HOW WILL THAWING PERMAFROST AFFECT THE MICROBIAL ABUNDANCE AND DIVERSITY IN THE ARCTIC?

BY MARIA RIGSTAD LANGVAD

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Abbreviations and Important Terms

BSA – Bovine Serum Albumin

CFU – Colony Forming Units

DAPI – 4´,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to adenine-thymine rich regions in DNA.

DNA – Deoxyribonucleic acid

ITS - Internal transcribed spacer, a region with information that lies between two fungal rRNA genes.

MMN – Melin-Norkrans media, used for cultivating fungi

OA – Oatmeal Agar (or Ottaviani & Agosti agar), used for cultivating fungi

OUT – Operational taxonomic unit, used as unknown sequenced isolate

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction, used to amplify a specific DNA sequence

PDA – Potato Dextrose Agar, used for cultivating fungi

Polygon – geometrical patterned ground formed due to frost heaving, in where the edges often consists of high amounts of ice

R2A – Reasoner's 2A agar, used for cultivating bacteria

TAE – Tris-Acetate-EDTA buffer

Summary

Permafrost, permanently frozen soil, constitutes a major portion of the Earth's terrestrial cryosphere and represents a unique niche for cold-adapted microorganisms. As the global temperature increases, it is still unknown what consequences this will have for the inhabiting microbial structure and diversity. We assessed the microbial diversity within permafrost in Adventdalen, Svalbard across two different years (2016 and 2019). Our results revealed that the living conditions of the indigenous microorganisms had changed, due to an increase in moisture and organic matter. Furthermore, we observed that the bacterial and fungal gene abundance had also increased. The majority of the microorganisms discovered within our samples are recognised as degraders of complex carbon compounds, indicating that a potential increase of carbon dioxide emissions is likely to be a consequence of the further increase in global temperatures. Due to differing study design across years, exact replicates were not possible, and most of the measured data would not be statistically reliable if compared directly to each other. However, one depth from each of the cores could be directly compared, and here we observed an overall increase in moisture content, organic matter, and in microbial abundance. We thus infer that there is a possibility of increased greenhouse gas release when the microbial living conditions have shifted.

1 Introduction

1.1 Permafrost

Permafrost is considered to be soil, rocks, and ice that has been permanently frozen (below 0°C) for a minimum of two consecutive years (Muller, 1947; Zhang *et al.*, 2008; Jansson and Taş, 2014). Depending on the thickness of the permafrost, it has an estimated cover of between 23,9% and 27,7% in the Northern Hemisphere (Zhang *et al.*, 2008). While the permafrost is constantly frozen, the overlying soil undergoes a seasonal freeze-thaw cycle and is referred to as the active layer. This layer varies in thickness between 30 – 60 cm in Arctic areas (Tarnocai, 2009), where it is influenced by seasonal air temperature, snow cover, vegetation, summer precipitation and topography (Hinkel and Nelson, 2003). Arctic soil development is dominated by cryogenic processes, where cryoturbation is the main mechanism (Peterson and Krantz, 2003; Tarnocai, 2009). This results in a movement of material both vertically and laterally. In general, there is a very low amount of nitrogen in the Arctic soil, limiting plant growth (Elser *et al.*, 2007), thus only a small amount of organic matter builds up annually. However, these soils have through thousands of years operated as carbon sinks (Bockheim and Tarnocai, 1998). Through gravity and water movement the organic matter is moved down into the frozen, deeper layers where there is limited biological decomposition and is subsequently stored for thousands of years (Tarnocai, 2009). However, such low temperatures also limits the water activity, and the availability of nutrients and metabolite transfer is extremely low, which are considered as extreme stressors for the indigenous organisms (Steven *et al.*, 2006; Margesin and Miteva, 2011).

1.2 Permafrost and climate change

The Intergovernmental Panel on Climate Change (IPCC, 2019) has reported that the temperatures of Arctic and Antarctic permafrost has increased to “record high levels”, due to global climate change, where the Arctic has been recorded the most affected. The arctic, subarctic, and boreal regions are thought to have onset and more severe impact by the projected climate changes compared to other regions (Chapin III *et al.*, 2000; Serreze *et al.*, 2000; Hinzman *et al.*, 2005). For the past two to three decades, there has been continuous monitoring of the thermal state of permafrost, including the thickness of the active layer in the Northern Hemisphere (Romanovsky, Smith and Christiansen, 2010). This has been done in order to observe the long-term effects of climate change on permafrost (Burgess *et al.*,

2000). Due to a strong increase in the mean annual surface air temperature (+6°C in the Arctic), it has been predicted that 25% of the Arctic permafrost could thaw by the end of this century (IPCC, 2019). The winter temperature in Svalbard can compared to other areas in the same altitude (e.g. Russia and Canada) be up to 20°C higher due to Svalbard's close proximity to the North Atlantic Current (Humlum, Instanes and Sollid, 2003). The permafrost in Svalbard is therefore likely to be more sensitive to changes in the temperature of this current as well as global temperatures (Humlum, Instanes and Sollid, 2003). Degradation of permafrost can both occur vertically from the seasonally thawed active layer, but also laterally due to ground- or surface waters flow paths (Grosse *et al.*, 2011). As a consequence of thawing permafrost, a decrease of microbial stress factors, such as the increase in water availability and nutrient access, can improve microbial living conditions. We know that there are large reservoirs of organic carbon, including methane, stored in the permafrost (Mackelprang *et al.*, 2011). There is therefore a concern if the microbes will be partaking in further release of greenhouse gases such as carbon dioxide or methane as global temperatures increases. In order to assess this concern and its potential magnitude, there is an urgent need to study these sites to gain a better understanding of the microorganisms inhabiting the permafrost and the functions they are performing (Schuur *et al.*, 2009).

1.3 Permafrost as a microbial habitat

For most of the year the Arctic soil is at sub-zero temperatures, but increases during summer where temperatures up to 15°C can be recorded. The freeze-thaw cycles during the spring melt cause changes in the soil that impacts the indigenous microbial community, carbon content, and nutrient transformations (Sulkava and Huhta, 2003; Grogan *et al.*, 2004). Rising temperatures leads to enhanced snow and ice melt, with water entering the lower soil layers through soil pores, frost-induced cracks, and dendritic channels (Kane and Stein, 1983; Marsh and Woo, 1984). This acts to enhance microbial respiration and nutrient availability for the microorganisms (Burton and Beauchamp, 1994; Schimel and Clein, 1996; Brooks, Williams and Schmidt, 1998). Additionally, studies have found that lysis of a substantial portion of microbial cells happens during freeze-thaw cycles, resulting in release of carbon and nitrogen to the surrounding soil (Ivarson and Sowden, 1970; DeLuca, Keeney and McCarty, 1992), making the carbon available for consumption by the surviving microorganisms (Morley *et al.*, 1983; Skogland, Lomeland and Goksøyr, 1988). Depending on

the composition and temperature of the soil, 93-99% of the water existing in permafrost is present as ice (Rivkina *et al.*, 2004). However, a thin layer of unfrozen water surrounds both soil particles and bacterial cells. Through these water channels, metabolic activities occur through diffusion. Yet, the thickness of this film of water decreases considerably at lower temperatures, representing a diffusion barrier at too low temperatures. The microorganisms are therefore dependent on the thickness of the liquid water film in order to handle the low nutrient conditions found in permafrost (Rivkina *et al.*, 2000).

1.4 Microbial activity and adaptations to cold temperatures

Within the depth of soil there are a vast number of biogeochemical processes, which are driven by the inhabiting microbial community. These microorganisms play a key role in processes such as degradation of organic carbon, methane oxidation, methane production, ammonium oxidation, nitrogen fixation, nitrification, denitrification, among others (Madsen, 2011). They are reliant on many environmental factors in order to proceed their part in the biogeochemical processes, where abiotic factors such as temperature can influence growth significantly (Collins and Margesin, 2019). In comparison to other surface environments, permafrost is relatively stable with a range of constant stress factors (Gilichinsky, 2002). Examples of these stress factors are; low temperatures slowing down physical and biological processes, low water availability and nutrient flow, high salinity levels, and high UV radiation (Collins and Margesin, 2019). The organisms living in permafrost are adapted to such edaphic conditions (Jansson and Taş, 2014). Studies analysing the reproduction and metabolism of bacteria inhabiting sub-zero temperatures in permafrost show there is a wide physiological and genomic heterogeneity, where most organisms are ubiquitously psychrotolerant/psychrophilic (Shi *et al.*, 1997; Rivkina *et al.*, 2000; Bakermans *et al.*, 2003). The adaptations for these constant stressors which bacterial cells exhibit while inhabiting permafrost is necessary for their survival. For instance, the conversion of saturated fatty acids to unsaturated fatty acids increases the flexibility of the cell membrane, reduction of acidic amino acids increases the protein flexibility, and mechanisms of DNA repair are some adaptations necessary for bacterial growth in permafrost with low temperatures and background radiation (Chattopadhyay, 2006; Ayala-Del-Río *et al.*, 2010).

Eukaryotic microbial cells, like mycelial fungi or yeasts, are not as adapted to long-term cryopreservation in permafrost compared to bacterial cells. While the fungal cell walls are well preserved, the internal structures can be damaged (Soina *et al.*, 1995). One adaptation they have that is similar to bacteria, is that some genera of fungi also produce higher amounts of unsaturated fatty acids (Robinson, 2001). Another adaptation found in some fungi is that they contain melanin within their cell wall components for protection against the low temperature, drying, high concentrations of salt, and radiation (Sterflinger, 1998; Robinson, 2001; Rosas and Casadevall, 2001; Gessler, Egorova and Belozerskaya, 2014).

The total number of microorganisms found in permafrost does not inform us on whether the organisms are active, dead or in a dormant state, and both bacteria and fungi are capable of entering dormancy. However, the frequency which lead to the induction of dormancy in permafrost is not well understood (Mackelprang *et al.*, 2017; Burket *et al.*, 2019). Bacteria are able to form endospores and cells with thick capsules, while fungi form spores called unicellular conidia to survive the stressors found in permafrost (Soina *et al.*, 1995; Dmitriev *et al.*, 2001; Ozerskaya *et al.*, 2009). The assessment of activity using a LIVE/DEAD staining method, determined only 26% of the total number of bacteria within the permafrost microbial community of Spitsbergen, Svalbard are viable (Hansen *et al.*, 2007). This indicates that microorganisms inhabiting permafrost do exist in an active state, though, there is a larger proportion that is either in a dormant state or dead.

1.5 Microbial diversity in permafrost

Presence of viable bacteria in permafrost was first recognised at the end of the 19th century by Omelyansky (Shi *et al.*, 1997). Since then, a significant number of microorganisms have been discovered and isolated in both northern and southern polar regions (Gilichinsky *et al.*, 2008). Bacteria is the more commonly assessed group of microorganisms within scientific literature, thus more data on their phylogeny and abundance exists (Jansson and Taş, 2014). The most commonly reported bacterial phyla in Arctic permafrost includes Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria, and Bacteriodetes, in addition to other uncharacterized and novel phyla (Steven *et al.*, 2007; Yergeau *et al.*, 2010; Wilhelm *et al.*, 2012; Taş *et al.*, 2014). Nonetheless, both fungi and archaea have also been discovered in Arctic permafrost, however, this is limited to only a few reported studies (Jansson and Taş,

2014). The most common fungal phyla found in Arctic permafrost are Ascomycota and Basidiomycota (Kochkina *et al.*, 2001; Gittel *et al.*, 2014; Zhang *et al.*, 2016; Inglese *et al.*, 2017), although the abundance of fungi have no good estimates.

The microbial diversity associated with permafrost, and their functional potential has been recognised in several studies within Siberia (Gittel *et al.*, 2014; Schnecker *et al.*, 2014), the Canadian Arctic (Yergeau *et al.*, 2010), and in Svalbard (Alves *et al.*, 2013; Tveit, Urich and Svenning, 2014; Müller *et al.*, 2018; Xue *et al.*, 2020). Origin, age, and physiochemical properties are highly variable in permafrost, and the variation of the microbial diversity and abundance are thus dependent on location. This variation does not necessarily have to be between countries, it can be changing in a relatively close proximity of the same area, both vertically and laterally (Schostag *et al.*, 2015). Early studies of the microbial diversity in permafrost were highly culture-dependent (Shi *et al.*, 1997; Vorobyova *et al.*, 1997; Vishnivetskaya *et al.*, 2000), and thus biased due to the well-known fact that only a small number of microorganisms are culturable (Staley and Konopka, 1985; Steven *et al.*, 2006). The use of culture-dependent methods has restricted the assessment of the overall microbial diversity found in permafrost, and the development of culture-independent methods including denaturing gradient gel electrophoresis (DGGE), sequencing, and the more recent high-throughput sequencing methods has revolutionised microbial community analysis in general.

Permafrost metagenomic studies assessing microbial functionality are few, however, these studies have revealed a high abundance of carbon-cycling genes, where genes for degrading various carbon compounds such as chitinase, cellulose and cellobiose has been detected (Yergeau *et al.*, 2010; Taş *et al.*, 2014). One of the dominant phyla that are to be found in permafrost, *Actinobacteria*, contains members recognized as degraders of these compounds, giving them a central role in turnover of organic matter and in the carbon cycle (Yergeau *et al.*, 2010). The methanotrophic bacterial functional gene (*pmoA*) has also been detected in permafrost, which are found in members of another dominant permafrost phyla, *Proteobacteria* (Mackelprang *et al.*, 2011; Taş *et al.*, 2014). These bacteria can utilize the trapped methane in permafrost, and thereby release carbon dioxide during respiration (Mackelprang *et al.*, 2011). Almost all genes related to the different steps in the nitrogen-

cycle (e.g. *amoA*, *camoA*, *nirK*) has been detected in permafrost (Yergeau *et al.*, 2010; Mackelprang *et al.*, 2011; Taş *et al.*, 2014). However, the relative amount of denitrifying bacteria was considered too low for N_2 production, which could either lead to accumulation of nitrous oxide (N_2O) or assimilation of nitrate (NO_3^-) for forming biomass (Yergeau *et al.*, 2010; Taş *et al.*, 2014). In order to better predict which microbial processes are likely to occur in the different Arctic permafrost locations upon thaw, more information on the microbial function is necessary.

The fungal diversity reported from permafrost is mostly based on cultivation studies and isolated species, rather than metagenomic studies. The reported fungal diversity within the Arctic permafrost reveals mostly plant pathogens, saprotrophs, or endophytes (Bellemain *et al.*, 2013). The most common species isolated are; *Geomyces pannorum*, *Cladosporium spp*., *Aspergillus spp*., and *Penicillium spp*. which all can reproduce asexually by producing small, single spores (Kochkina *et al.*, 2001; Gilichinsky *et al.*, 2005; Ivanushkina, Kochkina and Ozerskaya, 2005; Ali *et al.*, 2013; Zhang *et al.*, 2016). Though eukaryotic fungi and yeasts are not fit for long-term cryopreservation, they have been found to not only be metabolically active, but also able to propagate under the stressors observed within the permafrost (Ozerskaya *et al.*, 2009; Buzzini and Margesin, 2014).

Though research in recent years has provided more information about the microbial communities and their function in permafrost, there are still many uncertainties to be uncovered. The microorganisms inhabiting permafrost may be important contributors to global temperature increase through the transformation of soil carbon, but to what extent is uncertain. This is why it is of interest to assess the microbial community responses in a climatically sensitive area of Arctic permafrost along temporal and spatial gradients.

Project aims

The main objective of this master thesis was to elucidate potential microbial community responses within the Arctic permafrost along temporal and spatial gradients. I approached this objective by asking the following four research questions:

1) What are the differences in the microbial community composition within permafrost cores collected from Adventdalen in Svalbard in the years 2016 and 2019?
2) Is the abundance of microbial organisms different in time and space?
3) Does the function of the microbial community, measured by assessing bacterial, fungal, and functional gene abundance (*amoA*, *camoA*, *pmoA*, and *mcrA*) change with time and space?
4) What is the fraction of cultivable or "living" components of the microbial community within a permafrost core and who are they?

2 Materials and Methods

2.1 Site description and core retrieving

The permafrost material in this thesis was retrieved from two different years, 2016 and 2019 in Adventdalen Valley, Svalbard archipelago (78.18° N, 15.92° E) (Figure 1). Eight cores were collected June 12th, 2016 from three different sites, two at site one and three at each of the sites two and three. Only site three will be examined in this thesis.

Figure 1: Location of drill site in Adventdalen, Svalbard, marked with a blue pinpoint. Map is from <https://www.geoplaner.com>

For the cores collected in 2016, a pit was dug to where the permafrost started, so the active layer was not retrieved. From there two cores of approximately 50 cm each were drilled consecutively, where in total ~100 cm of permafrost was drilled out of each hole. These cores were then wrapped in aluminium foil, labelled (core 1-8) and kept frozen in coolers for transport. The samples were further stored in a freezing room (-20°C) until further processing.

The permafrost core collected in 2019 was retrieved as part of The University Centre of Svalbard (UNIS) AB-327/827 course, by the students under supervision from lecturers and technicians with extensive experience using coring equipment. A motorized drill was used to

collect a 2 m permafrost core. A hole measuring 25 cm^2 and 34 cm deep was removed from the active layer before drilling. The average air temperature was recorded at 6.7°C by the Adventdalen weather station (http://158.39.149.183/Adventdalen/index.html).

Five sections were collected with a 50 cm core bit (Figure 2) and measured. The sections were wrapped in aluminium foil, placed on ice, and stored at -20°C.

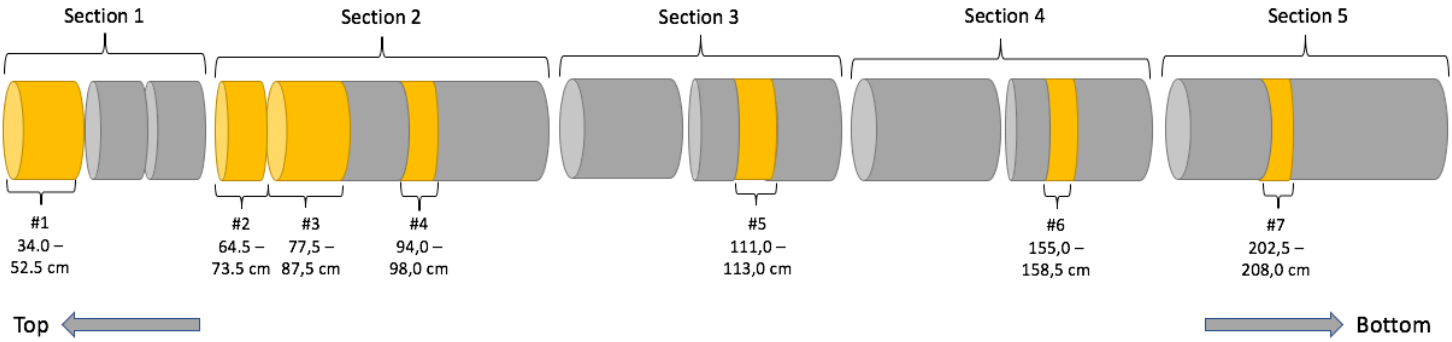

Figure 2: Scheme of the obtained soil core from 2019. An illustration of how the sections were split and where in the sections each of the samples were taken. The sample number is represented with a hash (#) and indicated with a yellow colour. Sample 1 is taken at depth 34,0 cm – 52,5 cm; sample 2 from depth 64,5 cm – 73,5 cm; sample 3 from depth 77,0 cm – 87,5 cm; sample 4 from depth 94,0 cm – 98,0 cm; sample 5 from depth 111,0 cm – 113,0 cm; sample 6 from depth 155,0 cm – 158,5 cm; sample 7 from depth 202,5 cm – 208,0 cm.

2.2 Subsampling of the core

The 2016 cores were opened at the University of Bergen in a fume hood. Core 6, 7, and 8 were cut in ~1 cm thick fragment at the depths 110 cm, 122 cm and 135 cm using a sterile chisel. The outer ~2 cm was cut off, leaving a non-contaminated fragment for DNA extraction. The soil from the outer part was used to measure geochemical data (Figure 3).

Figure 3: Process of subsampling the 2016 fragments. A. Samples cut by sterile chisel. B. ~1 cm thick fragment. C. Cutting of the outer part with sterile chisel.

Approximately 5 g of permafrost soil was collected to assess water content and organic matter content measurements, 2 g for pH measurements, 1 g for microbial cultivation, and lastly 0,7 g for DNA extraction. All samples were collected using a sterile spoon and placed in labelled tubes.

The five sections retrieved from the 2019 core were examined in the laboratory and visually inspected to determine where to perform subsampling. A total of seven samples from the five sections were obtained. The frozen core sections were broken into 2-3 cm fragments at the desired sampling site, using a sterilized chisel and mallet. It should be noted, however, that samples 1 to 4 were collected from 3 cm to 8 cm fragments of the internal core due to errors in breaking the sections open. The sample fragments were placed on sterile aluminium foil, and soil from the middle was collected to avoid possible contamination from the core fringes.

Approximately 5 g of permafrost soil was collected to assess water content and organic content measurements, 2 g for pH measurements, 1 g for microbial cultivation, and lastly 0,25 g for DNA extraction. All samples were collected using a sterile spoon and placed in labelled tubes.

For the rest of the analysis, the same protocols were used for both 2016 and 2019 samples.

2.3 Soil characteristics

2.3.1 Dry weight and organic matter

In order to measure water content and organic matter 5g soil was collected from each sample and placed in a muffle furnace (Nabertherm, Germany) at 105°C overnight, measured for water and moisture content and then subsequently at 450°C overnight, for organic matter measurements. The porcelain beakers were weighed before the soil was added with 0,1 mg accuracy. The samples were cooled down to room temperature in a desiccator, above beads of $CaCl_2$ to prevent moisture from the air to be absorbed back into the samples prior to weighing. The dry weight was calculated using equation 1-2, and the total organic matter was calculated using equation 3:

$$M_{water}\,(g) = (M_{beaker} + M_{soil}\,(g)) - M_{soil\,(105^oC)}\,(g) \quad \text{Equation 1}$$

$$Moisture\,(\%) = \frac{M_{water}\,(g)}{M_{soil}} \times 100 \quad \text{Equation 2}$$

$$T_{OM}(\%) = \frac{M_{soil\,(105^oC)}\,(g) - M_{soil\,(450^oC)}\,(g)}{M_{soil\,(105^oC)}} \times 100 \quad \text{Equation 3}$$

2.3.2 pH measurements

In a 50 ml Falcon tube, 2 g aliquots of soil sample were mixed with 20 ml MilliQ water by shaking, followed by ~30 min incubation at 4°C. The pH was measured using PHM210 Standard pH meter (Hatch®, Colorado) according to the manufactories protocol.

2.4 Enrichment media

2.4.1 Enrichment media for bacteria

Reasoner's 2A agar (R2A) is a low nutrient agar, and was used as enrichment medium because of its design to promote growth of bacteria living in oligotrophic environments (Reasoner and Geldreich, 1985). This medium has previously been observed as a preferred growth medium for microorganisms in permafrost (Hansen *et al.*, 2007). The agar was made by mixing 18,12 g R-2A Agar (Merck Life Science, 17209, Germany) in 1 l distilled water. This was then autoclaved at 121°C for 15 min and cooled down to 60°C before pouring the media into agar plates.

2.4.2 Enrichment media for fungi

To stimulate growth, three fungi specific media were used, Oatmeal Agar (or Ottaviani & Agosti) (OA), Melin-Norkrans media (MMN) and Potato Dextrose Agar (PDA). The first two media OA and MMN were used as isolation plates to target a broad range of fungi, typically living within edaphic conditions in soil, and antibiotics were added to prevent bacteria from growing on the plates. PDA was used as a secondary growth medium transferring the colonies previously isolated into pure cultures and this medium did not have any antibiotics added because of the assumption that there would not be any bacteria following the isolates.

OA was made by grinding 50 g oatmeal into a fine powder in a blender. The oatmeal was then added to ~500 ml water and brought to boil. The mixture was then strained through a muslin cloth, and the liquid was collected. 15 g agar was added to the liquid and brought up to a total volume of 1 L. This was autoclaved at 121°C for 20 min. The agar was set to cool to 50°C before 100 µg/mL tetracycline was added and the mix was poured into plates.

MMN was made by combining stock solution (1 ml $CaCl_2$, 1 ml NaCl, 10 ml $MgSO_4$, 5 ml $(NH_4)_2PO_4$, 10 ml KH_2PO_4, 1.2 ml $FeCl_3$, and 1 ml Thiamine), 5 g malt extract, 1,25 g D-glucose, and 15 g agar. Distilled water was added to make a total volume of 1 L. The mix was autoclaved at 121°C for 20 min. When this had cooled down to 50°C 100 µg/ml tetracycline was added, and the media was poured into Petri dishes.

The last media, PDA was used to ensure growth of isolated colonies from the two previous media. This was made by combining 39 g PDA powder with 1 L distilled water. This was autoclaved at 121°C for 20 min and cooled down to 50°C before it was poured into Petri dishes. This media did not contain any antibiotics because of the assumption that contamination by bacteria was unlikely at this point.

2.5 Enrichment and isolation

Culturable bacteria were enumerated by the spread plate method. A 1:10 dilution was made using 1 g of soil and 9 ml of sterile PBS. A dilution series up to 10^{-4} was made before plating 100 µl from the 10^{-2}, 10^{-3}, and 10^{-4} dilutions on the R2A plates (section 2.4.1). The 10^{-2} plates

were placed at 10°C to resemble a colder climate. The rest of the plates (10^{-3} and 10^{-4}) were incubated at room temperature.

For fungi enrichment, dilutions of 1:10 and 1:100 was used on the MMN and OA isolation plates, where 100 µl was spread on the plates. These were incubated at 14°C for 4 weeks before sub-culturing colonies onto the PDA plates, which again was incubated at 14°C. Equation 4 was used for calculating colony forming units per ml:

$$\frac{CFU}{ml} = \frac{number\ of\ colonies \times dilution\ factor}{volume\ of\ culture\ plate} \quad \text{Equation 4}$$

2.6 Total numbers

Total numbers of bacteria were estimated using fluorescent microscopy analyses. The DNA-specific 4'6-diamidino-2- phenylindole (DAPI) stain was used to give the cells a fluorescence of a bright blue colour (non-DNA would fluorescence a week yellow) as a result of being excited by light at a wavelength of 365 nm (Porter and Feig, 1980). The 1:10 dilution samples, from section 2.5 (~8 ml) made for culturing of bacteria was fixed by adding 889 µl 25% glutaraldehyde (final concentration ~2,5%) and well mixed. The samples were kept in the fridge at 4°C for two nights before a ten-fold dilution was made until 10^{-4}. Both dilution samples from 10^{-3} and 10^{-4} were used for DAPI staining.

A mounting solution of 2 ml (20 µl 0.10% p-phenylenediamine dihydrochloride in 1980 µl PBS:glycerol (1:1)) was made and stored at 4°C until use. The sample (3 ml of 10^{-4} dilution, and 2 ml of 10^{-3} dilution) was vacuum filtered onto Poretics Black Polycarbonate 0.22 Micron 25 mm Membrane Filters (OSMONICS INC, USA) by using the Carbon 14 Centrale (Denmark) filtration set up. A smaller volume was used for the lowest dilution to prevent clogging from the soil. After the samples were filtered, the filters were rinsed twice with 5 ml of Milli-Q water (filtered PBS was used for the first 10 samples). The filters were covered with DAPI and left to sit in the dark for 15 min, the suction was turned off to let the filters air dry at room temperature. Excess DAPI stain was then washed off with 2 rinses of 5 ml Milli-Q water. The filters were again set to dry, with low vacuum, for 5 min before mounting the filters. The filters were placed between a microscope slide and coverslip with mounting

solution on both sides of the filter before they were wrapped in aluminium foil and placed at -20°C until enumeration.

Equations 5-6 for calculating cell concentrations:

$$F\ value = \frac{Area\ of\ filtration}{Area\ of\ grid\ used\ for\ counting} \quad \text{Equation 5}$$

$$Concentration\ of \frac{cells}{ml} = \frac{\left(\frac{Average\ cell\ counts}{field\ of\ view}\right) \times F\ value}{(ml\ filtered) \times (fields\ counted)} \quad \text{Equation 6}$$

*Area of filtration (314 mm^2) and area of grid used for counting (1,44 x 10^2 mm^2) were the same for all samples.

2.7 DNA isolation

2.7.1 DNA isolation from soil

DNA isolation was conducted using the protocol from the DNeasy® PowerSoil® Kit (QIAGEN, 12888-100, Germany). A few modifications to the protocol were made for samples collected in 2016 due to the expected low concentration of DNA. Samples collected in 2019 followed the protocol by the manufacturer. Specific modifications included an increase in starting material using approximately 0,7 g of soil instead of 0,25 g as the protocol states. To prevent leakage when centrifuging the tubes, a maximum of 600 µl was loaded onto the spin column compared to 675 µl as stated in the protocol. After the flow through was discarded for the last time, the column was placed in a new collection tube to prevent traces of ethanol before elution. Lastly, when the 100 µL of Elution buffer "solution C6" was added to the membrane filter, it was left to incubate for 15 min in the first elution, and then the second elution with 50 µL of Solution C6 for 1 min. This extension of time was done to ensure that most of the DNA was filtered through, and any remnants of DNA was expected to be eluted in the second step. These two elutions were kept separate in collection tubes. The extracted DNA was quantified using Qubit® 2.0 Fluorometer (Invitrogen, Singapore) by following the manufacturer's instructions and then stored at -20°C. The principle behind the Qubit fluorometer is that the molecular probes, which emit fluorescent signals, binds to DNA, whereby the amount of DNA can be quantified based on a standard curve.

2.7.2 DNA isolation of bacteria from isolates

Bacterial colonies were collected from agar plates using a sterile toothpick and added into 10 µl of deionised water and kept frozen at -20°C until use. In order to ensure lysis of the bacterial cells, the samples were put on heating block at 80°C straight out of the freezer, then put on ice, both for 1 min, and repeated a second time (2x freeze-thaw cycles).

2.7.3 DNA isolation of fungi from isolates

Colonies of fungi were scraped from agar plates using a scalpel and placed in a 1,5 ml Eppendorf tube. 500 µl CTAB-buffer and 1 tungsten bead was added to the sample tubes before homogenizing in a "Tissue Lyser" set at 15 Hz for 20 seconds. The samples were then set to incubate at 60°C on a heat block for about 30 minutes. Two sets of new tubes were then prepared, one set with 500 µl Chloroform:isoamylate and one set with 500 µl isopropanol. The liquid from the sample was transferred to the tubes containing chloroform:isoamylate to lysis mixture. The tubes were then inverted for 5 minutes before spinning the samples for 1 minute at 12 000 rpm. The transparent aquatic phase was pipetted out and transferred to the tubes containing isopropanol and incubated at -20°C overnight. The following day the samples were spun for 10 minutes at 14 000 rpm before the supernatant was carefully poured out, leaving the pellet. 800 µl of 70% ethanol was added before the samples were inverted for 5 minutes. The samples were thereafter spun for 15 minutes at 14 00 rpm before the ethanol was poured out, leaving the pellet. The pellets were left to dry at 65°C on a heating block for about 1 hour. Lastly 100 µl AE elution buffer was added to the tubes and they were left over night in room temperature to resuspend the DNA pellet.

2.8 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed on the extracted DNA in order to amplify specific bacterial (16S rRNA genes), eukaryotic (18S rRNA genes), and fungal (ITS region) genes in addition to functional genes for ammonia oxidation in bacteria (*amoA*) and archaea (*camoA*), methane oxidation in bacteria (*pmoA*), and methane generation in archaea (*mcrA*). The amplification was performed using the Applied Biocycler™ Veriti™ 96 Well Thermal Cycler (Thermo Fisher Scientific, 4375786, USA).

All PCR reactions were set up in a total volume of 20 µl. A typical PCR reaction would then consist of 1-10 ng template DNA added to a master mix containing; 10 µL HotStarTaq MM, 0,5 µM of each primer, 2,5% Bovine Serum Albumin (BSA), 2 µl template DNA, and 5,5 µl nuclease free water. Cycling conditions for each gene can be found in Table 1. BSA was a crucial component in this master mix as it binds to inhibitory substances and thereby prevents binding and inactivation of the DNA polymerase (Kreader, 1996).

Table 1: Primers, target microorganism, gene sequence, PCR cycling conditions, and number of base pairs (bp) for bacterial, eukaryotic, fungal, and functional genes amplified.

Gene	16S rRNA				18S rRNA		*amoA*		*camo A*		*pmoA*		*mcrA*		ITS		
Name	Bac338f	H1542r	A8f	PRUN518r	Euk566f	Euk1200aR	amoA-1F	amoA-2R	Camo A-19F	TamA 629R-2	A189F	A682r	mcrAfornew	mcrArevnew	ITS1-F	ITS4-R	ITS3-F
Target	Bacteria				Eukaryote		Ammonium oxidising Bacteria		Ammonium oxidising Archaea		Methanotrophic Bacteria		Methanogenic Archaea		Fungi		
Sequence	ACTCC TACG GGAG GCAG CAG	AAGG AGGT GATCC AGCC GCA	GAGA GTTT GATCCTG GCTCAG	ATTAC CGCG GCTG CTGG	CAGC AGCC GCGG TAA TTCC	CCGGT GTTGA GTCAA ATTAA GC	GGGG HTYT ACTG GTGG T	CCCCT CKGSA AAGC CTTCT TC	ATGGT CTGGY TWAG ACG	GCCAT CCATC KRTAN GTCCA	GGNG ACTG GGAC TTCT GG	GAAS GCNG AGAA GAAS GC	GGTG TMGG DTTCA CH CARTA YGC	TTCAT NGCR T AGTTH GGRT AGTT	CTTGG TCATT TAGA GGAA GTAA	TCCT C CGCT T ATTG ATAT GC	GCAT C GATG AAGA ACGC AGC
Thermal profile	95°C-15min, [95 °C-45sec, 55 °C-45sec, 72 °C-1min], 72 °C-10min			For qPCR	95°C-15min, [95 °C-45sec, 56 °C-45sec, 72 °C-1min], 72 °C-10min		95°C-15min, [95 °C-30sec, 55 °C-1min, 72 °C-1min], 72 °C-10min		95°C-15min, [95 °C-30sec, 55 °C-1min, 72 °C-1min], 72 °C-10min		95°C-15min, [95 °C-30sec, 55 °C-1min, 72 °C-1min], 72 °C-10min		95°C-15min, [95 °C-1min, 55 °C-1min, 72 °C-1min], 72 °C-7min		95°C-15min, [95 °C-30sec, 55 °C-30sec, 72 °C-1min], 72 °C-7min		For qPCR
No. cycles	28				28		35		35		35		28		35		
Product size	1204 bp		1534 bp		650 bp		491 bp		415 bp		472 bp		488 bp		600 bp		
Reference	(Amann, Ludwig and Schleifer, 1995)	(Fierer *et al.*, 2005)	Modified from (Lane, 1991)	(Suzuki and Giovannoni, 1996)	(Hadziavdic *et al.*, 2014)		(Stephen *et al.*, 1999)	(Liesack, Werner; Jan-Henrich, 1997)	Alves et al. (unpublished)		(Holmes *et al.*, 1995)		(Gagnon *et al.*, 2011)		(White *et al.*, 1990)		

Presence of a PCR product of correct amplification was verified with 1% Agarose gel in Tris-acetate-EDTA. The DNA was stained with 3 µl 10 000X GelRed™ stain (Biotium, 41003, USA) which was added to the gel. A 2 µl GenRuler 1 kb DNA ladder (Thermo Fisher Scientific, SM0313, Lithuania) was used as ladder, 4 µl PCR product was mixed with ~1 µl DNA Gel Loading Dye (Thermo Fisher Scientific, R0611, Lithuania) and loaded into the wells. The gel was run for ~40 min at 200 V, allowing the bands to separate properly. The DNA fragments were visualised using BIO RAD Molecular Imager® (ChemiDoc XRS™), and using the Image Lab™ software to generate an image.

2.9 Quantitative Real-Time PCR

2.9.1 Preparations of standards

Quantitative Real-Time PCR (qPCR) was used to detect and quantify bacterial and fungal genes. Absolute quantification was performed, where samples with a known concentration were used to produce a standard curve. Based on the standard curve the concentration of the environmental samples could be determined by the sample's PCR signal (Ct). Standards for both bacterial and fungal genes were made from environmental samples using the samples with the highest measured original DNA concentration to ensure all samples were placed within the standard curve. Four samples were chosen for this (core 6 depth 122 cm extraction number 1, 2, 3 and core 7 110 cm extraction number 2).

All PCR reactions for the standards were set up in a total volume of 100 µl, divided in two batches of 50 µl in PCR reaction tubes. Eight µl DNA template were added to a master mix containing the following reagents; 50 µl HotStarTaq MM, 0,5 µM of each primer, 2,5% BSA, and 29,5 µl nuclease free water. See section 2.9.2 and 2.9.3 for the PCR programs used for bacterial and fungal genes, respectively. After the PCRs were run the corresponding standard samples were pooled together before running them on a 1% Agarose gel to examine the purity of the bands.

The DNA concentration of the standards was measured using Qubit® 2.0 Fluorometer (Invitrogen, Singapore) before cleaning them using the DNA Clean & Concentrator™ -25 (Zymo research, Irvine, Ca, USA) – kit. The protocol from the manufacture was followed,

although with minor modification; the DNA Elution Buffer stayed on the filter for 15 min instead of 1 min. This was done to ensure that most of the DNA was eluted from the filter.

A ten-fold dilution series was then made in triplicate before again measuring the DNA concentrations of the standards. These standards were stored at -20°C until the environmental samples were prepared and ready for qPCR. Equation 7 was used to calculate the number of copies of the products used in the qPCR for the standards:

$$\frac{amount\ of\ DNA\ (ng) \times Avogadro's\ number}{length\ of\ template\ (bp) \times (1 \times 10^9) \times 650\ Da} = Number\ of\ gene\ copies \qquad \text{Equation 7}$$

* 650 Dalton is the average weight of a base pair.

2.9.2 qPCR of bacterial genes

The primer pair used for bacterial standards was Bac338F/PRUN518r (Table 1) (Suzuki and Giovannoni, 1996; Lopez *et al.*, 2003). PCR amplification was done with the following programme: 95°C for 15 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min, and a single final extension at 72°C for 7 min.

The V3 region was targeted for the qPCR of the unknown environmental bacterial samples, where the primer pair used was Bac338F/PRUN518r (Table 1) (Suzuki and Giovannoni, 1996; Lopez *et al.*, 2003) which targets the small part of the 16S region (180 bp). The qPCR reaction mix was set up in a total volume 20 µl, where 1 ng/µl DNA template was added to a master mix containing the following reagents; 10 µl SsoFast™ EvaGreen® Supermix, 0,250 µM of each primer, 2,5% BSA, and 7 µl nuclease free water. qPCR amplification was done using the following programme: enzyme activation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and a single melt curve at 65 – 95°C with a 0,2°C increment for 5 sec per step.

2.9.3 qPCR of fungal genes

The primer pair that was used for fungal standards was ITS1-F/ITS4-R (Table 1). PCR amplification was done with the following programme: 95°C for 15 min; 35 cycles of

denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, and a single final extension at 72°C for 7 min.

The ITS2 region was targeted for the qPCR of the unknown environmental fungal samples, where the primer pair used was ITS3-F/ITS4-R (300bp) (Table 1) (White *et al.*, 1990). The qPCR reaction mix was set up in a total volume 20 µl in a 96 well plate (BioRad), where 1 ng/µl DNA template were added to a master mix containing the following reagents; 10 µl SsoFast™ EvaGreen® Supermix, 0,125 µM of each primer, 2,5% BSA, and 8 µl nuclease free water. qPCR amplification was done using the following programme: enzyme activation at 98°C for 2 min; 45 cycles of denaturation at 98°C for 20 sec, annealing at 55°C for 30 sec, and a single melt curve at 65 – 95°C with a 0,5°C increment for 10 sec per step.

2.10 Sanger sequencing

2.10.1 Purification of PCR product and preparation for sequencing

In order to verify that the DNA isolates from colonies (section 2.7.2 and 2.7.3) contained DNA of the desired base pair length, a PCR was set up for both the bacteria and fungi using the primer pairs A8f/H1542R and ITS1-F/ITS4-R, respectively (Table 1). The PCR product was then run on a 1% agarose gel electrophoresis.

The PCR products showing correct band size on the agarose gel, were then purified by adding 5 µl PCR product with 2 µl 1-step ExoStar (illustra™ ExoProStar™, US77705, GE Healthcare) in order to remove unincorporated nucleotides and primers. The samples were incubated at 37°C for 15 min for the enzymes in the ExoStar to be activated. Followed by incubation at 80°C for 15 min for deactivation of the enzymes. These two steps were done using the PCR machine; Applied Biocycler™ Veriti™ 96 Well Thermal Cycler (Thermo Fisher Scientific, 4375786, USA), where the end step was set to 4°C.

2.10.2 Sequencing

Using Big-Dye Cycling sequencing kit (Big-Dye version 3.1 and sequencing buffer provided by the sequencing Facility, MBI, UiB) a library preparation was set up. A Big-Dye master mix was prepared, where each of the samples contained; 1 µl Big-Dye (version 3.1), 1 µl sequencing buffer, 2 µl template (200 ng), 3,2 µl primer (1,0 µM), and 2,8 µl deionised water. The products were spun down and placed in the PCR machine on the following programme; 96°C

for 5 min, 25 cycles of 96°C for 10 sec, 55°C for 5 sec, and 60°C for 4 min. After the PCR reaction, 10 µl deionised water was added to each of the PCR-products. The samples were then sequenced using the Sanger sequencing method using Applied Biosystems 3730XL Analyzer (Thermo Fisher Scientific) at the Sequencing Facility, BIO, University of Bergen (Thormølensgate 55, 5008 Bergen, Norway).

2.10.3 Genetic distance

In order to compare the different strains similarities in the 16S rRNA gene and the conserved ITS region, consensus sequences were aligned and compared in MEGA7 for macOS X (Kumar, Stecher and Tamura, 2015) using the Muscle function.

The closest hits and identities of the isolated bacterial and fungal sequences was collected by BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the Nucleotide BLAST function, and from the reference database UNITE (https://unite.ut.ee), respectively.

2.10.4 Phylogenetic tree

The genetic distance between the operation taxonomic units and their closets hits were visualised by the construction of a phylogenetic tree. All of these sequences were aligned in MEGA7 for macOS X (Kumar, Stecher and Tamura, 2015) using the Muscle function. The tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model where the robustness was weighed using 500 bootstrap replications in MEGA7 for macOS X (Kumar, Stecher and Tamura, 2015).

2.11 Statistics, calculations, and software

All graphs and statistical analyses represented were created using R Software (R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/), where one-way ANOVA was used to find differences in depths of the permafrost. Calculations were completed in Microsoft® Excel for macOS, Version 16.

3 Results

3.1 Soil characteristics

The soil characteristics are summarised in Figure 4. Measurements of the samples collected in 2016 demonstrated a trend of higher overall average soil characteristics at 122 cm depth, while the lowest averages were observed at depth 135 cm. The percentage of water at the three different depths had an average value of 29,13%, 29,95%, and 27,85% at the depths 110 cm, 122 cm, and 135 cm respectively with a combined mean of 29,98% ± 0,50 (Figure 4A). No significance between the depths was found ($p = 0,73$). The organic matter content from the same year showed the same pattern as the water content, with an average percentage of 3,26%, 3,89%, and 3,26%, in respect to the depths. The overall mean was 3,59% ± 0,15 (Figure 4B). Significance between the depths was not found ($p = 0,37$). Likewise, the pH measurements showed a similar pattern. In the respective depths there was an average pH value of 5,56, 5,58, and 5,50, where the combined average was 5,55 ± 0,02 (Figure 4C). The difference between the depths was also not significant ($p = 0,21$).

No obvious trend was observed for the samples collected in 2019. The percentage of water ranged from 25,91% at the depth 64,5 – 73,5 cm to 55,7% at the depth 155,0 – 158,5 cm where the average for the entire core was at 38,03% ± 3,48 (Figure 4D). In the samples from the same year the average percentage of organic matter was 4,49% ± 0,15, where the sample containing the highest percentage was at the highest depth (34,0 – 52,5 cm) with 5,23%, and the lowest in the middle of the core (94,0 – 98,0 cm) with 3,82% (Figure 4E). The pH peaked at the depth 64,5 – 73,5 cm with a pH of 6,92, and the lowest pH measured was 5,88 at the depth 111,0 – 113,0 cm (Figure 4F), with an overall average of 6,39 ± 0,14.

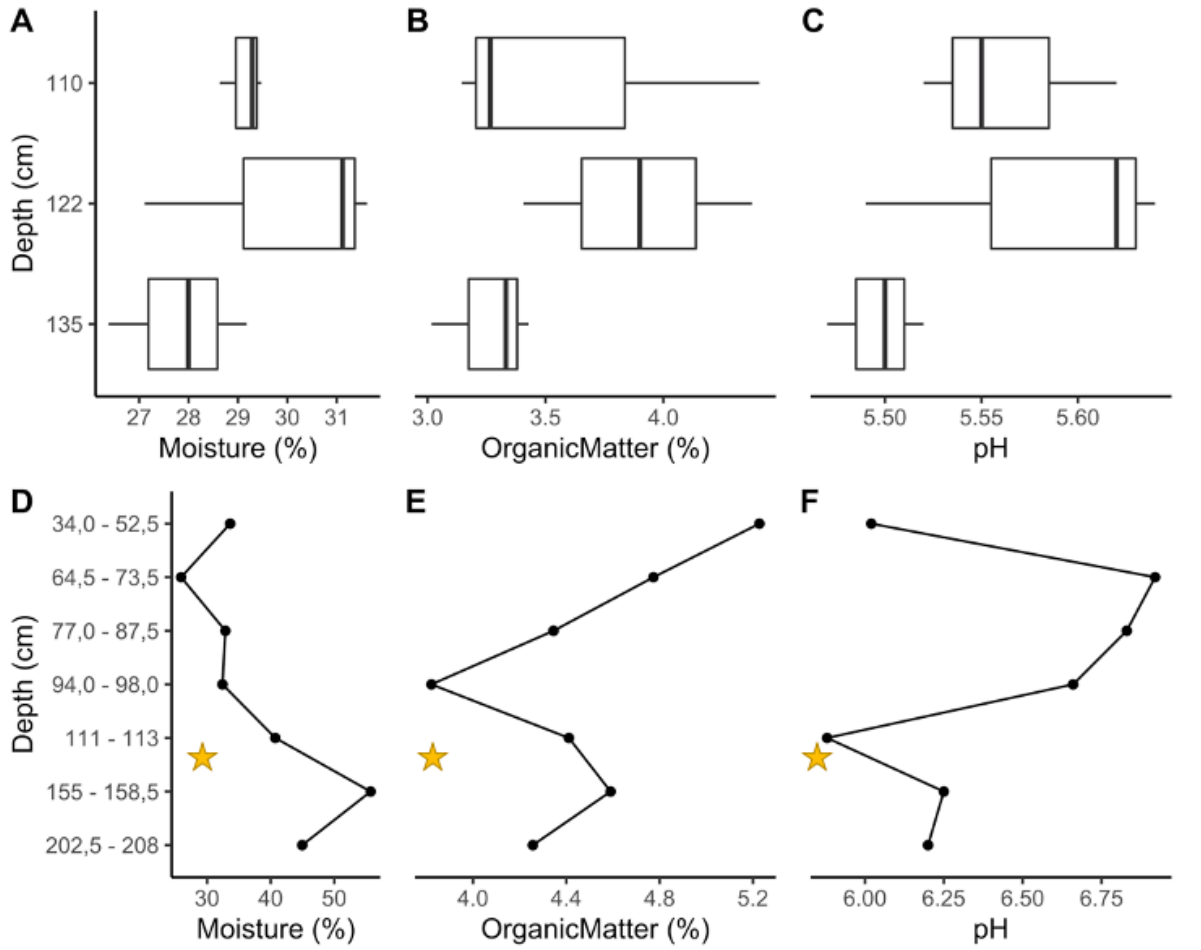

Figure 4: Soil characteristics of the permafrost cores collected in 2016 and 2019. Samples are arranged by soil characteristics on the x-axis and in descending order down the depth of the cores on the y-axis. A. Percentage of water in the soil from 2016 core B. Percentage of organic matter in the soil from 2016 core C. pH in the soil from 2016 core D. Percentage of water in the soil from 2019 core. The star indicates where the 2016 samples approximately is found. E. Percentage of organic matter in the soil from 2019 core. The star indicates where the 2016 samples approximately is found. F. pH in the soil from 2019 core. The star indicates where the 2016 samples approximately is found. Because of sampling design, samples from 2016 are represented by a boxplot (they were taken in triplicate per depth), while samples from 2019 only one sample per depth was collected and is therefore represented in a line plot.

3.2 Total bacterial numbers

Within samples collected in 2016 the bacterial cells were not homogenously distributed, they were mostly aggregated, which made them difficult to count accurately in the fluorescence microscope. There was in general very few cells to be found, combined with organic material in the samples. Both 10^{-3} and 10^{-4} dilutions were used for counting (Figure 5).

The 10^{-4} dilution samples had an overall higher number of cells where the average at the three different depths (110 cm, 122 cm, and 135 cm) was 6,77 x 10^8 cells/g (d.w), 8,77 x 10^7 cells/g (d.w), and 1,08 x 10^8 cells/g (d.w), respectively. The reason for the high average at depth 110 cm stems from high aggregation of cells at core 8 at this depth where the concentration of cells was at 1,96 x 10^9 cells/g (d.w), while in the same depth in core 6 and 7 the concentrations were 3,02 x 10^7 cells/g (dw) and 3,85 x 10^7 cells/g (d.w), respectively. The total number of cells at the 10^{-3} dilution samples had an average of 1,11 x 10^7 cells/g (d.w), 1,57 x 10^7 cells/g (d.w), and 6,87 x 10^6 cells/g (d.w) at the depths 110 cm, 122 cm, and 135 cm. There was no significant difference between the depths or the dilutions (p = 0,47, p = 0,22 respectively).

For the samples collected in 2019 the bacterial particles could not be properly quantified due to challenges when counting the specimens from the samples. At 100X magnification, the cells were very small and hardly distinguishable from other particles. Due to this, reliable cell counts could not be obtained.

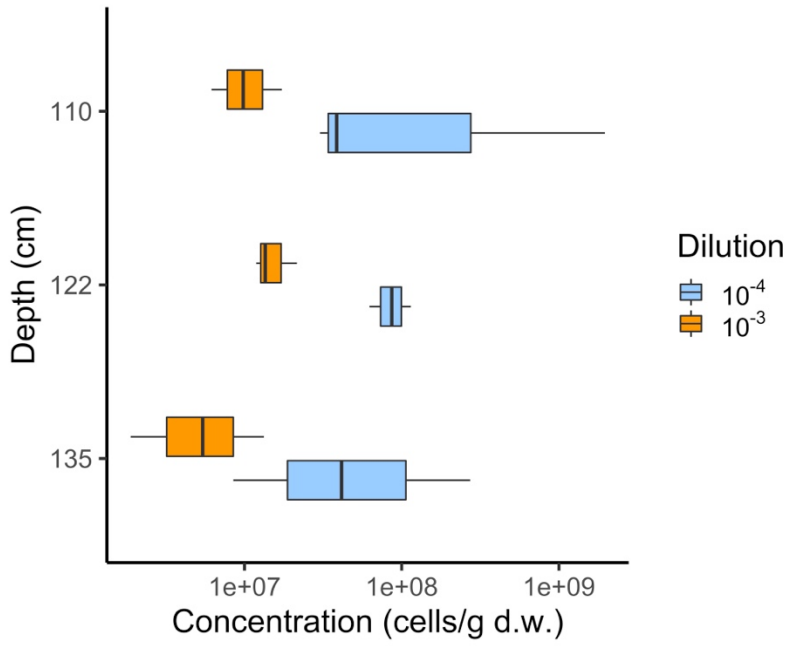

Figure 5: Total numbers from DAPI staining of the core collected in 2016, divided into two categories; 10^{-3} and 10^{-4} dilution. Samples are arranged by the concentration of cells per gram dry weight (d.w) on the x-axis, and in descending order of depth on the y-axis.

3.3 Bacterial colony forming units

Two different temperatures were used for cultivation of isolates on agar plates (R2A) from the 2016 samples, in order to see if there was a difference in both number of CFU and in diversity. One set of samples (the 10^{-2} dilution samples) was placed at 10°C, while another set (the 10^{-3} samples) was left at room temperature (20°C). A negative trend was observed in the descending depths (-6,82 x 10^4), however, there was no significant differences determined between depths or dilutions (p = 0,47) (Figure 6). The number of CFU per gram ranged from zero to 4,10 x 10^5 CFU/g (d.w). The 10^{-4} dilution was not taken into consideration as there were too low colony counts. Two plating attempts were conducted for the 2019 samples, but neither of the attempts yielded any CFU.

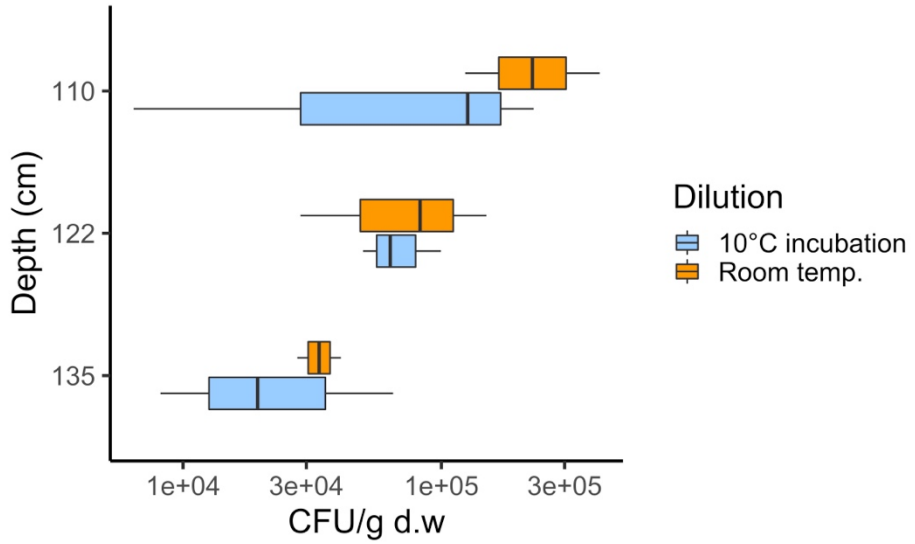

Figure 6: Colony forming units from samples collected in 2016 divided by incubation temperature; 10°C, and room temperature (20°C). Samples are arranged in order of colony forming units per g dry weight (d.w) on the x-axis, and in descending order of depth on the y-axis.

3.4 DNA

DNA extractions from the core taken in 2016 were conducted in triplicates per depth per core. In addition to the first elution a second elution was extracted from the same samples in order to check if all the DNA was extracted in the first elution. Most of the DNA was extracted in the first elution. The average amount of DNA from these samples were 2,50 ± 0,26 ng/g of soil dry weight. The majority of the samples ranged between 1 – 2 ng/g (d.w) DNA, but two samples (core 6 depth 122 cm, and core 7 depth 110 cm) deviates from this with a concentration above 4 ng/g (d.w) (Table 2).

Only one extraction per depth was collected from the 2019 samples. The amount of DNA was lower in the descending depths, with an exception of the sample collected at depth 34,0 – 52,5 cm. The average amount of DNA from these samples were 8,28 ± 2,12 ng/g of soil dry weight. The bottom four samples (below 94,0 cm) ranged between 2 – 5 ng/g (d.w), but the three top samples (above 87,5 cm) all had a concentration above 10 ng/g (d.w) (Table 2).

Table 2: DNA concentrations (ng) per gram of dry weight soil from samples collected in both 2016 and 2019. Only one sample per depth was extracted in 2019. Three samples were extracted per depth per core in 2016, where also a second elution was collected from each sample.

Year	Core	Depth (cm)	DNA concentration (ng/g soil extracted) First elution	DNA concentration (ng/g soil extracted) Second elution
2016	6	110	2,03 ± 0,33	0,23 ± 0,06
2016	6	122	5,08 ± 0,29	0,49 ± 0,07
2016	6	135	1,65 ± 0,13	0,50 ± 0,06
2016	7	110	4,43 ± 0,30	0,61 ± 0,08
2016	7	122	2,16 ± 0,37	0,29 ± 0,02
2016	7	135	1,75 ± 0,08	0,16 ± 0,01
2016	8	110	1,43 ± 0,19	0,22 ± 0,07
2016	8	122	2,52 ± 0,12	0,47 ± 0,02
2016	8	135	1,43 ± 0,10	0,16 ± 0,01
2019	1	34,0 - 52,5	10,18	NA
2019	1	64,5 - 73,5	19,05	NA
2019	1	77,0 - 87,5	13,11	NA
2019	1	94,0 - 98,0	4,56	NA
2019	1	111,0 – 113,0	4,43	NA
2019	1	155,0 - 158,5	3,79	NA
2019	1	202,5 – 208,0	2,85	NA

3.5 PCR

All molecular analysis was based on multiple DNA extractions from each of the samples. DNA from the second elution (2016 samples) was used in the PCR reactions. All samples resulted in positive bands of the 16S rRNA gene (Figure 7). Sample 110 cm from core 6 from the 2016 samples was done twice on the same gel as the first attempt was wrongly placed in the well.

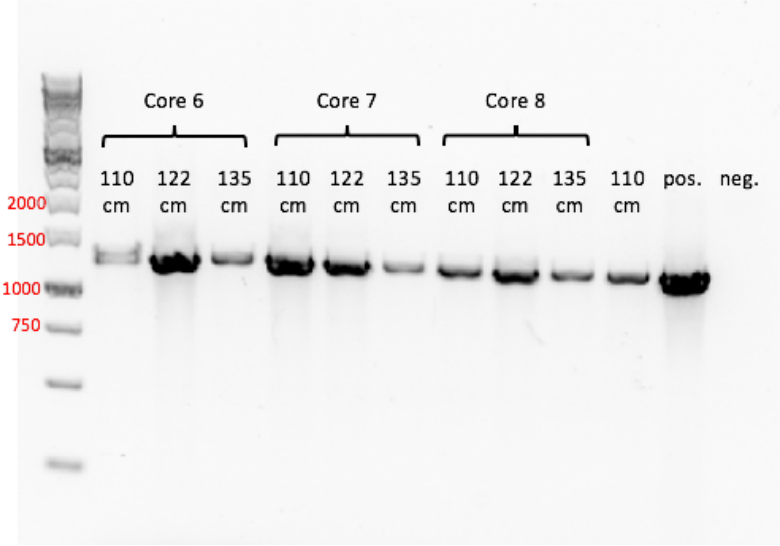

Figure 7: PCR gel results of 16S rRNA gene. Samples from 2016 in descending order in each of the cores. Sample 110 cm from core 6 was done twice.

The majority of samples from 2016 gave visible bands, indicating positive amplification of the fungal ITS region when using an annealing temperature of 55°C. However, sample 135 cm in core 6 did not have any visible bands before the annealing temperature was raised to 56,5°C. When combining the results from both annealing temperatures, it showed that all samples contained the genes for the fungal ITS region. All samples from 2019 had positive amplification of the fungal ITS region with annealing temperature of 55°C (Figure 8).

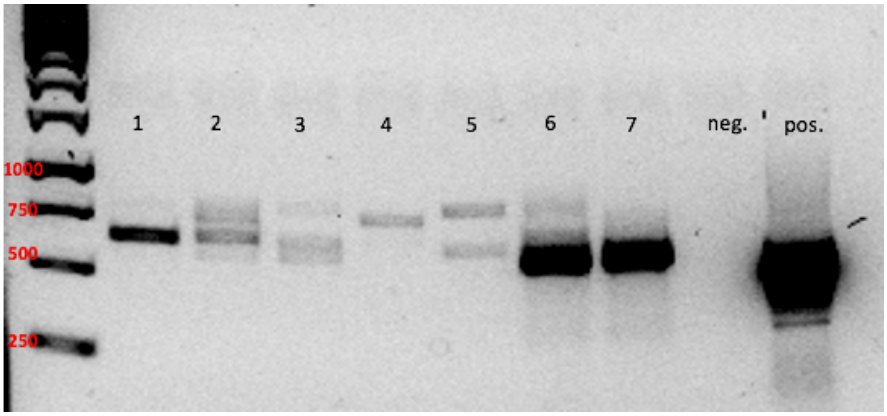

Figure 8: PCR gel results from the 2019 samples of the fungal ITS region. Samples are arranged in descending order (34,0 – 52,5 cm, 64,5 – 73,5 cm, 77,5 – 87,5 cm, 94,0 – 98,0 cm, 111,0 – 113,0 cm, 155,0 – 158,5 cm, and 202,5 – 208,0 cm) down the core. Annealing temperature was at 55°C.

The eukaryotic 18S rRNA gene was present in all samples from 2019 (Figure 9), while for the 2016 samples the gene seemed to be absent at the depths 110 cm and 135 cm in core 6.

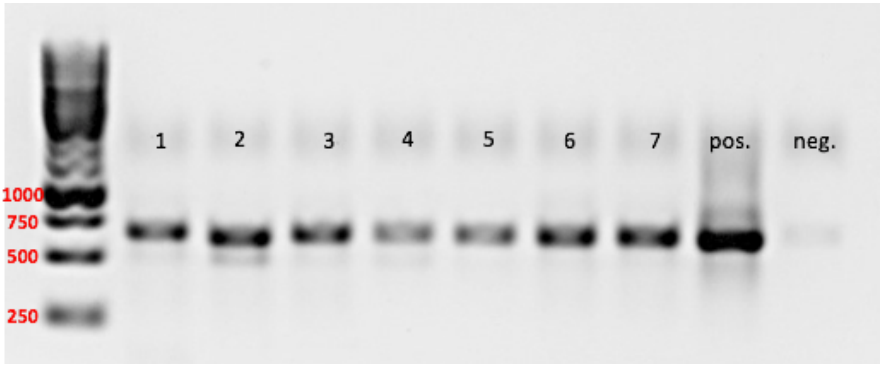

Figure 9: PCR gel results of 18S rRNA gene. Samples from 2019 in descending order (34,0 – 52,5 cm, 64,5 – 73,5 cm, 77,5 – 87,5 cm, 94,0 – 98,0 cm, 111,0 – 113,0 cm, 155,0 – 158,5 cm, and 202,5 – 208,0 cm) down the core.

Positive amplification of the bacterial ammonium oxidizing gene *amoA* was seen in both years. In the 2016 samples only depth 122 cm in core 8 showed a distinct band. However, there were some faint bands present in most of the samples, with an exception of the samples 110 cm in both core 6 and core 7. Most samples from 2019 showed positive amplification of the *amoA* gene, with an exception of the depths 64,5 – 73,5 cm and depth 111,0 – 113,0 cm (sample 2 and 5, respectively in Figure 10). The non-template control (NTC) used in 2019 was *Escherichia coli* which does not contain this gene.

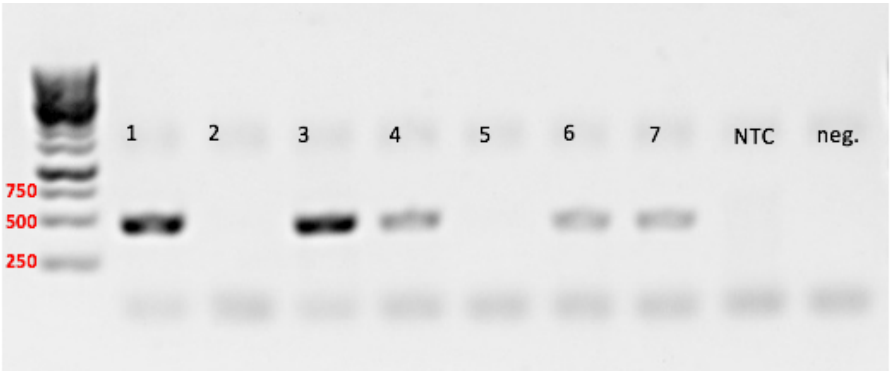

Figure 10: PCR gel results of functional gene *amoA*. Samples from 2019 in descending order (34,0 – 52,5 cm, 64,5 – 73,5 cm, 77,5 – 87,5 cm, 94,0 – 98,0 cm, 111,0 – 113,0 cm, 155,0 – 158,5 cm, and 202,5 – 208,0 cm) down the core.

In regard to the ammonium oxidizing archaea (*camoA* gene) there was an irregular pattern of positive bands at the various depths (Figure 11A). All depths had positive bands in core 7 from the 2016 samples, whereas, in core 6 only depth 135 cm and in core 8 depth 122 cm contained positive amplification on the gel (Figure 10A). Only the lowest depth (202,5 –

208,0 cm, sample 7 in Figure 11B) from the 2019 core had a distinct band on the gel. A faint band was shown on the gel at depth 111,0 – 113,0 cm (sample 5 in Figure 11B).

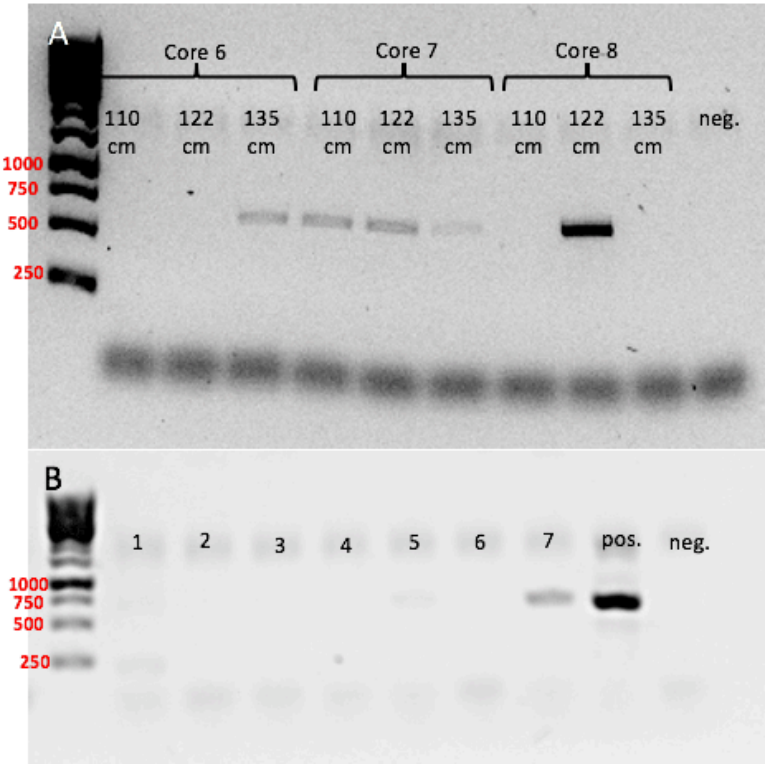

Figure 11: PCR gel results of functional gene *camoA*. A. Samples from 2016 in descending order in each of the cores. B. Samples from 2019 in descending order (34,0 – 52,5 cm, 64,5 – 73,5 cm, 77,5 – 87,5 cm, 94,0 – 98,0 cm, 111,0 – 113,0 cm, 155,0 – 158,5 cm, and 202,5 – 208,0 cm) down the core.

Multiple bands per column showed up on the gel with the primers for methane oxidizing bacteria (*pmoA*) in the 2016 samples. However, at the 500 bp mark of the ladder, there is a distinct pattern of which depths showed positive amplification; for all the cores, there were no bands present at depth 110 cm, while for the depths 122 and 135 cm positive amplification of the gene was seen in the gel. For the 2019 samples bands were seen in every depth with an exception of 64,5 – 73,5 cm (sample 2 in Figure 12). Nevertheless, there looks to be a smear throughout the columns, and a few faint bands at a higher base pair position in these samples also (Figure 12).

Figure 12: PCR gel results of functional gene *pmoA*. A. Samples from 2016 in descending order in each of the cores. B. Samples from 2019 in descending order (34,0 – 52,5 cm, 64,5 – 73,5 cm, 77,5 – 87,5 cm, 94,0 – 98,0 cm, 111,0 – 113,0 cm, 155,0 – 158,5 cm, and 202,5 – 208,0 cm) down the core.

None of the samples contained methane generating archaea, with the use of the primers for methane generation (*mcrA*).

We did not observe any differences in the depths using these functional genes.

3.5 Microbial abundance determined by quantitative Real-Time PCR (qPCR)

Abundance of bacterial genes throughout the soil core was determined by using primers targeting the 16S rRNA gene using qPCR. The overall trend showed that copy numbers were quite evenly distributed. The average abundance of genes for both bacteria and fungi were higher at 122 cm depth, and lowest at 135 cm depth. The presence of bacterial genes in the samples collected in 2016 were on average at the three different depths (110 cm, 122 cm, and 135 cm) 1,13 x 10^6 copies/g (d.w), 1,36 x 10^6 copies/g (d.w), and 6,98 x 10^5 copies/g (d.w), respectively. There was no significant correlation between abundance of bacterial genes and depth ($p = 0,39$). In the same respective samples, the abundance of fungi was 7,67 x 10^2 copies/g (d.w), 5,96 x 10^2 copies/g (d.w), and 3,19 x 10^2 copies/g (d.w). No significance was found between the abundance of fungal genes and depth ($p = 0,49$) (Figure 13A). Though quite evenly distributed, the samples from 2019 showed a slight decrease in the number of 16S rRNA gene copies through descending depths. These samples had a range from 2,23 x 10^6 copies/g (d.w) to 3,71 x 10^7 copies/g (d.w), where the overall average of the whole core was 1,56 x 10^7 copies/g (d.w) (Figure 13B). The number of fungal genes based on the qPCR number, was between 10^3 and 10^4 times lower than the bacterial 16S rRNA gene copy number. The presence of fungal genes ranged from 3,27 x 10^2 copies/g (d.w) to 1,64 x

10^4 copies/g (d.w) (Figure 13B). Supplementary information about the quality of the qPCR for both bacteria and fungi can be found in the Appendix.

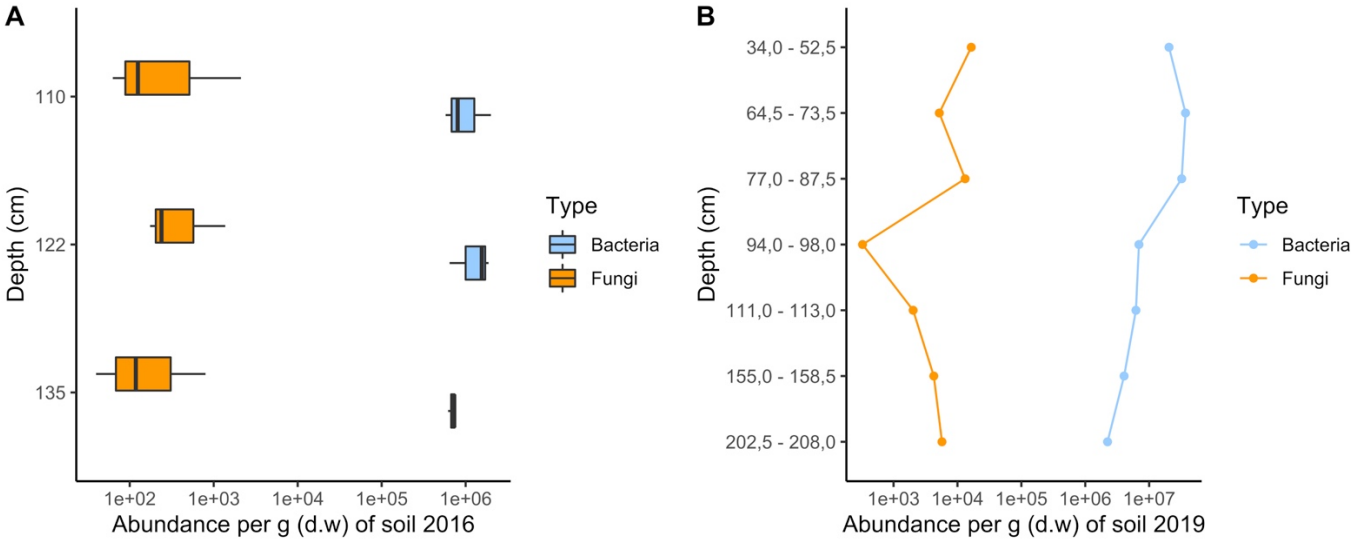

Figure 13: Bacterial and fungal gene abundance (per gram of dry weight (d.w)) from samples collected in 2016 (A) and in 2019 (B) arranged by abundance of genes present per gram of dry weight soil in log scale on the y-axis and in descending order of depth on the x-axis. Because of sampling design, samples from 2016 are represented by a boxplot (they were taken in triplicate per depth), while samples from 2019 only one sample per depth was collected and is therefore represented in a line plot.

3.6 Genetic diversity

3.6.1 Closest relatives

Nineteen bacterial isolates were selected based on colony morphology, with the attempt to produce a sequence from all the different culturable bacteria found in the two incubation temperatures (room temperature and 10°C incubation). The first eight isolates were obtained from the plates incubated at room temperature, where most had a closest match in BLASTn to a *Cellulomonas sp*. (Table 3). Isolates from 10°C incubation had a greater variability in species, though all species either belonged to the phylum Actinobacterium or Proteobacteria.

Fourteen fungal isolates were chosen, also based on morphology of the culture. All isolated species belonged to the phylum Basidiomycota, where the majority had a closest match to *Sistotrema sp*. within the UNITE database (Nilsson *et al.*, 2019; Table 4) and are categorised as wood decaying fungi. According to the Global Biodiversity Information Facility

(https://www.gbif.org) all species have been recorded in Norway previously, while none of them have previously been found in Svalbard.

Table 3: List of bacterial isolates (OTU) recovered form permafrost soils collected in 2016, their closest BLASTn hits, accession numbers, number of matching bases, and the habitat of where the closest hits have been found.

OTU	Blast hit	Identity	Accession nr	# bases	Habitat
1	Arthrobacter sp. Strain AQ5-06	100%	KX946127.1	1746/1746	Antarctic soil
2	Cellulomonas cellasea strain aBf15	100%	KJ194899.1	1633/1633	NA
3	Cellulomonas sp. KAR1	100%	EF451631.1	1753/1753	Permafrost
4	Cellulomonas sp. KAR4	100%	EF451634.1	1792/1792	Permafost
5	Cellulomonas sp. KAR4	100%	EF451634.1	1825/1825	Permafrost
6	Subtercola frigoramans strain QGC-5	100%	MK246112.1	1749/1749	Cryoconites
7	Cellulomonas sp. KAR4	100%	EF451634.1	1784/1784	Permafrost
8	Sphingomonas sp. BF14	100%	Z23157.1	1849/1849	NA
9	Mycobacterium sp. MN7-2	100%	JQ396585.1	1784/1784	Arctic rhizophere
10	Friedmanniella Antarctica strain AA-1042	100%	NR_026536.1	1712/1712	NA
11	Methylobacterium sp. Strain Alpha-35	100%	MH686087.1	1836/1836	Soil
12	Polaromonas sp. Strain M2-7	100%	MG757996.1	1851/1851	Moonmilk from Szczelina Chocholowska cave
13	Frondihabitans sp. A2-573	100%	KF441616.1	1783/1783	Urgeirica mine, water and sediments
14	Sphingomonas faeni	99%	JF970600.1	1832/1832	Ice core samples from the continental ice sheet, depth 10,5 m
15	Rhodoferax sp. KAR16	99%	EF451646.1	1875/1875	Permafrost
16	Sphingomonas sp. V29 gene	100%	AB534590.1	1875/1875	Grassland soil
17	Salinibacterium sp. TP-Snow-C10	100%	HQ327119.1	1820/1820	Tibetan Plateau
18	Glaciihabitans sp. strain CHu50b-6-2	100%	MF770244.1	1858/1858	Sediments
19	Sphingomonas mali gene, strain GM289	99%	AB740933.1	1844/1844	Grassland soil

Table 4: List of fungal isolates (OTU) recovered form permafrost soils collected in 2016, their closest UNITE hits, accession number, and their trait.

OTU	UNITE hit	Identity	Accession nr	Trait
1	Sistotrema brinkmannii	100%	JX535041	Decay wood
2	Sistotrema brinkmannii	100%	JX535041	Decay wood
3	Sistotrema brinkmannii	100%	LK052763	Decay wood
4	Sistotrema oblongisporum	99%	KF218970	Decay wood
5	Sistotrema brinkmannii	100%	LK052763	Decay wood
6	Peniophora incarnata	99%	MH860518	Decay wood
7	Thanatephorus cucumeris	99%	MK460832	Root pathogen
8	Sistotrema brinkmannii	100%	MN430946	Decay wood
9	Sistotrema oblongisporum	99%	KF218970	Decay wood
10	Chondrostereum purpureum	99%	UDB0754237	Tree pathogen
11	Thanatephorus cucumeris	99%	MK460832	Root pathogen
12	Sistotrema brinkmannii	99%	JX535041	Decay wood
13	Sistotrema brinkmannii	100%	MF511073	Decay wood
14	Sistotrema sernanderi	100%	UDB038251	Decay wood

3.6.2 Phylogenetic tree

A phylogenetic tree was produced in order to analyse the genetic distances between the colonies and their closest related sequences. A maximum likelihood tree was constructed for both bacterial (Figure 14) and fungal (Figure 15) sequences.

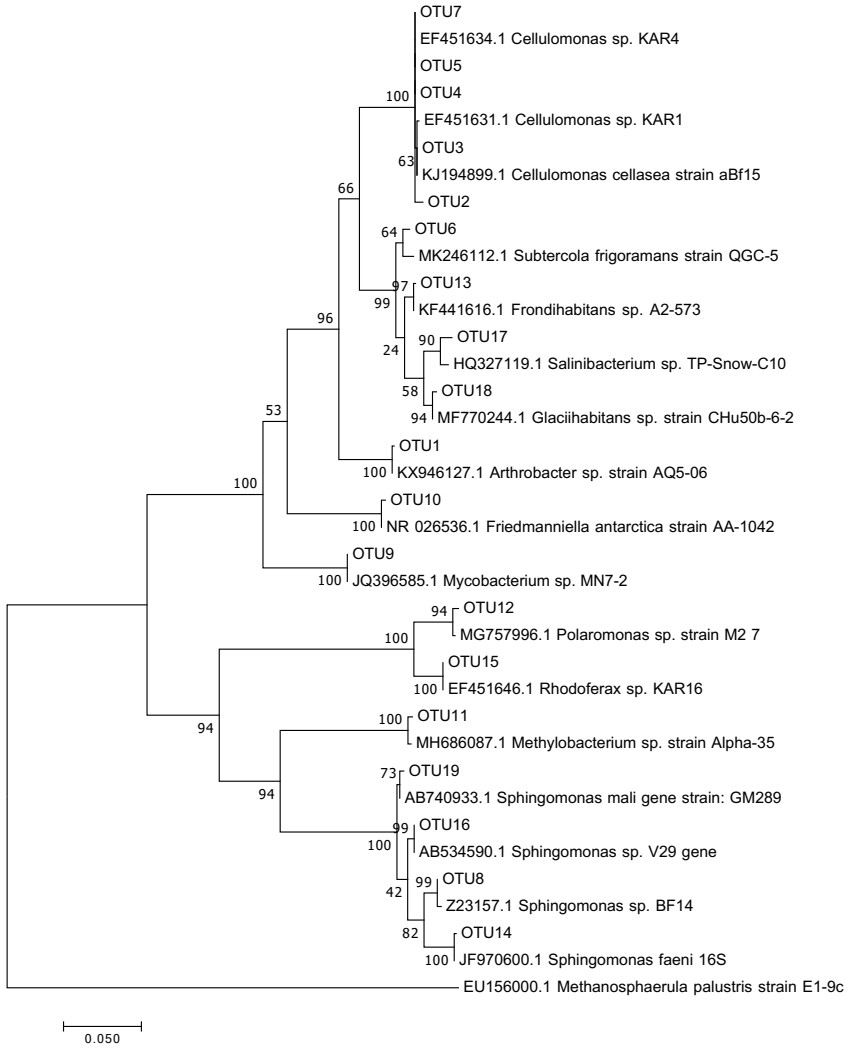

Figure 14: Maximum-likelihood phylogenetic tree of bacterial 16S rRNA gene. Analysis is based on 37 sequences and includes operational taxonomic units (OTUs) (≥99% nucleotide identity), their closest BLAST hits marked with accession number and species name, and an Archaea to achieve a more robust tree. The tree was built using the Tamura-Nei model (Tamura and Nei, 1993) with 500 bootstrap replications. Percentage of bootstrap is represented in percentage besides the nodes for the species.

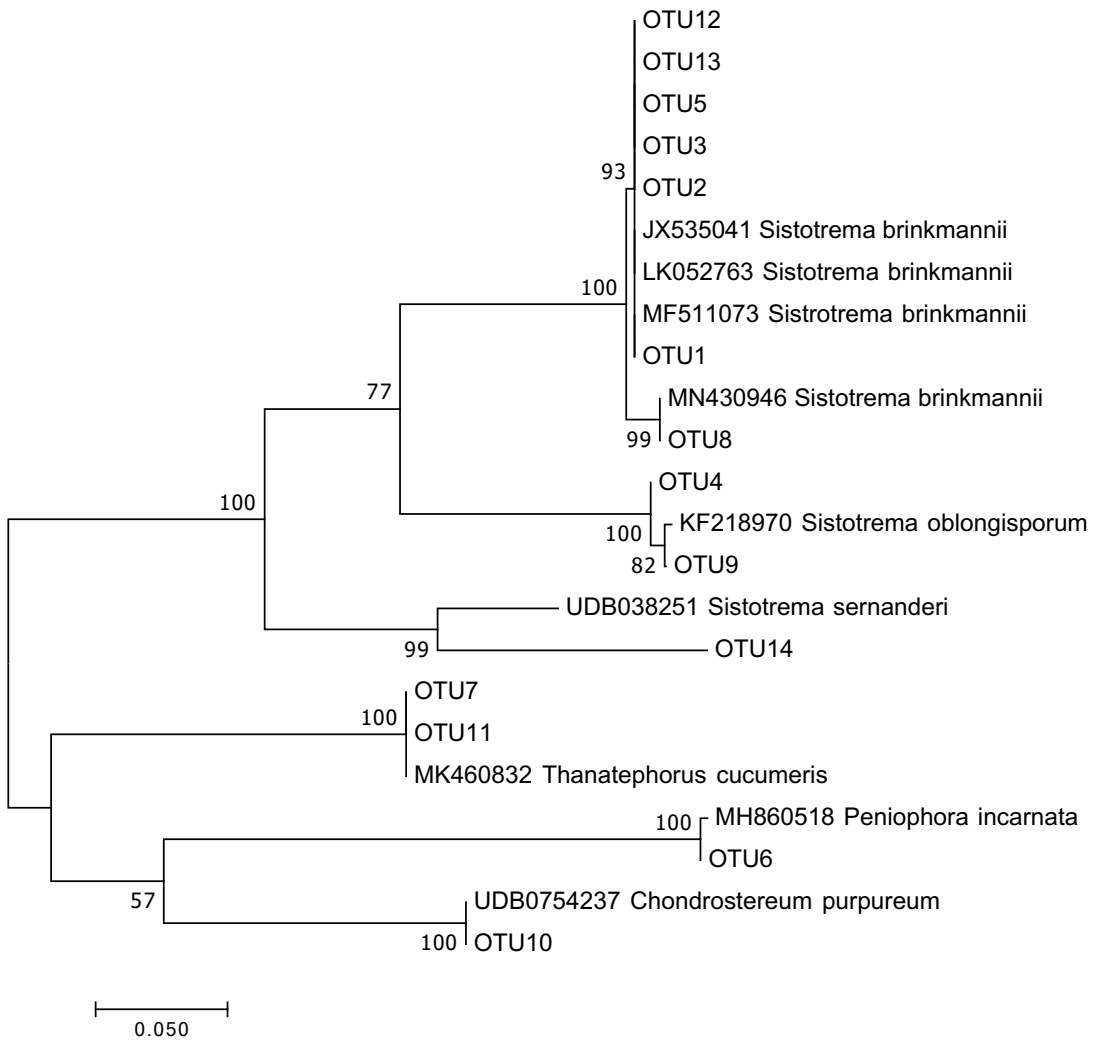

Figure 15: Maximum-likelihood phylogenetic tree of fungal ITS2 region. Analysis is based on 23 sequences and includes operational taxonomic units (OTUs) (>99% nucleotide identity), and their closest BLAST hits marked with accession number and species name. The tree was built using the Tamura-Nei model (Tamura and Nei, 1993) with 500 bootstrap replications. Percentage of bootstrap is represented in percentage besides the nodes for the species.

4 Discussion

4.1 Discussion of methods applied in the thesis

The sampling design of the cores collected in 2016 and 2019 was quite different. The samples from 2019 were collected at several depths at frequent intervals from the active layer to the permafrost itself, while only three specific depths within the permafrost were examined in 2016. Among these three depths only one overlapped with the 2019 sampling depths (110 cm and 111- 114 cm, respectively), which means it is not possible to directly compare the results recovered from 2016 and 2019 cores in respect to the exact sampling depths. As a result of this, comparison would be unprecise and not definitive. Therefore, the two sampling years are discussed separately and only compared when reasonable.

The exact position of where the active layer ends and the permafrost starts can be challenging to define. Nonetheless, looking at the soil characteristics from 2019 there is a definite change at depth 94 – 98 cm in both organic matter and pH values, which could indicate that the permafrost begins there. This also correlates with another study from Adventdalen, in which they also found that the active layer and transition layer transitions over to permafrost at about 90 cm depth (Müller *et al.*, 2018). The samples collected from a depth of 94 cm and deeper from 2019 are therefore considered as permafrost, and the overlying samples the active layer.

During DNA extraction it is expected that some of the DNA is lost, though the kit used in this study (DNeasy® PowerSoil® Kit) has been found to be the best for extracting DNA from low biomass samples (Vishnivetskaya *et al.*, 2014). By doing a second elution of the DNA extracted we found that on average almost 15% of the DNA was not eluted in the first step. A second elution of DNA is not commonly performed; however, due to the overall low abundance of DNA in permafrost, and the issue concerning trapped DNA on the filter, a second elution revealed in our study to be a simple way of collecting more DNA from the sample.

The primer pair used for detecting eukaryotic microorganisms (18S rRNA gene), has been confirmed by a study to be one of the best universal eukaryotic primers, despite that this

primer region only covers ~80% of all the tested eukaryotic sequences (Hadziavdic *et al.*, 2014). One genus of eukaryotes that was not covered by this primer pair was fungal (Hadziavdic *et al.*, 2014). We confirmed that this 18S primer pair indeed does not detect all fungal genes, as not all samples from this study had positive amplification of the 18S rRNA genes, however, with a specific fungal primer pair we detected fungi in all samples. This indicate the need to use organism specific primers in order to recover the total diversity within permafrost samples.

Counting total cell numbers using fluorescent staining from permafrost samples can be somewhat challenging. In this study the cells were very aggregated and not homogenously distributed whilst observing in the epifluorescence microscope. Observation of big lumps of cells were therefore highly visible and hard to avoid when counting. An overestimation of how many cells were present in these samples is therefore likely. In order for this method to be statistically reliable, improvements are essential. For instance, by adding a solvent that can separate the aggregated cells to enhance the accuracy of cell counting.

4.2 Microbial community responses

4.2.1 Total numbers and microbial abundance

Multiple approaches were used to determine the abundance of microorganisms in both 2016 and 2019 samples. Early studies of permafrost were highly culture-dependent, where direct total count of colony forming units (CFU) were performed in order to find the abundance of microorganisms (Shi *et al.*, 1997; Vorobyova *et al.*, 1997; Vishnivetskaya *et al.*, 2000). This method is considered very restrictive due to the well-established fact that only approximately 1% of bacteria can be cultivated *in vitro*. This is referred to as the "great plate count anomaly", which is based on observations in where microscopic counts are considerably higher than the total viable count (Staley and Konopka, 1985; Hugenholtz, Goebel and Pace, 1998; Ferrari, Binnerup and Gillings, 2005). An additional method to measure abundance and thereby find total numbers was by fluorescent microscopy using a DNA stain to count cells (Vorobyova *et al.*, 1997). Measuring the abundance of a gene with quantitative polymerase chain reaction (qPCR) is also a method used for finding the abundance of microorganisms. This method has a disadvantage in that there can be multiple copies of the same gene within the genome. One example of this is that there is in average

4,2 16S rRNA genes in one bacterial genome (Větrovský and Baldrian, 2013). Although all methods have their strengths and weaknesses, by combining them in one sample it can provide a better overview of the microbial abundance in the samples.

Total numbers

Previous studies from Ny-Ålesund, Svalbard reported an average of 4,01 x 10^5 cells/g soil (Singh *et al.*, 2017) by epifluorescence microscopy, while in the Siberian Arctic the total cell count ranged from 10^3 to 10^8 cells/g soil (Vishnivetskaya *et al.*, 2000), and in the Canadian High Arctic there were an average of 10^7 cells/g soil (Steven *et al.*, 2008). In my study the average bacterial count was within the same range as the bacterial count found in the Siberian Arctic and in the Canadian Arctic. This was, however, only true in the lowest diluted (10^{-3}) sample. The reason both dilutions (10^{-3} and 10^{-4}) were included in my results was due to the large differences between them, even though the two dilutions theoretically should have been equivalent after conversion. The highest dilution (10^{-4}) showed a higher number of cells, more aggregated cells, and greater variation within depths, compared to the lower dilution (10^{-3}). By comparing the total numbers from epifluorescence microscopy with the abundance of bacterial 16S rRNA genes from qPCR, we found that the gene abundance accounted for ~9% of the total numbers counted, which is rather low. Due to the uncertainties and challenges experienced using this method for counting bacterial cells, other methods can potentially have a higher accuracy towards the true value of bacterial numbers, or methodological improvements for distributing the cells should have been done.

Colony forming units

It has been suggested that an increase in the age of the permafrost decreases the number of viable bacteria (Steven *et al.*, 2006). This trend was detected in a study from Ny-Ålesund, Svalbard (Singh *et al.*, 2017). Our study also supports this theory, as the number of colony forming units decreased with descending depths in both incubation temperatures (10^{o}C and room temperature). Previous studies have reported that on average 0,1 – 10% of the microbial community was able to be recovered by standard culturing (Vorobyova *et al.*, 1997; Steven, Niederberger and Whyte, 2009). By comparing our measured abundance of bacterial 16S rRNA genes (qPCR) with the bacterial CFU (plate count), we discovered that 6 – 8% of the bacterial community could be cultured, which falls within the range detected of

previous studies. Another observation from the culture experiment revealed that lower temperatures gave greater diversity, though the average percentage of viable cells was lower. At higher incubation temperatures we observed a selection towards a smaller group of bacteria, limiting the diversity, suggesting that there is a high diversity among the psychrophilic bacteria, compared to psychrotolerant or even mesophilic bacteria in these permafrost samples.

qPCR

Detection of higher amounts of bacterial genes compared to fungal genes in permafrost has been reported by using qPCR (Waldrop *et al.*, 2010; Yergeau *et al.*, 2010; Jansson and Taş, 2014). In the High Canadian Arctic the fungal gene abundance accounted for only 0,6% when comparing the abundance of bacteria and fungi (Yergeau *et al.*, 2010). Although this is a very low percentage, in our study we found that the fungal abundance was even lower and accounted for only a tenfold (0,06%) of the genes detected compared to what was found in the Canadian permafrost. Why the fungal gene abundance was so low in our study is difficult to say, as a direct comparison between the gene abundance between bacteria and fungi is to my knowledge highly understudied.

The abundance of the genes detected within permafrost is lower compared to the active layer. In the High Canadian Arctic, there was 95% lower bacterial abundance and 90% lower fungal abundance in the permafrost (Yergeau *et al.*, 2010). Though quite similar, in the Alaskan permafrost the bacterial abundance was 70 – 90% lower and the fungal abundance was 66% lower in the permafrost (Waldrop *et al.*, 2010). The abundance of genes detected in this study is more similar to what has been found in the Alaskan permafrost, where here the bacterial abundance was 84% lower and the fungal abundance was 73% lower in the permafrost compared to the active layer of the 2019 samples. This difference in gene abundance is likely caused by the temperature and liquid water availability between the active layer and the permafrost (Hinkel and Nelson, 2003).

4.2.2 Microbial diversity

Microbial diversity in permafrost was culture-dependent in earlier studies, when the methodologies using DNA identifications were more limited (Shi *et al.*, 1997; Vorobyova *et al.*, 1997; Vishnivetskaya *et al.*, 2000). Using culture-independent methods, such as examining the diversity of genes by DNA based molecular analysis such as PCR, metabarcoding, and metagenomics, have proven in the later years to tell us more about the diversities within each sample, and subsequently the biodiversity within ecosystems (Yergeau *et al.*, 2010; Müller *et al.*, 2018; Xue *et al.*, 2020).

Sequencing

Previous studies have detected that the most common phyla to be found in the Arctic permafrost are Actinobacteria, Proteobacteria, Firmicutes, Chloroflexi, Acidobacteria, and Bacteriodetes, in addition to other uncharacterized and novel phyla (Steven *et al.*, 2007; Yergeau *et al.*, 2010; Wilhelm *et al.*, 2012; Taş *et al.*, 2014). Where Actinobacteria have been discovered to account for up to 80% of the entire community in permafrost collected from Adventdalen, Svalbard (Müller *et al.*, 2018). This high abundance of this phylum was also uncovered in this study, where about 70% of the isolates belonged to Actinobacteria, while the rest of the isolates were Proteobacteria. The members of Actinobacteria have been recognised to contribute to the degradation of plant biomass in soil, together with other microorganisms such as fungi (De Boer *et al.*, 2005; Lewin *et al.*, 2016). It has also been found that Actinobacteria are the primary contributors to cellulose decomposition in Arctic peat soil (Tveit, Urich and Svenning, 2014). Hypotheses have been made that members of Actinobacteria are globally successful in permafrost environments due to their adaptations to low carbon availability and energy sources, which explains their ubiquitous distribution in these habitats (Hansen *et al.*, 2007; Steven *et al.*, 2008; Yergeau *et al.*, 2010; Yang *et al.*, 2012; Gittel, Bárta, Kohoutová, Schnecker, *et al.*, 2014).

The most common fungal phyla found in Arctic permafrost are Ascomycota and Basidiomycota (Kochkina *et al.*, 2001; Gittel, Bárta, Kohoutová, Mikutta, *et al.*, 2014; Zhang *et al.*, 2016; Inglese *et al.*, 2017). Though there is usually found more than one phylum in the permafrost samples, all fungal isolates in this study were members of the Basidiomycota phyla, in which 70% of them were *Sistotrema sp*. The most common species found,

Sistotrema brinkmannii, has thus far not been isolated from Svalbard before, but has been isolated from Antarctic soils (Hao *et al.*, 2010). The low fungal diversity can be explained by the low number of colonies isolated. However, the colonies chosen for sequencing were based on morphology, in order to provide a good overview of the fungal diversity found in permafrost. The outcome of such a low diversity was therefore not expected. The great morphological and genetic variation of *Sistotrema brinkmannii* (Hao *et al.*, 2010) made it further quite difficult to distinguish them from other fungi with similar morphology.

4.2.3 Functions

In order to get insight into the microbial diversity found in the samples collected, a series of bacterial and fungal specific genes, and bacterial and archaeal functional genes were examined. No distinct pattern was found regarding the functional gene profiles, with an exception of a higher number of amplified genes in the lower depths (122 cm and 135 cm) within the 2016 samples (Figure 16). A summary of these data is presented in Figure 16. Genes for bacterial and archaeal ammonium oxidizing (*amoA* and *camoA*), and methane oxidising bacteria (*pmoA*) have previously been detected in the Arctic (Yergeau *et al.*, 2010; Jansson and Taş, 2014; Taş *et al.*, 2014). Using metagenomic data ammonium oxidisers were only observed in the active layer and no methane oxidizers, however through sequencing and qPCR they determined that ammonium oxidizing bacteria and archaea, and methane oxidising bacteria were present in the permafrost in the High Canadian Arctic (Yergeau *et al.*, 2010). Yergeau et al. (2010) demonstrated that ammonium oxidizing archaea dominated the active layer, while the dominance was lower in the permafrost. The same genes were detected in this study, although, it seems to be the opposite results in regard to the ammonium oxidisers in the 2019 core, where *amoA* was found in almost all depths and the *camoA* were only observed in the deepest layer of the 2019 core. While no distinct pattern was found regarding the *amoA* and *camoA* genes, the *pmoA* gene was detected only at the two lower depths (122 cm and 135 cm) in the 2016 samples (Figure 16).

Previous studies have found that there was little to no genes present in permafrost for methane production (*mcrA*) in the Arctic (Steven *et al.*, 2007; Waldrop *et al.*, 2010; Yergeau *et al.*, 2010; Taş *et al.*, 2014). This coincides with our findings, as none of our samples gave any positive amplification of the *mcrA* gene. Other studies have found that methane

production may be present, but it is highly variable in the permafrost layers and zones where these are detected (Rivkina *et al.*, 2007). The reason for these observed variations is yet not understood (Waldrop *et al.*, 2010).

Some of the genes (including functional genes) showed up as multiple bands on the electrophoreses gel (e.g. Figure 8). One possible explanation for this is that there can be differences in gene length depending on species as a result of insertions and deletions. Since we ran the PCR using environmental samples, multiple species could have been detected. Another possible reason is that certain primers have low specificity, and can pick up multiple variations of the gene sequence and/or non-targeted genes (Holmes *et al.*, 1995). For instance, both the ITS region of fungi and the gene for methanotrophy in bacteria can in different closely related species exhibit multiple gene lengths (Bourne, McDonald and Murrell, 2001; Aanen, Kuyper and Hoekstra, 2001; Brandon Matheny *et al.*, 2007; Vialle *et al.*, 2009). Additionally, the primer pair used for targeting methanotrophic bacteria (*pmoA*) also picks up the gene for ammonium oxidising bacteria (*amoA*) (Holmes *et al.*, 1995).

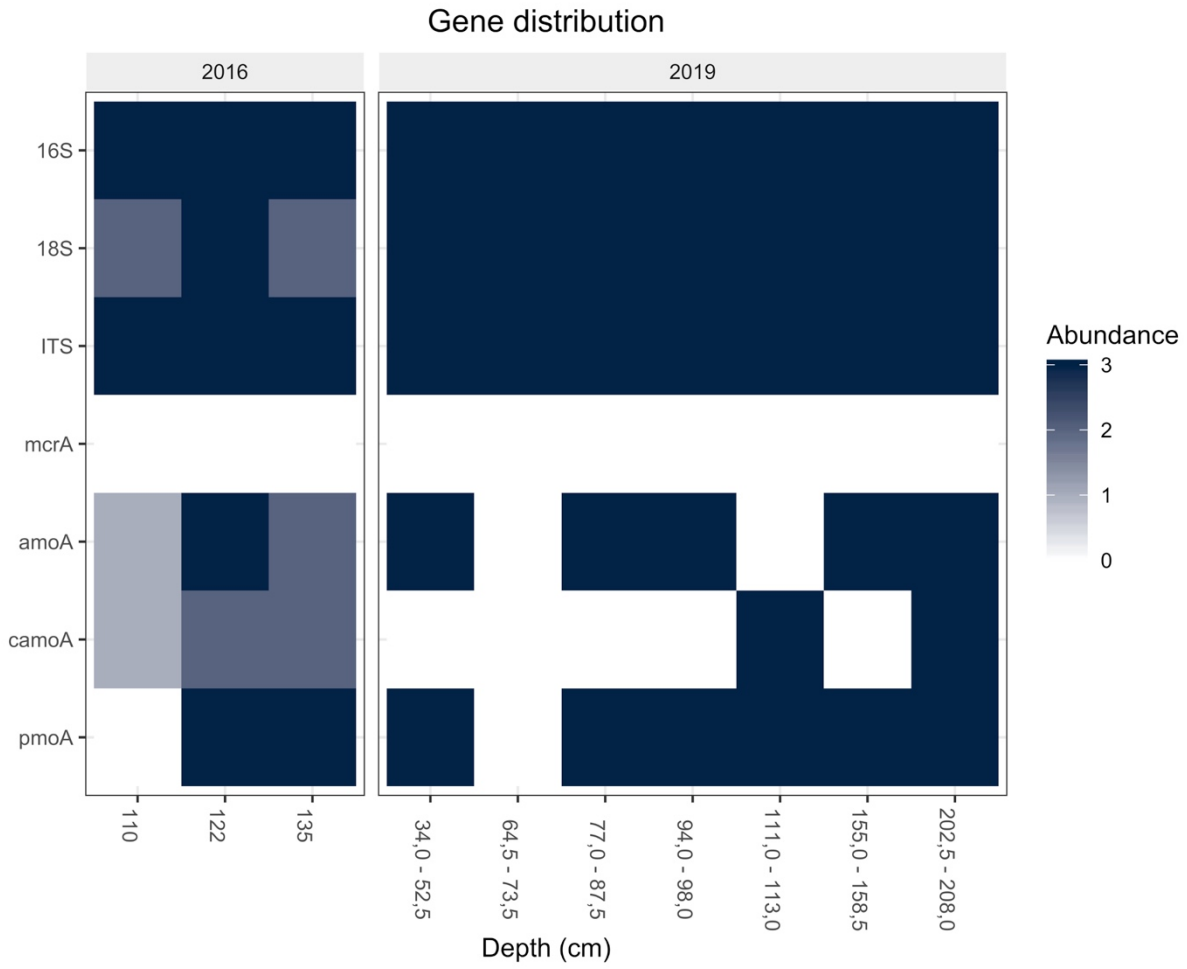

Figure 16: Heatmap of the permafrost core soil samples 2016, and 2019 samples arranged binary by present/absent bands of each of the PCR products gel bands. Samples are arranged in order of depth within each core on the x-axis and the genes tested for amplification are on the y-axis. Dark shade represents a band for the respective amplified gene product, while light shade represents no band for the respective amplified gene.

4.3 Implications of soil characteristics and climate

4.3.1 Soil characteristics

A distinct pattern was found in the soil characteristics from the 2016 samples, where all characteristics were higher at depth 122 cm (Figure 4A-C). This corresponds with the increased number of both bacterial and fungal gene abundances (Figure 13A). These results support the hypothesis of the microorganism's dependency on water and organic matter in their habitat. An increase of 1-2% in moisture can lead to a large difference as 93-99% of the water in permafrost consist as ice (Rivkina *et al.*, 2004), which increases the likelihood to see a difference in the microbial abundance with only a slight increase in moisture. Only a very

thin film of water is sufficient for microbial cells to get enough nutrients and deposit metabolic waste through simple diffusion mechanisms (Rivkina *et al.*, 2000). Whether or not the water was available as liquid water or as ice is unknown, but due to the increase in abundance we can argue that this specific depth likely contained more liquid water compared with the other two depths.

Another interesting observation from the 2016 cores was that the pH was slightly acidic. A low pH environment like this may indicate that the microbial community consisted of a significant number of decomposers, as the process of decomposing plant litter tends to lower the pH in the soil (Torsvik and Øvreås, 2008). This assumption was further strengthened by the high percentage of Actinobacteria isolated, where the members of this phyla are recognised as decomposers of different carbon compounds (Yergeau *et al.*, 2010).

The soil characteristics for the 2019 samples did not show any trend and could not be linked to neither the abundance of bacterial or fungal genes measured with qPCR nor the DNA concentration. However, we observed that the water content was significantly higher in the descending depths ($p < 0,05$), where the reason for this could have been due to being sampled at the edge of a polygon, where ice wedges are likely to find (Lachenbruch, 1962).

4.3.2 Climate

Comparing the permafrost samples of the two years showed some changes both in regards of the microbial abundance, and the soil characteristics. With regards to the microbial abundance, we found that the abundance of bacterial 16S rRNA genes increased by 358% (quadrupled), and the fungal gene abundance had an increase by 450% (quintupled) from 2016 to 2019. This is an enormous increase in such a short climatic time frame, which could be explained by the average moisture content increase of 50%, and the average moisture content increase of 19%. However, if this large change had to do with the slightly different collection locations or because the 2019 samples were almost one meter deeper than the 2016 samples and there in theory should be a higher ice content in the lower depths (Tarnocai, 2009) is unknown. By comparing the two samples from both years that were closest in depth (110 cm from 2016 and 111 – 113 cm from 2019) there was found that the number of bacterial genes quintupled (451%) and the fungal genes almost tripled (164%).

Even though the soil characteristics at this depth in theory should have been the same, we observed an increase of 40% in moisture content and a 13% increase in organic matter content. These findings support the normal biological response, where a decrease in stress factors will improve the microbial living conditions as they have been found to reproduce.

Whether the shift in microbial diversity observed within these samples is due to climate change are difficult to determine. All bacterial, eukaryotic, fungal genes, and functional genes tested were present in the permafrost samples with the exception of the gene for methanogenesis (*mcrA*). However, it would not be possible to determine if the function of the microbial community has changed with time and space due to the limited depths measured in the 2016 samples. Another concern regarding the functional groups, by just basing the gene diversity on the use of PCR it does not give any indication about whether or not the genes are actively expressed or not. Because of the sub-zero temperatures found in permafrost there is a good chance that some of the genes belong to non-living microorganisms (Mackelprang *et al.*, 2016). Microbial genes dating back about 3 million years have been detected in the Arctic permafrost, and under Antarctic climate conditions it has been estimated that there might be a factor of ten years older microbial genes found (Gilichinsky, 2002).

Even though the genes detected might not have been actively expressed at the sampling time, an increase in temperature might change this. We know there are big reservoirs of carbon (including methane) in permafrost (Mackelprang *et al.*, 2011), which through aerobic methane oxidation can be released as carbon dioxide to the atmosphere. Both the genes for methane oxidation and members of Proteobacteria were detected in these samples, where Proteobacteria are considered to be major sinks for methane in terrestrial habitats (Liebner and Wagner, 2007). A high proportion of our samples had genes for methane oxidation, indicating that this gene is widespread and important for this environment. Increasing temperatures in permafrost can therefore escalate the rate of carbon dioxide emissions, which could lead to further increases in global temperatures.

Many members of the dominating phyla, Actinobacteria, are recognised as degraders of different carbon compounds such as chitin, cellulose, and other complex carbon compounds.

Their role in the carbon cycle and turnover of organic material in the permafrost is therefore central (Yergeau *et al.*, 2010). The high percentage of Actinobacteria found in these permafrost samples could therefore indicate that thawing at this site could, after aerobic degradation of carbon soil, lead to carbon dioxide emissions.

Though research in recent years has provided more information about the microbial communities and their function in permafrost, there are still many uncertainties to be uncovered. The microorganisms inhabiting permafrost may be important contributors to global temperature increase through the transformation of soil carbon, as observed in this study, but to what extent is still uncertain. Gaining a better overview of how the indigenous taxa respond to environmental changes is essential for predicting how the microbial community in Arctic permafrost will further respond to global temperature change.

Conclusion

Using data from two different sampling years with a three-year gap, we have found that thawing permafrost affects the inhabiting microbial community. We measured that the living conditions of the microbes had improved, where both the amount of moisture and organic matter had increased. These environmental changes are likely the reason for the increase in both the amount of bacterial and fungal gene abundance. However, a clear change in presence of the selected bacterial, fungal, and functional genes within the microbial community could not be determined based on these samples. There was a low microbial diversity in these samples, where many of the microbes detected are recognised to be well adapted to low carbon availability and energy sources that are found in permafrost. In addition to this, the majority of the microorganisms discovered are recognised as degraders of different carbon compounds, meaning it is likely that high amounts of carbon dioxide will be released as a consequence of the further increase in global temperatures. Despite the difference in sampling design, it does not change the characteristics of the microorganisms discovered, and we can thus conclude that there is a possibility of increased greenhouse gas release, mostly as CO_2 when the microbial living conditions are improved.

Future work

Due to the issue of cell aggregation when using the DAPI staining method, questions the reliability and accuracy of this methods and furthermore indicate that we should be sceptical to other studies where this method is used. Aggregation or a non-uniform spread of the cells could lead to either over- or underestimations of the cell count. Additionally, the human factor adds a certain bias to this counting method. In order to separate the aggregated cells and make a homogenous cell distribution there should be used a solvent to improve this method. If not use of such a solvent, we should question if this method should be rejected all together as a way of presenting the total bacterial number found in such an environment.

The differences in sampling design made it hard to provide a definite answer to whether or not there was an effect on the microbial diversity and abundance due to thawing between the two sampling years. In order to provide a reliable answer to this question true replicates should have been made. Additionally, previous studies have used several different sampling designs to examine the permafrost in different areas, which makes it hard to compare to one another. The climate is changing on a global scale, and the effects seen at different locations can be just as important to understand as the local changes. A universal way of sampling should be made available in order to compare the different effects thawing has on permafrost in the Arctic.

In this study we did not measure how many microorganisms were active or viable in the permafrost samples. We know there is a high chance that most of the DNA measured does not belong to active microbes because of the sub-zero temperatures. Previous research done in Spitsbergen, Svalbard has found that only 26% are viable, where the rest is either dormant or dead (Hansen *et al.*, 2007). Whether or not this is the same in other samples is unknown. Further research on how large proportion of the microorganism are viable needs to be investigated. The functional genes discovered in these samples indicates there are microbes able to aerobically oxidise methane to carbon dioxide. However, we did not measure if these genes were actively expressed or not. Figuring out how many of the microorganisms are viable and are able to express these genes can give us an indication of to what extent the greenhouse gas release can be upon thawing.

In addition to this, our viable microbial diversity found from plate counts can indicate that there is a possibility of further increased carbon dioxide release. It is therefore important to continue the monitoring of permafrost to find long-term effects caused by global temperature increase. This will allow us to predict what the future may look like, and possibly prevent future greenhouse gas release.

Characterising both bacteria and fungi, and some of their functional genes have been investigated in this study. However, by using computational tools to aid in high-quality metagenome-assembled genome recovery and to find trends in the permafrost functional potential, we could maximise the information from these samples. Since permafrost community information is still lacking due to the soil's complexity and heterogeneity, the results from metagenomic analyses could be compared with geochemical analyses. This could provide a better connection with the biological, chemical and geological fields when analysing permafrost samples.

References

Ali, S. H. *et al.* (2013) 'Studies on diversity of soil microfungi in the Hornsund area, Spitsbergen', *Polish Polar Research*, 34(4), pp. 39–54. doi: 10.2478/popore.

Alves, R. J. E. *et al.* (2013) 'Nitrification rates in Arctic soils are associated with functionally distinct populations of ammonia-oxidizing archaea', *ISME Journal*. Nature Publishing Group, 7(8), pp. 1620–1631. doi: 10.1038/ismej.2013.35.

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995) 'Phylogenetic identification and in situ detection of individual microbial cells without cultivation', *Microbiological Reviews*, 59(1), pp. 143–169. doi: 10.1111/j.1439-0426.2012.01974.x.

Ayala-Del-Río, H. L. *et al.* (2010) 'The genome sequence of psychrobacter arcticus 273-4, a psychroactive siberian permafrost bacterium, reveals mechanisms for adaptation to low-temperature growth', *Applied and Environmental Microbiology*, 76(7), pp. 2304–2312. doi: 10.1128/AEM.02101-09.

Bakermans, C. *et al.* (2003) 'Reproduction and metabolism at -10°C of bacteria isolated from Siberian permafrost', *Environmental Microbiology*, 5(4), pp. 321–326. doi: 10.1046/j.1462-2920.2003.00419.x.

Bellemain, E. *et al.* (2013) 'Fungal palaeodiversity revealed using high-throughput metabarcoding of ancient DNA from arctic permafrost', *Environmental Microbiology*, 15(4), pp. 1176–1189. doi: 10.1111/1462-2920.12020.

Bockheim, J. G. and Tarnocai, C. (1998) 'Recognition of cryoturbation for classifying permafrost-affected soils', *Geoderma*, 81(3–4), pp. 281–293. doi: 10.1016/S0016-7061(97)00115-8.

De Boer, W. *et al.* (2005) 'Living in a fungal world: Impact of fungi on soil bacterial niche development', *FEMS Microbiology Reviews*, 29(4), pp. 795–811. doi: 10.1016/j.femsre.2004.11.005.

Bourne, D. G., McDonald, I. R. and Murrell, J. C. (2001) 'Comparison of pmoA PCR Primer Sets as Tools for Investigating Methanotroph Diversity in Three Danish Soils', *Applied and Environmental Microbiology*, 67(9), pp. 3802–3809. doi: 10.1128/AEM.67.9.3802-3809.2001.

Brandon Matheny, P. *et al.* (2007) 'Contributions of rpb2 and tef1 to the phylogeny of mushrooms and allies (Basidiomycota, Fungi)', *Molecular Phylogenetics and Evolution*, 43(2), pp. 430–451. doi: 10.1016/j.ympev.2006.08.024.

Brooks, P. D., Williams, M. W. and Schmidt, S. K. (1998) 'Inorganic nitrogen and microbial biomass dynamics before and during spring snowmelt', *Biogeochemistry*, 43(1), pp. 1–15. doi: 10.1023/A:1005947511910.

Burgess, M. M. *et al.* (2000) 'Geological Survey of Canada Current Research Global Terrestrial Network for Permafrost (GTNet-P): permafrost monitoring contributing to global', *Geological Survey of Canada, Current*

Research, 2000-E14(January).

Burket, A. *et al.* (2019) 'Changes in the Active , Dead , and Dormant Microbial Chronosequence', *Applied and Environmental Microbiology*, (November 2018), pp. 1–16.

Burton, D. L. and Beauchamp, E. G. (1994) 'Profile Nitrous Oxide and Carbon Dioxide Concentrations in a Soil Subject to Freezing', *Soil Science Society of America Journal*, 58(1), pp. 115–122. doi: 10.2136/sssaj1994.03615995005800010016x.

Buzzini, P. and Margesin, R. (2014) *Cold-Adapted Yeasts*, *Cold-adapted Yeasts: Biodiversity, Adaptation Strategies and Biotechnological Significance*. doi: 10.1007/978-3-642-39681-6_10.

Chapin III, F. S. *et al.* (2000) 'Arctic and boreal ecosystems of western North America as components of the climate system', *Global Change Biology*, 6(S1), pp. 211–223. doi: 10.1046/j.1365-2486.2000.06022.x.

Chattopadhyay, M. K. (2006) 'Mechanism of bacterial adaptation to low temperature', *Journal of Biosciences*, 31(1), pp. 157–65.

Collins, T. and Margesin, R. (2019) 'Psychrophilic lifestyles: mechanisms of adaptation and biotechnological tools', *Applied Microbiology and Biotechnology*. Springer Verlag, pp. 2857–2871. doi: 10.1007/s00253-019-09659-5.

DeLuca, T. H., Keeney, D. R. and McCarty, G. W. (1992) 'Effect of freeze-thaw events on mineralization of soil nitrogen', *Biology and Fertility of Soils*, 14(2), pp. 116–120. doi: 10.1007/BF00336260.

Dmitriev, V. V. *et al.* (2001) 'Ultrastructural characteristics of natural forms of microorganisms isolated from permafrost grounds of eastern Siberia by the method of low-temperature fractionation.', *Doklady biological sciences : proceedings of the Academy of Sciences of the USSR, Biological sciences sections / translated from Russian*, 378(6), pp. 304–306. doi: 10.1023/A:1019203800276.

Elser, J. J. *et al.* (2007) 'Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems', *Ecology Letters*, 10(12), pp. 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x.

Ferrari, B. C., Binnerup, S. J. and Gillings, M. (2005) 'Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria', *Applied and Environmental Microbiology*, 71(12), pp. 8714–8720. doi: 10.1128/AEM.71.12.8714-8720.2005.

Fierer, N. *et al.* (2005) 'Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays', *Applied and Environmental Microbiology*, 71(7), pp. 4117–4120. doi: 10.1128/AEM.71.7.4117-4120.2005.

Gagnon, N. *et al.* (2011) 'A novel fingerprint method to assess the diversity of methanogens in microbial systems', *FEMS Microbiology Letters*, 325(2), pp. 115–122. doi: 10.1111/j.1574-6968.2011.02418.x.

Gessler, N. N., Egorova, A. S. and Belozerskaya, T. A. (2014) 'Melanin pigments of fungi under extreme environmental conditions (Review)', *Applied Biochemistry and Microbiology*, 50(2), pp. 105–113. doi: 10.1134/S0003683814020094.

Gilichinsky, D. *et al.* (2005) 'Biodiversity of cryopegs in permafrost', *FEMS Microbiology Ecology*, 53(1), pp. 117–128. doi: 10.1016/j.femsec.2005.02.003.

Gilichinsky, D. *et al.* (2008) *Bacteria in Permafrost*, *Psychrophiles: From Biodiversity to Biotechnology*. doi: 10.1007/978-3-540-74335-4_19.

Gilichinsky, D. A. (2002) 'Permafrost Model of Extraterrestrial Habitat BT - Astrobiology: The Quest for the Conditions of Life', in Horneck, G. and Baumstark-Khan, C. (eds) *Astrobiology. The Quest for the Conditions of Life*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 125–142. doi: 10.1007/978-3-642-59381-9_9.

Gittel, A., Bárta, J., Kohoutová, I., Mikutta, R., *et al.* (2014) 'Distinct microbial communities associated with buried soils in the siberian tundra', *ISME Journal*, 8(4), pp. 841–853. doi: 10.1038/ismej.2013.219.

Gittel, A., Bárta, J., Kohoutová, I., Schnecker, J., *et al.* (2014) 'Site- and horizon-specific patterns of microbial community structure and enzyme activities in permafrost-affected soils of Greenland', *Frontiers in Microbiology*, 5(OCT), pp. 1–14. doi: 10.3389/fmicb.2014.00541.

Grogan, P. *et al.* (2004) 'Freeze-thaw regime effects on carbon and nitrogen dynamics in sub-arctic heath tundra mesocosms', *Soil Biology and Biochemistry*, 36(4), pp. 641–654. doi: 10.1016/j.soilbio.2003.12.007.

Grosse, G. *et al.* (2011) 'Vulnerability of high-latitude soil organic carbon in North America to disturbance', *Journal of Geophysical Research: Biogeosciences*, 116(3), pp. 1–23. doi: 10.1029/2010JG001507.

Hadziavdic, K. *et al.* (2014) 'Characterization of the 18s rRNA gene for designing universal eukaryote specific primers', *PLoS ONE*, 9(2). doi: 10.1371/journal.pone.0087624.

Hansen, A. A. *et al.* (2007) 'Viability, diversity and composition of the bacterial community in a high Arctic permafrost soil from Spitsbergen, Northern Norway', *Environmental Microbiology*, 9(11), pp. 2870–2884. doi: 10.1111/j.1462-2920.2007.01403.x.

Hao, Y. *et al.* (2010) 'Sistotrema brinkmannii, a psychrotolerant fungus from Antarctic soil', *Mycosystema*, 29(6), pp. 864–868.

Hinkel, K. M. and Nelson, F. E. (2003) 'Spatial and temporal patterns of active layer thickness at Circumpolar Active Layer Monitoring (CALM) sites in northern Alaska 1995-2000', *Journal of Geophysical Research D: Atmospheres*, 108(2), pp. 1995–2000. doi: 10.1029/2001jd000927.

Hinzman, L. D. *et al.* (2005) 'Evidence and implications of recent climate change in Northern Alaska and other Arctic regions', *Climatic Change*, 72(3), pp. 251–298. doi: 10.1007/s10584-005-5352-2.

Holmes, A. J. *et al.* (1995) 'Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related', *FEMS Microbiology Letters*, 132(3), pp. 203–208. doi: 10.1016/0378-1097(95)00311-R.

Hugenholtz, P., Goebel, B. M. and Pace, N. R. (1998) 'Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity', *Journal of Bacteriology*, 180(24), pp. 4765–4774. doi: 10.1128/JB.180.24.6793-6793.1998.

Humlum, O., Instanes, A. and Sollid, J. L. (2003) 'Permafrost in Svalbard: A review of research history, climatic background and engineering challenges', *Polar Research*, 22(2), pp. 191–215. doi: 10.3402/polar.v22i2.6455.

Inglese, C. N. *et al.* (2017) 'Examination of Soil Microbial Communities after Permafrost Thaw Subsequent to an Active Layer Detachment in the High Arctic', *Arctic, Antarctic, and Alpine Research*, 49(3), pp. 455–472. doi: 10.1657/AAAR0016-066.

IPCC (2019) *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate*, *Intergovernmental Panel on Climate Change*. Edited by H.-O. Pörtner et al. In press. Available at: https://www.ipcc.ch/site/assets/uploads/sites/3/2019/12/SROCC_FullReport_FINAL.pdf.

Ivanushkina, N. E., Kochkina, G. A. and Ozerskaya, S. M. (2005) 'Fungi in Ancient Permafrost Sediments of the Arctic and Antarctic Regions', in Castello, J. D. and Rogers, S. O. (eds) *Life in Ancient Ice*. Princeton Press, Princeton, NJ, pp. 127–139. doi: 10.1515/9781400880188-013.

Ivarson, K. C. and Sowden, F. J. (1970) 'Effect of Frost Action and Storage of Soil At Freezing Temperatures on the Free Amino Acids, Free Sugars and Respiratory Activity of Soil', *Canadian Journal of Soil Science*, 50(2), pp. 191–198. doi: 10.4141/cjss70-027.

Jansson, J. K. and Taş, N. (2014) 'The microbial ecology of permafrost', *Nature Reviews Microbiology*, 12(6), pp. 414–425. doi: 10.1038/nrmicro3262.

Kane, D. L. and Stein, J. (1983) 'Water movement into seasonally frozen soils', *Water Resources Research*, 19(6), pp. 1547–1557. doi: 10.1029/WR019i006p01547.

Kochkina, G. A. *et al.* (2001) 'Survival of micromycetes and actinobacteria under conditions of long-term natural cryopreservation', *Microbiology*, 70(3), pp. 356–364. doi: 10.1023/A:1010419831245.

Kreader, A. C. (1996) 'Relief of amplification inhibition in PCR with bovine serum albumin or T4 Gene 32 Protein', *Applied and Environmental Microbiology*, 62(3), pp. 1102–1106.

Kumar, S., Stecher, G. and Tamura, K. (2015) 'MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets', *Molecular Biology and Evolution*, 33, pp. 1870–1874. doi: https://doi.org/10.1093/molbev/msw054.

Lachenbruch, A. H. (1962) 'Mechanics of thermal contraction cracks and ice-wedge polygons in permafrost.',

Geological Society of America, 70. doi: https://doi.org/10.1130/SPE70.

Lane, D. J. (1991) '16S/23S rRNA Sequencing', in Stackebrandt, E. and Goodfellow, M. (eds) *Nucleic Acid Techniques in Bacterial Systematics*. New York: Wiley, pp. 115–175.

Lewin, G. R. *et al.* (2016) 'Evolution and Ecology of Actinobacteria and Their Bioenergy Applications', *Annual Review of Microbiology*, 70(1), pp. 235–254. doi: 10.1146/annurev-micro-102215-095748.

Liebner, S. and Wagner, D. (2007) 'Abundance, distribution and potential activity of methane oxidizing bacteria in permafrost soils from the Lena Delta, Siberia', *Environmental Microbiology*, 9(1), pp. 107–117. doi: 10.1111/j.1462-2920.2006.01120.x.

Liesack, Werner; Jan-Henrich, R. (1997) 'The Ammonia Monooxygenase Structural Gene', *Microbiology*, 63(12), pp. 4704–4712.

Lopez, I. *et al.* (2003) 'Design and Evaluation of PCR Primers for Analysis of Bacterial Populations in Wine by Denaturing Gradient Gel Electrophoresis Design and Evaluation of PCR Primers for Analysis of Bacterial Populations in Wine by Denaturing Gradient Gel Electrophoresis', *Applied and Environmental Microbiology*, 69(December 2003), pp. 6801–6807. doi: 10.1128/AEM.69.11.6801.

Mackelprang, R. *et al.* (2011) 'Metagenomic analysis of a permafrost microbial community reveals a rapid response to thaw', *Nature*. Nature Publishing Group, 480(7377), pp. 368–371. doi: 10.1038/nature10576.

Mackelprang, R. *et al.* (2016) 'Permafrost Meta-Omics and Climate Change', *Annual Review of Earth and Planetary Sciences*, 44(1), pp. 439–462. doi: 10.1146/annurev-earth-060614-105126.

Mackelprang, R. *et al.* (2017) 'Microbial survival strategies in ancient permafrost: Insights from metagenomics', *ISME Journal*. Nature Publishing Group, 11(10), pp. 2305–2318. doi: 10.1038/ismej.2017.93.

Madsen, E. L. (2011) 'Microorganisms and their roles in fundamental biogeochemical cycles', *Current Opinion in Biotechnology*. Elsevier Ltd, 22(3), pp. 456–464. doi: 10.1016/j.copbio.2011.01.008.

Margesin, R. and Miteva, V. (2011) 'Diversity and ecology of psychrophilic microorganisms', *Research in Microbiology*. Elsevier Masson SAS, 162(3), pp. 346–361. doi: 10.1016/j.resmic.2010.12.004.

Marsh, P. and Woo, M. K. (1984) 'Wetting Front Advance and Freezing of Meltwater Within a Snow Cover • SOIL T ADVANCING •' SOIL', *Water Resources Research*, 20(12), pp. 1853–1864.

Morley, C. R. *et al.* (1983) 'Effects of Freez-Thaw Stress on Bacterial Populations in Soil Micocosms', *Microbial Ecology*, 9, pp. 329–340.

Müller, O. *et al.* (2018) 'Disentangling the complexity of permafrost soil by using high resolution profiling of microbial community composition, key functions and respiration rates', *Environmental Microbiology*, 20(12), pp. 4328–4342. doi: 10.1111/1462-2920.14348.

Nilsson, R. H. *et al.* (2019) 'The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications', *Nucleic Acids Research*, 47(D1), pp. D259–D264. doi: 10.1093/nar/gky1022.

Ozerskaya, S. *et al.* (2009) *Fungi in Permafrost*. Edited by R. Margesin. Berlin, Heidelberg: Berlin, Heidelberg: Springer Berlin Heidelberg (Soil Biology). doi: 10.1007/978-3-540-69371-0_7.

Peterson, R. A. and Krantz, W. B. (2003) 'A mechanism for differential frost heave and its implications for patterned-ground formation', *Journal of Glaciology*, 49(164), pp. 69–80. doi: 10.3189/172756503781830854.

Porter, K. G. and Feig, Y. S. (1980) 'The Use of DAPI for Identifying and Counting Aquatic Microflora', *Limnology and Oceanography*, 25(5), pp. 943–948.

Reasoner, D. J. and Geldreich, E. E. (1985) 'A new medium for the enumeration and subculture of bacteria from potable water', *Applied and Environmental Microbiology*, 49(1), pp. 1–7. doi: 10.1128/aem.49.1.1-7.1985.

Rivkina, E. *et al.* (2004) 'Microbial life in permafrost', *Advances in Space Research*, 33(8), pp. 1215–1221. doi: 10.1016/j.asr.2003.06.024.

Rivkina, E. *et al.* (2007) 'Biogeochemistry of methane and methanogenic archaea in permafrost', *FEMS Microbiology Ecology*, 61(1), pp. 1–15. doi: 10.1111/j.1574-6941.2007.00315.x.

Rivkina, E. M. *et al.* (2000) 'Metabolic activity of Permafrost Bacteria below the freezing point', *Applied and Environmental Microbiology*, 66(8), pp. 3230–3233. doi: 10.1128/AEM.66.8.3230-3233.2000.

Robinson, C. H. (2001) 'Cold adaptation in Arctic and Antarctic fungi', *New Phytologist*, 151(2), pp. 341–353. doi: 10.1046/j.1469-8137.2001.00177.x.

Romanovsky, V. E., Smith, S. L. and Christiansen, H. H. (2010) 'Permafrost thermal state in the polar northern hemisphere during the international polar year 2007-2009: A synthesis', *Permafrost and Periglacial Processes*, 21(2), pp. 106–116. doi: 10.1002/ppp.689.

Rosas, Á. L. and Casadevall, A. (2001) 'Melanization decreases the susceptibility of Cryptococcus neoformans to enzymatic degradation', *Mycopathologia*, 151(2), pp. 53–56. doi: 10.1023/A:1010977107089.

Schimel, J. P. and Clein, J. S. (1996) 'Microbial response to freeze-thaw cycles in tundra and taiga soils', *Soil Biology and Biochemistry*, 28(8), pp. 1061–1066. doi: 10.1016/0038-0717(96)00083-1.

Schnecker, J. *et al.* (2014) 'Effects of soil organic matter properties and microbial community composition on enzyme activities in cryoturbated arctic soils', *PLoS ONE*, 9(4). doi: 10.1371/journal.pone.0094076.

Schostag, M. *et al.* (2015) 'Distinct summer and winter bacterial communities in the active layer of Svalbard permafrost revealed by DNA- and RNA-based analyses', *Frontiers in Microbiology*, 6(APR), pp. 1–13. doi: 10.3389/fmicb.2015.00399.

Schuur, E. A. G. *et al.* (2009) 'The effect of permafrost thaw on old carbon release and net carbon exchange from tundra', *Nature*. Nature Publishing Group, 459(7246), pp. 556–559. doi: 10.1038/nature08031.

Serreze, M. C. *et al.* (2000) 'Observational Evidence of Recent Change in the Northern High-Latitude Environment', *Climatic Change*, 46(1/2), pp. 159–207.

Shi, T. *et al.* (1997) 'Characterization of Viable Bacteria from Sibarian Permafrost by 16S rDNA Sequencing', *Microbial Ecology*, 33(3), pp. 169–179. doi: 10.1016/B978-008045405-4.00519-X.

Singh, P. *et al.* (2017) 'Bacterial communities in ancient permafrost profiles of Svalbard, Arctic', *Journal of Basic Microbiology*, 57(12), pp. 1018–1036. doi: 10.1002/jobm.201700061.

Skogland, T., Lomeland, S. and Goksøyr, J. (1988) 'Respiratory burst after freezing and thawing of soil: Experiments with soil bacteria', *Soil Biology and Biochemistry*, 20(6), pp. 851–856. doi: 10.1016/0038-0717(88)90092-2.

Soina, V. S. *et al.* (1995) 'Preservation of cell structures in permafrost: A model for exobiology', *Advances in Space Research*, 15(3), pp. 237–242. doi: 10.1016/S0273-1177(99)80090-8.

Staley, J. T. and Konopka, A. (1985) 'Microorganisms in aquatic and terrestrial habitats', *Annual Review of Microbiology*, 39, pp. 321–346.

Stephen, J. R. *et al.* (1999) 'Effect of toxic metals on indigenous soil β-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria', *Applied and Environmental Microbiology*, 65(1), pp. 95–101.

Sterflinger, K. (1998) 'Temperature and NaCl- tolerance of rock-inhabiting meristematic fungi', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 74(4), pp. 271–281. doi: 10.1023/A:1001753131034.

Steven, B. *et al.* (2006) 'Microbial ecology and biodiversity in permafrost', *Extremophiles*, 10(4), pp. 259–267. doi: 10.1007/s00792-006-0506-3.

Steven, B. *et al.* (2007) 'Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods', *FEMS Microbiology Ecology*, 59(2), pp. 513–523. doi: 10.1111/j.1574-6941.2006.00247.x.

Steven, B. *et al.* (2008) 'Microbial diversity and activity through a permafrost/ground ice core profile from the Canadian high Arctic', *Environmental Microbiology*, 10(12), pp. 3388–3403. doi: 10.1111/j.1462-2920.2008.01746.x.

Steven, B., Niederberger, T. D. and Whyte, L. G. (2009) 'Permafrost Soils', in Margesin, R. (ed.) *Permafrost soils*. Springer, pp. 59–72. doi: 10.1007/978-3-540-69371-0.

Sulkava, P. and Huhta, V. (2003) 'Effects of hard frost and freeze-thaw cycles on decomposer communities and N mineralisation in boreal forest soil', *Applied Soil Ecology*, 22(3), pp. 225–239. doi: 10.1016/S0929-1393(02)00155-5.

Suzuki, M. T. and Giovannoni, S. J. (1996) 'Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR', *Applied and Environmental Microbiology*, 62(2), pp. 625–630. doi: 10.1128/aem.62.2.625-630.1996.

Tarnocai, C. (2009) 'Arctic Permafrost Soils', in Margesin, R. (ed.) *Permafrost soils*. Berlin, Heidelberg: Springer-Verlag Berlin Heidelberg, pp. 3–16. doi: 10.1007/978-3-540-69371-0.

Taş, N. *et al.* (2014) 'Impact of fire on active layer and permafrost microbial communities and metagenomes in an upland Alaskan boreal forest', *ISME Journal*, 8(9), pp. 1904–1919. doi: 10.1038/ismej.2014.36.

Torsvik, V. and Øvreås, L. (2008) 'Microbial Diversity, Life Strategies, and Adaptation to Life in Extreme Soils BT - Microbiology of Extreme Soils', in Dion, P. and Nautiyal, C. S. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 15–43. doi: 10.1007/978-3-540-74231-9_2.

Tveit, A. T., Urich, T. and Svenning, M. M. (2014) 'Metatranscriptomic analysis of arctic peat soil microbiota', *Applied and Environmental Microbiology*, 80(18), pp. 5761–5772. doi: 10.1128/AEM.01030-14.

Větrovský, T. and Baldrian, P. (2013) 'The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses', *PLoS ONE*, 8(2), pp. 1–11. doi: 10.1371/journal.pone.0057923.

Vialle, A. *et al.* (2009) 'Evaluation of mitochondrial genes as DNA barcode for Basidiomycota', *Molecular Ecology Resources*, 9(SUPPL. 1), pp. 99–113. doi: 10.1111/j.1755-0998.2009.02637.x.

Vishnivetskaya, T. *et al.* (2000) 'Low-temperature recovery strategies for the isolation of bacteria from ancient permafrost sediments', *Extremophiles*, 4(3), pp. 165–173. doi: 10.1007/s007920070031.

Vishnivetskaya, T. A. *et al.* (2014) 'Commercial DNA extraction kits impact observed microbial community composition in permafrost samples', *FEMS Microbiology Ecology*, 87(1), pp. 217–230. doi: 10.1111/1574-6941.12219.

Vorobyova, E. *et al.* (1997) 'The deep cold biosphere: Facts and hypothesis', *FEMS Microbiology Reviews*, 20(3–4), pp. 277–290. doi: 10.1016/S0168-6445(97)00047-8.

Waldrop, M. P. *et al.* (2010) 'Molecular investigations into a globally important carbon pool: Permafrost-protected carbon in Alaskan soils', *Global Change Biology*, 16(9), pp. 2543–2554. doi: 10.1111/j.1365-2486.2009.02141.x.

White, T. J. *et al.* (1990) 'Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics', *PCR Protocols*, (January), pp. 315–322. doi: 10.1016/b978-0-12-372180-8.50042-1.

Wilhelm, R. C. *et al.* (2012) 'Life at the wedge: The activity and diversity of arctic ice wedge microbial communities', *Astrobiology*, 12(4), pp. 347–360. doi: 10.1089/ast.2011.0730.

Xue, Y. *et al.* (2020) 'Metagenome-assembled genome distribution and key functionality highlight importance of aerobic metabolism in Svalbard permafrost', *FEMS Microbiology Ecology*, 96(5), pp. 1–13. doi: 10.1093/femsec/fiaa057.

Yang, S. *et al.* (2012) 'Pyrosequencing Investigation into the Bacterial Community in Permafrost Soils along the China-Russia Crude Oil Pipeline (CRCOP)', *PLoS ONE*, 7(12). doi: 10.1371/journal.pone.0052730.

Yergeau, E. *et al.* (2010) 'The functional potential of high Arctic permafrost revealed by metagenomic sequencing, qPCR and microarray analyses', *ISME Journal*, 4(9), pp. 1206–1214. doi: 10.1038/ismej.2010.41.

Zhang, T. *et al.* (2008) 'Statistics and characteristics of permafrost and ground-ice distribution in the Northern Hemisphere', *Polar Geography*, 31(1–2), pp. 47–68. doi: 10.1080/10889370802175895.

Zhang, T. *et al.* (2016) 'Soil pH is a key determinant of soil fungal community composition in the Ny-Ålesund Region, Svalbard (High Arctic)', *Frontiers in Microbiology*, 7(FEB), pp. 1–10. doi: 10.3389/fmicb.2016.00227.

Aanen, D. K., Kuyper, T. W. and Hoekstra, R. F. (2001) 'A widely distributed ITS polymorphism within a biological species of the ectomycorrhizal fungus Hebeloma velutipes', *Mycological Research*, 105(3), pp. 284–290. doi: 10.1017/S0953756201003628.

Appendix

The quality of the bacterial (Figure 17 and Figure 18) and fungal (Figure 19 and Figure 20) qPCR.

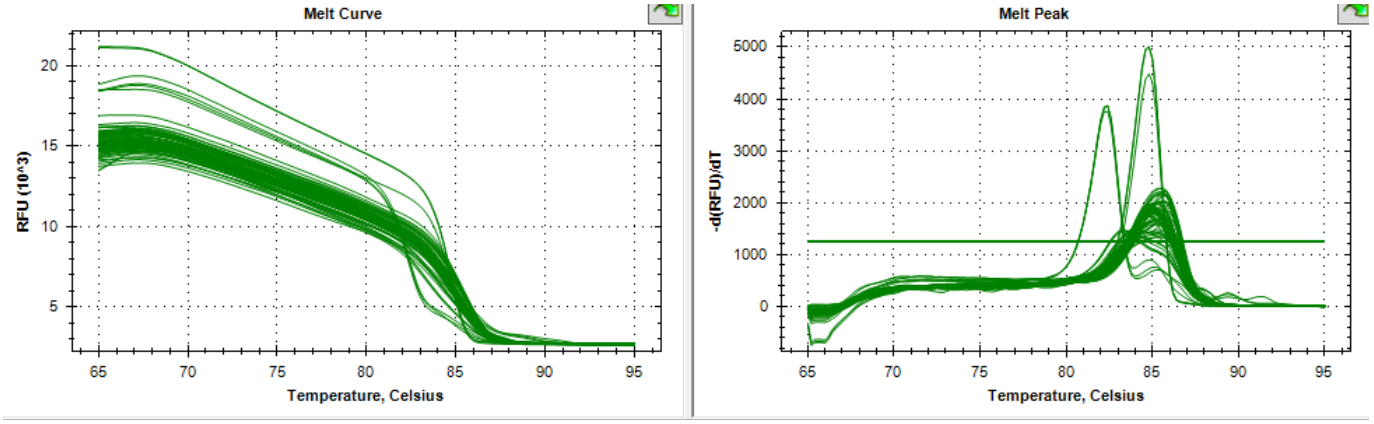

Figure 17: Melt curve and melt peak graph data for qPCR of the bacterial samples.

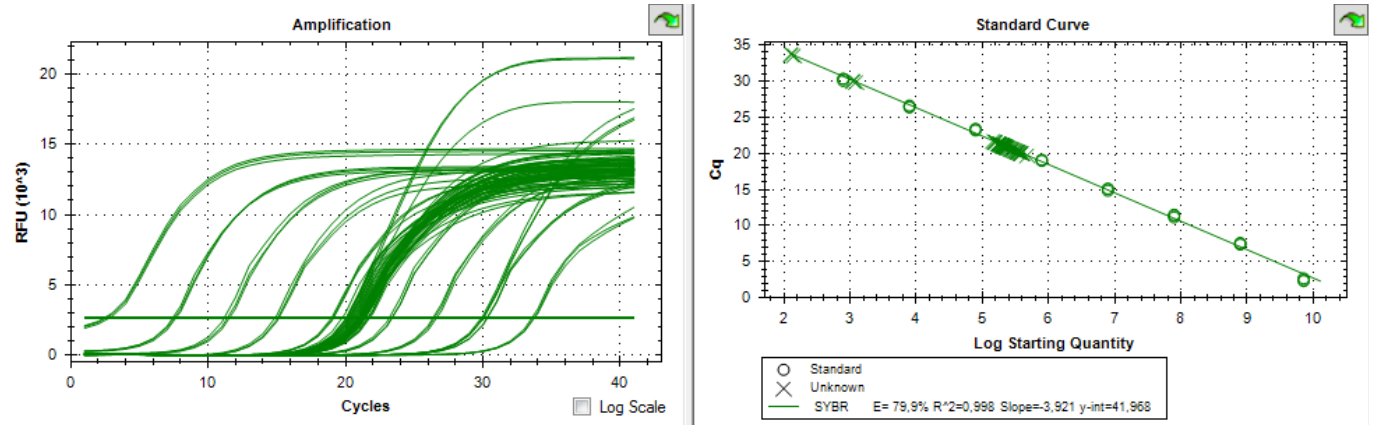

Figure 18: Amplification and standard curve for qPCR of bacterial samples.

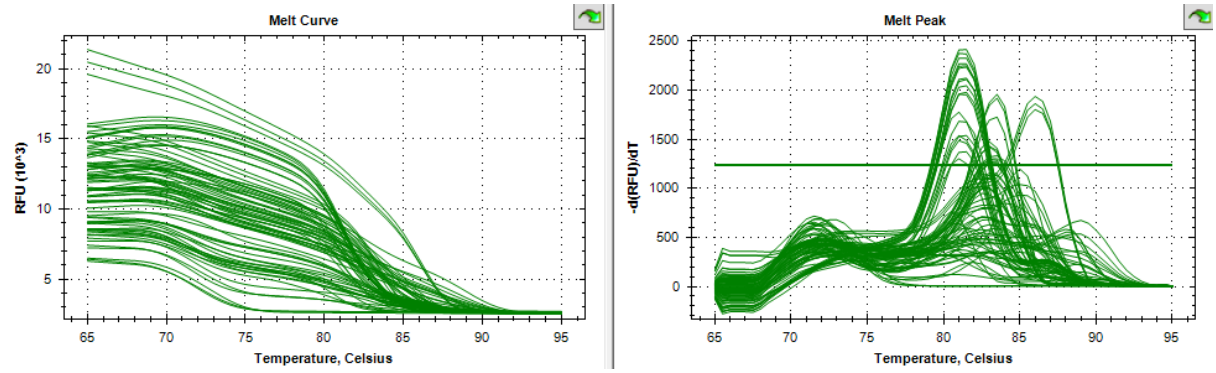

Figure 19: Melt curve and melt peak graph data for qPCR of the fungal samples.

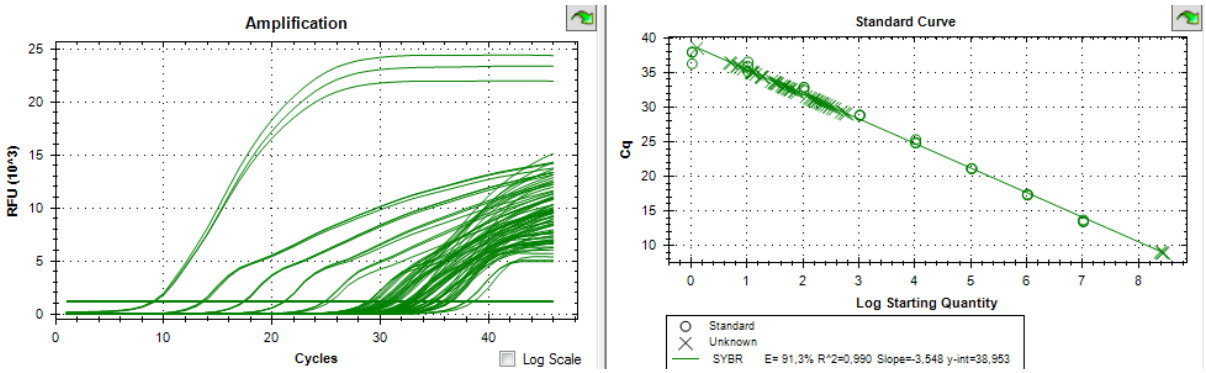

Figure 20: Amplification and standard curve for qPCR of fungal samples.