{-1} h^{-1}], the CO_2 increase per mL was divided by the incubation time [h]. CO_2 increase per h [nM mL^{-1} h^{-1}] multiplied with the headspace volume in the incubation flask [mL] then gives the total CO_2 increase per h [nM h^{-1}]. Now, to calculate the CO_2 emission in nM per h and per g soil, total CO_2 increase per h was divided by the amount of soil [g] in the incubation flask. Lastly, dividing CO_2 in nM h^{-1} g^{-1} by the soil DW:WW ratio gave CO_2 concentration in nM h^{-1} g^{-1} DW soil. The cumulative CO_2 accumulation is from now on referred to as CO_2 emission.

2.4 Metatranscriptomics analysis

In addition to the two main parts of this thesis, the seasonal ForHot forest survey and the incubation experiments with forest soils, a metatranscriptome analysis of long-term warmed ForHot grassland soils was conducted to study adjustments of the microbial protein

biosynthesis machinery throughout seasonal temperature changes. The employed double RNA approach allows studying of the community composition using rRNA and functional analysis using mRNA (Urich et al., 2008), the latter being the focus in this thesis.

2.4.1 Seasonal metatranscriptomics of grassland soils

In order to get further insights into how microbial communities change in response to warming, a seasonal grassland metatranscriptome dataset was processed and analyzed. The grassland soils were sampled from the ForHot long-term grassland warming site (GO) during the same period as the seasonal forest soil survey was conducted (2021 – 2022). The metatranscriptomes were generated from total RNA extractions by Mathilde Borg Dahl, our ForHot collaborator from the University of Greifswald, Germany, and raw Illumina reads were provided for analysis in this thesis. Before extraction, RNA from a spike organism (*Saccharolobus solfataricus*) was added to the soil samples to validate the performance of the sequencing but was filtered out for the analysis in this thesis.

The Life Science Computer Cluster (LiSC) run by CUBE (Division of Computational Systems Biology), Department of Microbiology and Ecosystem Science, University of Vienna, Austria, was used for bioinformatical processing of the metatranscriptome data.

It can be expected that microbial communities in forest soil differ substantially from the grassland communities regarding presence and abundance of taxa. However, responses to warming on a transcriptional level might be in general taxon-independent (Domeignoz-Horta et al., 2023), as already shown for the downregulation of the bacterial protein biosynthesis machinery by Söllinger et al., 2022. Thus, transcriptional changes during the seasons and between warmed and non-warmed soils from grasslands can give some understanding of a general transcriptional warming response in soils.

2.4.2 Processing of Metatranscriptome data

Fastq read files from Illumina paired-end sequencing were provided (sample processing, library generation, and sequencing were performed by our collaborators at the University of Greifswald, Germany). The data processing largely followed the processing pipeline used by Söllinger et al., 2022; scripts for all processing steps can be found in Appendix IV.

Since one sample consisted of several read files, the individual files were combined into one collective file per sample using the Linux command cat (concatenate). For pairing of the Illumina paired-end reads, the program PEAR v.0.9.10 (Zhang et al., 2014) (Paired End reAd mergeR, default settings) was used, but due to too little overlapping reads (<70%), it was decided to analyze the forward and reverse reads separately. A quality filtering was conducted with prinseq-lite v.0.20.4 (Schmieder & Edwards, 2011) and a minimum quality mean threshold of 30, that step also included the conversion from fastq files to the fasta format, that was used in the following steps. Non-rRNA and rRNA was separated with SortMeRNA v.4.1.0 (Kopylova et al., 2012) (default settings). Due to a high variance in the fraction of non-rRNA reads, an additional filtering step was included. A non-conservative DIAMOND (Buchfink et al., 2015) blastx search v.2.1.3 (-k 1, -e 10) against the National Center for Biotechnology Information (NCBI) non-redundant database ("Database resources of the National Center for Biotechnology Information," 2016), as of September 2019, verified the mRNA sequences within the non-rRNA reads (now referred to as mRNA). The mRNA sequences were extracted out of the non-rRNA fasta sequence files. Lastly, functional annotation of the metatranscriptomes was conducted with a DIAMOND blastx search against the KEGG database (Kanehisa & Goto, 2000), as of April 2019, using only the top hit and an e value of 0.0001 (-k 1, -e 0.0001). From here, data output was further processed in R.

The rRNA reads (obtained from SortMeRNA) were used to compile broad community profiles on domain level. Changes in the rRNA to mRNA ratio during the seasons, were analyzed for an overview of the investment into functional transcripts in response to warming, while mRNA reads annotated using the KEGG database were used for analyzing the investment in specific functions.

2.5 Figures and statistical analysis

Standard curves and standard regression lines for GC analysis were made using scatter plot charts in Microsoft® Excel® for Microsoft 365 MSO (Version 2212 Build 16.0.15928.20278) 64-bit. Other results were visualized using Rstudio and R version 4.2.0 (2022) from the R foundation for statistical computing (R Core Team, 2021). Functions in the package 'tidyverse' (Wickham et al., 2019) were used to reorder and structure data. The package 'ggplot2' (Wickham, 2016) was used to generate plots. Figures were design in Inkscape 1.2 (dc2aedaf03, 2022-05-15) (Harrington, 2023).

Regarding investigation of changes in average cellular ribosome contents, RNA and DNA concentrations in soil were analyzed. RNA per unit of soil was used to indicate the ribosome content, while DNA per unit of soil served as an indicator for the approximate number of microbial cells and the RNA:DNA ratio gives a proxy for the average cellular ribosome contents of the microbial population. To test for significant differences in RNA and DNA contents and RNA:DNA ratios between seasons an analysis of variance (ANOVA) was performed. Prior to conducting the ANOVA, normal distribution was tested with Shapiro-test and equal variance was tested with Bartlett- and Fligner-Killeen-tests. One-sided t-tests (equal variance) were used to test for differences in TNA concentrations between A_T and E_T within each season, as well as to test for differences in TNA concentration between winter and the other seasons. Since we hypothesized that ribosome reduction will occur in the warmed soils one-sided t-tests with the setting "alternative = greater" were used. For difference in TNA concentrations in grassland soils in winter a two-sided t-test (equal variance) was used, based on the observations made in the forest with no indications for a ribosome reduction during winter.

In order to investigate changes in CO_2 emission rates over the incubation time, plus changes in CO_2 emission rates between the different temperature treatments, ANOVA was used. Differences in TNA concentrations between incubation time points for each temperature treatment were tested with ANOVA and one-sided paired student's t-test since a reduction in ribosome content was hypothesized at warmer temperatures.

For the Metatranscriptome analysis, ANOVA and two-sided student's t-test were used to analyze differences in eukaryotic 18S rRNA transcript abundance, difference in mRNA content and differences in transcriptional investment into metabolism and *genetic information processing* between seasons and between A_T and E_T.

3 Results

In this thesis, warming effects on microbial physiologies and metabolism in naturally warmed forest soils were investigated, focusing on the reduction of average cellular ribosome contents of the microbial population as a proposed common response of soil microorganisms to warming (Söllinger et al., 2022). A seasonal survey was conducted to examine temperature effects on soil microorganisms throughout the seasons. Warmed and non-warmed soils from a long-term warming (~15 y) forest site were compared regarding average cellular ribosome contents of the microbial communities, using RNA:DNA ratios as proxy. Additionally, winter samples from two long-term warmed grassland sites (~15 y, >50 y) were analyzed to gain a better understanding of microbial adjustment to cold temperatures. To investigate a possible temperature threshold for microbial warming responses, a short-term warming experiment using forest soil was conducted. Furthermore, the effects of warming extent and duration as well as cooling of previously warmed soils on ribosome contents and CO_2 emission rates were tested. Seasonal changes in the microbial investment into the protein biosynthesis machinery (i.e., the cellular ribosome content) were further investigated by analyzing a metatranscriptomic dataset from the long-term warmed (>50 y) ForHot grassland site, obtained in parallel to the seasonal forest soil survey.

3.1 Seasonal warming effects on average cellular ribosome contents

Forest soils from non-warmed (A_T) and elevated temperature (+3°C, E_T) plots were compared to identify differences in the average cellular ribosome contents of the microbial population. To detect seasonal differences, samples from autumn (October 2021), winter (February 2022), spring (May 2022) and summer (July 2022) were compared. Total DNA and RNA contents from all samples were extracted and quantified, where RNA was used as an indicator for average ribosome content and DNA as an indicator for the number of microbial cells in the soil samples. The resulting RNA:DNA ratios were used as proxy for cellular ribosome contents, and thus represent the key parameter in this study.

DNA and RNA contents and the resulting RNA:DNA ratios were compared in absolute values between A_T and E_T over the seasons (**Figure 5**). Relative differences between RNA and DNA contents and the resulting RNA:DNA ratios in A_T and E_T within each season were compared

(**Figure 6)**. Lastly, grassland soil was added to the survey to examine differences in warming effects on RNA and DNA contents and the resulting RNA:DNA ratios in grassland and forest soils in winter (**Figure 7**).

3.1.1 Seasonal dynamics of RNA contents in soil were opposing seasonal temperature dynamics

Soil temperature was the highest in summer (July; A_T: 10.0 ± 1.3 °C; E_T: 12.9 ± 1.8 °C) and the lowest in winter (February; A_T: 1.0 ± 1.0 °C; E_T: 3.7 ± 1.7 °C), while temperatures in autumn (October; A_T: 5.5 ± 1.8 °C; E_T: 8.1 ± 1.7 °C) and spring (May; A_T: 5.0 ± 1.9 °C; E_T: 8.1 ± 2.0 °C) were very similar. Non-warmed and elevated temperature plots showed the same temperature profiles, with 2.8 ± 0.2 °C warming at E_T compared to A_T (**Figure 5 A**). Inspecting DNA and RNA contents over these four timepoints in A_T and E_T revealed a seasonal effect of temperature on nucleic acid contents in soil. Total RNA contents per g DW soil showed almost the opposite pattern of the temperature profiles, with the highest RNA contents at the lowest temperature in winter (**Figure 5 B**). However, the lowest RNA contents were observed in spring, followed by summer and autumn. The RNA content at E_T between winter and spring varied significantly. Total DNA contents per g DW soil showed the same pattern as the temperature profiles (**Figure 5 C**). DNA contents in A_T were significantly higher in summer than winter.

3.1.2 Average cellular ribosome contents were temperature dependent, being lowest at the highest temperatures

In the RNA:DNA ratios, our proxies for cellular ribosome contents, an opposite pattern to temperature, similar to the RNA profiles, could be observed, with the highest RNA:DNA ratios in summer and the lowest in winter (**Figure 5 D**). The RNA:DNA ratio at E_T showed a significant difference between winter and all other seasons (for p-values see Appendix III, Table 3, 4), while the RNA:DNA ratio at A_T showed the same pattern as in warmed soils but without significant differences.

3.1.3 Soil water contents and temperatures were positively related

The gravimetric water contents of the soils largely followed the seasonal temperature profiles, with lowest contents in winter and spring and significantly higher contents in summer, revealing that warmer temperatures did not lead to soil drying (**Figure 5 E**).

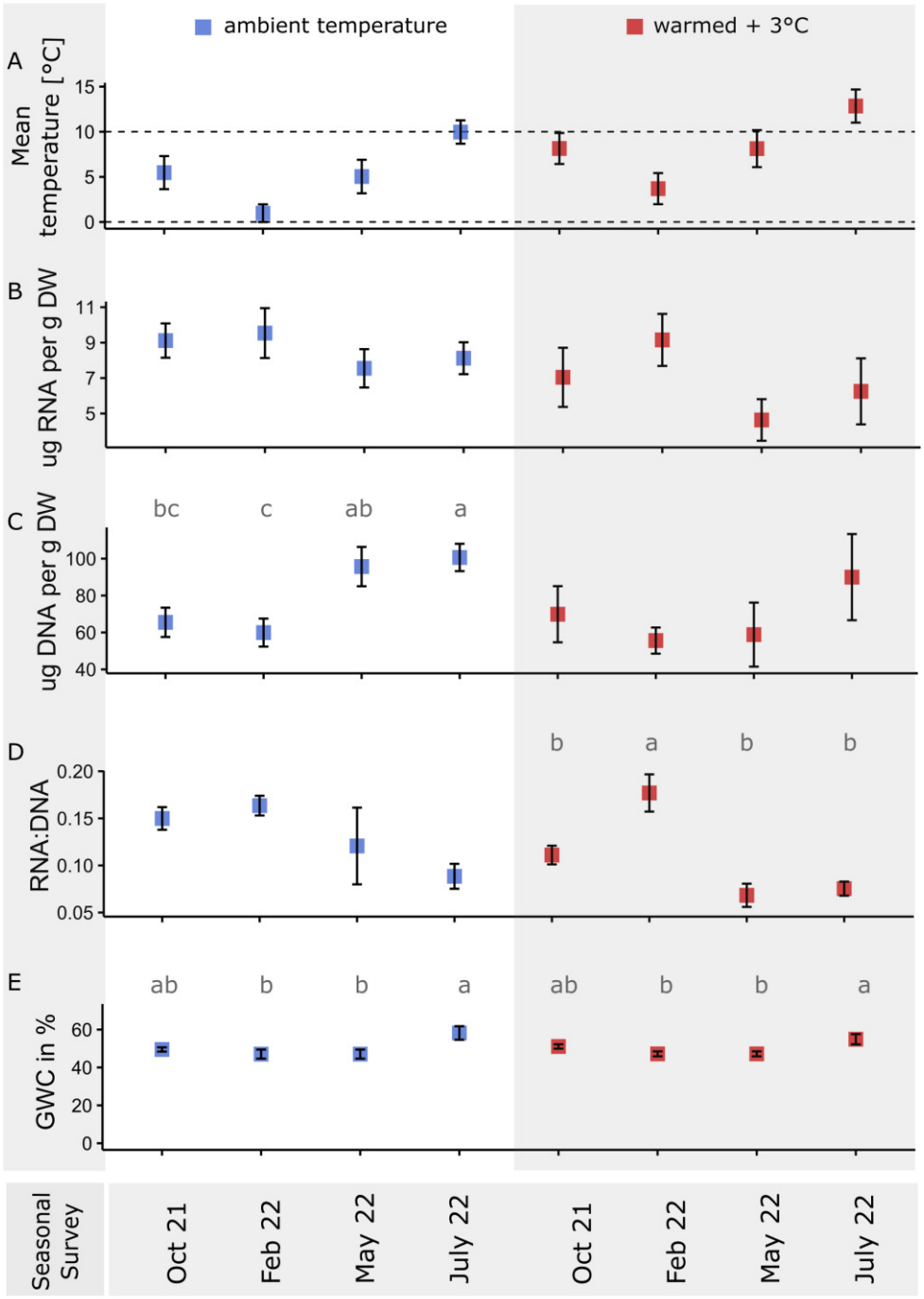

Figure 5. Change in absolute RNA and DNA contents per g dry weight (DW) soil, RNA:DNA ratios and water contents throughout the seasons between non-warmed and warmed ForHot forest soils. Soil temperature means **(A)** are presented in °C with error bars indicating the standard deviation. Means of absolute RNA (**B**) and DNA (**C**) contents are given in µg DNA and RNA per g soil dry weight, as well as means of RNA:DNA ratios (**D**) and mean gravimetric water contents (GWC) I in % with error bars indicating the standard error. Parameters for non-warmed and warmed temperatures (+3 °C) are displayed in October, February, May and July representing the four seasons. The letter code indicates significant differences ($p<0.05$), see Appendix III, Table 3 and 4 for exact p-values.

3.1.4 Long-term warming led to ribosome reduction throughout the seasons except in winter

To display relative differences in A_T and E_T soils within each season and compare those across seasons, the data was normalized by dividing DNA and RNA contents and RNA:DNA ratios from A_T and E_T by the mean DNA and RNA contents and RNA:DNA ratios of A_T (**Figure 6**). Within seasons, temperature comparison confirmed that the expected ~3 °C differences between A_T and E_T were present in all seasons (**Figure 6 A**). Relative differences in RNA contents showed lower RNA contents in warmed soils throughout the seasons except in winter (**Figure 6 B**). A similar picture could be observed in the relative DNA differences, except in the autumn samples where DNA was higher at A_T than E_T (**Figure 6 C**). The RNA:DNA ratio mirrored the RNA pattern, with a trend towards lower RNA:DNA ratios at E_T than A_T (**Figure 6 D**). The soil water content differed only marginally between A_T and E_T in all seasons (**Figure 6 E**). A significant difference between A_T and E_T (p-value <0.05) could only be observed in the RNA:DNA ratios in autumn and DNA contents in May (for p-values see Appendix III, Table 6). Additionally, a trend (p-value <0.1) towards lower RNA contents in spring was observed.

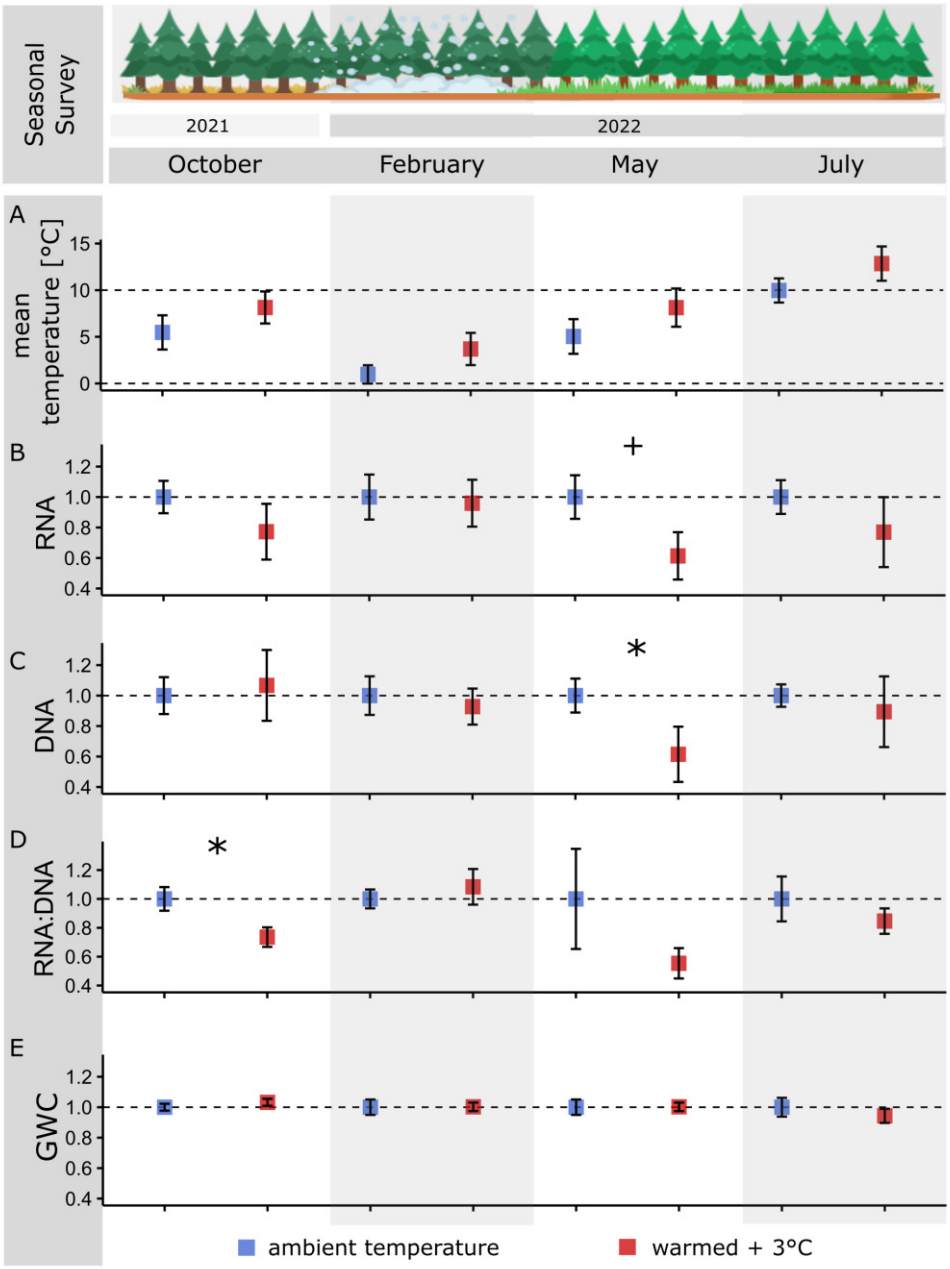

Figure 6. Relative differences in RNA and DNA contents, RNA:DNA ratios and water contents in non-warmed and warmed (+3°C) ForHot forest soil throughout the seasons. A schematic overview indicates the seasonal comparison with environmental alterations in October, February, May and July. Soil temperatures (**A**) are presented in °C with mean temperature with a square, including error bars for standard deviation. RNA (**B**) and DNA (**C**) contents, RNA:DNA ratios (**D**) and gravimetric water contents (GWC) I are normalized by the mean values of the non-warmed temperature soils and have no unit. The error bars indicate the standard error. Statistical significance of < 0.05 is marked with asterisks (*), trends with a p-value of < 0.1 are marked with plus (+), see Appendix III, Table 6 for exact p-values.

The seasonal survey of nucleic acid concentrations in non-warmed and warmed forest soil indicated a reduction in average cellular ribosome contents of the microbial population throughout the seasons except in winter (**Figure 6 D**). Thus, to get a more thorough picture of the winter season, soil samples from the two ForHot grassland sites were added to the survey and a second winter was sampled (**Figure 7**).

3.1.5 No reduction in average cellular ribosome contents in winter in forest and grassland soils

In samples from both winters, taken in February 2022 and 2023, the relative difference in DNA and RNA contents in grassland and forest soil were examined (**Figure 7**). One grassland (GN) had been warmed for ~15 years, while the other grassland (GO) had been warmed for >50 years. The mean warming (E_T) in the last ~ 9 years was +6 °C in GO and +9 °C in GN, notably higher than the warming of +3 °C in FN. RNA, DNA, and water content, varied between the sites as well as the years (**Figure 7 ABD**).

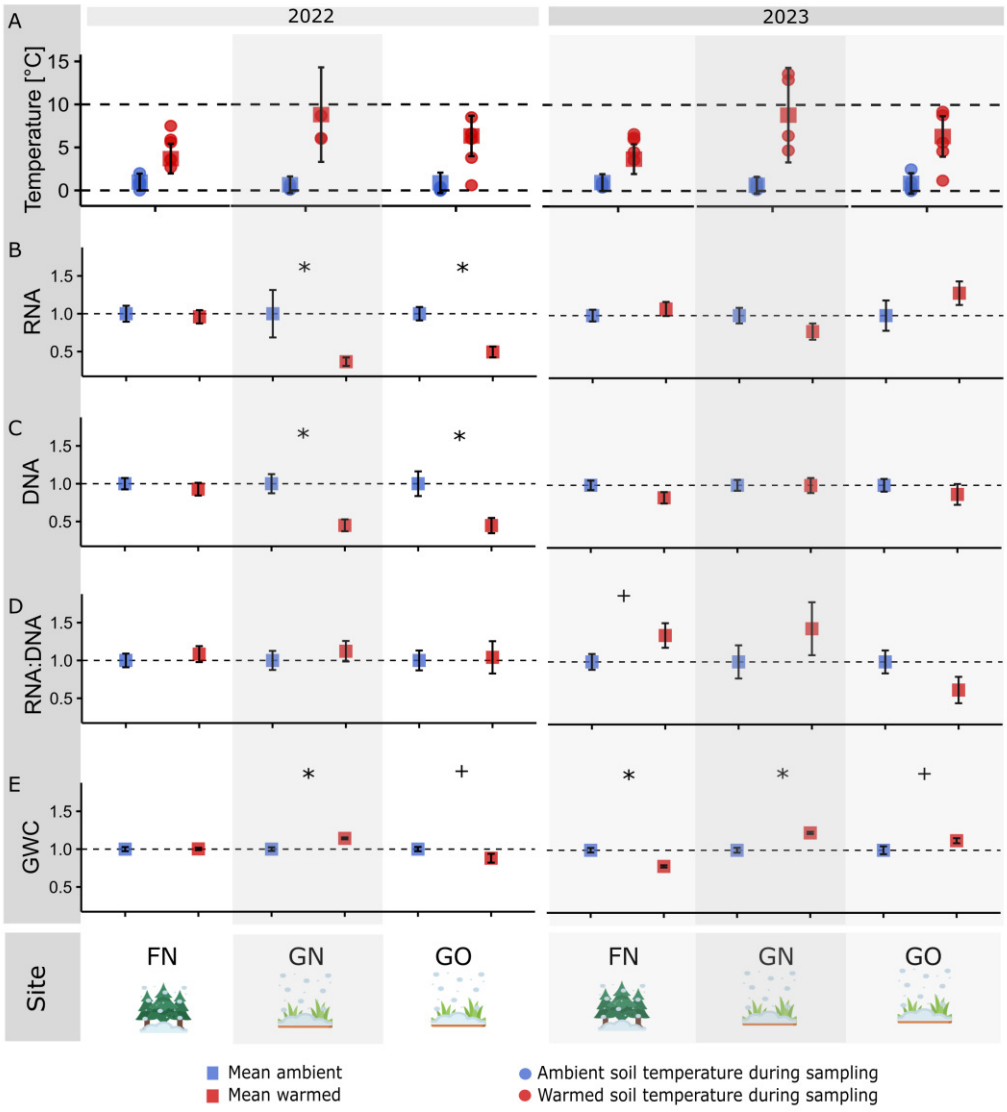

Figure 7. Relative differences in RNA and DNA contents, RNA:DNA ratio and water contents in non-warmed and warmed ForHot soils in winter 2022 and 2023. Soil temperatures (**A**) are presented in °C with mean temperatures indicated with a square, including error bars for standard deviation, and measured soil temperature during sampling shown by circles. RNA (**B**) and DNA (**C**) contents, RNA:DNA ratios (**D**) and gravimetric water contents (GWC) are normalized by the mean values of the non-warmed temperature soils and have no unit. The error bars indicate the standard error. FN and GN (~15 years of forest and grassland soil warming), as well as GO (>50 years of grassland soil warming) in February 2022 and 2023 are shown. Statistical significance of < 0.05 is marked with asterisks (*), trends with a p-value of < 0.1 are marked with plus (+), see Appendix III, Table 7 for exact p-values.

Differences in RNA and DNA contents between A_T and E_T were statistically significant (p-value of <0.05) in GN and GO in 2022 (for exact p-values see Appendix III, Table 7). Nevertheless, RNA:DNA ratios were not reduced in A_T compared to E_T at any site or any of the two winters, with the exception of GO in 2023 where a decrease in RNA:DNA in the warmed soil was observed, albeit not significant (**Figure 7 C**). There was also a trend towards higher RNA:DNA ratio and therefore higher cellular ribosome content at E_T (p-value of <0.1) in FN in 2023. However, the overall picture confirmed the observation of a lack of ribosome reduction in warmed soil during winter (**Figure 5, 6**). The water content differed between A_T and E_T in GN (<0.05) and GO (<0.1) in both years, while in FN the water content was significantly different (<0.05) only in 2023 (**Figure 7 D**).

All in all, the seasonal survey of forest soils indicated lower average cellular ribosome contents of the microbial population throughout the year except in winter, supported by a thorough study of winter samples from different soils (grassland and forest soils) and two different sampling years (2022 and 2023). This may indicate a temperature threshold where a cellular reduction of the ribosome content is not occurring. In the following, a short-term warming experiment was used to further investigate the possibility of a temperature threshold.

3.2 Short-term warming effects on average cellular ribosome contents and CO_2 production rates

A short-term incubation experiment was conducted to investigate possible temperature thresholds for ribosome reduction at cold temperatures, the effect of warming duration, and subsequent cooling on microbial metabolisms and physiologies (see **Figure 3** for details on the experimental setup). Non-warmed soil (2 °C) was warmed to three different temperatures for six weeks and consequently cooled to *in situ* temperature (2 °C) for eight weeks. As a proxy of the average cellular ribosome contents of the microbial population, RNA:DNA ratios were calculated from measured TNA concentrations, while CO_2 emission rates were investigated to detect changes in microbial activity. Measurements were conducted after 1 (t_7), 2 (t_{14}), 3 (t_{21}) and 6 (t_{42}) weeks of warming and after eight weeks of cooling pre-warmed soil (see 2.3.2, **Figure 3**). The temperature treatments were chosen according to *in situ* measurements and seasonal average temperatures. Non-warmed soil was sampled at 2 °C (measured *in situ* temperature) in April, 5 °C represents the approximate mean temperature in May, 8 °C reflects

the mean June temperature, while 11 °C represented an "extreme" warming since it is above A_T maximum mean temperatures (**Figure 1 C**).

3.2.1 Transects showed replicability in physicochemical soil properties

Before incubating at different temperatures, the transects were tested for replicability regarding their physicochemical properties. Soil pH, gravimetric water content and bulk density were measured in triplicates in all five transects (**Figure 8**). Measurements showed a high consistency within replicates as well as between the transects, supporting the eligibility of the transects as biological replicates.

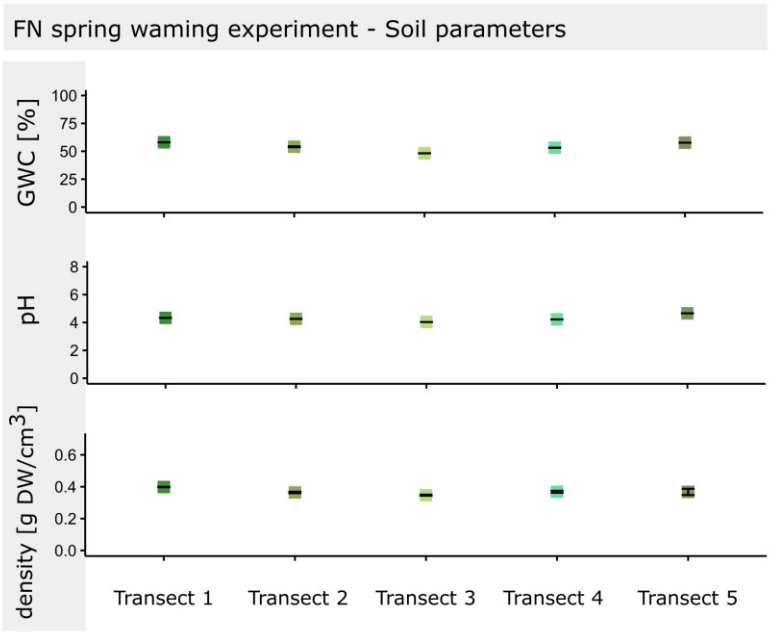

Figure 8. Comparison of physicochemical soil properties in ForHot forest transects (non-warmed plots). Gravimetric water content (GWC), pH and bulk density in forest soil were measured in preparation of the short-term warming experiment. All values are shown as means of three technical replicates with error bars indicating the standard error.

3.2.2 Warmer incubation temperatures led to higher CO_2 emissions

To investigate warming effects on microbial metabolisms (activities), CO_2 emission rates were measured over the incubation period (**Figure 9**). Differences in gas production rates are displayed using standardization by the control at *in situ* temperature (2 °C). Gas measurements were conducted using gas chromatography (see 2.3.5) right after the incubation started and after 7 (t_7), 14 (t_{14}), 21(t_{21}) and 42 (t_{42}) days of warming and after eight weeks of cooling (t_{98}). The temperature treatments were: 5 °C, 8 °C and 11 °C, plus a 2 °C control treatment (see **Figure 3** for details on the experimental setup).

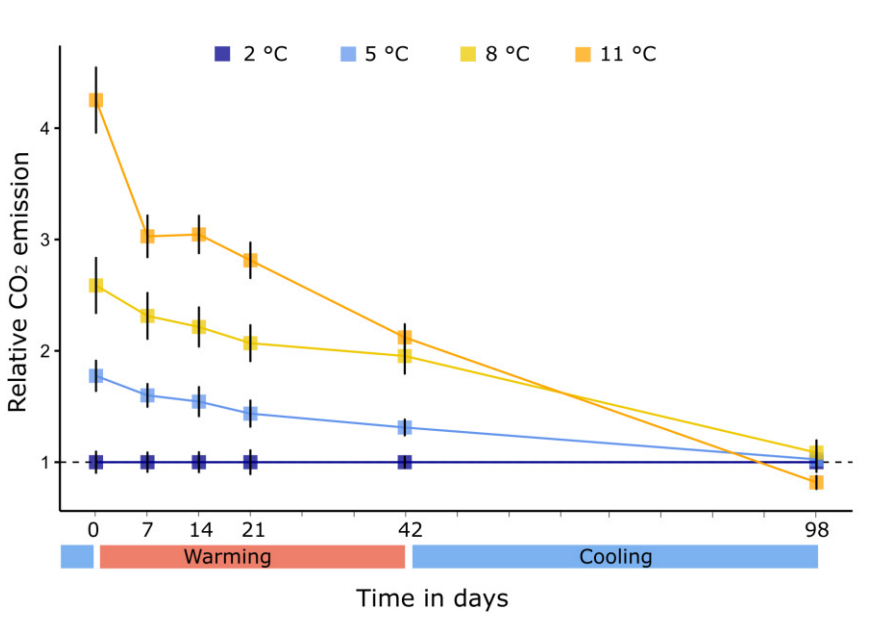

Figure 9. Relative CO_2 emissions from soils at different temperatures during the short-term warming experiment and after subsequent cooling. The main incubation included warming at different temperatures for six weeks (0 – 42 days) and cooling at 2 °C for eight weeks (42 – 98 days) with prior pre-incubation for three weeks at 2°C. All values are shown as means with error bars indicating the standard error. The emissions were normalized by the emissions of the 2 °C control to show differences between emission rates in warming treatments. See Appendix III, Table 8 – 11 for significant differences.

Higher gas emission rates were observed at warmer temperature treatments throughout the warming incubation, with the highest production rates at the highest temperature (11 °C) (**Figure 9).**

In all warming treatments (2 °C, 5 °C, 8 °C, 11 °C) the CO_2 emission rates differed significantly between start and end of the total incubation period (t_0 vs t_{98}), with lower rates at the t_{98} (for exact p-values see Appendix III, Table 8,9). Only in the warmest treatment (11 °C), differences between start of incubation (t_0) and every other warming timepoint were detected, with overall decreasing rates over time. Looking at the CO_2 emission rates between incubation temperatures, significant differences at all warming timepoints were observed (for exact p-values see Appendix III, Table 10,11). At t_0, t_7 and t_{14} all temperature treatments had significantly different emission rates (p-value <0.05) or a trend towards higher emission at warmer temperatures (p-value <0.1). Only 2 °C and 5 °C did not differ significantly during the warming incubation at t_{21} and at t_{42}; likewise, 8 °C and 11 °C were not significantly different at the end of the warming incubation (t_{42}).

3.2.3 Cooling pre-warmed soil resulted in a return to pre-warmed CO_2 emissions

After cooling pre-warmed soil (t_{98}) a notable drop in CO_2 emissions of the warmest treatment occurred, that even undercut the control treatment (**Figure 9**). A return to pre-warming (control) emission rates was observed for the intermediated warming treatments (5 °C and 8 °C). During the cooling incubation, a significant difference between start and end of cooling (t_{42} vs t_{98}) in all temperature treatments was detected (for exact p-values see Appendix III, Table 8,9).

Besides determining warming effects on microbial metabolisms (microbial activities), physiological adjustments were examined by using DNA and RNA contents to investigate cellular ribosome reduction (decreasing RNA:DNA ratios) at different temperatures (compare 2.3.3). Relative differences in DNA and RNA contents and RNA:DNA ratios were investigated after warming of three (t_{21}) and six weeks (t_{42}) (**Figure 10**). The data was normalized by dividing absolute values at t_{21} and t_{42} by the starting point (t_0).

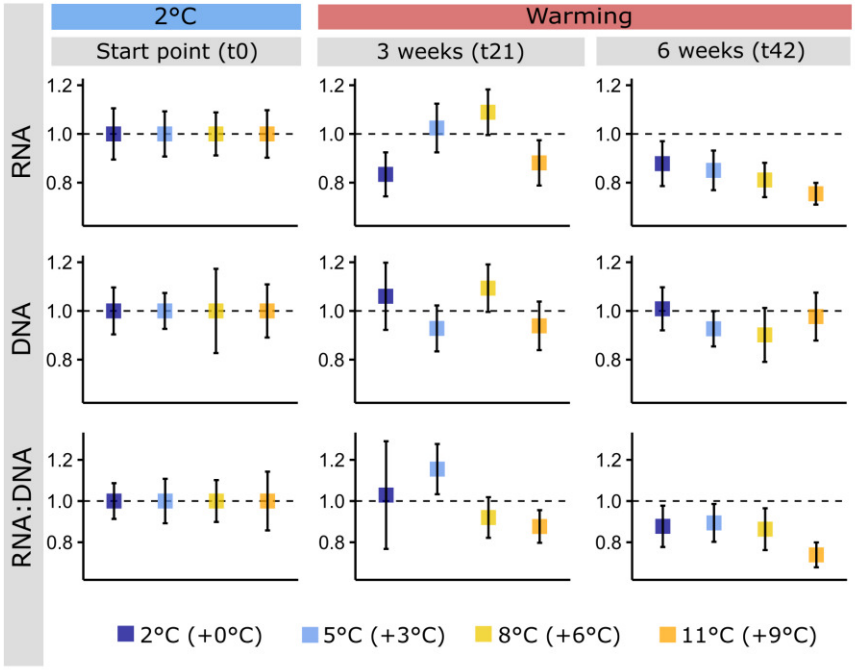

Figure 10. Relative difference in DNA contents, RNA contents and RNA:DNA ratios during the short-term warming experiment. Nucleic acid concentrations are compared between starting point (t_0), after three weeks (t_{21}) and six weeks (t_{42}) of short-term warming. All values are shown as means with error bars indicating the standard error. The RNA contents (**A,B,C**), DNA contents (**D,E,F**) and RNA:DNA ratios (**G,H,I**) were normalized by the control (2 °C) contents at the starting point (t_0) to show differences over incubation time. The dashed line represents the mean of the non-warmed temperature at t_0 (control contents). The colors indicate different warming treatments with the temperature increase compared to the control temperature in parentheses. See Appendix III, Table 12 and 13 for significant differences.

3.2.4 No average cellular ribosome content reduction after three weeks of short-term warming

At the starting point t_0 all treatments have been pre-incubated at 2 °C for three weeks, which is reflected in the similar DNA and RNA contents (**Figure 10 ADG**). The four warming treatments at 2 °C (control), 5 °C, 8 °C and 11 °C started at t_0 and lasted until t_{42}. After three weeks of warming (t_{21}) RNA contents and DNA contents did not show a temperature-dependent pattern (**Figure 10 BE**). The RNA:DNA ratio shows higher RNA content per cell at 5 °C, but lower at the high temperatures relative to t_0 (**Figure 10 F**).

3.2.5 RNA and DNA contents were variable between short-term warming of three and six weeks

After warming incubation for six weeks (t_{42}) a decrease in RNA contents compared to the starting point could be observed in all treatments including the control, with stronger decrease at higher temperatures (**Figure 10 C**). The DNA content of the control remained unchanged, 5 °C and 8 °C decreased very slightly, while the 11 °C treatment did not (**Figure 10 F**). Significant differences in RNA and DNA contents between timepoints were only detected in the two warmest treatments (8 °C, 11 °C) (for exact p-values see Appendix III, Table 12,13). At 8 °C there was a significant (p-value <0.05) reduction in RNA content between three and six weeks of warming (t_{21} and t_{42}). However, at the same time, there was a drop in the DNA content at 8 °C, reflected in a stable RNA:DNA ratio from three to six weeks of warming at 8 °C. At 11 °C RNA contents differ significantly (p-value <0.05) between start and end of the warming treatment (t_0 and t_{42}).

3.2.6 A microbial short-term warming response could be observed after six weeks in the warmest temperature treatment

The RNA:DNA decreased at 2 °C, 5 °C, 8 °C and the strongest at 11 °C relative to t_0 (Figure 10I) and may indicate a reduced cellular ribosome content in the warmest temperature treatment (11°C), after both three and six weeks of warming (t_{21} and t_{42}) (Figure 10BC). The RNA:DNA at 11°C showed a trend (p-value < 0.1) towards reduced RNA:DNA ratios between the start and end of the warming treatment (t_0 and t_{42}).

3.2.7 Cooling pre-warmed soil revealed no change in average cellular ribosome contents

After the warming incubation, a cooling of the previously warmed soil was conducted. While CO_2 emission rates were measured for all temperature treatments, TNA extractions were only carried out on the samples exposed to the highest temperature (+9 °C) and the control. No

significant differences in DNA and RNA contents and the resulting RNA:DNA ratios between start and end of the cooling incubation (t_{42} and t_{98}) could be observed (data not shown).

3.3 Metatranscriptomics results

In addition to the seasonal ForHot forest survey and the incubation experiments using forest soils, metatranscriptome analysis of ForHot grassland soils was conducted. The long-term warmed (>50 y) grassland (GO) was sampled seasonally from summer 2021 to summer 2022 by collaborators from the University in Greifswald, Germany. As part of this thesis, the metatranscriptomes were processed (see 2.4) using the LiSC computer cluster, University of Vienna, Austria, to study adjustments of the microbial protein biosynthesis machinery throughout seasonal temperature changes and to compare the long-term warmed plots (+6 °C) with the non-warmed control plots over the seasons.

To compile a broad community profile on domain level, rRNA reads obtained during the data processing were used (**Figure 11**). The rRNA to mRNA ratios in warmed (E_T) and non-warmed soils (A_T) were analyzed to get an impression of the investment into functional transcripts in response to warming (**Figure 12**). Lastly, mRNA reads were used for analyzing the transcript abundance annotated to specific functions (**Figure 13**).

3.3.1 The seasonal community profile on domain level was similar between warmed and non-warmed soils and across seasons

Investigation of archaeal and bacterial 16S rRNA transcripts and eukaryotic 18S rRNA transcripts revealed no consistent changes in community composition on domain level between warmed and non-warmed plots and throughout the seasons (**Figure 11**).

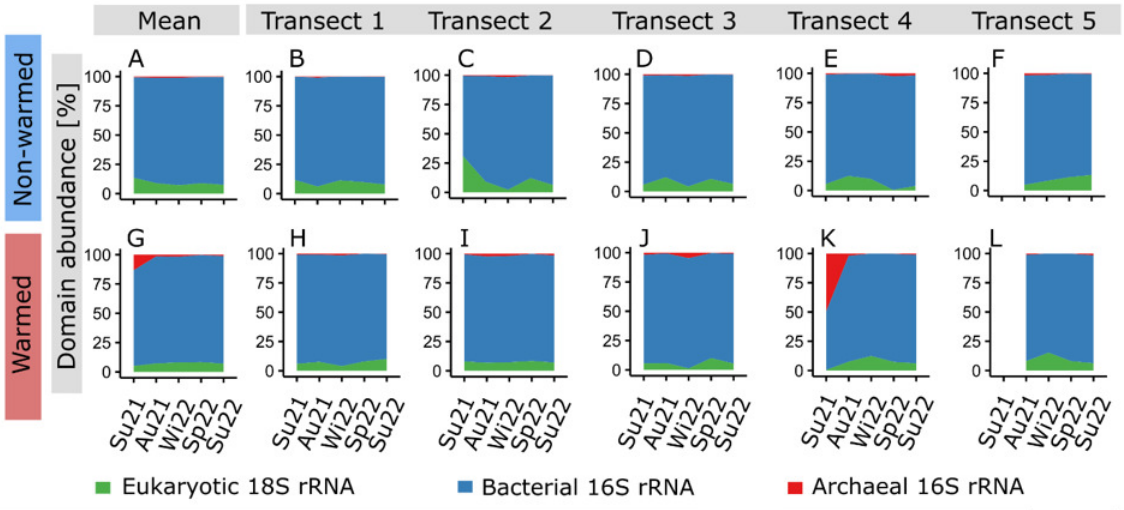

Figure 11. Seasonal community profiles on domain level in warmed (**G – L**) and non-warmed grassland soils (**A – F**). The relative abundance of archaeal and bacterial 16S rRNA transcripts and eukaryotic 18S rRNA transcripts is displayed in % for each transect (**B – F, H – L**) and as mean % of all transects (**A, G**). The seasons start with summer 2021 (Su21), continue with autumn 2021 (Au21), winter 2022 (Wi22), spring 2022 (Sp22) and end in summer 2022 (Su22). See Appendix III, Table 14 and 15 for significant differences.

While the grassland soils were dominated by bacteria, a small fraction of archaea was present, and a variable larger fraction of eukaryotes could be observed in all transects. The relative archaeal 16S rRNA transcript abundance was the highest in the warmed transect 4 in summer 2021 (**Figure 11 K**, Su21), while in other warmed (**Figure 11 G – L**) and non-warmed transects (**Figure 11 A – F**) little variability was detected. The extreme archaeal 16S rRNA transcript abundance in the warmed transect 4 (**Figure 11 K**) can likely be explained with a mistake in the num of spike organism (*Saccharolobus solfataricus*), that was added before the analysis.

The relative abundance of eukaryotic 18S rRNA transcript in winter (Wi22) peaked in some transects (**Figure 11 BKL**), while it was lower in others (**Figure 11 CDHJ**). Significant changes in the relative abundances of eukaryotes between seasons could not be observed. In summer 2021 a trend (p-value <0.1) towards lower eukaryotic abundance in the warmed soil was found (see Appendix III, Table 14 and 15 for exact p-values).

3.3.2 No seasonal effects on microbial transcript abundance occurred at mRNA and rRNA level

To identify whether there was a change in the expression of gene-coding functional transcripts (mRNAs) in response to warming, the mRNA to rRNA ratios in warmed and non-warmed soils across seasons were investigated (**Figure 12**).

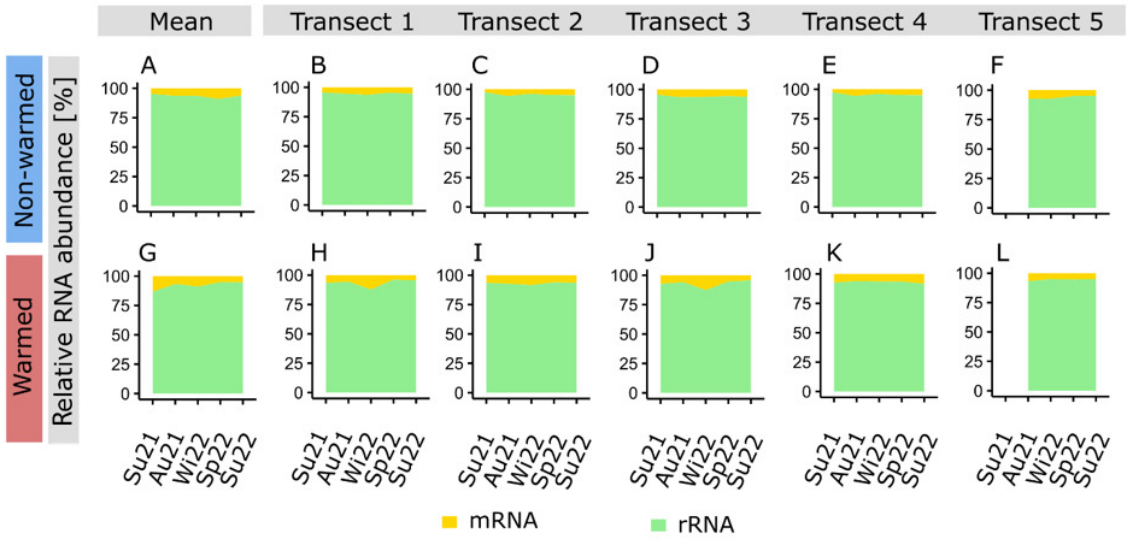

Figure 12. Seasonal mRNA to rRNA ratios in warmed (**G – L**) and non-warmed grassland soils (**A – F**). The relative abundance of rRNA and mRNA transcripts is displayed in % for each transect (**B – F, H – L**) and as mean % of all transects (**A, G**). The seasons start with summer 2021 (Su21), continue with autumn 2021 (Au21), winter 2022 (Wi22), spring 2022 (Sp22) and end in summer 2022 (Su22). See Appendix III, Table 16 – 18 for significant differences.

The ratio between mRNA and rRNA in warmed (**Figure 12 G – L**) and non-warmed soils (**Figure 12 A – F**) showed no consistent differences between the seasons. The mRNA content at E_T (**Figure 12 G**) varied more over time compared to A_T (**Figure 12 A**). Tests revealed no significant differences between seasons at A_T (**Figure 12 A – F**). However, in summer 2021 (Su21) at E_T (**Figure 12 G – L**), a significantly (p-value <0.05) higher mRNA content compared to spring (Sp22), and summer (Su22), plus a trend (p-value <0.1) towards a higher mRNA content in summer 21 compared to autumn (Au21) could be observed (see Appendix II, Table 16 and 17 for exact p-values). When comparing mRNA contents at A_T (**Figure 12 A – F**) and E_T (**Figure 12 G – L**) over the seasons, a trend (p-value < 0.1) towards higher mRNA contents in warmed soil was observed in summer 2021, while in winter (Wi22) there was a significant difference (p-value <0.05) (see Appendix III, Table 18 for exact p-values).

All in all, no seasonal effects on microbial transcript abundance were observed, and thus a more detailed investigation of transcript abundance was necessary.

3.3.3 Transcript abundance of broad functional categories showed no consistent pattern

For a more thorough investigation of transcriptional activity related to specific microbial functions, annotated mRNA reads were used. Annotating the transcripts using the KEGG

database and its hierarchical structure allowed an overall study of transcription for major functional categories. Seasonal changes in transcription for different functional categories were analyzed on the first level of KEGG orthologies (KO): *Metabolism*, *genetic information processing*, *cellular processes*, and *environmental information processing* (**Figure 13**). More detailed information about changes in transcript abundance between A_T and E_T per season was investigated at the second KEGG level, including information about pathways annotated to the four KO groups (**Figure 14**).

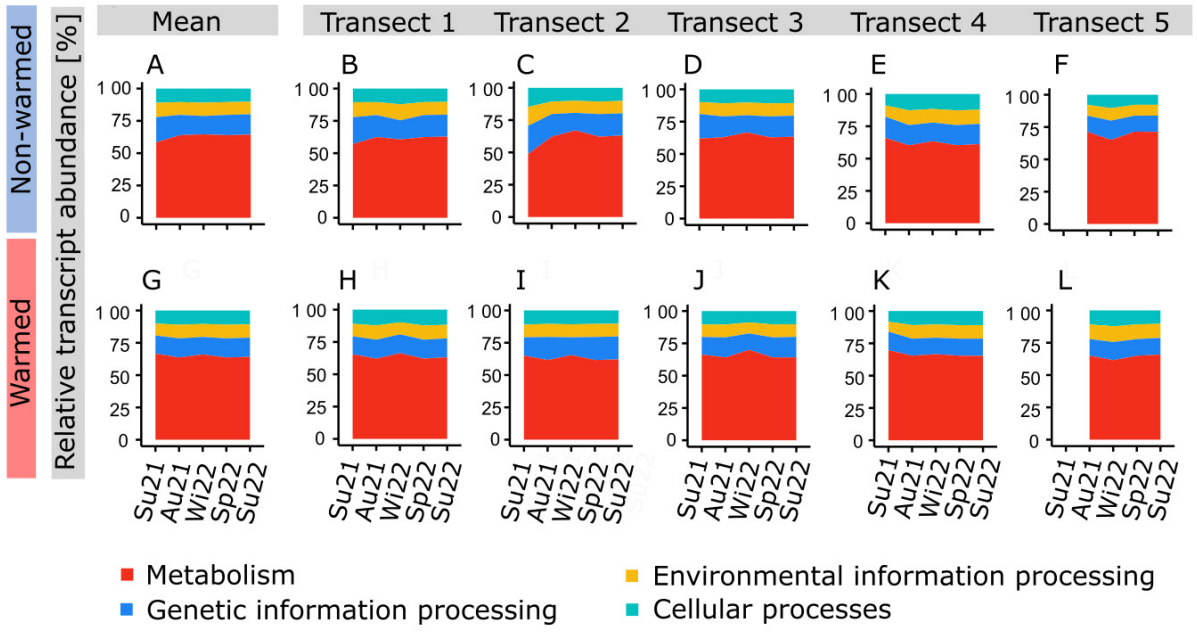

Figure 13. Seasonal transcriptional investment of the microbial communities in warmed (**G** – **L**) and non-warmed grassland soils (**A** – **F**). The relative transcript abundances were normalized to counts per million and are displayed in % for each transect (**B** – **F, H** – **L**) and as mean % of all transects (**A,G**). The seasons start with summer 2021 (Su21), continue with autumn 2021 (Au21), winter 2022 (Wi22), spring 2022 (Sp22) and end in summer 2022 (Su22). See Appendix III, Table 19-21 for significant differences.

A comparison between the seasons showed a peak in transcripts annotated to *metabolism* in winter (Wi22) in several transects (**Figure 13 CDHIJ**), that was only reversed in transect 5 (**Figure 13 FL**). However, no statistically significant changes in transcription for the *metabolism* category were detected between seasons. At A_T higher relative abundances of transcripts for *genetic information processing* were detected between summer 2021 (Su21) and autumn (Au21) (p-value <0.05), spring (Sp22) (p-value <0.05), and winter (Wi22), the latter being a weaker trend (p-value <0.1) (see Appendix III, Table 19 and 20 for exact p-values).

Looking at mean transcript abundances within the transects, a peak in transcripts assigned to the *metabolism* category in winter at E_T was observed, while no peak occurred in any season at A_T. The mean transcript abundance over seasons at A_T (**Figure 13 A**) was affected by a lower

transcript abundance assigned to the *metabolism* category in two transects (**Figure 13 BC**). Significant difference between A_T and E_T (p-value <0.05) occurred in in summer 2021 with a higher abundance of *metabolism* transcripts together with a lower abundance of *genetic information processing* transcripts (see Appendix III, Table 21 for exact p-values).

3.3.4 Down-regulation of protein biosynthesis machinery as possible common warming response, except in winter

Changes in abundance of transcripts annotated to sub-categories within the four KO groups were compared between A_T and E_T within seasons (**Figure 14**), looking for consistent patterns. Overall, a higher relative abundance of transcripts annotated to sub-categories within the category *genetic information processing* was found in grassland soils at A_T, when compared to E_T, except in winter (**Figure 14**). Furthermore, in summer 2021, a lower abundance of transcripts annotated to *metabolism* sub-categories was found at A_T, whereas in other seasons no clear trend was observed. Transcriptional abundance of *environmental information processing* sub-categories showed no clear pattern between seasons or warming, while abundance of *cellular processes* sub-categories was higher at E_T in autumn, spring and summer 2022. Transect 4 (GO4A/E) seemed to be an outlier in all seasons at A_T and E_T (**Figure 14**).

Lower investment into transcripts associated with *genetic information processing* sub-categories, especially with *Translation* (which includes ribosomal proteins), in warmed grassland soils indicate a downregulation of the protein biosynthesis machinery and occurred in all seasons except winter (**Figure 14**).

This observation is in line with the key observation made in the main parts of this thesis indicating that the average cellular ribosome contents of microbial populations in forest soils are reduced at higher temperatures, except in winter, and highlight the presence of a temperature threshold for this response.

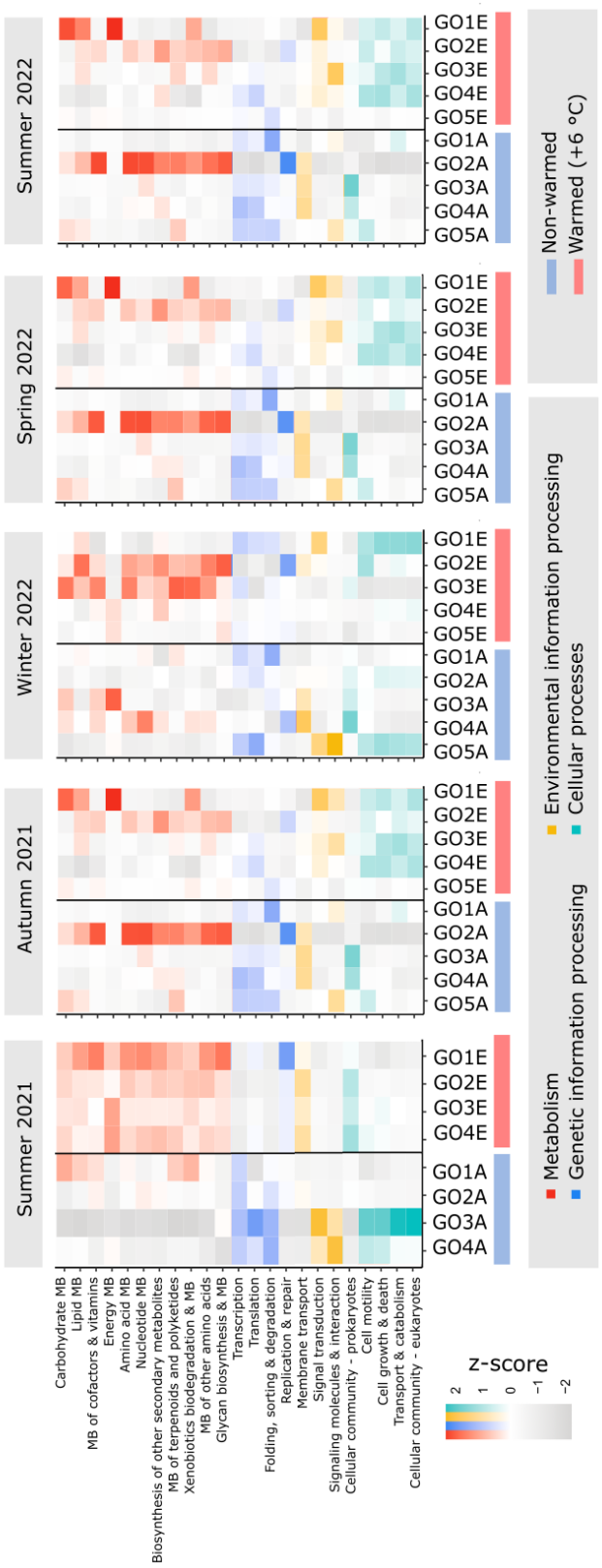

Figure 14. Warming effects on transcriptional investment of the microbial communities in warmed and non-warmed grassland soils. The transcript abundances were normalized to counts per million and transformed to z-scores. Five transects (GO1 – 5) are compared; A indicates the non-warmed plots (blue), while E indicates the +6 °C warmed (red) transects. The transcriptional investment is compared in five seasons (Summer 2021 – Summer 2022), with the exception of transect 5 that was not sampled in summer 2021. The colors in the heatmap indicate the annotation to the four KO: *Metabolism* (MB, red), *genetic information processing* (blue), *environmental information processing* (yellow) and *cellular processes* (turquoise).

4 Discussion

In this thesis, non-warmed (A_T) and warmed (E_T) forest soils were investigated to reveal if the common microbial warming response proposed by Söllinger et al. (2022), namely a reduction in average cellular ribosome contents of the microbial population, is also triggered in forest soil. It was hypothesized that cells exposed to warming reduce their ribosome content, due to higher enzymatic reaction rates, including higher protein biosynthesis rates per ribosome, at increased temperatures. A more efficient protein biosynthesis machinery would allow soil microorganisms to produce sufficient numbers of proteins with a reduced number of ribosomes. Thereby, energy and matter previously used for ribosome production could be liberated and re-allocated to metabolic pathways, possibly resulting in higher microbial growth and CO_2 emission rates at warmer temperatures. These physiological adjustments were hypothesized to occur throughout the year in varying degrees depending on the season and the effective temperatures (seasonal hypothesis). Thus, a seasonal survey with forest soils was performed. Furthermore, a short-term warming experiment with forest soils was conducted to investigate the effect of the temperature range on the magnitude of a reduction in cellular ribosome content. It was proposed that a reduction in cellular ribosome content can be observed after three to six weeks of warming with less or no reduction at the low warming extends, and that warmer temperatures would lead to higher CO_2 emissions (i.e., higher microbial activity). During the experiment a cooling incubation was added after the warming incubation, to investigate the effect of cooling pre-warmed soils on cellular ribosome contents and microbial activity. CO_2 emission rates were hypothesized to return to pre-incubation values, while the reduction in ribosome content would be reversed. In addition, a metatranscriptomics analysis of long-term warmed grassland soil was used to investigate the effects of warming and seasonal temperature changes on microbial ribosome reduction on a transcriptional level. This approach allowed testing the above-mentioned seasonal hypothesis using a different site and other methods (gene expression data instead of RNA:DNA ratios).

4.1 Estimating average cellular ribosome contents

To detect changes in the approximated average cellular ribosome contents of the microbial populations between warmed and non-warmed soils, RNA and DNA contents were quantified (see 2.2.2). Despite the fragility of RNA molecules, measuring RNA concentrations is a reliable method for determining average cellular ribosome contents of the microbial population

(Blazewicz et al., 2013). While the DNA concentration is more stable due to higher robustness of the DNA double helices (Blazewicz et al., 2013), it is not suitable as an indicator for biomass, but it may serve as a proxy for cell counts. This was demonstrated in the ForHot grassland soils where considerable differences between patterns of DNA content and microbial biomass were observed (Söllinger et al., 2022). Further limitations of DNA quantification are extracellular DNA (necro mass DNA), the ratio between intracellular and extracellular DNA that potentially vary between seasons or with temperature, and variations in the cellular DNA concentration depending on the growth and DNA replication rate (Blazewicz et al., 2013). In pure cultures one can quantify the microbial biomass by combining cell counts with dry weight estimates, but this is not possible for mixed soil communities (Blazewicz et al., 2013). Thus, the RNA:DNA ratio was used in this thesis to give an approximation of the average cellular ribosome contents of the microbial population. However, as indicated above, this estimate can be influenced by the genome size and growth phase of a cell, as well as the amount of extracellular DNA, and thus, needs to be considered as an estimate, not a quantitative value, and be treated with care. Originally, we aimed to determine the microbial biomass (mass of microbial carbon and nitrogen from intact cells), to get a second proxy for changes in cellular composition. As a large proportion of cells consists of ribosomes (Lafontaine & Tollervey, 2001) it would allow a more careful consideration of the RNA estimates and calculation of ribosome contents per soil microbial biomass, which would allow an additional evaluation of the RNA:DNA ratios as proxies for cellular ribosome contents. Unfortunately, due to technical issues, biomass extracts could not be analyzed. However, the study by Söllinger et al. (2022) conducted on the ForHot grassland soils showed a strong correlation between the RNA content per microbial biomass and RNA:DNA ratios, both decreasing in warmed soils. This suggests that the average number of ribosomes in soil microorganisms is reduced with warming and that RNA:DNA ratios can be used as a proxy for cellular ribosome content.

4.2 Warming effects on soil microorganisms

4.2.1 Long-term warming and seasonal warming effects

Lower RNA:DNA ratios, indicating lower ribosome content per cell at E_T compared to A_T were observed in long-term warmed forest soils in spring, summer, and autumn, but not in winter. This suggests that ribosomal downregulation demonstrated in grassland soils (Söllinger et al., 2022) also takes place in forest soils. The lack of cellular ribosome content reduction in winter

was also observed in two grasslands (GO, GN) and in a second winter comparison of forest soils. Thus, while ribosomal downregulation at warming occurs in different soil types during spring, summer and autumn, the winter observations point towards a low-temperature threshold for ribosomal downregulation. Perhaps, at cold temperatures, the advantages of down-adjusting microbial ribosome contents do not apply, as demonstrated by the lack of ribosomal content reduction at +3 °C warming during winter (see also 4.2.4 for a further discussion).

In should be noted that the studied forest soils were only warmed at +3 °C, while the grassland soils were warmed at +6 °C (GO) and even +9 °C (GN), showing that the physiological warming response (ribosome reduction) in the forest is triggered by soil warming that is well within the predicted warming range of +6 °C in arctic regions (IPCC, 2013b). While the extend of warming required to trigger this mechanism will certainly vary between different soil types and climatic regions, the possible implications for the terrestrial C cycle, such as accelerated decomposition rates facilitated via cellular resource re-allocations, are worrying, especially in northern regions storing large amounts of soil C (Jansson & Hofmockel, 2020; Tiedje et al., 2022).

Furthermore, seasonal changes in ribosome content were observed in both the non-warmed and warmed soils, suggesting that in addition to long-term warming, seasonal temperature changes can also influence cellular ribosome contents. Thus, greenhouse gas fluxes and other microbial activities in soil environments that experience increased seasonal temperature variation and perhaps less prominent long-term increases in average temperatures, might also be influenced by microbial physiological adjustments to warming. Such seasonal shifts might lead to accelerated microbial activity, potentially with consequences for the global C budget (Jansson & Hofmockel, 2020; Tiedje et al., 2022).

4.2.2 Short-term warming effects

The microbial warming response was further investigated in a short-term warming experiment with a focus on warming duration and a possible temperature threshold for a reduction of the average cellular ribosome contents of the microbial population. Based on results from an incubation experiment with ForHot grassland soils, a first microbial physiological warming response in forest soil was expected after three weeks of warming (Söllinger et al., 2022). However, in the short-term forest soil warming experiment, a reduction in ribosome content

was only observed after six weeks of +9 °C warming (from the *in situ* temperature of 2 °C to 11°C). Below an effective temperature of 11 °C (here 5 °C and 8 °C) warming did not result in a clear trend, suggesting that in the forest soils, warming for more than three weeks at temperatures above at least 8 °C are necessary to trigger a physiological acclimation in form of cellular ribosome reduction. However, it cannot be excluded that thresholds may be different when starting from an initial temperature above 2 °C. In a similar short-term warming experiment with grassland soils, temperatures of 7 °C to 13 °C were used (Söllinger et al., 2022). The authors observed indications for a reduction in cellular ribosome contents already after one week of warming (+6 °C warming; from 7 °C to 13 °C). Comparing these observations to the short-term warming experiment with forest soils conducted in this thesis, we see that a shorter incubation time of one week and an increase of only 6 °C is needed when the initial temperature was 7 °C, as opposed to three weeks and +9 °C when the initial temperature was 2 °C. This suggests that at lower temperatures (e.g., 2 °C), the Icelandic soils may require a larger temperature increase, more time, or both, before a ribosomal downregulation is triggered (see 4.2.4 for a further discussion).

4.2.2.1 CO_2 emission rates and substrate limitation

Short-term warming led to elevated CO_2 emissions relative to the controls already after 24 h of warming, suggesting that this microbial warming response happens within the first day. However, CO_2 emission rates decreased over time, which may be explained by depletion of substrates in the incubation flasks, perhaps of dissolved and easily available substrates. Direct temperature effects on enzymes, resulting in higher reaction rates at warm temperatures (*Effect of Temperature on Enzymatic Reaction*, 2022) might have caused the initial increase of CO_2 emission rates, while a possible microbial acclimation to both temperature and altered substrate concentrations or other environmental factors, might have led to a temporal decrease in CO_2 emissions. In addition, the amount of soil in the flasks was little (~40 g) and regularly sampled (~2 – 8 g/week during warming incubation), adding a stress factor by mixing, aerating, and decreasing the soil mass in the flasks. These are limitations that highlight how experiments in laboratories are always only an approximation of natural *in situ* conditions, emphasizing the need of combining laboratory experiments with *in situ* measurements and studies. Unfortunately, measuring CO_2 emissions from ForHot soils *in situ* is not trivial since a considerable amount of gases emitted from these Icelandic soils is of geogenic origin. Thus, no *in situ* measurements have been conducted in the course of this thesis.

4.2.2.2 Connection between cellular ribosome reduction and microbial activity

Traditionally, high ribosome contents are associated with high metabolic activities (Kjeldgaard & Kurland, 1963; Schaechter et al., 1958) and faster growth rates (Lankiewicz et al., 2016). However, these studies focus on pure cultures under optimal growth conditions. Blazewicz et al. (2013) found that the use of rRNA or ribosome content as an indicator of the metabolic state in microbial populations had serious limitations and the relationship between rRNA, growth and activity was often contradictory.

Short-term warming of forest soils for six weeks showed a possible connection between reduced average cellular ribosome contents of the microbial population and increased metabolic activities for the highest warming extend (+9 °C warming; from 2 °C to 11°C). At 11 °C the cellular ribosome contents were lower after 6 weeks of warming compared to the starting point and the CO_2 emission rates were higher than in the other temperature treatments, indicating that warming leads to accelerated metabolic activities, despite or maybe even because of lower ribosome contents. That warming might accelerate metabolic activities not only despite a lower ribosome content, but possible *because* of the cellular ribosome content reduction, can be explained with the hypothesis that the energy and matter saved by reducing the ribosome content in a cell could be re-allocated to metabolic processes and increase microbial activities (Söllinger et al., 2022). However, since higher CO_2 emission rates were observed right after the onset of warming before any changes in ribosome contents could be observed, the relationship between warming-induced ribosome reduction, microbial activities, and microbial-derived CO_2 emissions from soil remain elusive.

4.2.2.3 Lacking ribosome reduction at low temperatures

When increasing the temperature from 2 °C to 5°C and 8 °C in the short-term experiment, we observed a ribosomal reduction that was similar to the ribosomal reduction in the 2 °C control. This might be explained by limitation of substrate availability in the incubation flasks, as also mentioned as a possible explanation for the reduction in CO_2 emission over time, above. A general limitation of easily degradable substrates could force a majority of the microorganisms to reduce their ribosomal content and general activity to save mass and energy. The reason for a substrate limitation that influences all temperatures, including the control, is that the soil microbial network that have established over a long time *in situ*, and includes roots and hyphal networks, is likely to have been damaged during sampling and experiment preparation. Thus, our soil processing may have severely limited biological interactions that are crucial for efficient decomposition and sharing of different substrates and nutrients between the members

of the soil community. Such networks take a long time to establish, and include connections between different microbial populations, fungi, and plants, for example between bulk soil microorganisms and the root rhizosphere (Baldrian, 2016). Boreal forest soil communities are dependent on interaction with fungal hyphae of ectomycorrhizal fungi between roots and bulk soil. Ectomycorrhizal fungi represent up to 30 % of the microbial community in boreal forest soil (Baldrian, 2016), while the grasslands under investigation in this thesis are dominated by bacteria. The higher abundance of fungi in forest soils and the disruption of hyphal networks might have led to a weaker pattern and lack of cellular ribosome reduction after short-term warming of forest soils compared to short-term warming of grassland soils (Söllinger et al., 2022), as warming also effects the structure of forest communities, especially the fungi:bacteria ratio (Baldrian et al., 2023).

4.2.2.4 Cooling pre-warmed soil had no effect on average cellular ribosome contents but CO_2 emissions decreased drastically

The cooling of previously warmed soils resulted in a return to pre-warming CO_2 emission rates, except for the highest warming extent of +9 °C where the CO_2 emission rates dropped below non-warmed control values. This suggests a connection between the reduction in average cellular ribosome contents of the microbial population at 11 °C and its response during subsequent cooling. Possibly, the ribosome reduction during warming prevented the re-establishment of pre-warming respiration rates during cooling. The reason for this might be that the cells with downregulated protein biosynthesis machineries are unable to produce sufficient numbers of proteins to obtain the pre-warming substrate CO2 production rates, indicating that more time at the low temperature or a slower transition between temperatures might be needed to acclimate back to the cold conditions.

It was hypothesized that the return to *in situ* temperature (2 °C) would lead to a higher ribosome content in cells than during the warming treatment. Such an increase in ribosome content triggered by cooling was previously observed in an incubation experiment with peat soil performed in our lab (cooled from 10 °C down to 2 °C; unpublished data from Yngvild Bjørdal et al.). However, such a physiological response to cooling was not found after the 8 weeks of cooling pre-warmed forest soils, suggesting that the increase in ribosome content after cooling may be an effect of particular conditions in peat soil, such as altered substrate availability co-occurring with the cooling. In forest soil the observed seasonal dynamics in the approximated average cellular ribosome contents, with an increase after the transition from autumn to winter

("cooling") and a gradual decrease after winter ("warming"), suggest that an increase in cellular ribosome content might be a general response to cooling, nevertheless.

4.2.3 Seasonal metatranscriptomics after long-term warming

The study of microbial warming responses in forest soil and the underlying hypotheses were based on observations from a metatranscriptomics analysis of grassland soils from one single summer timepoint (Söllinger et al. 2022). The proposed ribosome reduction with warming seems to be a mechanism that occurred also in the seasonal forest survey, except in winter. It was not possible to obtain seasonal forest metatranscriptomes and investigate transcriptional evidence for a ribosome reduction across seasons (except winter) during the course of this thesis. However, seasonal ForHot grassland metatranscriptomes were available and investigated for a seasonal transcriptional pattern indicating a downregulation of the protein biosynthesis machinery instead.

The seasonal relative abundance patterns of transcripts associated with *genetic information processing* observed comparing long-term warmed (>50 y) grassland soils with non-warmed grassland soils were in line with the seasonal patterns of ribosome reduction and the absence of a temperature effect in winter seen in the forest soils. Thus, the observed downregulation of the protein biosynthesis machinery at E_T in spring, summer and autumn supports previous findings (Söllinger et al. 2022) and suggests that warming leads to a reduction in cellular ribosome contents in grassland soils and forest soils, possibly leading to a reallocation of energy resources into metabolic activity. However, the winter exception highlights that the previous understanding (Söllinger et al., 2022) of this mechanism was incomplete, and furthermore suggests the existence of temperature thresholds or other mechanisms that could inhibit the temperature-driven reduction of cellular ribosome contents.

4.2.4 Community composition and microbial warming responses

Nevertheless, another reason for both, the observed changes in the approximated average cellular ribosome contents in the seasonal study and the lack of changes after cooling connected to the decreased CO_2 emission rates in the short-term experiment, could be a shift in the microbial community composition. Unfortunately, the ForHot forest microbiome was only investigated regarding fungal community changes (Rosenstock et al., 2019). The authors found

that warming had only limited effects on ectomycorrhizal community composition and mycelial growth, which may suggest that changes in the bacterial community composition are also minor, or at least that the fungi:bacteria ratio may not have changed with warming. However, indications of lower cellular ribosome contents at warm temperatures were present in this master thesis and other studies found that functional changes in response to warming, including the down-regulation of the protein biosynthesis machinery, were not related to a shift in taxonomic compositions (Söllinger et al., 2022; Tveit, 2014).

A study of long-term warmed Harvard forest soil found a decrease in fungal abundance, a community shift towards gram-positive bacteria and an increase in abundance of bacteria with low rRNA operon copy numbers (Melillo et al., 2017). Cells with less rRNA operons might tend to have a lower ribosome content, since the number of ribosomes that can be produced by the cells is amongst others regulated by the number of transcripts that can be initiated at an rRNA operon promoter (Fegatella et al., 1998; Klappenbach et al., 2000). Thus, a lower cellular ribosome content may be in general favorable under warming conditions and can occur via physiological adjustments (ribosome reduction) of a broad range of community members (Söllinger et al., 2022) but also via a restructuring of the microbial community (Melillo et al., 2017).

4.2.5 Temperature threshold

Based on the warming response that was observed at E_T in grassland and forest soils and the consistent absence of this effect in winter, it was hypothesized that a temperature threshold might exist, below which a reduction in average cellular ribosome contents of the microbial population with increasing temperature does not occur. In addition to our observations, the rationale for this was that the physiological adjustments required for growth optimization by any given microorganism is not the same at all temperature ranges (Tveit et al., 2023).

In the short-term warming experiment, carried out to test this hypothesis, soils were incubated at 2 °C, 5 °C, 8 °C and 11 °C, but a reduction in ribosome content could only be detected in the warmest treatment. The winter soils that were analyses as part of the seasonal survey, had temperatures of ~4 °C (FN), ~6 °C (GO) and ~9 °C (GN) at E_T (see Appendix V, Figure 16). Observations from the warming experiment suggest that a temperature threshold lies between

8 °C and 11°C, where a warming response did not occur at and below 8 °C, but was pronounced at 11°C.

Tveit et al. (2023) suggested that ribosome content adjustments do not correlate linearly with temperature. In the methanotrophic organism *Methylobacter tundripaludum*, they observed a maximal ribosome content at 15 °C and a decline in ribosome content both below and above that temperature. At 21 °C growth rates and ribosomal RNA concentrations were maintained at numbers close to the growth observed at 15 °C, while below 15 °C growth rates declined together with ribosome content. This observation demonstrates that changes in temperature range affect adjustments of ribosome contents, with consequences for growth rates and substrates consumption, but depending on the temperature range, different effects are observed (Tveit, 2023). In line with this, a temperature change of 6 °C from 2 °C to 8 °C in the forest warming experiment did lead to a different microbial response than a temperature change of 6 °C from 7° C to 13 °C in the grassland warming experiment by (Söllinger et al., 2022). This is a strong indication for the existence of a temperature threshold in the ForHot forest and grassland soils at around 10 °C, below which ribosome reduction does not provide an overall physiological advantage for the microorganisms acclimating to the temperature increase.

A possible reason why a ribosome reduction is not occurring at low temperatures, is that a temperature increase of 6 °C within a low-temperature range of 0 – 10 °C, does not lead to a temperature that is sufficiently high to support the fast enzymatic reaction rates required for a downregulation of the protein biosynthesis machinery. Based on the Arrhenius equation for temperature-dependance of reaction rates, enzymatic reaction rates will increase predictably with temperature increase within a certain threshold of enzyme functioning (Cammack et al., 2008), but towards the lower limits of the growth range of an organism, a combined sub-optimal function of multiple enzymes required for growth could mean that the organism reacts differently to a certain temperature increase than at a higher temperature. This is in line with studies on pure cultures, showing that within a certain range of tolerable temperatures for microorganisms, there were temperatures where an effect on ribosome content was larger, smaller or absent (Mairet et al., 2021; Tveit et al., 2023). Despite the potential interference of an effect of substrate saturation in these studies, the magnitude of the temperature increase and the range relative to optimal growth temperatures of the populations impacted strongly how they adjusted their ribosome contents. Thus, the existence of a group-specific thresholds where a downregulation of the protein biosynthesis machinery is not efficient, that is regulated by temperature, is strongly indicated. Furthermore, other factors such as nutrient and water

availability could be affected by the cold temperatures and thus indirectly affect microbial physiologies and prevent a warming response.

4.3 Temperature, substrate availability and moisture

The effect of warming on microbial physiologies and metabolisms might be driven not only by temperature but by change in nutrient availability and quality, as well as moisture in the soil. Whether the reduction in average cellular ribosome contents of the microbial population in response to warming and the changes in respiration rates are due to a combination of these factors, and if one factor is dominating the response, is uncertain.

4.3.1 Temperature and substrate availability in warmed soil

Temperature controls several elements of the microbial metabolism (Allison et al., 2010; Walker et al., 2018). Walker et al. (2018) described microbial metabolism as intrinsically temperature sensitive and not acclimating to warming over weeks and decades of warming, but they also mentioned substrate depletion in combination with temperature sensitivity as actors that affect warming responses. Another study found that the efficiency of soil organic matter usage was connected to the decline in substrate quality with higher temperatures (Frey et al., 2013), showing that effects of temperature and substrate availability are not easy to disentangle. Domeignoz-Horta et al. (2023) suggested that the reduced C availability, as an indirect effect of warming on microbial physiology, overrules the direct warming effects, by limiting microbial growth, respiration, and metabolic activity.

Ribosome content is a physiological component that is possibly influenced by temperature and substrate availability. Bosdriesz et al. (2015) found that fast-growing bacteria, such as *Escherichia coli*, used tuning of ribosome concentration to optimize growth rates and concluded that inactive ribosomes indicate the nutritional state of a cell. Previous studies on the ForHot grassland soils showed nutrient depletion and restricted substrate availability (Marañón-Jiménez et al., 2018; Walker et al., 2018) and a downregulation of the protein biosynthesis machinery in warmed soil (Söllinger et al., 2022). This demonstrates the possible connection between cellular ribosome content and substrate availability that could also be the reason for the reduction in cellular ribosome contents observed in this thesis, given that substrate depletion occurs in the warmed ForHot forest soils. Unfortunately, up to now no data on nutrient concentrations and substrate availabilities in the ForHot forest soils were available.

The depletion of nutrients in the warmed ForHot grassland soils along with the observed reduction in ribosome contents may suggest that also the warmed ForHot forest soil microbial communities have a restricted access to substrates. In the seasonal survey of forest soils, an increase in DNA contents could be observed in autumn. The high DNA content might be connected to an increase in substrate availability due to the litter input from trees and understory in this season, as observed previously in other soils where enriched microbial biomass was observed after litter input in soil (Jin et al., 2010). However, even if substrate availabilities increased in autumn, it did not prevent a reduction in cellular ribosome contents. If that's the case that would suggest a less tight connection between substrate availabilities and ribosome reduction than discussed above. Regarding the effects on CO_2 emissions, a meta-analysis on the effect of litter inputs on forest soil microorganisms reported a strong positive impact of litter input on soil respiration and labile C availability, while they described the impact of soil moisture and temperature as less important (Zhang et al., 2020). This might also be reflected in the decrease in CO_2 emission rates over time in the warming incubation experiment, where no substrates were added to the incubation flasks and nutrients might have depleted over time.

4.3.2 Temperature and moisture in warmed soil

Soil water content is essential for substrate transport, important for hydrolysis processes, and controls microbial activity, thus it determines the rates of mineralization (Paul et al., 2003). Depending on seasonal patterns of rainfall and temperature, activities of decomposers vary across the seasons (Manzoni et al., 2012). How tightly interwoven temperature and water availability are, was stressed by a study conducted on frozen soils that found water availability largely controlled microbial temperature sensitivity (ÖQUIST et al., 2009). Extreme temperatures in both ways, cold and heat, lead to restricted water availability and stress for soil microorganisms (ÖQUIST et al., 2009, Manzoni et al., 2012). With warming, evaporation of water increases and soil dries (Davidson et al., 2000), while a combination of wet conditions and warming leads to higher microbial respiration (Fei et al., 2015).

While no significant differences in soil water contents could be observed comparing non-warmed (A_T) and warmed (E_T) forest soils at the selected seasonal timepoints, soil water contents differed significantly comparing seasons. Furthermore, water content differed between A_T and E_T in grassland winter soils: A significantly higher soil water content at E_T was present in one grassland (GN) in both years, and in the other grassland in one year (GO, 2023), whereas in the previous year (2022) a significantly lower water content was present. However, in winter

forest soil (2023) significant lower water content co-occurred with significantly higher cellular ribosome content and in winter grassland soils higher water content co-occurred with both lower (GO, 2023) and higher (GN, 2022 and 2023) ribosome content. Thus, a direct connection between approximate average ribosome contents and water contents could not be found in our soils.

5 Summary and conclusion

This thesis demonstrates that a reduction in average cellular ribosome contents of the microbial population is an important microbial response to forest soil warming that occurs as fast acclimation after weeks, across seasons and as well after years and decades of warming, but its magnitude is limited by a temperature threshold. Our winter observations indicated the existence of a temperature threshold, below which a downregulation of the protein biosynthesis machinery is not efficient. The analysis of the metatranscriptomes of long-term warmed (>50 y) grassland soils confirmed the seasonal pattern in the downregulation of the protein biosynthesis machinery as observed in warmed forest soils. The existence of a temperature threshold was furthermore corroborated by the short-term warming experiment and a second winter sampling including grassland soils, further suggesting the threshold temperature is around 10 °C.

A reduction in cellular ribosome contents was observed in forest and grassland soils and across seasons, except in winter, suggesting that this is a widespread microbial warming response. Possible consequences of such a physiological response might be a resource re-allocation leading to increased activities beyond a linear temperature response and underestimation of microbial activities in climate change predictions. These results strengthen the hypothesis that cells exposed to warming reduce their ribosome content, since higher enzymatic reaction rates at increased temperatures allow a more efficient protein production and liberate energy. The re-allocation of energy and matter to metabolic activity can lead to potentially large consequences for the terrestrial C cycle and the global greenhouse gas budget.

6 Outlook

In the following, possible further directions that could be explored in the future are summarized.

In order to examine the temperature threshold for cellular ribosome reduction more closely, a new incubation experiment with more temperatures could be conducted including smaller temperature steps and a focus on the range between 9 and 11°C. Additionally, improving frequency of sampling timepoints and a focus not on the early weeks but between three and six weeks, could help to investigate the required acclimation time for a microbial warming response. Observations about CO_2 emission rates from the short-term warming experiment could be backed up with *in situ* measurements of respiration rates in forest soil and help to determine seasonal changes and warming effect on *in situ* respiration rates. This would furthermore allow to draw a more direct connection between cellular ribosome contents and *in situ* CO_2 emission and their consequences for the global soil C stock. However, a considerable amount of gases emitted from these Icelandic soils is of geogenic origin, making measuring *in situ* CO_2 emissions not trivial as isotope measurements would be necessary to detect the biogenic emissions.

Regarding the metatranscriptomics analysis, the very first insights from this thesis should be investigated further to gain a deeper understanding of transcriptional changes between warmed and non-warmed soil communities and over a seasonal timescale. Filtering for the spike organism, can be optimized and the normalization to counts per million for comparison in transcripts should be reconsidered since individual counts are very low and potentially produce a skewed picture when multiplied to millions. Furthermore, a metatranscriptomics analysis of ForHot forest soils would be required to verify that observations made in this study on grassland soils apply to forest soils as well. In line with that, the microbial community in the forest soils needs to be investigated to rule out that shifts in microbial community composition are the underlying cause of the observed warming responses.

It has to be kept in mind that few of the differences in approximate average cellular ribosome contents between warmed and non-warmed soil were statistically significant (p-value <0.05). In this thesis, five replicated forest transects (biological replicates) of naturally warmed soils and non-warmed counterparts were sampled and technical triplicates were used to replicate sample processing. Increasing the number of replicates for a higher statistical reliability is difficult as biological replicates are naturally limited and technical replication must be within a

reasonable scope. Despite the lack of significant differences, a trend towards a reduction of cellular ribosome contents in warmed soil could be observed across different seasons and years, as well as in different soils (forest and grassland soils) and indicated with different methods (using RNA:DNA ratios as proxies and metatranscriptomics), which overall strengthens the reliability of the observations made and conclusions drawn in this thesis.

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Appendix

I. Total nucleic acid (TNA) extraction

Solutions for TNA extraction

Henckel *et al.* (1999)

1. Phosphate buffer (120 mM PB pH 8.0)

$K_2HPO_4 \cdot 3\ H_2O$ (M.W 228.22)	6.215 g
KH_2PO_4 (M.W 136.09)	0.376 g
RNase-free water (MiliQ)	-> 300 ml

Dissolve and autoclave

2. TNC Henckel *et al.* (1999)

500 mM TRIZMA (M.W 121.14)	23.76 g
100 mM NaCl (M.W 58.44)	1.76 g
10% CTAB (M.W 364.45)	30.00 g
RNase-free water (MiliQ)	-> 300 ml

Dissolve and autoclave

3. Precipitation solution

30% PEG (M.W 7000-9000)	90.00 g
1.6 M NaCl (M.W 58.44)	28.05 g
RNase-free water (MiliQ)	-> 300 ml

Shake well by hand, autoclave and mix well while hot (solution turns milky when hot, but turns clear when cooled to room temperature)

4. DEPC treated water (0.1%)

MiliQ water	900 ml
DEPC (1%)	900 µl

Incubate 3 h at room temperature, then autoclave

TNA extraction protocol for seasonal survey

Procedure for approximately 0.3 g of soil following Angel et al. (2012)

1. Weigh approximately 0.3 g of soil into a Lysis E Tube and place tube on ice (If prepared beforehand, collect samples from -80 °C freezer and thaw tubes on dry ice)
2. Add **375 µl of PB**, **125 µl of TNC** and **300 µl of PCI** (phenol/chloroform/isoamylalcohol; 25:24:1)
3. Immediately place tube in a bead beater CoolPrep adapter with dry ice and process for 30 sec at 6.5 m s^{-1}
4. Centrifuge (13,200 rpm) at max speed (13,200 rpm) at 4 °C for 3 min
5. Transfer **450 µl supernatant** (extraction buffer and the phenol phase) to a fresh 2 mL tube by pipetting, retain the lysing tube. Keep samples on ice

6. Repeat steps 4 to 7 using the same lysing tube.
   ! In third extraction round decrease volume down to **200 µl PCI** (Supernatant in third round is 350 µl). Use a fresh 2 mL tube to collect the supernatant from every extraction round
7. After the **third extraction** divide the supernatant between the two tubes from the previous extractions to achieve equal volumes (2x 140 µl). This means that each 2 mL tube will contain a total of 590 µl supernatant
8. Add 1 volume **(590 µl) PCI** to each of the tubes containing the extract
9. Mix phases by vortexing for 30 sec
10. Centrifuge (13,200 rpm) at max speed at 4 °C for 3 min
11. Transfer the supernatant (**500 µl**) from each tube to fresh 2 mL tubes and add 1 volume **(500 µl) CI** (chloroform/ isoamylalcohol 24:1). Mix phases by inverting the tubes for 30 sec
12. Centrifuge (13,200 rpm) at max speed at 4 °C for 3 min
13. Transfer supernatant (**350 µl**) from each tube to fresh 2 mL non-stick silicon tubes
14. Add **2 µl of glycogen** and **700 PEG** Precipitation Solution (2 volumes) to each tube
15. Centrifuge (13,200 rpm) at max speed for 60 min at 4 °C
16. Decant the supernatant by pipetting, shortly (30 sec) centrifuge again to collect the drops and remove with a pipette as much as possible from the remaining precipitation solution. Be careful not to disturb the pellet
17. Wash once with **2 mL of ice cold 75% EtOH**, invert the tube several times for 30 sec
18. Centrifuge (13,200 rpm) at max speed for 10 min
19. Remove the supernatant, shortly centrifuge again to collect the drops and remove with a pipette as much as possible from the remaining precipitation solution
20. Leave tubes open at room temperature for approximately 5 min to evaporate the remaining ethanol (note: pellets might not be completely dry at this point).
21. Resuspend the pellets in 50 µl nuclease free water and combine both subsamples into one of the non-stick tubes (total volume 100 µl)
22. Add 1 µl RiboLock in each of the combined samples
23. Keep 20 µl at -20 °C for further analysis (Qubit Analysis, Gel) and the rest (80 µl) store at -80 °C

Agarose gel electrophoresis:

Small 1% agarose gel (BioRad, 8 samples):

1. Prepare gel:
   a. 1x TAE (Tris-acetate-EDTA) buffer 40 ml

b. Agarose 0.4 g

2. Boil in microwave and let cool, then add
    a. GelRed (in fridge) 2 µl
3. Caste gel in fume hood and let dry for ca. 30 min
4. Prepare samples
    a. 5 µl samples + 2 µl 6x loading buffer (DNA gel loading dye, ThermoFisher, MA, USA)
5. Load samples and marker
6. Run gel at 60 V, 400 (max) mA for 40 – 60 min

Example picture for quality check after TNA extraction:

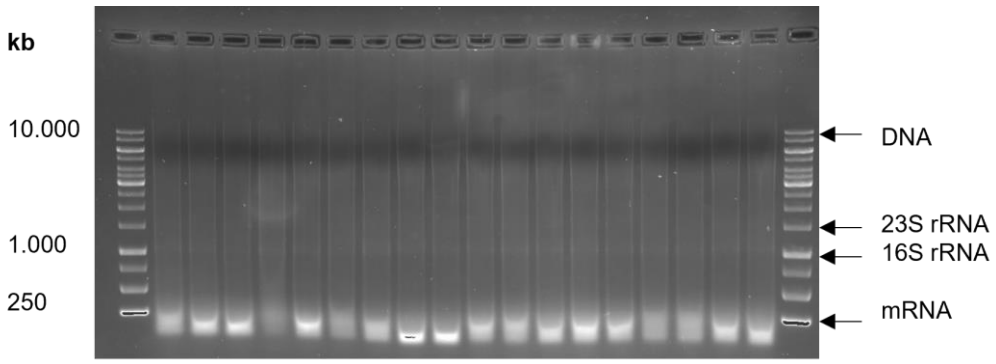

Figure 15. Agarose gel electrophoresis of TNA extractions. Example for a quality control of extraction products after total nucleic acid extraction from forest soils.

The gel in Figure 15 shows weak bands because the concentration in samples is generally low. A band at ~1.000 kb, the 16S rRNA representing the smaller subunit of bacterial ribosomes, is visible in all samples. The strong bands at the bottom of the gel shows degraded nucleic acids and small RNAs (mRNA, tRNA), that are not filtered out in the used TNA extraction method. Together with the Qubit™ analysis that evaluates DNA and RNA concentrations, the agarose gel electrophoresis gives a reliable quality check.

II. KCl extraction protocol (chloroform fumigation)

Controls and fumigation start (Day 1):

Soil sampling:

1. Weigh in 2 g of soil in an aluminum dish
2. Place the aluminum dish in the desiccator containing the chloroform
3. Weigh in 2 g soil in a 50 mL falcon tube (for controls)
4. After the sampling return the bottle plus the 50 mL tube to the incubator

KCl extractions:

Before start, the filtration unit needs to be cleaned with MiliQ water and 70% EtOH

1. Add 15 mL KCl to each tube
2. Close the tubes properly and place them in/on the shaker
3. Shake them at 125 rpm for 30 minutes
4. Turn on the vacuum pump
5. Fill the samples into the cylinders
6. Wait until all samples went fully through the filter
7. Turn off the vacuum pump and disassemble the filtration unit
8. Now pour the filtrate in the pre-labelled 15 mL tubes
9. After all 8 samples + 2 blank are poured bring the 15 mL tubes to the -20 degree room
10. Before the next samples can be processed the filtration unit needs to be cleaned

Fumigated samples (Day 2):

1. Transfer fumigated soil from aluminum dishes into 50 mL tubes
2. Clean filtration unit
3. Extract as in protocol for Day 1
4. Store samples at -20°C

III. Statistical analysis and significance

Seasonal Survey:

1. Difference in nucleic acid and water contents between seasons

Table 3. ANOVA (p-values): Test for significant difference in RNA contents, DNA contents, RNA:DNA ratios and gravimetric water contents (GWC) between seasons in non-warmed (A_T) and warmed soils (E_T). Green = p-value < 0.05.

	A_T	E_T
RNA	0.5800	0.3100
DNA	0.0064	0.4840
RNA:DNA	0.1370	0.0001
GWC	0.0195	0.0172

Table 4. ANOVA and post-hoc Tukey test (p-values): Test for significant difference in RNA contents, DNA contents, RNA:DNA ratios and gravimetric water contents (GWC) between all four seasons seasons in non-warmed (A_T) and warmed soils (E_T). Green = p-value < 0.05, light green = p-value < 0.1.

		DNA		
		October	February	May
A_T	October			
	February	0.9663		
	May	0.0957	0.0401	
	July	0.0443	0.0178	0.9759

		RNA:DNA		
		October	February	May
E_T	October			
	February	0.0132		
	May	0.1714	0.0003	
	July	0.2554	0.0003	0.9829

		GWC		
		October	February	May
A_T	October			
	February	0.9017		
	May	0.9017	1.0000	
	July	0.1095	0.0301	0.0301
E_T	October			
	February	0.4107		
	May	0.4107	1.0000	
	July	0.4281	0.0285	0.0285

2. Difference in nucleic acid and water contents between winter and other seasons

Table 5. One-sided student's t-tests (p-values): Test for significant differences in RNA contents, DNA contents, RNA:DNA ratios and gravimetric water contents (GWC) in forest soils between winter and other seasons. Green = p-value < 0.05.

	Season	RNA	DNA	RNA:DNA	GWC
A_T	**Autumn**	0.4055	0.6865	0.2082	0.8095
	Spring	0.1475	0.9871	0.1689	0.5000
	Summer	0.2102	0.9975	0.0011	0.9839
E_T	**Autumn**	0.1852	0.7899	0.0088	0.9723
	Spring	0.0272	0.5660	0.0017	0.5000
	Summer	0.1281	0.9017	0.0007	0.9836

3. Difference in nucleic acids and water contents between A_T and E_T within each season

Table 6. One-sided student's t-tests (p-values): Test for significant differences in RNA contents, DNA contents, RNA:DNA ratios and gravimetric water contents (GWC) in non-warmed (A_T) and warmed forest soil (E_T) throughout the seasons. Green = p-value < 0.05, light green = p-value <0.1.

Season	RNA	DNA	RNA:DNA	GWC
October	0.1568	0.5976	0.0187	0.8236
February	0.4271	0.3438	0.7173	0.5171
May	0.0556	0.0539	0.1530	0.5171
April	0.1962	0.3375	0.2047	0.2415

4. Difference in nucleic acid and water contents between A_T and E_T in winter

Table 7. Two-sided student's t-test (p-values): Test for significant differences in RNA contents, DNA contents, RNA:DNA ratios and gravimetric water contents (GWC) in grassland and forest soils between non-warmed (A_T) and warmed soil (E_T) in winter 2022 and 2023. Green = p-value < 0.05, light green = p-value <0.1.

		RNA	DNA	RNA:DNA	GWC
2022	**GN**	0.0010	2.0600E-05	0.5154	1.2700E-05
	GO	0.0001	0.0075	0.8713	0.0739
2023	**FN**	0.477	0.1017	0.0812	6.38E-07
	GN	0.1759	0.9826	0.2762	4.4970E-07
	GO	0.2664	0.4615	0.1187	0.0501

Warming Experiment:

1. Difference in relative CO_2 emission rates over time

***Table 8*.** ANOVA (p-values): Test for significant difference in CO_2 emission rates between non-warmed (a), +3 °C (d), +6 °C (e) and +9 °C (x) warmed soil. Green = p-value < 0.05.

	a	d	e	x
CO2	1.00E+00	2.68E-03	2.51E-04	1.26E-10

Table 9. ANOVA and post-hoc Tukey test (p-values): Test for significant difference in CO_2 emission rates in non-warmed (a), +3 °C (d), +6 °C (e) and +9 °C (x) warmed soil between all sampling timepoints (t0 = starting point, t7 = 1 week, t14 = 2 weeks, t21 = 3 weeks, t42 = 6 weeks). Green = p-value < 0.05, light green = p-value <0.1.

d (5°C)

	t7	t14	t21	t42	t98
t0	0.8874	0.7181	0.3330	0.0852	0.0015
t7		0.9993	0.9127	0.5052	0.0194
t14			0.9847	0.7134	0.0419
t21				0.9711	0.1597
t42					0.5141

e (8°C)

	t7	t14	t21	t42	t98
t0	0.9052	0.7298	0.4035	0.2060	0.0001
t7		0.9990	0.9384	0.7565	0.0015
t14			0.9935	0.9209	0.0036
t21				0.9979	0.0133
t42					0.0357

x (11°C)

	t7	t14	t21	t42	t98
t0	0.0012	0.0014	0.0002	0.0000	0.0000
t7		1.0000	0.9611	0.0222	0.0000
t14			0.9466	0.0191	0.0000
t21				0.1262	0.0000
t42					0.0006

2. Difference in relative CO_2 emission rates between temperature treatments

Table 10. ANOVA (p-values): Test for significant difference in CO_2 emissions from non-warmed (a), +3 °C (d), +6 °C (e) and +9 °C (x) warmed forest soil between all sampling timepoints (t0 = starting point, t7 = 1 week, t14 = 2 weeks, t21 = 3 weeks, t42 = 6 weeks). Green = p-value < 0.05, light green = p-value <0.1.

	t0	t7	t14	t21	t42	t98
CO2	8.75E-08	9.36E-07	3.91E-07	9.71E-07	8.10E-06	0.237

Table 11. ANOVA and post-hoc Tukey test (p-values): Test for significant difference in CO_2 emission rates at different timepoints (t0 = starting point, t7 = 1 week, t14 = 2 weeks, t21 = 3 weeks, t42 = 6 weeks) between +3 °C (d), +6 °C (e) and +9 °C (x) warmed forest soils. Green = p-value < 0.05, light green = p-value <0.1.

t0

	d	e	x
a	0.0910	0.0005	0.0000
d		0.0730	0.0000
e			0.0003
x			

t7

	d	e	x
a	0.0782	0.0002	0.0000008
d		0.0302	0.0001
e			0.0300
x			

t14

	d	e	x
a	0.0937	0.0002	0.0000
d		0.0306	0.0000
e			0.0069
x			

t21

	d	e	x
a	0.1896	0.0005	0.0000
d		0.0333	0.0000
e			0.0112
x			

t42

	d	e	x
a	0.2604	0.0001	0.0000
d		0.0057	0.0007
e			0.7365
x			

3. Difference in TNA content during warming and cooling incubations

Table 12. ANOVA (p-values): Test for significant difference in RNA contents, DNA contents and RNA:DNA ratio between non-warmed (a), +3 °C (d), +6 °C (e) and +9 °C (x) warmed forest soils. Green = p-value < 0.05.

	a	d	e	x
RNA	0.6090	0.3570	0.0714	0.1080
DNA	0.7322	0.7660	0.5900	0.9650
RNA:DNA	0.5510	0.2390	0.6310	0.0944

Table 13. One-sided paired student's t-test (p-values): Test for significant difference in RNA contents and RNA:DNA ratios between +6 °C (e) and +9 °C (x) warmed forest soils between all sampling timepoints (t0 = starting point, t7 = 1 week, t14 = 2 weeks, t21 = 3 weeks, t42 = 6 weeks, t98 = 8 weeks of cooling). Green = p-value < 0.05, light green = p-value <0.1.

e (8°C)
RNA

	t21	t42
t21		0.0018

x (11°C)
RNA

	t0	t42	t98
t0		0.0090	0.0200

RNA:DNA

	t0	t42	t98
t0		0.0714	0.0296

Grassland metatranscriptomics:

1. Difference in relative eukaryotic 18S rRNA transcript abundance between seasons and warming

Table 14. ANOVA (p-values): Test for significant difference in eukaryotic 18S rRNA transcript abundance (euk) between seasons in non-warmed (A_T) and +6 °C (E_T) warmed soil.

	A_T	E_T
euk	0.17	0.166

Table 15. Two-sided student's t-test (p-values): Test for significant difference in eukaryotic 18S rRNA transcript abundance (euk) between non-warmed (A_T) and +6 °C warmed (E_T) grassland soil throughout the seasons. Light green = p-value <0.1.

	euk
summer21	0.0572
autumn21	0.1225
winter22	0.6848
spring22	0.7102
summer22	0.6278

2. Difference in mRNA contents between seasons and warming

Table 16. ANOVA (p-values): Test for significant difference in mRNA content between seasons in non-warmed (A_T) and +6 °C (E_T) warmed soil. Green = p-value < 0.05.

	A_T	E_T
mRNA	0.24	0.01

Table 17. ANOVA and post-hoc Tukey test (p-values): Test for significant difference in mRNA content in non-warmed (A_T) and +6 °C warmed (E_T) grassland soil tbetween the seasons. Green = p-value < 0.05, light green = p-value <0.1.

E_T

	autumn21	winter22	spring22	summer22
summer21	0.0710	0.3772	0.0153	0.0171
autumn21		0.8826	0.9650	0.9727
winter22			0.5139	0.5412
spring22				1.0000
summer22				

Table 18. Two-sided student's t-test (p-values): Test for significant difference in mRNA content between non-warmed (A_T) and +6 °C warmed (E_T) grassland soil throughout the seasons. Green = p-value < 0.05, light green = p-value <0.1.

	mRNA
summer21	0.0636
autumn21	0.2758
winter22	0.0236
spring22	0.1789
summer22	0.2944

3. Differences in relative transcript abundance between seasons and warming

Table 19. ANOVA (p-values): Test for significant differences in relative transcript investment into *metabolism* and *genetic information processing* in non-warmed (A_T) and +6 °C warmed (E_T) grassland soils between seasons. Green = p-value < 0.05, light green = p-value <0.1.

	A_T	E_T
Metabolism	0.227	0.644
Genetic Information processing	0.017	0.471

Table 20. ANOVA and post-hoc Tukey test (p-values): Test for significant differences in relative transcript investment into *metabolism* and *genetic information processing* in non-warmed (A_T) and +6 °C warmed (E_T) grassland soils between seasons. Green = p-value < 0.05, light green = p-value <0.1.

Genetic information processing

	Autumn 21	Winter 22	Spring 22	Summer 22
Summer 21	0.0708	0.0087	0.0708	0.8004
Autumn 21		0.8221	1.0000	0.9999
Winter 22			0.8221	0.7872
Spring 22				0.9999

Table 21. Two-sided student's t-test (p-values): Test for significant differences in relative transcript investment into *metabolism* and *genetic information processing* in non-warmed (A_T) and warmed (E_T) grassland soils throughout the seasons. Green = p-value < 0.05, light green = p-value <0.1.

	Metabolism	***Genetic Information Processing***
Summer 21	0.03407	0.0085
Autumn 21	0.735	0.6039
Winter 22	0.3649	0.2225
Spring 22	0.735	0.6039
Summer 22	0.8014	0.5667

IV. Data and scripts for metatranscriptomics analysis

Table 22. Additional information for metatranscriptomics analysis (processing results). Temp = temperature, Trans = transect, SSU = Small subunit of ribosome, bac = bacterial, euk = eukaryotic, arc = archaeal, # = number.

Season	Temp	Trans	Read	Prinseq Quality filtering		rRNA vs. mRNA		SSU taxonomy		
				# of fastq sequences	% passed	% rRNA	% mRNA	% bac of SSU	% euk of SSU	% arc of SSU
Summer 2021	Non-warmed	1	R1	1.6E+07	95.8	95.6	4.4	88.0	11.7	0.3
			R2	1.6E+07	94.8	95.6	4.4	87.9	11.7	0.3
		2	R1	1.2E+07	95.9	97.6	2.4	68.6	30.9	0.4
			R2	1.2E+07	94.2	97.6	2.4	68.5	31.1	0.4
		3	R1	1.2E+07	94.8	95.5	4.5	93.3	5.6	1.1
			R2	1.2E+07	94.4	95.4	4.6	93.2	5.7	1.1
		4	R1	1.7E+07	96.1	92.6	7.4	93.4	5.5	1.1
			R2	1.7E+07	95.3	92.6	7.4	93.4	5.5	1.1
	Warmed (+ 6°C)	1	R1	1.4E+07	96.0	93.3	6.7	93.2	5.9	0.9
			R2	1.4E+07	95.1	93.3	6.7	93.1	5.9	0.9
		2	R1	1.2E+07	95.9	93.5	6.5	91.3	8.0	0.7
			R2	1.2E+07	94.8	93.5	6.5	91.3	8.0	0.7
		3	R1	1.7E+07	95.7	92.6	7.4	92.5	5.6	1.9
			R2	1.7E+07	95.0	92.6	7.4	92.5	5.6	1.9
		4	R1	1.7E+07	94.5	67.2	32.8	50.3	0.6	49.1
			R2	1.7E+07	94.0	67.1	32.9	49.9	0.6	49.5
Autumn 2021	Non-warmed	1	R1	1.2E+07	95.9	94.6	5.4	93.0	6.1	0.9
			R2	1.2E+07	94.8	94.6	5.4	93.0	6.1	0.9
		2	R1	1.5E+07	95.9	94.3	5.7	90.1	9.1	0.7
			R2	1.5E+07	94.9	94.3	5.7	90.1	9.2	0.7
		3	R1	1.5E+07	95.6	93.5	6.5	87.2	12.1	0.8
			R2	1.5E+07	94.1	93.4	6.6	87.1	12.1	0.8
		4	R1	1.6E+07	95.8	93.1	6.9	87.2	12.3	0.5
			R2	1.6E+07	94.9	93.1	6.9	87.1	12.4	0.5
		5	R1	1.6E+07	95.7	92.8	7.2	93.5	4.8	1.7
			R2	1.6E+07	94.8	92.8	7.2	93.5	4.8	1.7
	Warmed (+ 6°C)	1	R1	1.6E+07	95.8	94.3	5.7	91.5	7.6	0.9
			R2	1.6E+07	94.8	94.3	5.7	91.5	7.6	0.9
		2	R1	1.6E+07	95.6	92.6	7.4	90.9	6.8	2.4
			R2	1.6E+07	94.7	92.6	7.4	90.8	6.8	2.4
		3	R1	1.7E+07	95.6	94.2	5.8	93.6	5.8	0.6
			R2	1.7E+07	94.7	94.2	5.8	93.6	5.8	0.6
		4	R1	1.7E+07	96.0	91.7	8.3	90.4	7.6	2.0
			R2	1.7E+07	94.9	91.7	8.3	90.4	7.7	2.0
		5	R1	1.3E+07	95.9	93.2	6.8	90.7	8.0	1.2
			R2	1.3E+07	94.9	93.2	6.8	90.7	8.1	1.2

Season	Temp	Trans	Read	Prinseq Quality filtering		rRNA vs. mRNA		SSU taxonomy		
				# of fastq sequences	% passed	% rRNA	% mRNA	% bac of SSU	% euk of SSU	% arc of SSU
Winter 2022	Non-warmed	1	R1	1.2E+07	96.7	93.6	6.4	88.5	11.3	0.1
			R2	1.2E+07	95.4	93.6	6.4	88.5	11.4	0.1
		2	R1	1.7E+07	95.8	96.1	3.9	96.0	2.2	1.8
			R2	1.7E+07	94.9	96.1	3.9	96.0	2.2	1.8
		3	R1	1.5E+07	95.9	93.6	6.4	94.4	4.2	1.4
			R2	1.5E+07	95.0	93.5	6.5	94.4	4.2	1.4
		4	R1	1.4E+07	96.9	92.8	7.2	89.9	9.9	0.2
			R2	1.4E+07	95.6	92.8	7.2	89.9	9.9	0.2
		5	R1	1.5E+07	95.5	92.8	7.2	90.4	8.2	1.4
			R2	1.5E+07	94.6	92.8	7.2	90.4	8.2	1.4
	Warmed (+ 6°C)	1	R1	1.4E+07	95.8	87.7	12.3	94.6	4.0	1.5
			R2	1.4E+07	94.8	87.7	12.3	94.5	4.0	1.5
		2	R1	1.6E+07	96.1	91.6	8.4	90.8	7.2	1.9
			R2	1.6E+07	95.3	91.6	8.4	90.7	7.3	2.0
		3	R1	1.3E+07	95.8	87.4	12.6	94.1	1.2	4.7
			R2	1.3E+07	94.6	87.4	12.6	94.1	1.2	4.7
		4	R1	1.1E+07	96.0	93.7	6.3	87.4	12.4	0.1
			R2	1.1E+07	94.8	93.7	6.3	87.4	12.5	0.2
		5	R1	1.2E+07	97.0	94.9	5.1	84.6	15.3	0.1
			R2	1.2E+07	95.9	94.9	5.1	84.6	15.3	0.1
Spring 2022	Non-warmed	1	R1	1.1E+07	96.8	95.4	4.6	90.0	9.8	0.1
			R2	1.1E+07	95.4	95.4	4.6	90.0	9.9	0.1
		2	R1	1.4E+07	96.9	95.1	4.9	87.8	12.1	0.1
			R2	1.4E+07	95.9	95.1	4.9	87.8	12.1	0.1
		3	R1	1.2E+07	96.7	94.3	5.7	89.3	10.6	0.1
			R2	1.2E+07	95.5	94.3	5.7	89.3	10.6	0.1
		4	R1	1.2E+07	96.3	75.0	25.0	97.0	0.5	2.4
			R2	1.2E+07	95.3	74.9	25.1	97.0	0.5	2.5
		5	R1	1.3E+07	97.0	95.0	5.0	88.4	11.4	0.2
			R2	1.3E+07	95.9	95.0	5.0	88.4	11.4	0.2
	Warmed (+ 6°C)	1	R1	1.9E+07	96.3	96.1	3.9	91.9	8.0	0.1
			R2	1.9E+07	95.3	96.1	3.9	91.9	8.0	0.1
		2	R1	1.4E+07	96.4	93.9	6.1	91.4	8.4	0.2
			R2	1.4E+07	95.3	93.9	6.1	91.4	8.3	0.2
		3	R1	1.6E+07	96.0	94.7	5.3	89.7	10.0	0.3
			R2	1.6E+07	94.2	94.7	5.3	89.7	10.0	0.3
		4	R1	1.2E+07	96.1	94.3	5.7	92.2	7.4	0.4
			R2	1.2E+07	95.0	94.3	5.7	92.2	7.4	0.4
		5	R1	1.2E+07	96.7	94.5	5.5	91.7	8.0	0.3
			R2	1.2E+07	95.5	94.5	5.5	91.7	8.0	0.3

Season	Temp	Trans	Read	Prinseq Quality filtering		rRNA vs. mRNA		SSU taxonomy		
				# of fastq sequences	% passed	% rRNA	% mRNA	% bac of SSU	% euk of SSU	% arc of SSU
Summer 2022	Non-warmed	1	R1	1.5E+07	96.6	94.5	5.5	91.9	7.8	0.3
			R2	1.5E+07	95.3	94.6	5.4	91.9	7.8	0.3
		2	R1	1.3E+07	96.5	94.8	5.2	93.4	6.3	0.3
			R2	1.3E+07	95.4	94.9	5.1	93.4	6.3	0.3
		3	R1	1.1E+07	96.8	93.9	6.1	93.2	6.4	0.4
			R2	1.1E+07	95.5	93.9	6.1	93.2	6.4	0.4
		4	R1	1.2E+07	96.8	91.0	9.0	94.4	3.9	1.7
			R2	1.2E+07	95.8	91.0	9.0	94.4	3.9	1.7
		5	R1	1.3E+07	97.0	95.4	4.6	86.0	13.3	0.7
			R2	1.3E+07	95.9	95.4	4.6	86.0	13.3	0.7
	Warmed (+ 6°C)	1	R1	1.7E+07	96.8	95.4	4.6	89.5	10.0	0.5
			R2	1.7E+07	95.6	95.4	4.6	89.5	10.0	0.5
		2	R1	1.0E+07	96.9	93.5	6.5	91.3	7.0	1.6
			R2	1.0E+07	95.6	93.5	6.5	91.3	7.0	1.6
		3	R1	1.4E+07	97.2	95.8	4.2	93.8	5.3	0.9
			R2	1.4E+07	96.1	95.8	4.2	93.8	5.3	0.9
		4	R1	1.4E+07	96.9	93.4	6.6	92.6	6.1	1.2
			R2	1.4E+07	95.5	93.4	6.6	92.6	6.1	1.2
		5	R1	1.1E+07	97.1	94.9	5.1	92.1	6.3	1.6
			R2	1.1E+07	96.2	94.9	5.1	92.1	6.3	1.6

Scripts for Summer 2021 as an example for the seasonal metatranscriptomics analysis:

1. Prinseq-lite

```
for samplename in $(ls /scratch/visitors/forhot/seasonal_metat_1/summer21/names_);
do
        sbatch 000_prinseq_qual_fq2fasta.sh $samplename
done
```

```
#!/bin/bash
#
#SBATCH --job-name=prinseq
#SBATCH --cpus-per-task=1
#SBATCH --mem=1g
#SBATCH --time=00-1:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=lah005@post.uit.no
#SBATCH --output=log/prinseq_qual-%j.out
#SBATCH --error=log/prinseq_qual-%j.err

SAMPLENAME=$1

module load prinseqlite

cp
/scratch/visitors/forhot/seasonal_metat_1/summer21/fastq_cat_R1_R2_files/${SAMPLENA
ME}* $TMPDIR
cd $TMPDIR

gunzip ${SAMPLENAME}* # prinseq -fastq does not take fastq.gz
```

```
prinseq-lite.pl -fastq ${SAMPLENAME}* -min_qual_mean 30 -out_format 1 -out_good
${SAMPLENAME}.min_qual_m_30 -out_bad ${SAMPLENAME}.low_qual

cp *min_qual*
/scratch/visitors/forhot/seasonal_metat_1/summer21/prinseq_fasta_files_qual30
cp *low*
/scratch/visitors/forhot/seasonal_metat_1/summer21/prinseq_fasta_files_qual30

# clean up temporary files
rm -rf $TMPDIR/*
```

2. SortMeRNA

```
for samplename in $(ls
/scratch/visitors/forhot/seasonal_metat_1/summer21/names_fasta); do
        sbatch 001_sortmerna_summer21.sh $samplename
done
```

```
#!/bin/bash
#
#SBATCH --job-name=sortmerna
#SBATCH --cpus-per-task=8
#SBATCH --mem=30g
#SBATCH --time=00-05:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=lah005@post.uit.no
#SBATCH --output=log/sortmerna-%j.out
#SBATCH --error=log/sortmerna-%j.err

SAMPLENAME=$1

module load sortmerna

cp
/scratch/visitors/forhot/seasonal_metat_1/summer21/prinseq_fasta_files_qual30/${SAM
PLENAME} $TMPDIR
cd $TMPDIR

sortmerna --ref /apps/sortmerna/4.1.0/sortmerna/data/rRNA_databases/silva-arc-16s-
id95.fasta --ref
/apps/sortmerna/4.1.0/sortmerna/data/rRNA_databases/silva-arc-23s-id98.fasta --ref
/apps/sortmerna/4.1.0/sortmerna/data/rRNA_databases/silva-bac-16s-id90.fasta --ref
/apps/sortmerna/4.1.0/sortmerna/data/rRNA_databases/silva-bac-23s-id98.fasta --ref
/apps/sortmerna/4.1.0/sortmerna/data/rRNA_databases/silva-euk-18s-id95.fasta --ref
/apps/sortmerna/4.1.0/sortmerna/data/rRNA_databases/silva-euk-28s-id98.fasta --
reads ${SAMPLENAME} --threads 8 --workdir $TMPDIR --fastx --other

cd out/
mv aligned.fasta ${SAMPLENAME}.sortmerna_rRNA.fasta
mv other.fasta ${SAMPLENAME}.sortmerna_non_rRNA.fasta
mv aligned.log ${SAMPLENAME}.sortmerna.log

cp * /scratch/visitors/forhot/seasonal_metat_1/summer21/sortmerna_results

# clean up temporary files
rm -rf $TMPDIR/*
```

3. DIAMOND blastx against NCBI database

```
for samplename in $(ls
/scratch/visitors/forhot/seasonal_metat_1/summer21/sortmerna_results/names);
do
    sbatch 003_diamond_1hit_10evalue_summer21.sh $samplename
done
```

```
#!/bin/bash
```

```
#
#SBATCH --job-name=diamondblast
#SBATCH --cpus-per-task=14
#SBATCH --mem=12g
#SBATCH --time=00-3:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=lah005@post.uit.no
#SBATCH --output=log/diamondblast-%j.out
#SBATCH --error=log/diamondblast-%j.err

SAMPLENAME=$1

module load conda
#module list
#conda info --envs
conda activate diamond-2.1.3
#diamond --help

cp
/scratch/visitors/forhot/seasonal_metat_1/summer21/sortmerna_results/non_rRNA/${SAM
PLENAME} $TMPDIR
cd $TMPDIR

/usr/bin/time diamond blastx -d
/scratch/visitors/grazing_2020/06_diamond_blast/060_diamond_nr_db/nr -q
${SAMPLENAME} -o ${SAMPLENAME}.10evalue_nr.m8 -f 6 -k 1 -e 10 -p 14 --max-hsps 1 -b
2 -c 4

cp *.m8
/scratch/visitors/forhot/seasonal_metat_1/summer21/mRNA_diamond_1hit_10evalue_resul
ts

# clean up temporary files
rm -rf $TMPDIR/*
```

4. “get mRNA script” to extract mRNA sequences from non-rRNA sequences

```
for samplename in $(ls
/scratch/visitors/forhot/seasonal_metat_1/autumn/sortmerna_results/non_rRNA); do
        sbatch 004_get_mRNA_fastas_based_on_m8_files_autumn.sh $samplename
done
```

```
#!/bin/bash
#
#SBATCH --job-name=get_mRNA_seq
#SBATCH --cpus-per-task=1
#SBATCH --mem=250M
#SBATCH --time=00-1:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=lah005@post.uit.no
#SBATCH --output=log/get_mRNA_seq-%j.out
#SBATCH --error=log/get_mRNA_seq-%j.err

SAMPLENAME=$1

module load conda
#conda info --envs
conda activate seqkit-2.3.1

awk -F '\t' '{ a[$1]++ } END { for (b in a) { print b } }'
/scratch/visitors/forhot/seasonal_metat_1/summer21/mRNA_diamond_1hit_10evalue_resul
ts/$SAMPLENAME.10evalue_nr.m8 >
/scratch/visitors/forhot/seasonal_metat_1/summer21/mRNA_diamond_1hit_10evalue_resul
ts/$SAMPLENAME.10evalue_nr.m8.unique.ids



/usr/bin/time seqkit grep -f
/scratch/visitors/forhot/seasonal_metat_1/summer21/mRNA_diamond_1hit_1
```

```
0evalue_results/$SAMPLENAME.10evalue_nr.m8.unique.ids
/scratch/visitors/forhot/seasonal_metat_1/summer21/sortmerna_results/non_rRNA/$SAMP
LENAME  >
/scratch/visitors/forhot/seasonal_metat_1/summer21/mRNA_diamond_1hit_10evalue_resul
ts/$SAMPLENAME.10evalue_nr.m8.unique.ids.fa
```

5. DIAMOND blastx against KEGG database for functional annotation

```
for samplename in $(ls
/scratch/visitors/forhot/seasonal_metat_1/mRNA_all_seasons/summer21); do
  sbatch 005_KEGG_diamond_blastx.sh $samplename
done
```

```
#!/bin/bash
#
#SBATCH --job-name=diamond_blastx_kegg
#SBATCH --cpus-per-task=14
#SBATCH --mem=5g
#SBATCH --time=00-01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=lah005@post.uit.no
#SBATCH --output=log/blastx_kegg-%j.out
#SBATCH --error=log/blastx_kegg-%j.err

SAMPLENAME=$1
module load conda
#conda info --envs
conda activate diamond-2.1.4

cp /scratch/visitors/forhot/seasonal_metat_1/mRNA_all_seasons/summer21/$SAMPLENAME
$TMPDIR
cd $TMPDIR

/usr/bin/time diamond blastx -d
/scratch/visitors/forhot/seasonal_metat_1/mRNA_all_seasons/databases/cat_EPT1T4.pep
.gz.diamondDB.dmnd -q $SAMPLENAME -o $SAMPLENAME.KEGG.m8 -f 6 -k 1 --evalue 0.0001
-b 0.9 -c 4 --threads 14

cp $SAMPLENAME.KEGG.m8
/scratch/visitors/forhot/seasonal_metat_1/mRNA_all_seasons/KEGG_results/summer21/

# clean up temporary files
rm -rf $TMPDIR/*
```

V. Additional Information

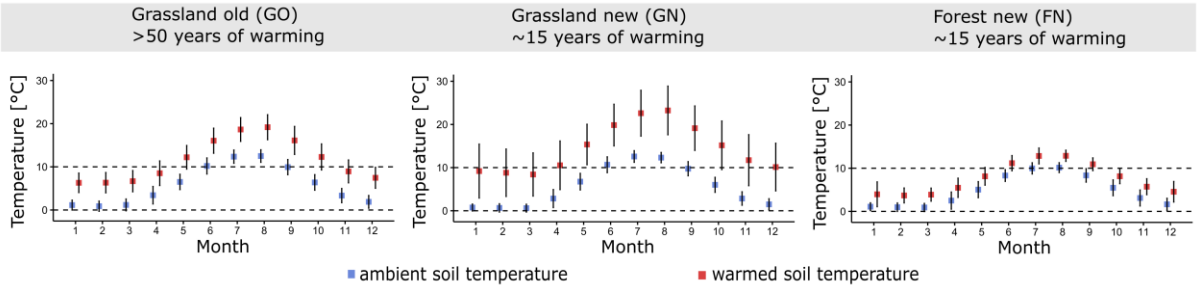

Figure 16. Annual temperature profiles of the ForHot grassland and forest sites. Mean monthly temperatures in non-warmed and warmed plots are displayed with standard deviation. Mean warming differences between the non-warmed and warmed plots are +6 °C for GO, +9 °C for GN and +3 °C for FN.