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ARTICLE

Bacterial and protozoan dynamics upon thawing and freezing of an active layer permafrost soil

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Abstract

The active layer of soil overlaying permafrost in the Arctic is subjected to annual changes in temperature and soil chemistry, which we hypothesize to affect the overall soil microbial community. We investigated changes in soil microorganisms at different temperatures during warming and freezing of the active layer soil from Svalbard, Norway. Soil community data were obtained by direct shotgun sequencing of total extracted RNA. No changes in soil microbial communities were detected when warming from -10 to $-2\,°C$ or when freezing from -2 to $-10\,°C$. In contrast, within a few days we observed changes when warming from -2 to $+2\,°C$ with a decrease in fungal rRNA and an increase in several OTUs belonging to Gemmatimonadetes, Bacteroidetes and Betaproteobacteria. Even more substantial changes occurred when incubating at $2\,°C$ for 16 days, with declines in total fungal potential activity and decreases in oligotrophic members from Actinobacteria and Acidobacteria. Additionally, we detected an increase in transcriptome sequences of bacterial phyla Bacteriodetes, Firmicutes, Betaproteobacteria and Gammaproteobacteria—collectively presumed to be copiotrophic. Furthermore, we detected an increase in putative bacterivorous heterotrophic flagellates, likely due to predation upon the bacterial community via grazing. Although this grazing activity may explain relatively large changes in the bacterial community composition, no changes in total 16S rRNA gene copy number were observed and the total RNA level remained stable during the incubation. Together, these results are showing the first comprehensive ecological evaluation across prokaryotic and eukaryotic microbial communities on thawing and freezing of soil by application of the TotalRNA technique.

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Introduction

Permafrost-affected soil systems cover up to 20% of the total land area in the northern hemisphere [1] and are esti-mated to contain up to 472 Pg of organic carbon in the first upper meter [2], which is equivalent to more than half of the atmospheric carbon content [3]. The Arctic is currently experiencing a temperature increase at a higher rate than the global average [4, 5], with documented impacts on permafrost-affected soils via thawing and increasing depth of the above active layers [6, 7]. Active layer soil is defined as the overlaying permafrost soils being exposed to seasonal freezing and thawing. Permafrost thaw will likely lead to an enhanced degradation of the large carbon pool in the newly established active layer by a wide diversity of micro-organisms, including bacteria, archaea, fungi and protozoa [8–10]. Thus, there is a growing interest in incorporating microbial parameters into modelling of the changing Arctic environments [11, 12]. Even though active layer permafrost soils are exposed to harsh conditions like freeze/thaw

cycles, sub-zero temperatures and low nutrient availability most of the year, these soils harbour a high diversity of bacteria [13, 14], fungi [15, 16] and protozoa [8]. Microbial communities in active layer soils are dynamic and show relatively large annual changes in biomass [17–20], with a peak before thawing of the soil followed by a fast decline shortly after [17, 18]. Accordingly, bacterial community composition in active layer soils is also dynamic [21, 22], with distinct winter and summer bacterial communities [22], and altered soil fungal community structures between seasons in the Arctic and at lower latitudes [20, 23].

Active layer soil may be exposed to several freeze/thaw cycles during spring and autumn, and it has been shown that these cycles change the microbial community composition [24, 25]. Soil freezing may result in lysis of microbial cells, resulting in available and easily degradable organic substrates from dead microorganisms for survivors upon thawing [26].

Several authors have proposed the concept of copiotrophic/oligotrophic lifestyles to classify bacterial groups as a response to nutrient concentration in soils ecosystems, and that large phylogenetic groups fall into these lifestyle groups [27–31]. Copiotrophic bacteria respond relatively quickly to an increase in labile nutrient availability, show a high growth rate and have a high (>5) copy number of the 16S rRNA gene, whereas oligotrophic bacteria typically have low-growth rates, respond slowly to an increase in labile nutrient availability and have few (</=<2) copies of the 16S rRNA gene [27]. These trophic lifestyles may indicate which bacterial groups will respond as the soil thaws and nutrient availability increases.

Cultivation biases, providing access to only a minority of soil microorganisms [32], has spurred the use of molecular techniques to study microbial communities, especially in soil where biodiversity levels are relatively high [33]. The majority of these studies are based on DNA, such as direct shotgun metagenomics and analysis of PCR amplicons from amplified 16S rRNA gene fragments or fungal ITS2, but interpretation in terms of activity and functions is hampered by a large fraction of extracellular DNA [34]. In combination with the low turnover of microbial DNA, the extracellular DNA pool may obscure short-term changes in microbial communities caused by thawing. In contrast, RNA has a faster turnover than DNA and is presumed to be produced only by metabolically active microorganisms [35]. Studies of the active soil microbial community using RNA-based techniques were pioneered by Urich et al. [36], who isolated total soil RNA and used shotgun cDNA sequencing and bioinformatics tools to annotate putative rRNA reads against databases using BLAST. This approach has subsequently been used to study microbial communities of Arctic soils [37, 38]. These studies mainly focused on

microbial community changes following thawing and did not reveal the microbial responses to (i) increasing temperatures before transition from frozen to thawed conditions (an important aspect because the main future temperature increase in the Arctic is expected to happen during winter [39]), and (ii) re-freezing. In addition, it has been demonstrated that predation of certain bacteria by protists may change the composition of the microbial community [40], but little is known about interactions among different trophic levels of soil microorganisms following short-term (days) perturbations, like thawing.

In order to understand the microbial response to not only thawing but also to increasing sub-zero temperatures and to freezing, we investigated the changes in rRNA-based active layer soil microbial communities to gradually increasing and decreasing temperature in the range from −10 to 2 °C. We hypothesized that only a subset of bacterial and fungi taxa would respond to an increase in temperature from −10 to −2 °C, and that bacterial–protozoan interactions will influence the microbial response to thawing events.

Materials and methods

Soil sampling and preparation

An active layer soil core was collected on 18th of April 2014 in Sassendalen, Svalbard, Norway (Latitude 78.270961, Longitude 17.228315, 5 m above sea level). The area has a mean annual air temperature of −6 °C and a mean annual precipitation of ~200 mm water equivalent [41]. After removal of the snow cover, the soil core (0–14-cm depth) was sampled using a core catcher (8-cm diameter) with a motorized hand drill. The core was transported in a cooling box to a −18 °C freezer at the University Centre in Svalbard and then to Denmark by airplane in a Styrofoam box with cooling elements to avoid thawing. Measurements by a temperature logger and visual inspection of the core indicated that it did not thaw during transport. At the University of Copenhagen, the core was kept at −18 °C until sample preparation, which was conducted in a −15 °C freezelab. Contamination during sample preparation was avoided by sterilizing all surfaces by UV exposure and washing all equipment in ethanol before autoclaving. In addition, we avoided soil potentially contaminated during drilling by only sampling the unexposed interior of the core as described by Bang-Andreasen et al. [42]. In brief, plant cover was scraped off the surface. Then the soil core was broken and the inner core soil was aseptically sampled with a sterilized bore head (16 mm) from the newly exposed surface and homogenized by smashing the soil in a sterile plastic bag with a hammer, obtaining an appropriate grain size range of 1–10 mm.

Soil incubation

Two-gram subsamples were placed in 5-ml sterile conical plastic tubes (Eppendorf AG, Hamburg, Germany) to a total of 40 replicates. The samples were incubated in a custom-made temperature chamber based on a recirculating cooling liquid (Bath fluid Thermal G, JULABO GmbH, Seelbach, Germany) with a recirculating Cooler (FL300, JULABO GmbH, Seelbach, Germany). The base of the device was an aluminium plate ($28 \times 15.5 \times 4$ cm, $L \times W \times H$) with drilled-in canals for the cooling liquid. On top of this, a steel box ($21.5 \times 14.5 \times 9.5$ cm, $L \times W \times H$) was placed and filled with Ottawa sand (Fisher Scientific, Leicestershire, UK) to evenly distribute the temperature. The entire device was then covered with Styrofoam to stabilize the temperature, which was monitored by five temperature sensors distributed in the sand. During incubation, the standard error of the mean of the temperature measurements by the five sensors was ≤ 0.1 °C. Samples were incubated for 26 days, starting with a pre-incubation step at -10 °C for 48 h, then a gradual increase to $+2$ °C over 6 days, followed by $+2$ °C for 16 days and a freezing phase with decreasing temperature over 6 days to -10 °C. Samples were obtained at two-day intervals at -10, -6, -2 and $+2$ °C during the thawing phase; these samples will be referred to as $T_{-10°C}$, $T_{-6°C}$, $T_{-2°C}$ and $T_{2°C}$, respectively. Following the 16 days at $+2$ °C, samples were obtained at two-day intervals $+2$, -2, -6 and -10 °C in the freezing phase; these samples will be referred to as $F_{2°C}$, $F_{-2°C}$, $F_{-6°C}$ and $F_{-10°C}$, respectively (see Fig. 1). At each of the eight sampling points, five replicates were snap-frozen in liquid nitrogen and subsequently stored at -80 °C until RNA extraction.
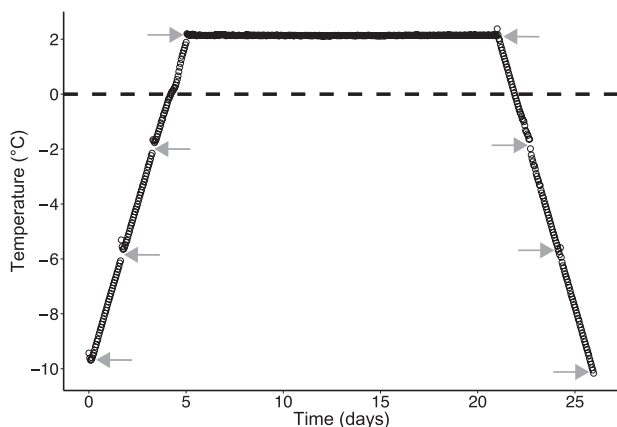

Fig. 1 Measured incubation temperature of active layer permafrost samples shown as average of five temperature loggers (black circles); Standard error of mean ≤ 0.1 °C. Grey arrows show time of sampling for RNA extraction

Soil analyses

Grain-size distribution was analysed by dissolving soil samples in 0.01 M tetrasodium pyrophosphate and demineralized H_2O with 2 min of ultrasound and then analysed by laser diffraction on a Mastersizer 2000 (Malvern Instruments, Malvern, UK). Soil pH was measured in a second incubation run, using the same incubation chamber. This was done because the whole sample needed to be snap-frozen to preserve the RNA in the first incubation run. The second run had the same temperature setup as the first run. A total of 36 samples of 2 g soil was incubated in same type of 5-ml plastic tubes, as described above. We selected four time points for the pH measurements: $T_{-6°C}$, $T_{2°C}$, $F_{2°C}$ and $F_{-6°C}$. A total of 16 g was collected at each time point: four replicates with 4 g (two tubes pooled per replicate) of soil were each extracted in 20 mL ddH2O for 10 min at 5 °C on a shaker. All extracts were filtered through Whatman GF-D filters and measured on pH meter. The total carbon and nitrogen contents were measured in solid samples by Dumas combustion (1020 °C) on an elemental analyser (CE 1110, Thermo Electron, Milan, Italy). Subsamples of 10 mg of dried and finely homogenized (ball mill) material were weighed out into tin combustion cups for elemental analysis. Soil standards (Elemental Microanalysis, Okehampton, UK) were used for elemental analyser mass calibration. For pH measurement, the temperature incubation was repeated with the sample experimental setup using a subset of the homogenized soil.

RNA and DNA isolation and sequencing library preparation

Total RNA isolation was done from 2 g of soil using RNA PowerSoil total RNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) with phenol:chloroform:isoamyl alcohol in the ratio 25:24:1 (Sigma-Aldrich, Copenhagen, Denmark) following the instructions of the manufacturer, resulting in 100-μl RNA extracts. DNA was co-isolated with RNA PowerSoil DNA Elution Accessory Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the instructions of the manufacturer. DNase treatment on a 10-μl subsample was performed using DNase max (MO BIO Laboratories) by following the manufacturer's instructions. The quality of DNase-treated RNA was checked with a Bioanalyser 2100 (Agilent Technologies, Glostrup, Denmark). This step was used to exclude one replicate from each time point, by selecting the replicate with the lowest RNA integrity number (RIN) number (28S/23S to18S/16S ratio). RNA quantity was estimated using Qubit 2.0 (Thermo Fisher Scientific, Hvidovre, Denmark) with the Qubit RNA HS Assay Kit. For sequencing library preparation, the NebNext Ultra directional RNA Library prep

kit for Illumina (New England Biolabs, Herlev, Denmark) was used with 100 ng of RNA from each sample. Library preparation was carried out according to the manufacturer's instructions. Samples were sequenced using a HiSeq 2500 (Illumina) in rapid mode, 150 base-paired end reads, at the National High-throughput DNA Sequencing Centre, University of Copenhagen.

Quantitative PCR

Quantitative PCR was performed to estimate the numbers of 16S rRNA gene and transcript copies in the soil samples. A 17-µl aliquot of each RNA extract was DNase treated as described above and reverse transcribed using random hexamer primers (Thermo Scientific, Schwerte, Germany) and RevertAid Reverse Transcriptase (Thermo Scientific). Reverse transcription was performed by incubation at 25 °C for 10 min, 50 °C for 30 min and 85 °C for 5 min. cDNA samples were stored at −80 °C until further analysis. Three replicate PCR amplifications were performed on each DNA and cDNA sample using the primers 341 F (5′-CCTACGG-GAGGCAGCAG-3′) and 518 R (5′-ATTACCGCGGCTGG-3′) [43]. For producing a standard, *E. coli* K27 was grown in Luria–Bertani medium for 2 days at room temperature to a desired density, after which DNA was isolated and the number of cells were counted in a microscope.

Bioinformatics framework

Quality filtering, merging and ribosomal filtering of reads were done using BioDSL (http://maasha.github.io/BioDSL/). In brief, raw sequences were filtered into two parts, one for potential mRNA and one for ribosomal RNA separated by SortMeRNA v. 2.0 [44] (http://bioinfo.lifl.fr/sortmerna/sortmerna.php). Reads were then cleaned by removing Illumina adapter sequences and low-quality reads (>20 Phred score). Finally, reads were paired using the following parameters: minimum overlap = 20 bp, and 20% mismatch allowed. Reads that could not be paired were discarded. From each sample, 2,000,000 quality checked and cleaned reads were randomly picked in order to reduce the dataset for computational efficiency. Potential ribosomal reads were used to find the closest matching microorganism based on ribosomal small subunit (SSU) in the SILVA database release 111, using Metaxa2 version 2.1.3 [45] with default settings (Reliability score cutoff = 80). For this study, we only used the results matching SSU. The complete OTU table can be seen in a supplementary datasheet.

Data and statistical analyses

Statistical analyses of the Physiochemical parameters were done with the *multcomp* package in R using ANOVA with a post-hoc Tukey HSD correction test ($p < 0.05$). The alpha diversity analysis was carried out as described in previous work [46]. R software version 3.0.2 (R Development Core [47]) and PAST software [48] were used to calculate the alpha diversity indices, which was done on rarefied data at 750,000 reads per sample in order to avoid biases that may come from uneven read numbers. Alpha diversity was assessed by measuring observed richness and the Shannon index (H). Statistical analysis was carried out as described for the soil Physiochemical parameters.

The beta diversity and multivariate analyses were done with the raw and non-rarefied contingency tables using the R with the package *vegan* [49] and *ade4* [50]. Because the contingency tables have features with abundance variation larger than 1000-fold, a log_{10} transformation was applied to account for this dispersion. Principal component analysis (PCA) was performed after centre-scaling normalization. A pattern search was applied to the original PCAs by grouping replicates together in order to perform a between-group analysis (BGA). The statistical significance of the selected grouping factor was tested with a Monte–Carlo simulation involving 10,000 permutations. Complementing PERMA-NOVA tests were performed on the Bray–Curtis dissimilarity profiles using 10,000 permutations in order to assess the significance of the tested factors. The major relative abundance-driven changes in the phylogenetic composition of profiles were identified by means of ANOVA with post-hoc Tukey HSD correction test and false discovery rate (FDR) multiple correction test ($p < 0.05$). Significant changes in large taxonomic groups were also investigated separately for each thaw and freezing treatments to identify potential temperature-related effects. Finally, fine changes in the soil microbiome at the operational taxonomic unit (OTU) (97% identity) level were extracted using generalized linear model and negative binomial regression (nbGLM) using the R package edgeR [51]. This was conducted in order to determine OTUs responding in a similar manner to thawing and freezing. The significance of changes in OTU abundance was inferred with a likelihood ratio test (LRT) under correction by a post-hoc false discovery rate test with the package edgeR in R ($p < 0.05$ following FDR correction). Groups of OTUs that changed their abundance upon thawing and freezing were plotted in a heatmap of centred and scaled counts using a combination of the following R packages: *gplots*[52], *vegan* [49], *rioja* [53] and *RcolorBrewer* [54], as previously described [33]. OTUs were clustered and classified in functional response groups (FRGs, i.e., groups of organisms responding similarly as a function of environmental conditions) as previously proposed [46], using complete hierarchical cluster dendrograms based on Euclidean distance. Functional response groups were manually determined with the lowest

number of groups possible. The selected groups were validated via a Monte–Carlo simulation involving 10,000 permutations between groups and the remaining non-significant OTUs, and visualized in a BGA.

Average 16S rRNA gene copy numbers in the microbial community were calculated to test the hypothesis that bacteria with a high number of 16S rRNA gene copies respond quickly to thawing. From Metaxa2 output, the bacterial fasta file (average of 711,123 reads per sample) was used to characterize the bacterial composition using QIIME v. 1.9.1 [55] to find the closest matching bacteria using closed-reference OTU-picking with default settings. 16S rRNA copy number for Greengenes v13.5 OTUs was obtained from the PICRUSt pipeline [56]. For each sample, we calculated the mean 16S rRNA gene copy number weighted by the relative abundances of each OTU; an R script for this analysis is available at: https://github.com/Russel88/MicEco. Significant differences between the thawing and freezing event was tested using Welch's t test (p <0.05). The estimate of phylum level copy numbers was obtained by finding the median and 90% quantile copy number of the OTUs in our dataset. The phylum-level fold change was estimated with the $\log_2$ ratio of the mean relative abundance for all freeze samples divided by the mean relative abundance for all thaw samples.

Results

Soil Physiochemical parameters

The average temperature during incubation and time of sampling can be seen in Fig. 1. The temperature measurements during incubation had a standard error of the mean of ≤0.1 °C. The slight increase in temperature after each sampling point is due to the opening of the chamber. Physiochemical properties of the soil measured prior to incubation were as follows. Water content was 39.6% (±1.4 SE), total carbon and nitrogen were 7.19% (±0.32 SE) and 0.48% (±0.02 SE) of soil dry weight, respectively. Soil texture distribution was clay (<2 μm) 3.1% (±0.4 SE), silt (2–63 μm) 55.4% (±5.7 SE) and sand (>63 μm) 41.5% (±6.2 SE). The pH of the soil was measured to be 7.5 (±0.02 SE), 7.6 (±0.04 SE), 7.4 (±0.03 SE) and 7.4 (±0.0 SE) for $T_{-6°C}$, $T_{2°C}$, $F_{2°C}$ and $F_{-6°C}$, respectively.

RNA concentration and 16S rRNA gene transcripts

RNA concentration after DNAse treatment showed no difference between samples (ANOVA, $p = 0.06$), though with a slightly higher concentration in the freezing samples. The mean ± standard error of the mean (SE) RNA concentration of all samples was $1.19 ± 0.04 \, \mu g \, g^{-1}$ of soil (fresh weight).

The 16S rRNA copy number did not differ between sampling points (ANOVA, $p = 0.06$), and the mean ± SE 16S rRNA transcript was $2.92 ± 0.5 × 10^8 \, g^{-1}$ of soil (fresh weight). The same observation for 16S RNA gene copies was seen with no difference between samples (ANOVA, $p = 0.23$). The mean ± SE 16S rRNA gene was $1.60 ± 0.5 × 10^8$ g-1 soil.

Overall microbial community composition

To study the microbial community, we screened two million randomly selected reads from each sample for rRNA transcripts, which resulted in 785,989 ± 2972 reads (average ± standard error of the mean, $n = 32$) per sample matching 16S or 18S rRNA transcripts. A total of 9763 distinct OTUs were detected of which 7154 belonged to Bacteria and Archaea, 651 to Fungi and 1958 to other Eukaryotes. The taxonomic composition of the community, including prokaryotes, Fungi and microeukaryotes (aka non-fungal/non-plant sequences) can be seen in Fig. 2. Bacteria constituted 84.0−94.1% of the total number of SSU reads, Archaea 0.05−0.15%, Fungi 0.6−6.7%, Rhizaria 0.5−2.6%, Viridiplantae 0.2−5.2% and other Eukaryotes 2.7–3.3%. At the highest taxonomic level (see Fig. 2a), only the relative abundance of Fungi significantly decreased in relative abundance from 6.7 ± 0.5 to 4.1 ± 0.1% when the temperature increased (ANOVA, $p = 0.037$ following the FDR correction, see Supplementary Figure S2). In contrast, the relative abundance of all these major taxonomic groups changed significantly when kept at 2 °C for 16 days. Three groups increased in relative abundance over the two sampling points, including Bacteria (88.5 ± 0.4 to 93.6 ± 0.3%), Chromalveolata (0.16 ± 0.01 to 1.16 ± 0.06%) and Rhizaria (0.62 ± 0.04 to 2.61 ± 0.23%).

The three groups that decreased most drastically during the period at 2 °C for 16 days were Fungi (4.08 ± 0.10 to 0.58 ± 0.10%), Metazoa (0.81 ± 0.28 to 0.12 ± 0.01%) and Viridiplantae (3.23 ± 0.34 to 0.30 ± 0.06%). The ratio between reads assigned to bacteria and fungi as calculated on real counts, increased from 17.4 ± 1.0 to 156.8 ± 9.4 (n = 16) between $T_{2°C}$ and $F_{2°C}$ (ANOVA, $p < 0.0001$), but did not change within the thaw and freeze events, see Fig. 3.

Prokaryote community composition

Major changes in the relative abundance of prokaryotic phyla and proteobacterial classes were found between the two sampling points at 2 °C (Fig. 2b). While most of these groups significantly decreased in abundance during this period, the majority (58.3%) of the 16S rRNA sequences belonged to a few phylogenetic groups enriched after 16 days at 2 °C. These were Beta- and Gammaproteobacteria, Firmicutes, Bacteroidetes and candidate division
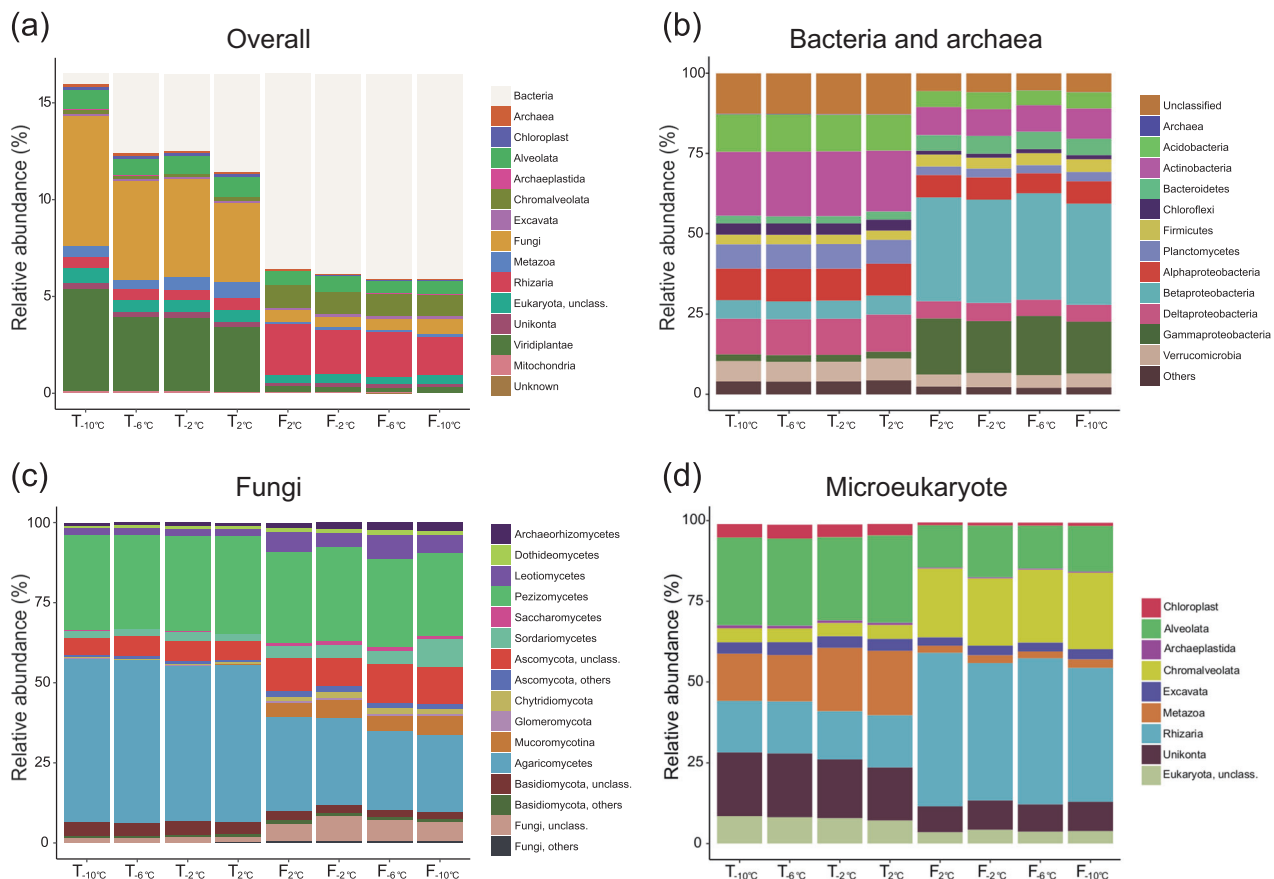
(a) Overall

(b) Bacteria and archaea

(c) Fungi

(d) Microeukaryote

Fig. 2 Changes in three-domain community profile of the microbiota in active layer permafrost during a thawing and a freezing event. The taxonomic profile are based on small subunit ribosomal RNA, extracted from total RNA sequences. The bar charts display the relative abundance in each taxonomic group. **a** Overall soil community, **b** bacterial and archaeal community, **c** fungal community and **d** microeukaryotic community
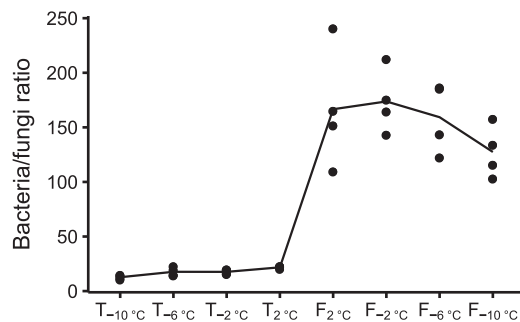

Fig. 3 Ratio between bacterial and fungal small subunit (SSU) rRNA reads calculated on raw sequence counts. Black dots are the ratio between bacteria- and fungi-assigned reads calculated from four replicates. Black line is mean of four replicates

TM7 (Figure S1). The Firmicutes were dominated by the genus *Paenibacillus* in the freezing samples, representing $37.3 \pm 1.20\%$ of the reads. The relative abundance of Betaproteobacteria increased from 5.95 ± 0.09 to $32.26 \pm 0.44\%$ and Gammaproteobacteria increased from 2.15 ± 0.05 to $17.48 \pm 2.29\%$. In contrast, Acidobacteria and Actinobacteria decreased from 11.20 ± 0.09 to $4.91 \pm 0.34\%$

and from 18.98 ± 0.14 to $8.75 \pm 0.71\%$, respectively, between the two time points at 2 °C. Only Gemmatimonadetes, Bacteroidetes and Betaproteobacteria responded to the increase in temperature from -10 to $+2$ °C (Supplementary Figure S2), and Aquificae was the only bacterial phyla that significantly responded to the freezing event as its relative abundance decreased between -2 and -6 °C ($p = 0.03$). Significant changes among prokaryotes at OTU level are described later.

Fungal community composition

The fungal community was dominated by two classes, Pezizomycetes (relative abundance of 29.8 ± 0.8 and $27.8 \pm 2.3\%$ in thawing and freezing samples, respectively) and Agaricomycetes (49.8 ± 1.3 and $26.3 \pm 2.3\%$ in thawing and freezing samples, respectively), see Fig. 2c. The overall trend of fungi was a decrease in 18S rRNA copies (Fig. 2a), which was also confirmed by an EdgeR test showing that fungal OTUs totalling 97.2% of the relative abundance at $T_{2°C}$ significantly decreased
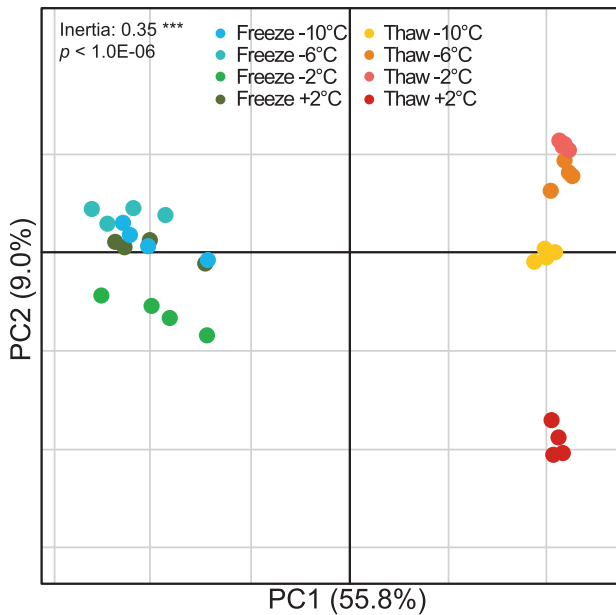
Fig. 4 Between-group analysis (BGA) of the soil microbial communities. The figure shows constrained principal component analysis (PCA) of the microbiome profiles after applying sample grouping, according to replicates for frozen and thaw samples along recorded temperature. Non-random distribution of the BGA grouping was tested using a Monte–Carlo simulation with 10.000 permutations ($p < 1.0E-04$***)

after 16 days at 2 °C. In contrast, only six fungal OTUs corresponding to a total of 0.2% of the relative abundance, significantly increased between the two sampling times. These OTUs were assigned to *Unclassified Mucoromycotina* ($0.0224 \pm 0.002\%$ at $F_{2°C}$), zygomycete sp. AM-2008a ($0.002 \pm 0.0002\%$ at $F_{2°C}$), *Tetracladium marchalianum* ($0.001 \pm 0.0001\%$ at $F_{2°C}$), *T. setigerum* ($0.0008 \pm 0.0002\%$ at $F_{2°C}$), *Mortierella parvispora* ($0.0003 \pm 0.0001\%$ at $F_{2°C}$) and *Nephridiophaga blattellae* ($0.0002 \pm 0.000\%$ at $F_{2°C}$), see supplementary datasheet.

Microeukaryote community composition

Microeukaryote community is the eukaryote community, excluding fungi and plants. None of the major groups significantly changed within the thawing or the freezing event (Fig. 2d). However, the relative abundance of all these groups, except *Excavata*, changed between the two events ($p < 0.05$, ANOVA with a FDR correction). These changes included a major increase in the relative abundance of *Rhizaria* from 16.0 ± 0.9 to $45.3 \pm 2.73\%$ and of *Chromalveolata* from 4.3 ± 0.2 to $22.4 \pm 1.3\%$ following 16 days at 2 °C. During this period, the relative abundance of *Alveolata* and *Metazoa* decreased from 27.1 ± 1.3 and $17.3 \pm 2.9\%$ to 13.7 ± 2.0 and $2.3 \pm 0.4\%$, respectively.

Alpha and beta diversity

A clear effect in favour of higher diversity in thaw profiles compared with the freeze profiles was observed. No clear effect of the temperature changes on alpha diversity was found (Supplementary Figure S3), but observed richness and Shannon index decreased significantly ($p < 0.05$) between thawing and freezing samples from 4075 ± 31 to 3761 ± 49 and from 5.1 ± 0.0 to 4.5 ± 0.1, respectively.

Beta diversity analysis based on all OTUs was applied in order to study the clustering of samples in the experimental setup. The constrained ordination analysis by grouping replicates under Monte–Carlo simulation revealed a significant, non-random distribution of the samples according to each temperature condition ($p < 0.0001$; Fig. 4). The first component explained most of the variance (55.67%) and clearly segregated the RNA profiles based on the treatment (freeze vs. thaw). The second component explained only 8.96% of the variance and was mainly separating the $T_{2°C}$ samples from the $T_{-10°C}$, $T_{-6°C}$ and $T_{-2°C}$ samples.

These observations were confirmed by the PERMANOVA on Bray–Curtis dissimilarity, where 62% of the variance was attributed to thaw versus freeze ($p < 0.0001$, Supplementary Table S1), although the changing temperature did not show any significant trend (4–8% of the variance). Similar analyses were conducted on the fungal and microeukaryote communities, and the same pattern was observed with clustering of the thawing samples and freezing samples and no clear trend within each event (Supplementary Figure S4).

Prokaryotic functional response groups (FRGs)

We investigated the changes in relative abundance at OTU level for prokaryotes within the thawing samples ($T_{-10°C}$, $T_{-6°C}$, $T_{-2°C}$ and $T_{2°C}$) and freezing samples ($F_{-10°C}$, $F_{-6°C}$, $F_{-2°C}$ and $F_{2°C}$). The relative abundance of 62 OTUs changed significantly within the thawing event (negative binomial regression, $p < 0.05$, see Supplementary Figure S5). Four different groups were identified representing four different responses upon thawing of the soil. Each of the prokaryotic functional response groups (FRGs) contained unique phylum-level specific OTUs (Fig. 5).

Both FRG 1 and FRG 3 responded negatively to increasing temperature when above -2 °C. FRG 1 had an increase until $T_{-2°C}$ and then decreased at $T_{2°C}$, whereas the OTUs in FRG 3 had a steady decrease during the entire thawing event. FRG 1 included 16 OTUs and was dominated by Actinobacteria, representing 92.1% of all OTUs within the group. FRG 3 consisted of 10 OTUs and was dominated by Acidobacteria, which represented 83.5% of the OTUs in the group. FRG 2 and FRG 4 represented OTUs, which increased in relative abundance when
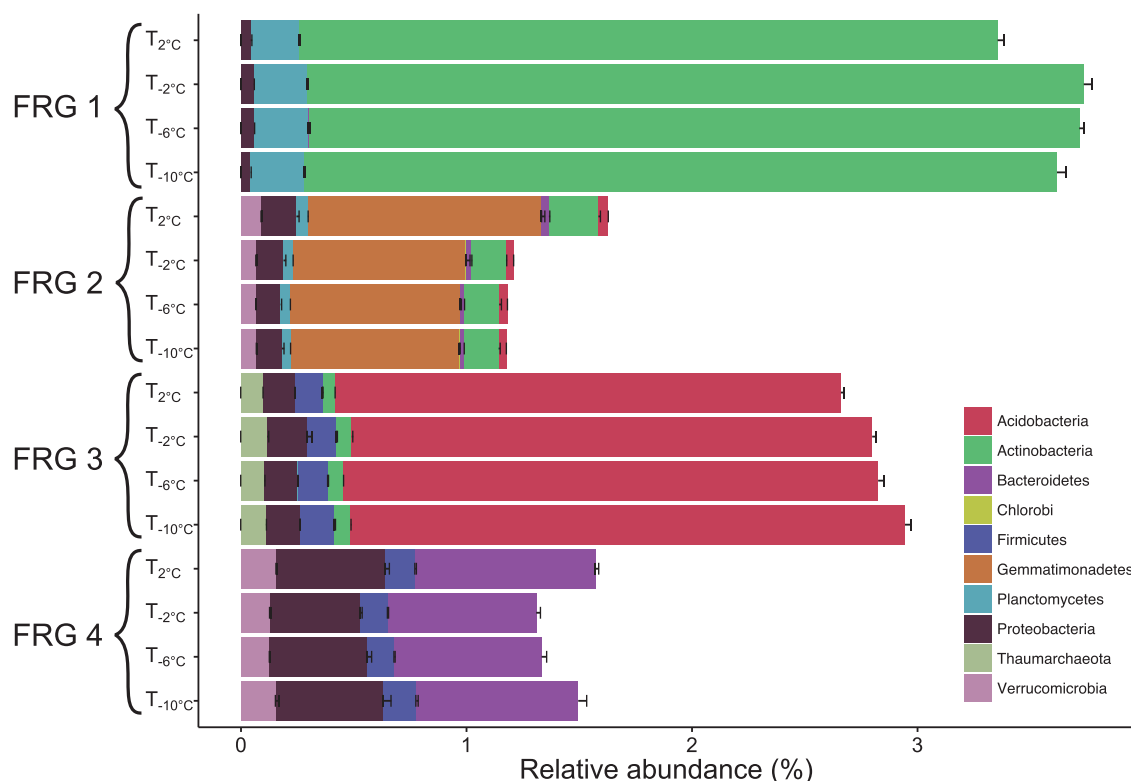
Fig. 5 Composition and abundance of the four functional response groups (FRG) representing a group of organisms that respond similarly to environmental changes. The figure represents the weighted abundance of phylogenetic groups in each FRGs for each temperature during both thawing and freezing events. Statistical coherency of the four FRGs was verified under a Monte–Carlo simulation results, where random groups of the same size were sampled from the whole operational taxonomic unit (OTU) pool to test the robustness of defined FRGs ($p < 1.0E-5$)

temperature increased from −2 to +2 °C. FRG 2, with 19 OTUs, increased throughout the thawing event and was dominated by Gemmatimonadetes. The relative abundance of OTUs in FRG 4 decreased between $T_{-10°C}$ and $T_{-2°C}$ and then increased at $T_{2°C}$. This group was represented by 16 OTUs within Bacteroidetes and Proteobacteria representing 49.5 and 31.4% of OTUs, respectively.

In the freezing event, the relative abundance of 39 OTUs changed significantly and four FRGs were established, but no phylum-level patterns within these groups were revealed (Supplementary Figure S6).

16S rRNA gene copy number and response to thawing

In order to test the hypothesis that the number of 16S rRNA gene copies within a bacterium correlates with its response to thawing, the average 16S rRNA gene copy number weighted by the relative abundance of each OTU was calculated. The average 16S rRNA gene copy number significantly increased from 2.19 ± 0.00 at the thawing to 3.35 ± 0.05 at the freezing event (Welch's test, DF = 15.02, $p = 1.14E-12$, Fig. 6).

A linear regression of the $\log_2$-fold change in relative abundance of each OTU between thawing and freezing
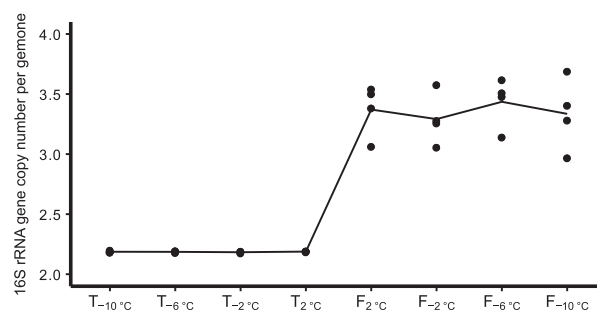

Fig. 6 Mean 16S rRNA gene copy number per genome of the four replicates from each time point. Calculated as mean 16S rRNA gene copy number weighted by the relative abundances of each operational taxomomic unit (OTU) based on the PICRUSt pipeline

samples against the 16S rRNA gene copy number revealed that the gene copy number explained 10.2% of the variance in the $\log_2$-fold change ($p < 0.0001$, Fig. 7).

Discussion

Here, we present the first detailed analysis of changes in microbial and microeukaryotic communities during thawing and freezing of an active layer permafrost soil using a direct
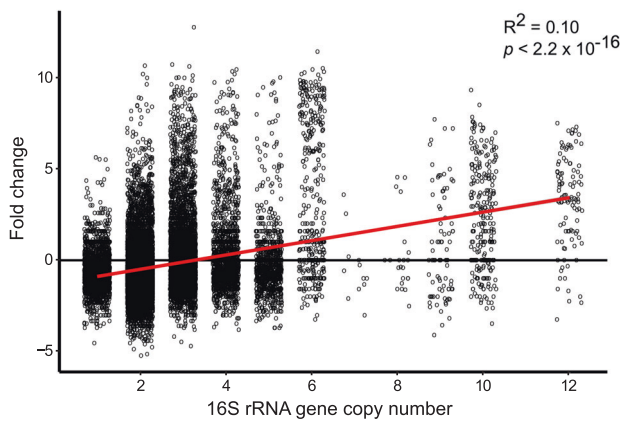
Fig. 7 Dot plot of the $\log_2$-fold change between $T_{2°C}$ and $F_{2°C}$ and the 16S copy number for each operational taxonomic unit (OTU) based on the PICRUSt pipeline. Red line are linear regression

shotgun RNAseq procedure, which is not biased by PCR amplification. The rRNA level cannot directly be linked to cell activity; instead it should be seen as an indicator for potential cell activity [35]. Further, we are aware that the rRNA content in cells differs considerably and that the number of rRNA transcript copies cannot be directly related to the number of cells. However, rRNA transcript copy numbers can be used for relative comparison of the different experimental conditions on each microbial taxa. We show that major changes in the soil microbiome occur after thaw with only small alterations at sub-zero temperatures, indicating that fluctuation in water availability is a main driver for changes in the community composition. Despite the knowledge that microbes are active and able to grow under sub-zero temperature [57, 58], we observed no changes in the microbial community at changing temperatures below the freezing point. In a previous study, looking at season changes in a potentially active microbial community over a course of one season, we observed very little variation during the winter months, November till April [22]. During this period, the temperature ranged from -14.3 to -3 °C. We believe that the lack of changes in the microbial composition could be due to time of incubation at sub-zero degrees and additionally, the turnover time of rRNA transcripts is decreased at low temperatures.

For all temperatures, the majority of rRNA was produced by bacteria. A large shift in the bacterial community was observed between $T_{2°C}$ and $F_{2°C}$, which may be linked to the environmental selection for different life strategies conserved at phylum level, and potentially linked to trophic strategies as previously shown [27]. During the experiment, we did not detect any changes in RNA concentration and 16S rRNA copy number. This was likely due to an increase in abundance of heterotrophic flagellates predating on bacteria and thereby limiting potential increases in total RNA concentration and 16S rRNA copy number due to

enhanced bacterial activity. The present data enabled us to reveal the dynamics of the soil microbial community upon thawing, which may have implications for our understanding of the degradation of soil organic matter during the short Arctic summer.

Temperature response of the bacterial community

The clear microbiome structure differences observed between thawing and freezing events were linked to the incubation time at 2 °C. Indeed, the molecular pictures obtained via RNA from the same communities ended up looking completely different based on this decisive 2 °C threshold. Even though this separation was mainly driven by large abundance changes in bacterial community composition, fungi and microeukaroytes also showed the same trend, indicating that all the microbial components of this system were impacted. These changes in bacterial composition upon a thawing event are consistent with other studies involving thaw of Arctic soils [25, 59–61]. Furthermore, the overall composition of the bacterial community is consistent with other studies of Arctic soil bacterial communities [13, 22, 37]. The relative abundance of most sequences from potentially active bacterial phyla decreased between $T_{2°C}$ and $F_{2°C}$, whereas only sequences from Bacteriodetes, Firmicutes and the Beta-Gammaproteobacterial classes increased. The shifts in the potential active bacterial composition at phylum level and proteobacterial class level may be explained by the copiotrophic and oligotropic lifestyle proposed by several authors [27, 28, 30, 31, 62]. Members of the phyla Bacteriodetes and Beta- and Gammaproteobacteria have previously been associated with a copiotrophic lifestyle [27, 63]. We found that their increase between samples $T_{2°C}$ and $F_{2°C}$ when thawing, likely corresponds to enhanced nutrient availability. There is little agreement on whether members of the Firmicutes have a copiotrophic or oligotrophic lifestyle. Another explanation for the increase in Firmicutes rRNA copy number is the capacity of several members within this phylum to produce endospores, including *Paenibacillus* [64, 65]. In endospore-forming bacteria, the rRNA level is as high or even higher in dormant cells compared with vegetative cells [66]. Thus, the increase in rRNA affiliated to Firmicutes may be a conserved response characteristic from this phylum to an initial increase in nutrient availability and/or due to preparation for dormancy as key nutrients are depleted.

The relative abundance of Actinobacteria and Acidobacteria decreased between the sampling points $T_{2°C}$ and $F_{2°C}$. Several Acidobacteria taxa have generally been reported as oligotrophic [27, 67, 68], and may be outcompeted by fast-growing bacteria when thawing enhances nutrient availability. In contrast, Actinobacteria have been reported as partly copiotrophic [69–71], which could

explain the decrease in potential activity of this phyla. Actinobacteria have been shown to be sensitive to freeze–thaw cycles in active layer soil [25], and to seasonal changes on glacial forefields [72] and in active layer soil [22].

In addition to the change in the prokaryote community during incubation at 2 °C for 16 days, we observed an increase in the relative abundance of Gemmatimonadetes, Bacteroidetes and Betaproteobacteria when temperature increased from −2 to 2 °C. Members of these three taxa responded quickly, within 1.5 days, to soil thawing. The increase in Bacteroidetes and Betaproteobacteria can again be explained by their rapid response to increased nutrient availability [27]. The life strategy of the phylum Gemmatimonadetes have not been studied in soil as far as we know; nevertheless, a study by Aanderud and Lennon [73] showed that Gemmatimonadetes responded rapidly to re-wetting of a soil by incorporating $H_2^{18}O$ into DNA. A more refined analysis at the OTU level using the Functional Response Group (FRG) analysis proposed by Nunes et al. [74] also showed a rapid response in the relative abundance of rRNA for some bacterial taxa. The analysis resulted in four functional response groups (FRGs) of which two were related to thawing. OTUs belonging to FRG 1 and FRG 3, decreased as the soil thawed, i.e., when temperature increased from −2 to 2 °C. Most OTUs in FRG 1 belonged to Actinobacteria, while FRG 3 was dominated by OTUs affiliated with Acidobacteria. Actinobacteria have been shown to respond negatively to an increase in soil moisture content [75–77], which could explain the decrease in relative abundance of the actinobacterial OTUs when water availability increases upon thawing. In contrast, members of FRG 2 and FRG 4 increased as the temperature increased from −2 to 2 °C. FRG 2 was dominated by the genus *Gemmatimonas (*Gemmatimonadetes*)*. The ecological role of Gemmatimonas in soil systems is not well-understood due to the lack of strains isolated from soil: the only representative of Gemmatimonas, *G. aurantica* was isolated from a wastewater treatment plant [78]. Further, even though OTUs assigned to Gemmatimonas have been found in Arctic soils [22, 78, 79], little is known about the response of *Gemmatimonas* spp. to thawing of soil and changes in nutrient availability. FRG 4 was dominated by unclassified Bacteriodetes and an unclassified betaproteobacterium. The response of these OTUs may be explained by their lifestyle as explained above.

In contrast to our initial hypothesis, very few members of the bacterial community responded to the temperature increase from −10 to −2 °C. We also observed negligible changes in the relative abundance of rRNA-based OTUs when the temperature decreased from −2 to −10 °C. Freezing is likely resulting in drastic decrease in nutrient availability and hence induce starvation. Bacteria decrease

the number of 16S rRNA upon starvation [80], but the low temperature may also lower the turnover of rRNA.

Average 16S rRNA copy number in bacterial community

The number of rRNA operons in bacterial genomes is correlated with growth rates [81, 82], and during early succession, soils are dominated by bacteria with a high number of rRNA operons, whereas bacteria with low numbers dominate during late succession phases [83, 84]. In a glacier forefield, the average rRNA gene copy number per cell was highest close to the glacier and decreased with distance from the glacier, which indicates that copiotrophic bacteria have an advantage in the initial phases of soil development [83]. To further investigate our hypothesis that the relative abundance of copiotrophic *r*-selected bacteria increase upon thawing of the soil, we estimated the number of 16S rRNA gene copies for each OTU, and then found the average 16S rRNA gene copy number for each sample. We observed an increase in the average 16S rRNA gene copy number per cell in the bacterial community after thawing, indicating a dominance of copiotrophic bacteria within 16 days of thawing. We hypothesize that a decrease in the 16S rRNA gene copy number per cell would occur if the soil was incubated at 2 °C for longer than 16 days as oligotrophic bacteria are expected to dominate later.

A linear regression between the $\log_2$-fold change in relative abundance of each OTU due to thawing and the 16S rRNA gene copy numbers revealed that 10.2% of the variation in the change could be explained by the copy number. Also, the average 16S rRNA gene copy number per cell of the 10 most abundant phyla and their $\log_2$-fold changes revealed that phyla assumed to be dominated by copiotrophic taxa increased the most upon thawing (Supplementary Figure S7). This further contributes to the hypothesis that copiotrophic bacteria dominated the soil within the first 16 days of thawing.

Temperature response of microeukaryotes

As seen for bacteria, thawing caused a marked shift in the microeukaryote community. Freeze–thaw cycles disturb the physical structure of the soil and hereby liberate organic substrate for the primary decomposers, for example bacteria, which will subsequently be grazed by protozoa that, along with the metazoan nematodes, are the primary grazing predators [85]. The thawing and the changed soil structure will create a larger water filled environment likely leading to an increased predation. Hence, to understand changes in abundance and diversity in the bacterial community, we must focus on the dynamics of their grazing predators. The microeukaryotes that increased during the thaw period can

all be included in the large paraphyletic group called heterotrophic flagellates. This group includes various stramenopiles, for example bicocecids, a group containing many unknown species in soil [86], and the common heterotrophic chrysophyte *Spumella*. Kinetoplastids, e.g., *Neobodo*, and in particular cercozoa, a very abundant group in soil Cercozoa [87], also strongly increased; in particular glissomonads—mainly *Neoheteromita* (formerly *Heteromita*) [88]. Heterotrophic flagellates are smaller than other protozoa, i.e., amoebae and ciliates [89] and therefore on average they have much shorter doubling times—down to a few hours, which is comparable with those of bacteria [90]. These small flagellates are faster colonizers and flourish at an earlier successional stage than larger protozoa [91]. Therefore, they are able to take advantage of the short period of thaw. In contrast, the other bacterivorous protozoa as ciliates and amoebae, and also metazoan nematodes, annelids and arthropods declined during the thaw period. Soil protozoa builds up populations during periods of growth and concomitantly form cysts; cyst formation is an intricate process and can hardly be done in response to the onset of unfavourable conditions but must rather be accomplished before initiation of a new frost period. Thus, a certain percentage of the actively growing cells are transferred to a cyst-bank, a percentage that increases drastically when cells are crowded [92]. Not all cysts will survive a frost period; they will be degraded by bacteria after onset of thaw and time is required to build up new populations. The thaw period in this study was only long enough to build up populations of heterotrophic flagellates. Longer thaw periods are most likely needed to keep up the population of the larger microeukaryotes.

We observed no change in 16S rRNA copy number using quantitative PCR or total RNA concentration after extraction and DNase treatment, which indicate that the number of potentially active microorganisms remained stable during the experiment. Furthermore, we detected a decline in dissolved organic carbon and an increase in CO_2 production between the two events indicating microbial activity and potential growth. We suggest that the stable total RNA and 16S rRNA concentrations are a consequence of grazing of bacteria by heterotrophic flagellates [85]. As discussed above, the relative abundance of potentially active copiotrophic bacteria increased upon thawing, which likely leads to growth of these bacteria and initiates grazing by the fast-growing heterotrophic flagellates. Even though protozoa are known to prey selectively on specific phylogenetic bacterial groups [93, 94], their grazing may not be limited to fast-growing bacteria but may also involve slow- or non-growing bacteria. This could explain the decrease in the relative abundance of oligotrophic bacteria following thaw and the stable RNA level.

Temperature response of the fungal community

The fungal community structure showed high similarity to communities in other Arctic soils [15, 95, 96], with a dominance of Agaricomycetes (Basidiomycota) and Ascomycota. However, in contrast to other studies, we found that the dominant class of Ascomycota was Pezizomycetes and not Leotiomycetes.

The fungal proportion of the microbial community declined dramatically when the soil started to thaw. In alpine soil systems, the ratio between fungi and bacteria shows strong seasonal variation with fungal dominance during winter, which indicates that fungi respond more dramatically to changes in soil temperature than bacteria [19, 20]. In accordance, Lipson et al. [19] showed that fungal hyphal length decreased during summer. This may explain the decline in the relative abundance of fungi following thaw. However, destruction of hyphae during our soil homogenization and disruption of the connection between plant-associated fungi and their plant partner may also explain the decline. Physical disturbance and soil handling can lead to breakage of fungal hyphae [97–99] and may expose fungal rRNA to degradation by other soil microorganisms. Of the 58 fungal OTUs that decreased between $T_{2°C}$ and $F_{2°C}$, 17 were assigned as ectomycorrhizal fungi (according to Tedersoo et al. [15]). These 17 OTUs correspond to a total relative abundance of 3.2%, but this percentage is a low estimate, as 20 of the 58 OTUs could not be assigned to an ecological function. This may be explained by the homogenization of the samples and removal of plants from the soil core prior to homogenization, since ectomycorrhizal fungi form symbiotic relationship with plants. Furthermore, we were unable to detect any significant changes in single celled fungal group Saccharomycotina using nbGLM between the $T_{2°C}$ and $F_{2°C}$, indicating that this fungal group were unaffected by the changes. Despite the general decline in the relative abundance of fungi, five fungal OTUs increased between $T_{2°C}$ and $F_{2°C}$. The two most dominating of these were an OTU assigned to an unclassified Mucoromycotina and an OTU assigned to zygomycete sp. AM-2008a. Zygomycetes and members of Mucoromycotina have been described as fast growing and are often among the primary fungal colonizers in systems with high concentrations of simple carbohydrates [100–103]. They increase their abundance if mycorrhizal fungi are disconnected from their plant partner [104]. These groups also harbour members that proliferate in low-temperature environments. Thus, fungi belonging to the Zygomycete orders Mucorales and Mortierellales that were isolated from snow in a subalpine forest in Colorado, USA, had fast growth rates at −2 °C [102, 105], and fungi isolated close to a Svalbard glacier all belonged to Zygomycetes [106]. The combination of activity at low temperature and

fast growth rates enables these fungi to increase their potential activity soon after soil thawing. The dramatic decline in fungal rRNA reads may have implications for the functionality of newly thawed soil because fungi play important roles in the degradation of recalcitrant soil organic matter, such as lignin, hemicellulose and cellulose [107]. Concomitant to the fungal decline, two bacterial phyla with members known to degrade complex organic matter, Acidobacteria [68, 108, 109] and Actinobacteria [107, 110, 111], declined. This decline could be caused by the increased flagellate grazing on these slow growing taxa following the thawing induced increase in fast-growing copiotrophic bacteria. The increase in potentially copiotrophic bacteria and fungi degrading mainly labile soil organic matter [27], and the concomitant decrease in oligotrophic bacteria, may delay degradation of recalcitrant soil organic matter when active layer soil thaws.

Conclusions

The RNAseq-based method allowed us to investigate the dynamics of all major members of the soil microbial community during a thawing and freezing event. Pronounced changes were observed in the microbial communities after the soil was thawed and kept at 2 °C for 16 days, for example a drastic decline in fungal potential activity. For bacteria, these changes could be related to the potential copiotrophic and oligotrophic lifestyle of the different bacterial taxa, with the relative abundance of potentially active copiotrophic bacteria increasing even within 1.5 days of thawing, showing a fast response of the these bacteria groups to thaw. As copiotrophic bacteria increased, heterotrophic flagellates grazing on bacteria also increased. These observations led us to hypothesize that protozoan grazing was not limited to the surge in copiotrophic bacteria, but also affected presumed oligotrophic bacterial taxa that are likely to possess important roles in the degradation of plant litter and soil organic matter. Thus, the combination of a decrease in potential fungal activity and likely grazing of bacteria by protozoa could potentially delay the degradation of more complex organic matter upon thawing of active layer soil. This may have implications for our understanding of plant nutrient availability and CO_2 emission from Arctic soils.

Data availability

Complete OTU tables are available in the Supplementary datasheet. Sequence data generated in this study was deposited in the NCBI Sequence Read Archive and are accessible through accession number SRP124869.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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