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Department of Soil and Environment

Microbial Community Responses to Freeze

– thaw cycles in active layer soils of permafrost tundra, Disko Island, Greenland

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1 Abstract

Permafrost affected soils are a vast pool of Carbon, storing around 1300Pg below the ground. As a result of climate change, permafrost soils that have been frozen for thousands of years are thawing, causing the ancient organic matter to be metabolised by microorganisms. Moisture content of soils has an impact on microbial community activity, but the mechanism of this effect is poorly understood. This study sought to explore the effect of microbial alpha and beta diversity, microbial activity, and greenhouse gas production respond to thaw in wet and dry soils in the Arctic. Soils were selected from a wet fen and dry sedge heath from Disko Island, Greenland to test the effect of soil type on thaw. Microbial community dynamics were studied over the thaw period using amplicon sequencing of 16S (bacteria) and ITS (fungi). It was found that although alpha and beta diversity did not significantly respond to thaw, enzyme activity did have an effect, suggesting that microbial community behaviour had been affected. The impact of this on greenhouse gas fluxes were that the dry soil became a sink of CO_2, N_2O and CH_4, whereas the wet soils became a source of all three gases. This finding indicates that moisture regime will play a large role in whether artic soils will contribute to climate feedbacks upon thawing.

Keywords: microbial ecology, active layer soil, bacteria, fungi, permafrost system, amplicon-sequencing, QIIME2, FUNguild, greenhouse gas fluxes, fluorogenic enzyme assays.

Popular Scientific Summary

Arctic soils are a vast pool of carbon, estimated to hold 1,330-1,580 Pg in the Northern Permafrost zone. This material has been kept under cold and frozen conditions for tens of thousands of years and therefore been largely immobile. However, due to increasing global temperatures and permafrost thaw, this carbon store is being released. The loss of this ancient carbon to the atmosphere poses a 'tipping point' threat to the earth that could result in runaway climate change and has therefore been nicknamed the 'carbon bomb'. In polar regions, temperatures are increasing twice as fast as the global average, causing Arctic soils to thaw rapidly on a large scale. Therefore, there is scientific interest in the factors that affect the behaviour of this soil upon thaw. The activities of the microbes that inhabit the soil will have influence on the fate of this material, therefore understanding its drivers is key. Soil hosts a diverse plethora of microorganisms, each playing a role in the digestion of material and pushing forward nutrient cycles, therefore the species present in the microbial community can indicate certain functions of the soil. Moisture content of the soil can have huge impacts on soil microorganisms, in some cases even switching their degradation pathways from oxygen dependant to oxygen independent, changing the final excretion of the organism.

With this in mind, we investigated the links between microbial activity and production of greenhouse gases in a Greenlandic Arctic soil, under differing moisture regimes. From a timepoint sub-sampling experiment, we were able to generate community, enzyme activity and chemical data that can inform us as to how the soil is responding to thaw. It was found that immediately after the soil was thawed, greenhouse gas levels increased dramatically, regardless of soil type. Production

continued to increase in the wet soil type. However, after a week the dry soil began to act as a sink for greenhouse gases. In spite of this significance, we did not find sufficient evidence that microbial communities were affect by the thaw, or that moisture regime interacted with this.

Despite the data gathered, it is assumed that the intense changes caused by freezing and thawing would cause a significant die off and have large impacts on the microbial community. However, due to the lack of quantitative data, it is difficult to determine how the community is reacting in real time, and which organisms are living or dead, and in what numbers. In order to get a complete picture of the microbial community response to thaw, more quantitative data such as biomass studies or qPCR would be required.

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2 Introduction

The ‘Permafrost Carbon Bomb’

Arctic soils are characterised by a layer of year-round frozen soil called permafrost, overlain by an active layer of intermittently freezing and thawing soil. Soils in the Northern permafrost region are a significant pool of carbon (C). These soils are estimated to hold around 1300 Pg of organic carbon, of which 800 Pg is perennially frozen in the permafrost [1]. This vast pool of C has been referred to in the past as the ‘permafrost carbon bomb’, alluding to the threat that permafrost soils pose to global C budgets [2]. In polar regions, temperatures are increasing as a result of climate change at a higher rate than the global average due to various feedback mechanisms. These warming temperatures cause the permafrost to thaw, causing a deepening of the active layer and the permafrost line to recede northwards [3]. This thawing of Arctic soil exposes ancient organic matter to a metabolically active consortium of microbes, ready to decompose and release it as the potent greenhouse gases (GHG), CO_2, CH_4, and N_2O [4]. This potential release of GHG from thawing permafrost would reduce the global carbon budget by up to 100Gt, as estimated by the latest Intergovernmental Panel on Climate Change (IPCC, 2018).

Although it is accepted that thawing soil microorganisms play a role in global carbon feedbacks, the exact mechanisms in response-to-thaw are poorly

understood [5]. Several studies have explored the effect of thawing circum-arctic soils on their microbial communities [6-9], and the effect of thaw on enzyme activity [4, 10]. Studies have also found that this effect interacts with gas production of soils [6, 11]. Interest in how soil physical characteristics, including changes in moisture regime, may affect response-to-thawing has also been explored [12, 13]. Another area of interest in global feedbacks is the vegetation response and the sequestration potential of Arctic greening [14]. Due to the methodological challenges of studying thawing communities, climate models are poorly constrained in this area. However, by pursuing a mechanistic understanding of these processes, we can inform climate models and build confidence in their robustness.

Microbial community response to thaw

Despite harsh climate and oligotrophic conditions, active layer and even permafrost soils have been shown to contain a diverse and active microbial community [15, 16]. The anticipated increase in active layer thickness and full seasonal thaw of permafrost regions has been hypothesised to increase in microbial activity in Northern latitude soils [11]. This increase in microbial activity will likely cause a net loss of C from the organic matter-rich subsoil, as the bacteria, fungi and archaea that make up the microbial community carry out the aerobic and anaerobic processes that make up the complex degradation pathways. However, how this will affect the net balance of C in the overall tundra system is less clear due to the offset effect of increased biomass [17]. Aerobic metabolic pathways result in the net loss of carbon from soil, in the form of CO_2. Under wet, anaerobic conditions, archaea become active and reduce CO_2 to CH_4, a process known as methanogenesis [18]. Other anaerobic microorganisms can cause a net loss of N from the soil in the form of N_2O, during incomplete denitrification. However, this process has shown to be limited in prevalence in Arctic soil [19].

Active layer soils are exposed to multiple freeze thaw cycles during the change in seasons. This exposes the soil environment to many dramatic physical and chemical changes, such as fluctuations in temperature, changes in moisture regime and associate osmotic stress, pH fluctuations, and mechanical breakdown by freeze-thaw activity. Microbial communities are sensitive to these changes in their immediate environment [8, 9]. Microbes have many physiological adaptations to survive the stresses of the frozen soil: synthesis of antifreeze proteins, shifting biochemical pathways to deal with low nutrients, and synthesis of molecules to control osmolality during drought [20]. These adaptations have intense ecosystem costs, which must then be reversed in order to survive a thawing soil, causing a die-off of microbial life [4]. Repeated freeze-thaw action has been shown to reduce microbial biomass by one third and basal respiration by up to 30% [8].

After thawing, pulse increases in microbial activity have been observed [21]. This may be explained by the release of fresh nutrients resulting from the necromass of cells killed by the thawing process. This stimulates microbial growth, but may also cause a decrease in alpha diversity due to strategic opportunistic species dominating the community [21]. Another effect of thawing is soil rewetting, causing microbes to rapidly dispose of their osmotic protectants. This has been observed to produce a pulse of CO_2 whilst the microbial diversity is reduced [21, 22]. Loss of microbial activity has been suggested to have been compensated by increases in activity due to increased soil temperatures in the summer period [23]. However, under ambient Arctic temperature conditions, this effect has not been shown to match the loss from freezing [4].

Assessing microbial communities from community to production

Microbial communities are characterised by a number of features. Their composition, defined by the different taxa that make up the community, can suggest a particular functionality of a soil, such as degradation of C [24]. Alpha diversity, meaning the number and abundance of taxa within a particular community or habitat, is another key characteristic of microbial communities which can be affected by stress [25]. The response of alpha diversity to thaw is an interesting one as it may also impact microbial community activity, which can have intense ecological consequences.

Studying the microbial community responses to freeze-thaw action is challenging due to most of the soil analytic techniques requiring the soil to be thawed completely. As a result of this, most study efforts of thaw involve studying the integrated freeze-thaw effect [8]. To try to circumvent this problem, here we freeze samples at key timepoints from across the thaw period, and analyse the microbial community using metagenomics. This effort is backed up by observing the environmental change over the crucial timepoints in thaw.

The purpose of the study was to expand understanding of the impact of freeze-thaw on the alpha diversity of microbial communities in active layer soils. Moreover, how the community interacts with the downstream processes of exo-enzyme activity and GHG fluxes. Moisture regime of the soil was another important consideration, as this has considerable effects on the soil thawing. Therefore, soil cores were collected from both wet and dry tundra on Disko Island, Greenland, frozen, and consecutively thawed *in vitro* over 28 days. Microbial community, enzyme activity and GHG fluxes were the measured variables of the study. On the impact of thaw to the microbial community, we hypothesised that (i) post thaw, there will be a loss of alpha diversity, followed by a marginal increase over time, this effect will be more pronounced in the site with a higher water content due to a longer period of thaw and therefore a longer period of recovery for alpha diversity (Figure 1). We further hypothesised that (ii) the release of greenhouse gases post thaw would be stronger in the more microbially active soil and that moisture regime would strengthen this effect.

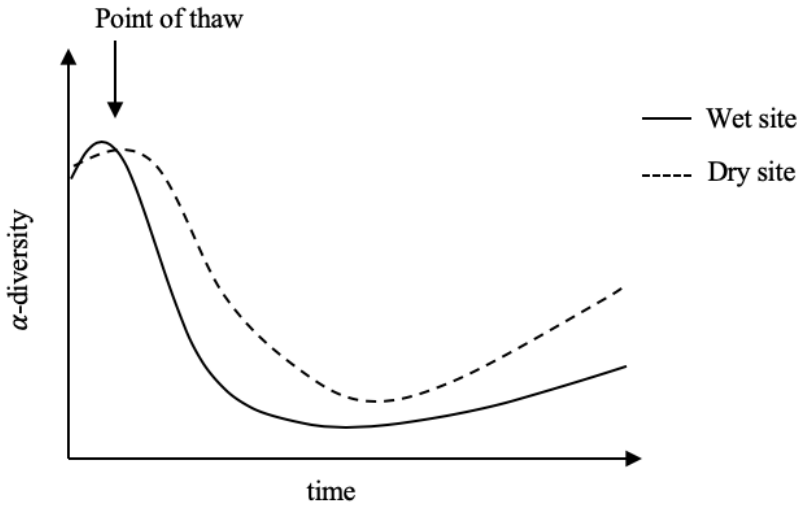

Figure 1 *Conceptual figure predicting changes in alpha diversity estimates over a period of thaw. Different line textures indicate effect of soil moisture type. It is expected that over time alpha diveristy will decrease due to a die off of generalised microorganisms, replaced by specialised and opportunistic individuals. This effect is expected to be more significant in the wet soil than the dry soil due to a prolonged thaw period and increased moisture.*

3 Method

Study Site

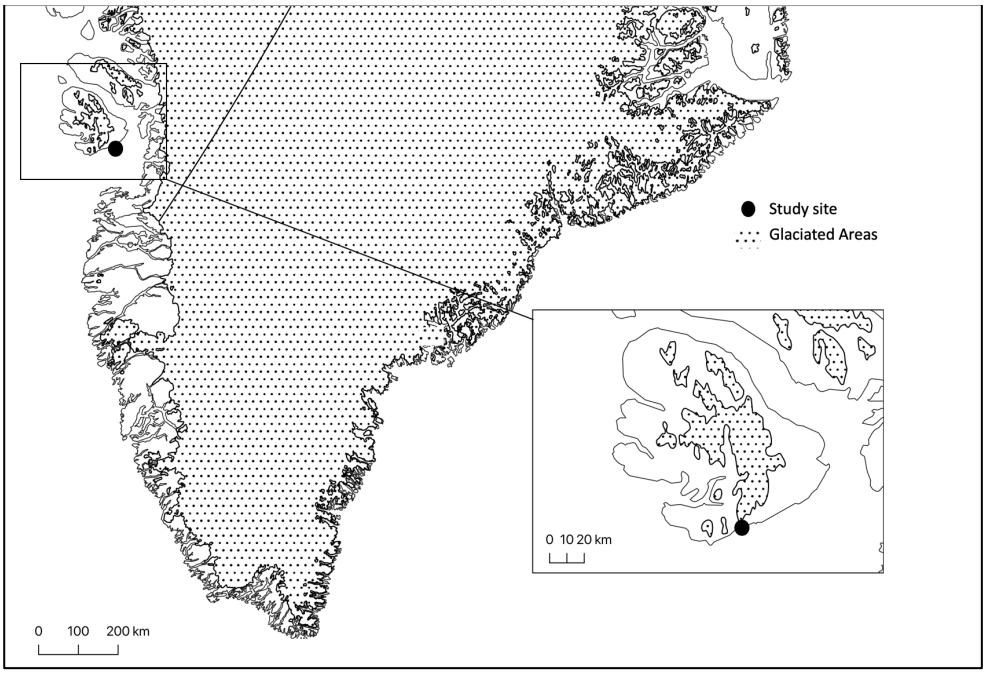

Figure 2 Map of Disko Island, Greenland. Samples were taken from a wet fen and dry heath on the South of the island.

The study site was located on Disko Island, in central West Greenland (69.16'N, 53.49W). The area is situated on a ridge of Precambrian

gneisses, overlain by basalts that originated from the Tertiary era (Inter-act, 2017). The climate of the area is categorised as a low, coastal arctic climate (Inter-act, 2017), and the soil is a weakly developed cryosol with basaltic parent material, in a transition zone between continuous and discontinuous permafrost (Inter-act, 2017).The site was located on a mesic tundra heath, and samples were taken from a dry heath area and an adjacent wet fen area at the base of a slope. The active layer thickness in the wet area averaged at 39.7cm (SE= 0.58, n=6), and in the dry area at 33.2cm (SE=0.55, n=6).

The wet soil area was a herbaceous fen dominated by graminoids, 2 sedges, Eriophorum angustifolium and Carex rariflora were identified as well as the forb Equisetum arvense and some Salix spp (Figure 3). In the dry heath area, dwarf shrubs dominated, in particular Cassiope tetragona, Empetrum nigrum, Betula nana, Pyrola grandiflora, Vaccinium uliginosum and Salix glauca were identified (Figure 4).

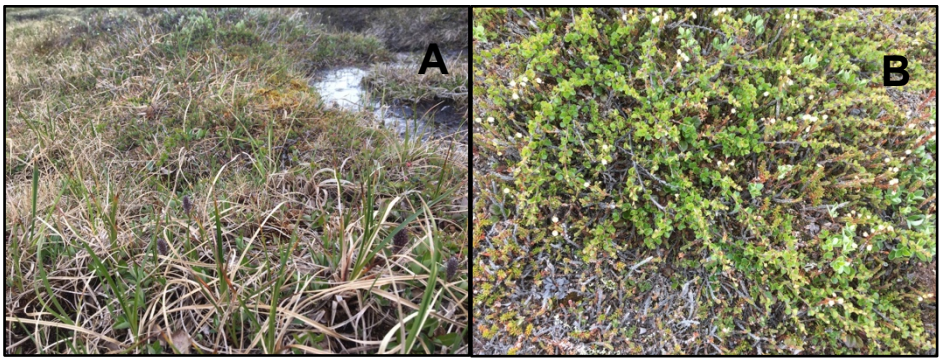

Figure 3 *Photographs taken at the sampling site on Disko Island, West Greenland displaying vegetation in both sites.* ***A*** *shows the sedge-dominated wet fen site,* ***B*** *shows a dry heath dominated by dwarf shrubs.*

Sampling strategy

Cores of 15-20cm length and 6cm diameter were taken in both wet and dry locations. The active layer depth was tested at each site using a permafrost probe. Replicates were taken in triplicate from the wet soil and in pentuiplicate for the dry soil. Samples were immediately sealed in plastic bags and

placed in a polystyrene cool box to maintain temperature and structural integrity. Samples were then stored at -4°C from June to October 2018, when they were processed.

During experimentation, samples were left to thaw in sealed 1L jars at room temperature and then kept at 4°C in a dark freezer for 28 days. Gas and 1g subsamples from the upper and lower core were taken at time of thaw, and at 1, 2, 7 and 28 days post thaw

Gas Measurements

Gas production of the frozen cores was monitored for 6 days. A gas sample was taken from each core at the beginning of this timepoint (T_{-6}) and 3 days post moving the cores to jars (T_{-3}), as well as the main timepoints (T_0-T_4). Samples were taken using a syringe, 3ml of gas was extracted from the jar head, and deposited into an evacuated vial for later analysis. All samples taken post thaw were taken over $4_{1/2}$ hours, at $1_{1/2}$ hour intervals so that the rate could be generated. Gas Chromatography (GC) set up with a Flame Ionization Detector (FID) was used to analyse CH_4 and CO_2. Electron Capture Detector (ECD) technology was used to analyse N_2O levels.

Chemical Analysis

For analysis of water extractable nutrients, 5g soil was shaken (120 rpm) in 20 mL ddH_2O for 10 min at 5 °C. All extracts were filtered through Whatman GF-D filters (Sigma-Aldridge, Copenhagen, Denmark) and frozen at -18°C until analysis. A subset of the filtered extracts was used for pH measurements with a pH meter. Dissolved organic carbon (DOC) was analyzed with a Shimadzu TOC-L CSH/CSN total organic carbon analyzer (Shimadzu, Kyoto, Japan), while dissolved organic nitrogen (DON) was analyzed using a FIAstar 5000 (FOSS Tecator, Höganäs, Sweden) after digesting the extracts in 2 M HCl with selenium as a catalyst. Ammonium (NH_4^+) was analyzed using the indophenol blue method and nitrate (NO_3^-) colorimetrically using the cadmium reduction method, both with a FIAstar 5000 flow injection analyser (Foss, Hillerød, Denmark).

Enzyme Analysis

Soil samples were assayed for activity of hydrolytic enzymes following the protocol of Kyachenko and colleagues (2017). Fluorometric assays of hydrolytic enzymes were performed with 0.5g of material in 50ml suspension of sodium acetate buffer (50mM, pH=5). The samples were homogenised at full speed using a handheld mechanical homogeniser (IKA Ultra-Turrax, Werke GmbH & Co., Germany) for 30 seconds and diluted to a concentration of 1g of soil per L of buffer. Fluorogenic substrates of N-acetyl-glucosaminidase (NAG), cellobiohydrolase (CBH), acid phosphatase (AP) and β-glucosidase (BG), were added to 200 µl of sample, resulting in a 250µl reaction volume. Samples were incubated at room temperature for 1 hour after which the reaction was stopped by the addition of 10µl of NaOH (0.5mM). The assay was performed with 5 technical repeats for each samples and 3 negative controls (boiled soil aliquots), 2 concentration series of non-quenched reference standards, and 2 quenched standards. Fluorescence was then be measured using a luminescence spectrometer, with wavelength set to 365nm for excitation and 450nm for emission. After subtraction of negative controls, enzyme activity was be calculated as $\mu molh^{-1}$ per gram soil based off the measured quenched standards.

DNA analysis

To this end, DNA was extracted from a 1g subsample using DNeasy PowerSoil kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions. 16S amplicons were produced using the forward primer 341F (CCTAYGGGRBGCASCAG) and the reverse primer 806R (GGACTACHVGGGTWTCTAAT). ITS amplicons were produced using the forward primer gITS7 (GTGARTCATCGARTCTTTG) and the reverse primer ITS4ngs (TTCCTSCGCTTATTGATATGC). PCR reactions were carried out using the PCRBIO Taq DNA polymerase kit (PCR biosystems Ltd, London, UK), in a 25 µl volume using 5 µl of PCRBIO Reaction buffer, 0.25 µl of PCRBIO Taq DNA polymerase, 2 µl of forward and reverse primer at 400 nM, and 1 µl of DNA template, with the following thermocycler conditions: 1 min at 95, followed by 30 cycles with 15 seconds at 95°C, followed by 15 seconds at 55°C and 15 seconds at 72°C, followed by 10 minutes at 72°C, and held at 10°C. The same conditions were used for 16S and ITS. After amplification, samples were checked for size on an agarose gel, before being sent for barcoding and Illumina MiSeq v3 sequencing at The University of Copenhagen.

Statistical analysis

Metagenomic data was processed using QIIME2 (Version 2019.4, Vitrual Box image 1.91 64-bit) [26]. Sequences were denoised, using DADA2 [27]. Taxonomic reference data was assigned to the amplicon sequence variants (ASVs) using the scikit-learn [28] classifier with UNITE (v8 18.11.2018) [29] and Greengenes (v13_8) [30], for fungi and bacteria, respectively. The phyloseq [31] and vegan [32] packages in R (version 3.5.3)[33] were used to analyse both the bacterial and fungal data, respectively. The 'veg-dist' function was used to compute a Bray-Curtis dissimilarity matrix based on fungal ASV table (rarefied at 1989 sequences per sample). Phyloseq 'ordinate' function was used to compute a weighted UNIFRAC distance matrix from the bacterial ASV table (rarefied at 1456 sequences per sample) and phylogeny data. Fungal OTUs were parsed into functional guilds using FUNGuild v(1.1) annotation tool [34]. Pipelines for these processes can be found in the supplementary material.

Linear models were created for all measured variables for the interaction of time (response to thaw), moisture (soil type) and time*moisture. Normality assumptions were tested graphically using R. Each model was also tested with the Shapiro-Wilk normality test, any variable with a p value > 0.05 was tested using Kruskal-Wallis pairwise comparison test. Variables with normal distribution according to the Shapiro-Wilk output were tested using one way ANOVA. The Adonis PERMANOVA from the vegan package in R was used to test significance of beta diversity plots. Data was presented using Rstudio ggplot2.

4 Results

Microbial Community Diversity

Both alpha and beta diversity were not significantly affected by time ($p > 0.2$), but were significantly affected by soil type ($p = 0.001$ and below) (Table 1, Figure 4). Although fluctuations appear in alpha diversity over time, this relationship was found to be insignificant. However, the ANOVA confirmed an interaction between soil moisture type and diversity. Bacterial community richness in the wet soil was found to be higher than in the dry soil, wet community richness peaking at 320 and dry community at 226. Similarly, for the fungal community, richness peaks at 149 in the wet community and 64 in the dry community.

Beta diversity in both the fungal and bacterial communities varied strongly with soil moisture type (Figure 4A, B, Table 1). Samples were separated by moisture, whereas timepoints can be seen to be scattered across the plot, and a p value greater >0.05 was generated using the ADONIS analysis for both communities. Nutrient measures (TOC, TON and NH_4^+) and most enzymes (NAG, BG and CBH) were most associated with PC1 in the bacterial community, indicating that they were strongly affected by soil moisture type. AP may associate more with the PCo1 in the case of the bacterial diversity, suggesting that it may have more of a relationship with the thawing effect. However, in the fungal community, all nutrients and NAG associated with PC2.

Microbial Community Structure

As reflected by the diversity analysis (Figure 4), bacterial community structure changed very little over time (Figure 5A, B). The main dominating phyla in both soil types were Acidobacteria, Actinobacteria and Proteobacteria.

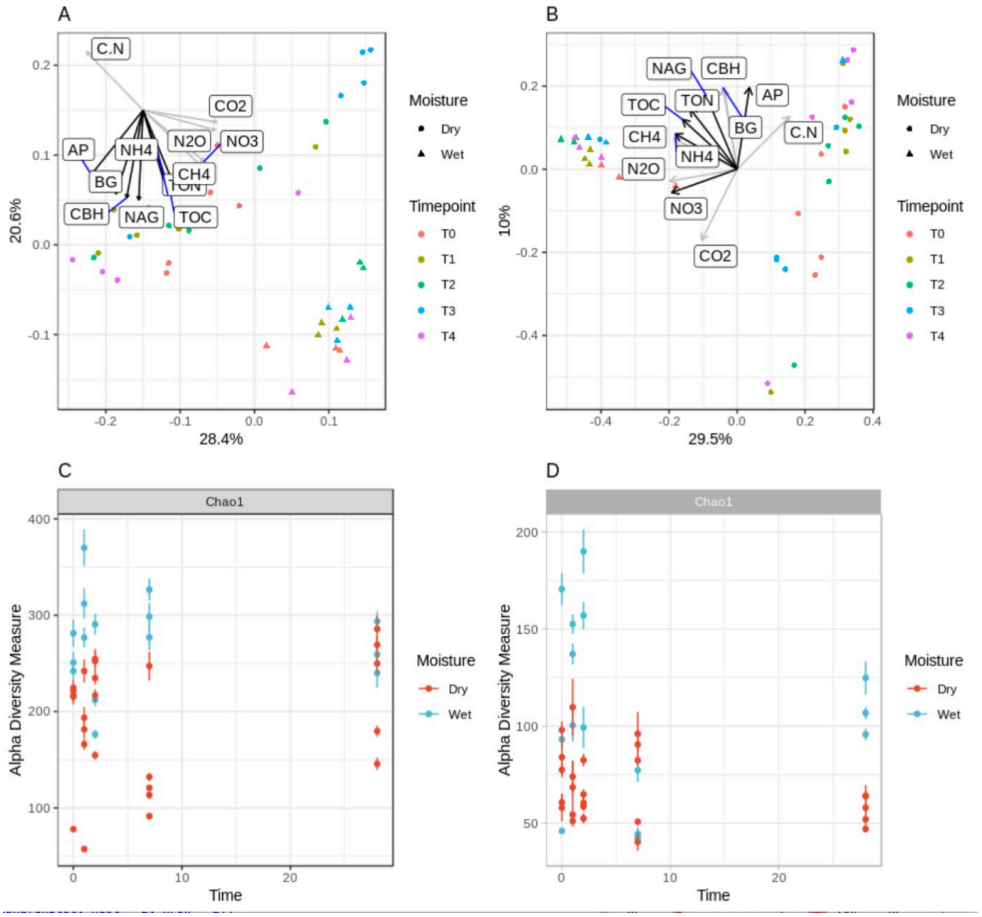

Figure 4 A *Principal coordinate analysis of weighted UniFrac distances of bacterial communities. Colour denotes timepoints as days post thaw: 0 days - 'T0', 1 day - 'T1', 2 days 'T2', 7 days 'T3' and 28 days 'T4'. Shape denotes soil environment from which samples were taken ('Wet' samples from a wet fen area, 'Dry' samples from a shrubbed heath). Arrows indicate the relationship of measured environmental variables* ***B*** *Principal coordinate analysis of Bray-Curtis distances of fungal communities. Colour, shape, and arrows as in A.* ***C*** *Line plot showing relationship of Chao1 species richness over time in bacterial community. Points indicate averages of replicates for each soil type and time points (for wet soil type n =3, for dry*

soil type n=5). ***D*** *Line plot showing relationship of Chao1 species richness over time in fungal community. Points as in C.*

Nitrospirae were found in both soil types, with a stronger presence in the wet soil. Cyanobacteria were found in the dry soil but became less than 1% abundant post-thaw. Planctomycetes were also found in the dry soil, but were missing from the wet soil, this is surprising as Plantcomycetes are aquatic by nature. Presence of Crenarchaeota was picked up in the wet soil, however the 16S primers that were used were not designed to test for the presence of Archaea, therefore this observation may not be reliable. It is possible that an increase of Proteobacteria can be seen in the wet soil type, however this is quite minimal. In the dry soil type an increase of Actinobacteria dominance can be seen, however this is reduced from day 7 to day 28.

Similar to the bacteria, the fungal taxonomic structure showed little response to thaw (Figure 5C, D). Minor differences might include an increase in Rozellomycota in the wet soil. This is logical as Rozellomycota contain many species that associate with algae, which are more likely to bloom in aquatic environments. Chytridmycota, a motile, fungal phyla that prefer aquatic habitats, are also present in the wet soil but absent from the dry soil. Overall, the dry soil was dominated by Ascomycetes, whereas the wet soil had a more even community, dominated by Ascomycetes and Basidiomycetes in balance.

The FUNGuild results matched 45% of the data successfully, the distribution of the data between samples can be found in the supplementary material. The matched remaining data confirmed the presence of several functional guilds (Figure 5E, F). Here they have been split into 3 main trophic strategies, indicated by colour: pathogens in shades of blue, mycorrhiza in shades of green, and saprotrophs in shades of red. The wet soil shows a variable functionality with time, mainly dominated by the Ectomycorrhiza, with the exception of 7 days post thaw in which the saprotrophs seemed to be more dominant. The dry soil has a strong presence of pathogenic species, and more animal pathogenic species when compared to the wet soil. Saprotrophs increase in abundance over time from thaw to day 7, after which they appear to decline to day 28. The presence of ectomycorrhiza seems to decline immediately after thawing, recovering at day 7, before declining again at day 28.

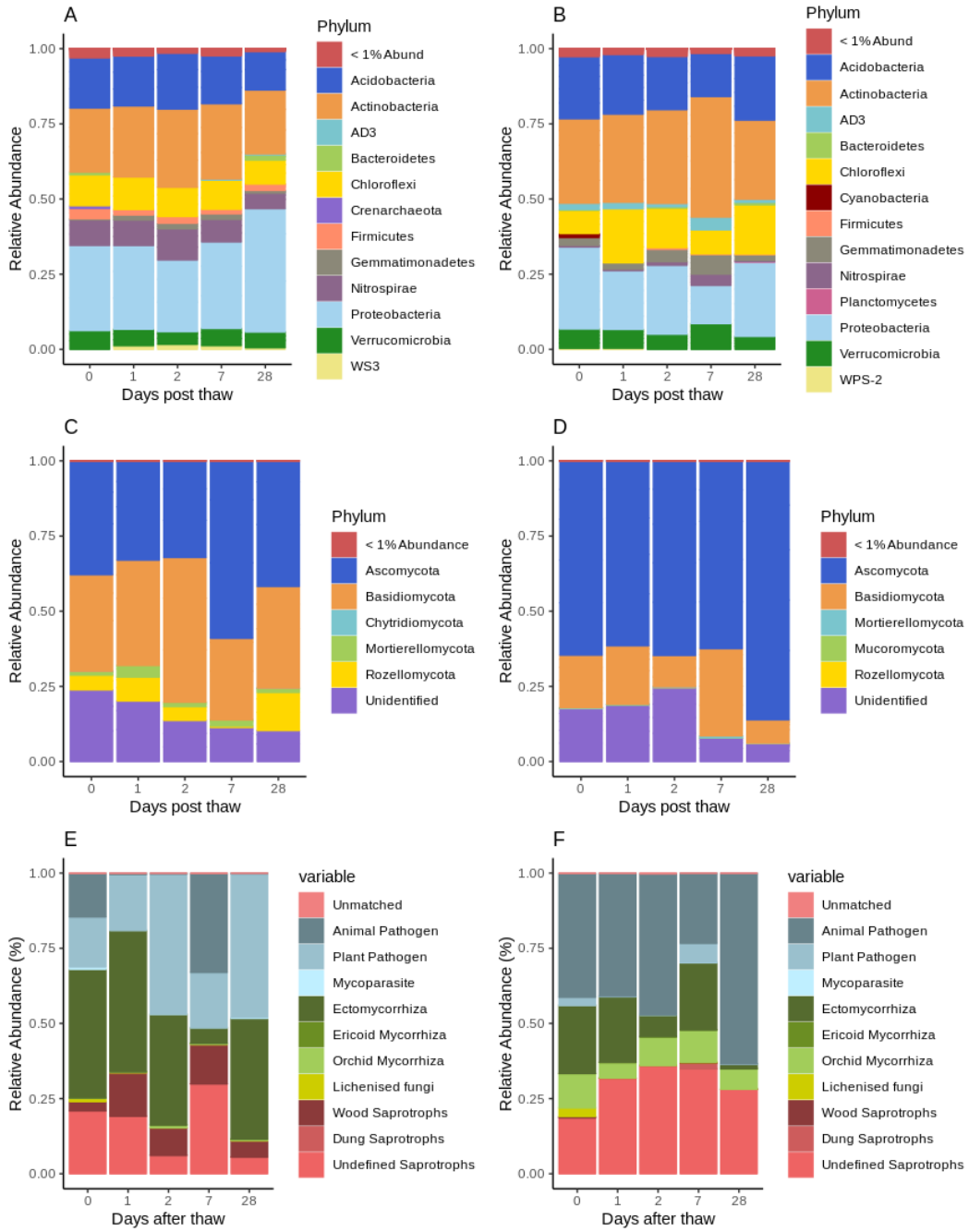

Figure 5 *Taxonomic bar plots generated from the metagenomic data, plotted over time post thaw and for each soil type. In A, C and E n=3, and in B, D, and F n = 5.* ***A*** *indicates the bacterial community composition in the wet soil* ***B*** *indicates bacterial community composition in the dry soil type* ***C*** *indicates fungal community composition by phyla in the wet soil D indicates fungal community composition by phyla in the dry soil* ***E*** *indicates the results of OTUs matched to function by FUNGuild in the wet soil, which represents 41% of rarefied sequences* ***F*** *indicates the results of matched OTUs by FUNGuild in the dry soil.*

Interestingly orchid mycorrhiza were found in the dry soil, although orchids were not identified in the vegetation survey. After investigating the raw data, it was found that the orchid mycorrhiza was *Serendipita* spp., formerly *Sebacina* spp. Which has been described as having both orchid and non-orchid hosts [35]. Animal pathogens that were identified in the dry soil were found to be dominated by *Aurobasidum pullulans*, a yeast-like opportunist which is found ubiquitously in oligotrophic environments [36][37]. In addition to this, *Alternaria* spp was also classified as an animal pathogen, despite being widely known as a plant pathogen. Although the genus is varied and contains species with plant, animal, soil and atmospheric hosts, it is likely in this case that it is a wrongly classified plant pathogen due to the abundance of plant hosts and relative scarcity of animal hosts [38].

Enzyme Activity

All four hydrolytic enzymes tested interacted significantly with time and moisture (Figure 6, Table 1). In all measured enzymes, activity was higher in the wet soil than the wet soil directly post thaw. 24 hours post thaw, cellobiohydrolase (CBH), β-glucosidase (BG) and acid phosphatase (AP) all decreased in activity in both soils. This was also observed for N-acetyl-glucosaminidase (NAG) in the wet soil type, but not in the dry. After 7 days, enzyme activity dropped for all enzymes and soil types, suggesting a decrease of microbial activity. After 28 days, NAG and BG activity increased to it highest point in the wet soil, but only marginally increased in the dry. The inverse was true for AP activity, and CBH activity increased by around the same amount I both the wet and dry soil.

Greenhouse Gas Flux

Significant interaction with time was found in all 3 measured greenhouses gases, CO_2, N_2O and CH_4 (Figure 6, Table 1). In both the wet and the dry soils, CO_2 increased sharply post thaw, showing the expected pattern of a 'pulse' of respiration post thaw. In both the wet and dry soils, an increase of N_2O can be seen from negative to net-zero over the thaw period. Although the interaction of CH_4 with time and moisture had a p value of >0.05, CH_4 fluxes interaction was significant with time and moisture separately. It can be seen that the CH_4 flux increases from negative to 0.05µg during the initial thaw period in the dry soil. Much higher CH_4 production was observed in the wet soil, with fluxes increasing from zero to over 1µg.

After 48 hours, CO_2 production in both soils sharply decreased. This pattern was also observed in the CH_4 in the dry soil, however a continued increase was observed in the wet soil, and for N_2O in both soils. After 7 days post thaw, decreases in gas production were observed for most soils and gases, with the exception of CO_2 and CH_4 in the dry soil and N_2O in the wet, which stayed constant. After 4 weeks, CO_2 production dropped to close to zero in the wet soil. However, in the dry soil, a negative flux was observed. As the cores were kept in the dark, this flux is not likely to be explained by photosynthetic activity, but more likely some kind of dark CO_2 fixation. Consumption of N_2O and CH_4 was also observed in the dry soils, however in the wet soil N_2O increased to its peak and CH_4 remained closed to zero.

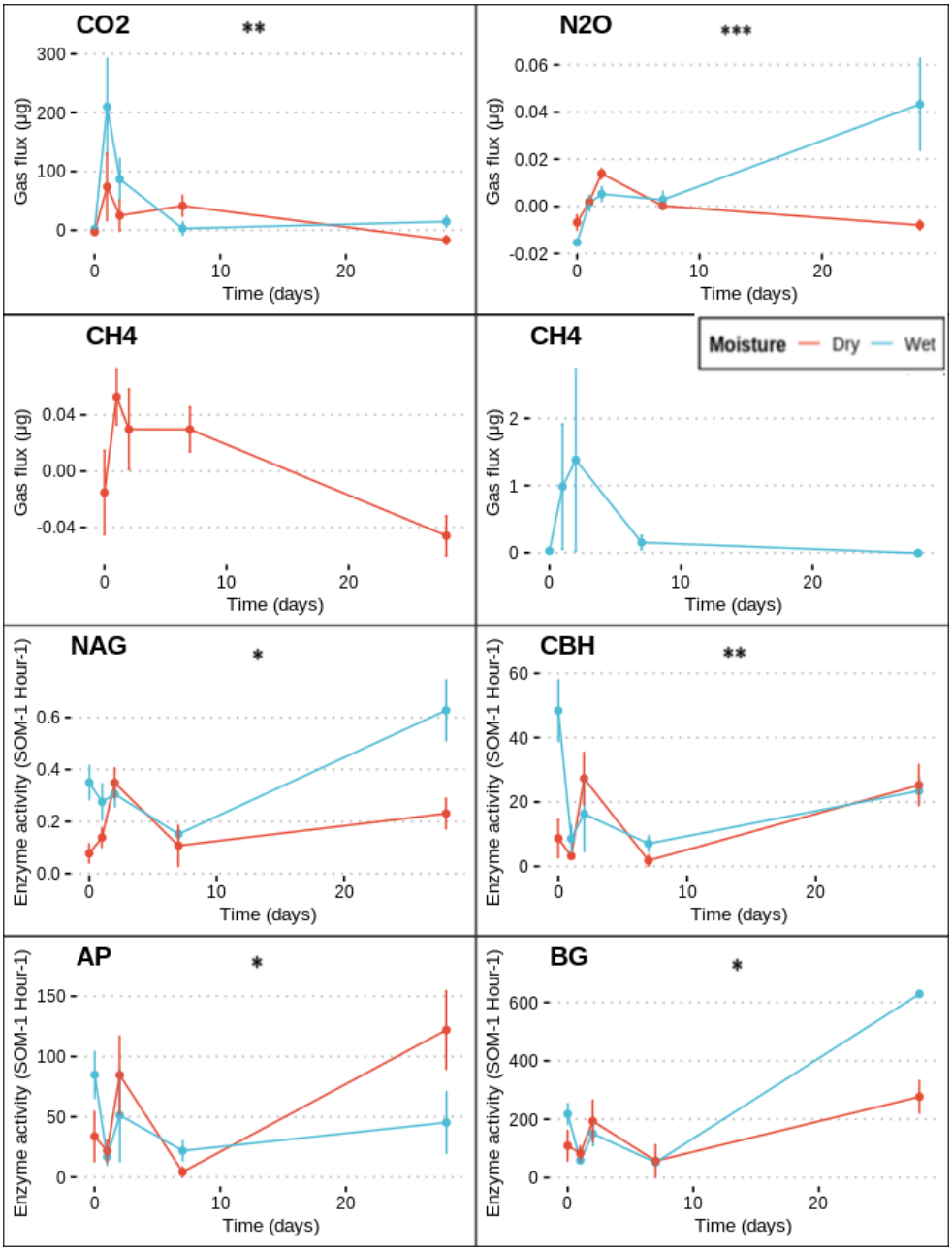

Figure 6 *Line plots indicating gas fluxes and enzyme activity responses to thaw. Line colour pertains to soil moisture type (red for dry soil moisture type, blue for wet soil moisture type), points indicate the an average for each time point (for wet soil types n=3, for dry n=5), and error bars indicate -/+ the standard error. Significant relationships between time and moisture are indicated by asterisks pertaining to p values (0-0.001 '***', 0.001-0.01 '**', 0.01-0.05 '*'). Abbreviations of enzymes are as follows:N-acetyl-glucosaminidase (NAG), cellobiohydrolase (CBH), acid phosphatase (AP) and β-glucosida*

	Time			Moisture			Time:Moisture		
Microbial Community	**F-value**	**df**	**Significance**	**F-value**	**df**	**Significance**	**F value**	**df**	**Significance**
Bacterial α-diversity	0.68	1	0.42	19.27	1	$9.52x10^{-5}$***	0.71	1	0.40
Fungal α-diversity	1.17	1	0.29	15.81	1	$3.24x10^{-4}$ ***	0.00	1	0.99
Bacterial β-diversity ϕ	0.94	1	0.41	12.95	1	0.001 ***	1.04	1	0.35
Fungal β-diversity ϕ	0.81	1	0.64	11.05	1	0.001 ***.	0.71	1	0.76
Enzymes	**F-value**	**df**	**Significance**	**F-value**	**df**	**Significance**	**F value**	**df**	**Significance**
NAG	6.36	1	0.016 *	10.49	1	0.0026 **	4.17	1	0.048 *
CBH χ	12.79	4	0.012 *	2.48	1	0.11	22.67	9	0.007 **
AP χ	11.67	4	0.02 *	0.33	1	0.56	18.09	9	0.035 *
BG χ	11.68	4	0.019*	1.09	1	0.29	20.74	9	0.014 *
Greenhouse gas flux	**F-value**	**df**	**Significance**	**F-value**	**df**	**Significance**	**F value**	**df**	**Significance**
CO_2 χ	18.195	4	0.0011 **	0.65	1	0.42	22.06	9	0.0087 **
CH_4 χ	12.22	4	0.016 *	4.22	1	0.040 *	16.67	9	0.054 .
N_2O χ	17.27	4	0.0017 **	0.51	1	0.48	31.70	9	0.00022 ***

Table 1 *Results of two way ANOVA test indicating interaction between the selected variable and time (effect of thaw) and moisture (soil moisture type). Significance codes indicate the following P-value ranges: 0-0.001 '***', 0.001-0.01 '**', 0.01-0.05 '*', 0.05-0.1 '.', 0.1-1. For variables marked with 'χ' a Kruskal-Wallis significance test was performed. For variables marked with 'ϕ' an ADONIS permutation test was performed.*

5 Discussion

Does the effect of thaw impact alpha diversity?

The results of the microbial community analysis show that alpha diversity did not change significantly with time, in either wet nor dry soil types. Therefore, we must reject the first hypothesis, which suggested that alpha diversity would decrease initially, and recover. However, part of this hypothesis stated that wet soil would have a stronger effect on alpha diversity, and we do find interaction between the soil moisture and alpha diversity (Table 1). This is likely due to variance in microbial communities between the two soil types, rather than the effect of thaw.

Although we see no significant changes in alpha diversity here, this is not definitive proof that community changes are not occurring. Measuring microbial communities using DNA has its limitations, for example the persistence of DNA molecules in soil. DNA can also be adsorbed into the soil matrix, the strength of the effect depending on soil pH, chemistry and texture [39, 40]. DNA has been demonstrated to persist in the soil for several months or even years [41, 42]. Therefore, OTUs that are not active may still be identified, which may be the case in our results. Removal of relic DNA had been demonstrated to reduce the richness estimates of some microbial communities [43] and have no significant effect in others [44]. This means that for some communities, DNA from non-viable has little effect on community analysis. However, due to the impact of stress in this study, it is likely that many non-viable cells are present in the sample at time of analysis that could overestimate diversity.

A few different approaches may have been taken in order to more accurately assess the effect of thaw on microbial diversity. One improvement may have been to take steps to remove DNA from non-viable cells before amplification by PCR. This is possible using Ethidium Monoazide and Propidium Monoazide extraction techniques. However, this method may overestimate viable cells, particularly in complex samples with high cell densities [45]. A metatranscriptomics-based approach would circumvent the problem of relic nucleic acids due to the short lifespan of RNA molecules. This technique has been used to successfully link increases in microbial processes to effect of thaw in permafrost soils [46] and in active layer soils [47]. However, metranscriptomics would also introduce similar biases based on the physiological state of the cell producing the RNA. As well as problems with accessibility of primers to the target region of the mRNA, all the associated PCR biases [48].

Another approach of analysing the community response to thaw may be to take samples from a thaw gradient *in situ,* rather than replicating its effect *in vitro*. This approach gives a long term view of how thaw affects a community and has demonstrated significant differences in community composition post thaw [49] and changes in alpha and beta diversity [50]. However, this doesn't address our query on the immediate mechanistic effect of thaw on microbial communities and its relationship with metabolising newly thawed organic matter. This method also has an added bias of differing soil type effect on the community, in addition to thaw. Another sampling condition to consider may be a controlled *in situ* thaw, for example snow fence manipulation. This method gives a multitude of advantages such as; implementing exact environmental conditions, ability to manipulate warming conditions, integrated vegetation and a less destructive sampling technique [51].

Microbial community response-to-thaw

The results of the enzyme assays give a varied response of microbial exoenzyme production to thaw and soil moisture (Table 1, Figure 6). The enzymes that were monitored are involved in the acquisition of essential nutrients for microbial growth. N-acetyl-glucosaminidase (NAG) is a vital enzyme in the degradation of chitin, which also allows for acquisition of N [52]. Cellobiohydrolase is a sequential hydrolyser of cellulose chains consisting of a

tunnel-like active site, which cleaves the glycosidic bonds to produce cellobiose [53]. The enzyme that then breaks down cellobiose to glucose is β-glucosidase (BG) [54]. Acid phosphate is an enzyme causing the hydrolysis of ester-phosphate bonds, causing the release of Phophate (P) [55]. These ubiquitous enzymes are powerful degradation tools for microbes to acquire nutrients. By measuring the activity of these enzymes, we gain an understanding of the major hydrolytic decomposition processes happening in the soil, performed by the microbial community.

Hydrolases are good indicator for changes over time as they are bound to organic matter which is broken down rapidly, after which they themselves are also broken down [56]. This gives us sensitivity whilst detecting changes in potential microbial activity over the thaw period. What we observe in our samples is that both NAG and BG had an initial decrease followed by a much larger increase in the wet soil rather than the dry soil. This initial decrease may be explained by the structural flexibility of the cold adapted enzymes being impaired by a sudden increase in temperature [57]. As these enzymes break down complex molecules for the acquisition of C and N respectively, we can infer that an increased activity indicates a shift in microbial demand for growth [58]. We can also see this trend is stronger in the wet soil than the dry soil, indicating that the wet soil is more microbially active. This may also be due to the increased water content of the wet soil, increasing the mobility of the substrate to the enzymes [56]. It may also be that low water content in the dry soil limits the intracellular water potential of the microorganisms, reducing the hydration and efficiency of the enzymes [59].

We also see in the reverse in the case of AP, and an equal distribution in wet and dry soils for CBH activity. This may suggest that another factor is driving the increase in activity over time, such as changes in nutrient concentrations or pH. We do find that TOC and TON are increasing over time in the dry soil, and interact significantly with Time*Moisture (Appendix 1). This may cause a shift in the C:N:P stoichiometry, limiting microorganisms for P, and increasing P harvesting. Hydrolases have been used as indicators for soil stochiometry, NAG and AP have been used as indicators of stochiometric limitations, for C, N and P, respectively [52, 58, 60]. It was demonstrated by Sinsabaugh and colleagues (2002) that increasing concentrations of N can stimulate the breakdown of labile organic matter by cellulolysis. It is therefore a consideration that changing nutrient content may also be influencing the change in enzymatic activity. However, likely due to the short sampling period, C:N stochiometery did not change significantly with time (Appendix 1). We can therefore not attribute the significant changes in potential

NAG an BG activity to stochiometry, in this instance. However, long term studies have made this link [61].

It may also be that increase in decomposition enzyme activity is driving increases in TOC and TON. However, we observe the opposite when considering the differences in soil type. In the dry soil we see and weaker increase in activity from 7 to 28 days when compared to the wet (Figure 6), but a more stronger increase in TOC and TON with time (Appendix 1). This may be explained by increased growth of microorganisms causing uptake of C and N for anabolic processes. This is logical as extracellular enzyme activity has been shown to correlate positively with resource demand [52]. Biomass data would been an interesting addition to this data set, to deepen the understanding of the microbial growth response over the thaw period.

As hydrolytic enzymes can only break down substrates with regularly arranged, easily broken down bonds they are associated with the availability of labile C. Their activity tends to correlate positively with dominance of saprotrophic fungi and bacteria [62]. Oxidative enzymes on the other hand have been shown to correlate positively with the presence of Ectomycorrhizal (ECM) fungi [62, 63]. Although potential Mn-peroxidase activity was we tested, we were not able to see peroxidase activity in our assay (Appendix 6). We therefore cannot link oxidase activity to the ECM we find in the metagenomic data (Figure 5). As for the hydrolytic activity, we do not observe a positive correlation between the hydrolytic enzymes and presence of saprotrophs, and the maximum r^2 value in the dry soil was 0.23 and 0.098 in the dry (Appendix 5). Moreover, when we compare the highest value of C and N acquiring enzymes (BG and NAG) in the wet soil, which occurs at 28 days, we see the lowest value of saprotrophs in the community. Additionally, at 7 days post thaw in the wet community, the number of observed saprotrophs is at its highest, whereas BG and NAG activity falls.

Difficulties arise when comparing stress responses of slow of microbial community changes to the rapid response of enzymes. This, in addition to the problems previously described with the interreference of relic DNA, may be why we are unable to correlate fungal community changes to enzyme activity. Other problems may stem from FUNGuild's parsing system. Of the sequences supplied, FUNGuild was able to match 42% to a function with a confidence rating of 'highly probable' or 'probable'. This is because FUNGuild relies heavily on exact sequence identity for taxonomic identification [34]. It can be seen that data loss was not homogenous through samples, therefore this may introduce a bias in the data set (Appendix 3). Additionally, as we do not know the quantity of the biomass in the samples, we are not

able to distinguish what proportion of decomposition is being carried out by saprotrophs, or bacteria.

Greenhouse Gas response to thaw

Our second hypothesis states that GHG fluxes would initially decrease, followed by a large increase in production. What we generally observe instead is a pulse of production during thaw, followed by a loss of production, and in some cases consumption. We can therefore reject the second hypothesis. All gases responded significantly to thaw, and CO_2 and N_2O had a p value >0.05 for the interaction of Time*Moisture. Our results suggest then that moisture does impact the response of gas fluxes from soils over a period of thaw. This has been already been demonstrated through field studies [64-66], and in models [67, 68].

The initial pulse of CO_2 from the soils we see immediately post thaw has been previously described in microcosm experiments [21, 66]. We can attribute this to a flush of nutrients entering the soil from the necromass of cells killed by thaw, which then stimulates the metabolism of the remaining microorganisms [21]. This effect is much stronger in the wet soil than the dry soil, this may be due to an increased osmotic toxicity causing a higher proportion of necromass for the microbial consortia to consume. We observe an increase in C:N ratio in the wet soil, but a decrease in the dry soil over initial period of thaw (Appendix 1) However, it should be noted that C:N ratio did not have a significant relationship with Time*Moisture (p=0.2).

After 2 days of incubation, CO_2 flux drops in both soils. This reduction can also been seen in the nutrient data, suggesting that the resources of the necromass begin to deplete and limit microbial processes. After 7 and 28 days TOC concentrations rise in the dry soil. However, CO2 flux do not rise and subsequently fall. Possible reasons for this may be the fixation of CO_2. As microcosms were incubated in darkness, and with very few plants left alive at the top of cores, photosynthetic activity is unlikely to be responsible. Another possibility is dark CO_2 fixation, which has been demonstrated to be unbiquitous in Arctic soils due their favourable geological conditions [69]. This often-overlooked phenomena may play an important role in Arctic CO_2 balances, especially considering the generally low net primary production and soil carbon input of Arctic soils.

CH4 fluxes varied significantly with both Time and moisture separately, but the interaction of Time*Moisture was slightly over the boundary of significance (p=0.054). However, a clear difference in flux can be seen between the wet and dry soils. It has been demonstrated that methanogenic Archaea are found ubiquitously in all soil types, but are mainly inactive unless put under wet, anoxic conditions [70]. Although methanogens are strict aerobes, they can survive long periods of time under oxic conditions, until they become wet [71]. It has been suggested that active layer deepening could increase CH_4 production due to the response of microbial communities to changes in hydrology, topography (such as thermokarst formation) and soil chemistry [11, 47].

In the dry soil, we observe fluctuations above and below zero. This suggests that there may methane consumption and production are occurring simultaneously. Methane oxidation is a strictly aerobic process, therefore a dry, well aerated soil provides favourable conditions for this [72]. Transcriptomics studies have highlighted the abundance of methanotrophs in active layer soils [73-75] and it has been suggested that tundra sites could be a methane sink due to their largely dry and mineral conditions [72, 73]. However, changes in soil moisture regime due to climate change may reverse this effect.

As with CO_2 and CH_4, N_2O fluxes respond to thaw with an initial pulse of production due to nutrient derived from necromass [76]. N_2O flux is one of the net outcomes of aerobic and anaerobic processes in the nitrogen cycle. Nitrification is a soil process that produces N2O, this is carried out by the phylum Nitrospirae, which we observe in both the wet and dry soil communities (Figure 5). However, in most cases the main source of N_2O stems from the incomplete denitrification of NO_3^- by denitrifying microorganisms [77]. Increases of denitrifying genes of up to 10 fold have been reported directly after thaw, coinciding with an exponential rise in N_2O production. After 9 days, the transcription levels decreased, as well as N_2O fluxes [78]. This is consistent with our results in the dry soil, however in the wet soil we observe a large increase from 7 to 28 days. Soil type and moisture content has been shown to be a key factor in the production of N_2O from soils, with wet soils acting as much larger sources of N_2O [79]. Here we observe the same, as N_2O production significantly reacts to soil moisture (p=0.0002).

Negative fluxes of N_2O were also observed in both soils at the time of thaw, and in the dry soil after 28 days.N_2O consumption is a highly specialised process, with few enzymes identified that can perform its reduction [80]. However, despite this niche ability net consumption of N_2O has been observed in soils [81, 82]. The complex nature of the nitrogen cycle makes it difficult

to make judgements based on net measurements due to organisms acting as both consumer and producers of N2O, and multiple processes contributing to the flux. To understand all these processes fully, measurements of anaerobic and aerobic mineralisation processes will need to be made.

Studying microbes in a changing climate

Here we notice that although community structure appears to not change significantly with time, we observe changes in functionality. There is currently debate about the importance of community structure in microbial ecology, with trait-based approaches becoming more popular [83]. It is more likely that a fluid, complementary consortium of organisms are contributing to ecological processes such as gas production, rather than abundance of specific OTUs [84]. Functional responses to freeze-thaw stress may have critical implications for GHG feedback systems, essential for the informing of climate change models.

Changes in environment can have very abrupt consequences for microbial communities, as we see here. Climate change can cause extensive ecosystem shifts, and Arctic systems are particularly sensitive to this [5, 85]. We see here that soil moisture type has significant impacts on microbial diversity and activity. As the Arctic soils become increasingly thawed, changes in soil hydrology are very likely to occur. Such changes as thawing of permafrost, increases in precipitation, formation of thermokarst lakes, and other meterological changes resulting from climate change [13]. Microbial communities have the potential to shift soils from C sink to a source of greenhouse gases, contributing to feedback mechanisms that could worsen the effects of climate change. It is therefore imperative that this topic is investigated further.

Choice of replication strategy is very important when designing the study. An ideal methodology would be to sample multiple wet and dry sites in triplicate to give true biological replication. This would allow for a confident judgement on the effect of moisture on thaw taking into account of factors in the soil that may have additional effects. However, here we chose to take multiple replicates from one wet site and one dry site, as this was within the boundaries of possibility for the study, when considering practical limiting factors such as time and expense. This may be considered psuedoreplication due to the single site chosen to compare wet and dry sites. Despite this bias our results give a confident indication that moisture has a differential

effect on production of greenhouse gas, which can be explored further in future studies.

The study of soil microbial communities poses many challenges, from initial sampling, through processing, to the interpretation of data. We recognise the multitude of biases introduced to our data set and here we suggest ways to circumvent them. Our suggestions for further investigation of microbial community response to thaw are as follows:

(i) More sensitive techniques for the study of microbial diversity such as transcriptomics. This also gives more insight into the changes in functional diversity for communities.

(ii) In situ studies, preferably with automated measurements, to give a non-destructive representation of microbial communities with exact environmental conditions.

(iii) Studies including multiple sites and replication at each site, to give true biological replications.

(iv) Longer time frame studies are an important consideration for identifying soil-atmosphere feedbacks. These studies give more accurate insights into long term effects on microbial communities and biogeochemical cycling. Additionally, here we present the effects of only one thawing episode which may misrepresent the long term response to thaw and GHG production.

6 Conclusion

Microbial communities are highly sensitive to environmental change and, as we have demonstrated here, can change behaviour very rapidly. Stresses imposed on a community from thaw can produce rapid shifts in microbial activity and biogeochemical cycles. We observe that over a short timeframe of 28 days, soils may act as both a sink and a source of GHGs in response to thaw. We find wet soil microbes may respond to thaw by mineralising organic matter to GHGs, whereas dry soils may become a C and N sink from the consumption of GHGs by microbes. Here we do not observe changes in diversity or community structure. However, we cannot rule out the possibility of changes derived from thaw. We suggest that further studies should include more sensitive techniques, such as metatranscriptomics, to gain more precise insights into the functional diversity of the community and its response to thaw.

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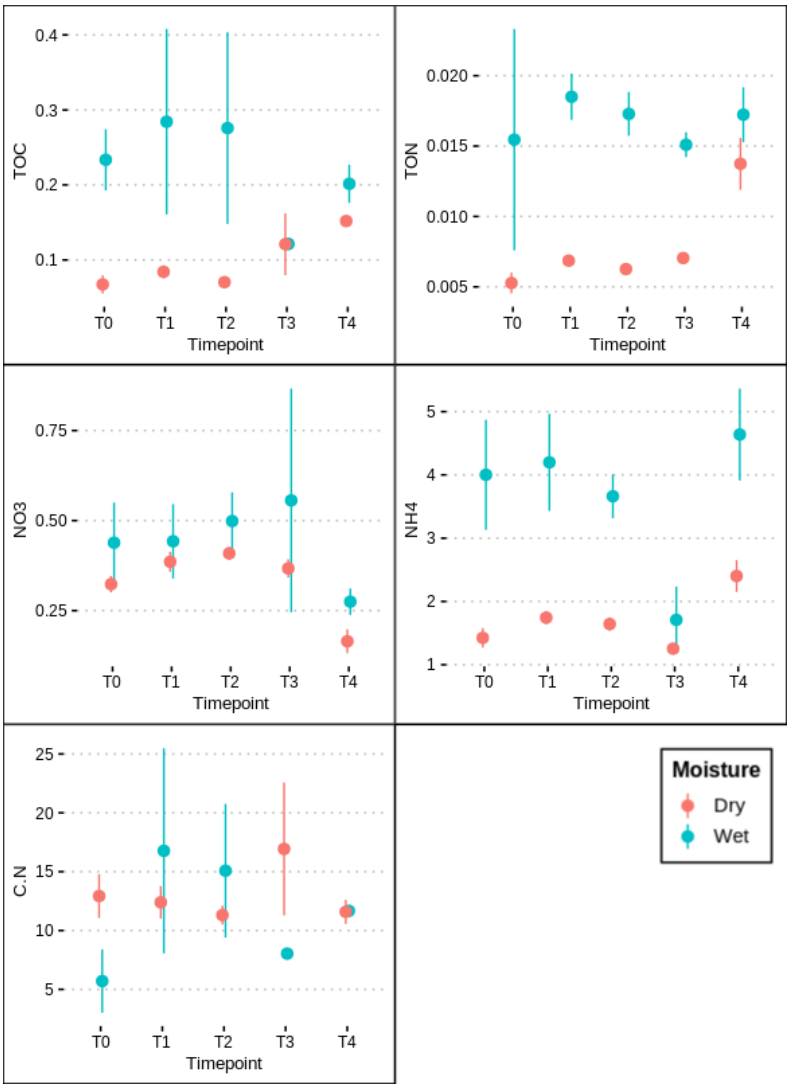
Appendix

Appendix 1



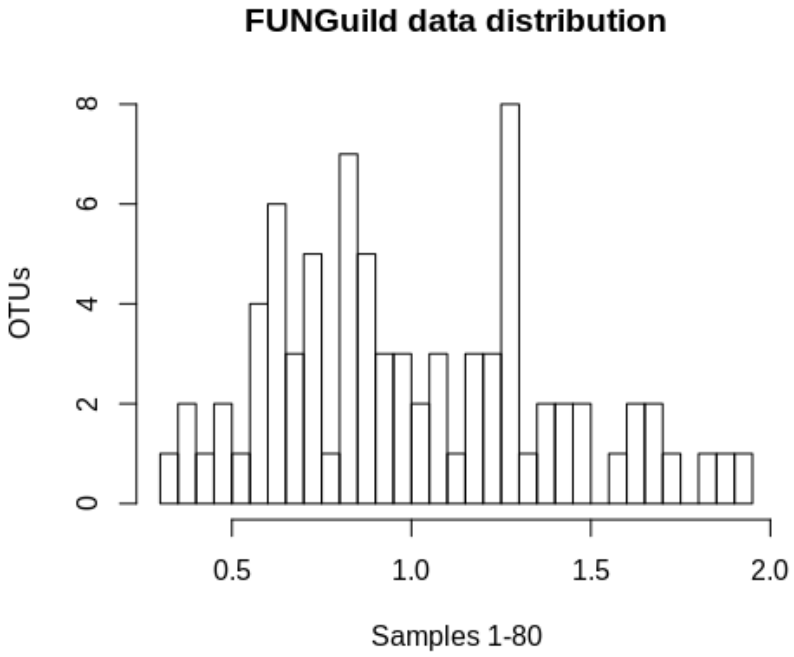
Plots indicating the change in nutrient content over the time series. Nutrients are given in ug/g dry soil. For wet samples n=3, for dry n=5. Abbreviations for the nutrients are as follows: total organic carbon (TOC), total organic nitrogen (TON), nitrates (NO3), ammonia (NH4) and the C:N ratio (C.N). Abbreviations for time series (TO, T1, T2, T3, T4) are 0, 1, 2, 7, and 28 days post thaw, respectively.

Appendix 2

	Time			**Moisture**			**Time*Moisture**		
TOC	5. 7	4	0.2	17.9	1	2.311e-05***	28.895	9	0.000675***
TON	6.8	4	0.1	17.9	1	2.311e-05***	27.135	9	0.001329**
C:N	2.0	4	0.7	4.8	1	0.0283*	11.272	9	0.258
NH_4^+	14.1	4	0.007**	14.5	1	0.000137***	30.734	9	0.000329***
NO_3^-	18.1	4	0.001**	0.8	1	0.3	20.014	9	0.01783*

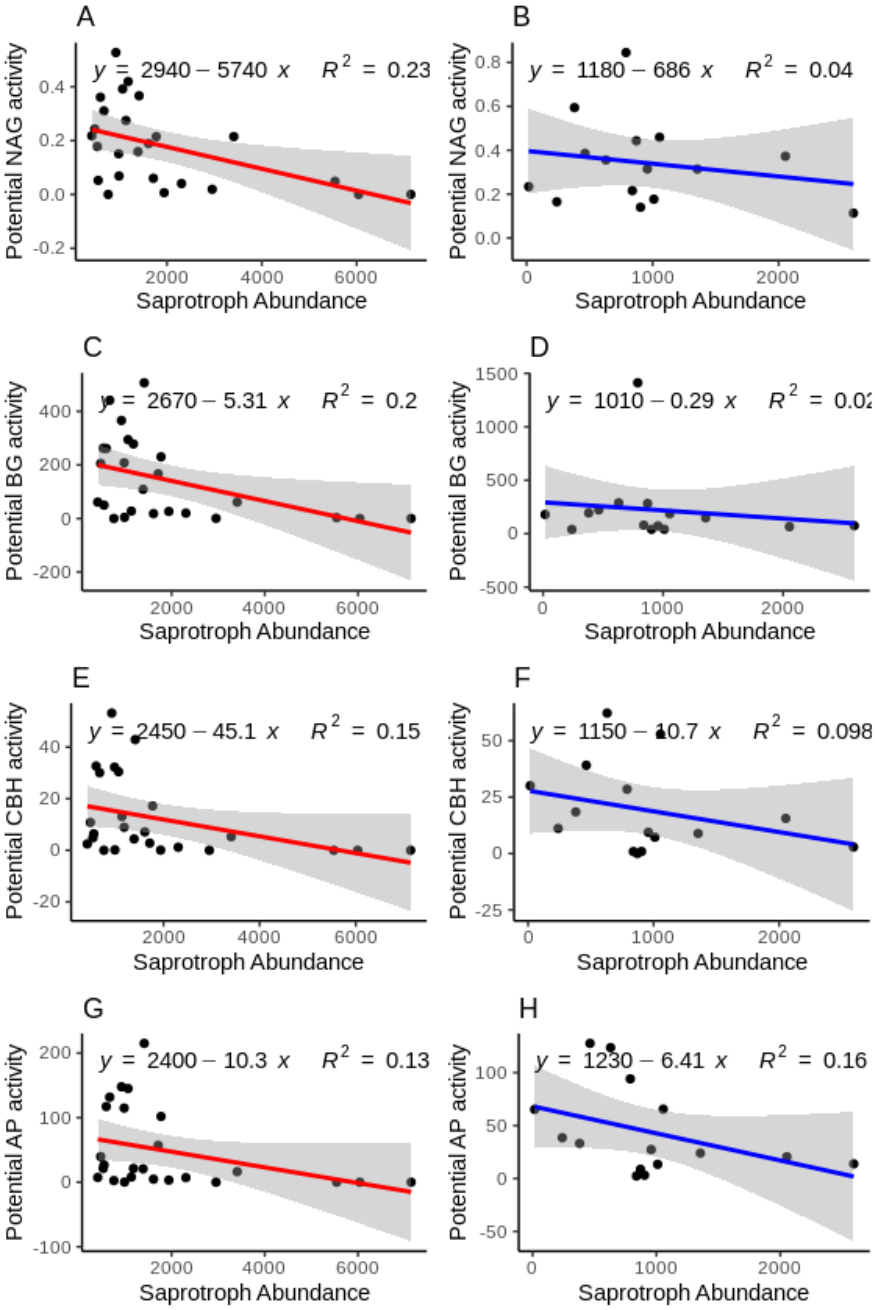
Table indicating the results of the Kruskal-Wallis significance test. Significance codes indicate the following P-value ranges: 0-0.001 ‘***’, 0.001-0.01 ‘**’, 0.01-0.05 ‘*’, 0.05-0.1 ‘.’, 0.1-1.

Appendix 3



Histogram showing the distribution of OTUs parsed into a guild by FUNGuild amongst the 80 samples. The number if breaks here is 30.

Appendix 4



Linear models showing relationships of each enzyme (NAG,CBH,BG, and AP) against the abundance of saprotrophs in each samples.

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