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Bacterial community composition changes independently of soil edaphic parameters following localized permafrost disturbance

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Abstract. Microbial degradation of frozen organic carbon increases with permafrost thaw, resulting in greater fluxes of the greenhouse gases CO_2 and CH_4. To examine the effect of disturbance-induced permafrost thaw on microbial communities, we assessed the microbial diversity of soils near a gold mine where thaw was induced by stripping the vegetation and topsoil at Dominion Creek, Yukon, Canada. Bacterial metabarcoding and soil physicochemical parameters were assessed across this disturbance including surface samples and three cores which included active layer and permafrost horizons. Bacterial communities changed in the absence of physicochemical parameter shifts after only 6 weeks of thaw, with a high proportion of active layer indicator species becoming more abundant with permafrost thaw. Three distinct communities emerged: (1) undisturbed active layer, (2) lower active layer, disturbed active layer, and disturbed permafrost samples, and (3) intact permafrost. Community composition shifts correlated with pH, Zn and community cohesion. These results suggest that active layer communities rapidly colonized thawed permafrost at our sample site, combining with and replacing many resident permafrost taxa. Disturbances may induce a strong microbial community change in permafrost-affected soils before micronutrient, organic carbon, and pH shifts as measured in this study.

1 Introduction

There is roughly 1700 Gt of carbon in northern circumpolar soils, more than twice as much as currently in the atmosphere (Schuur et al., 2015; van Huissteden and Dolman, 2012). Although increased vegetation and greening of the Arctic may offset carbon output from permafrost thaw, the increased rate of forest fires (Chen et al., 2021) and thermokarst formation as a result of abrupt permafrost thaw (Turetsky et al., 2020) may already have shifted the Arctic from a net carbon sink to a net carbon source as carbon mineralization outpaces fixation (Schuur et al., 2021). Models suggest that thawed permafrost sediments will release 41 Pg C per 1 °C of warming as greenhouse gases by the year 2100, representing one of the most significant biospheric sources of atmospheric carbon (IPCC, 2023). Newer models suggest that of this predicted 208 Pg C yr^{-1}, approximately 80 Pg C will be sourced from abrupt thaw areas which cover only 5 % of global permafrost-affected areas (Turetsky et al., 2020). However, there is significant uncertainty in the timing and mechanisms of these models. A significant source of uncertainty is how the microbial communities will respond to permafrost thaw.

Permafrost thaw acts as a disturbance to the environment, changing the ice and water content, leading to subsidence or mass wasting of overlying layers, and releasing preserved organic material (Kokelj and Jorgenson, 2013). Such disturbances can be the result of the rapidly rising temperatures in the Northern polar regions (i.e. polar amplification of climate warming) or industrial activity such as mining or road construction (Richter-Menge and Overland, 2010). The extent and severity of these disturbances can vary from relatively small scale and minor (e.g. subsidence and ponding due to road building) to large scale and extreme (e.g. active layer detachment and mass wasting due to the formation of retrogressive thaw slumps) (Kokelj and Jorgenson, 2013). These disturbances strongly affect bacterial communities in the permafrost, leading to shifts in both the structure and function of bacterial communities residing in permafrost

(Wu et al., 2018). However, our knowledge of such shifts is mostly limited to laboratory-based experiments, where there are no other sources of bacteria than the permafrost itself.

Bacterial community activity regulates the rate of carbon mineralization from permafrost, and so the characterization of these communities is paramount to modelling carbon flux from thawing permafrost soils (Monteux et al., 2018; McCalley et al., 2014). Although permafrost microbial communities are not as diverse as in the seasonally thawing active layer, permafrost microbial diversity is substantial (Hultman et al., 2015). Within the last decade, an abundance of information has been collected regarding the microbial response to in situ permafrost thaw (Taş et al., 2014, 2018; Singleton et al., 2018; Woodcroft et al., 2018; Emerson et al., 2018; Hultman et al., 2015). Field experiments have shown contradicting results; either that (1) thaw shifts the permafrost microbial community to become similar to the overlying active layer (Taş et al., 2014; Monteux et al., 2018), or that (2) subsidence creates an altogether new microbial community not previously seen (Mondav et al., 2014; Woodcroft et al., 2018).

Soil microbial activity refers to the growth and metabolic processes undertaken by soil microbial communities (Nazir et al., 2024). Increased microbial activity in thawing permafrost has been observed through increases in respiration, methanogenesis, and denitrification (Hultman et al., 2015; Palmer et al., 2012). While specific taxa upregulate broad transcriptional activity (with increased total RNA production) during permafrost thaw, only a fraction of the total community participates in biogeochemical cycling (Coolen and Orsi, 2015). Despite low levels of activity under frozen conditions, the fraction of viable microorganisms in permafrost is highly variable: 18 %–63 % (La Ferla et al., 2017; Hansen et al., 2007; Mackelprang et al., 2017); much lower than the 91.3 % viability ratio found in thawed temperate soils (Janssen et al., 2002). It is unknown whether increased microbial activity with thaw is driven by an increase in microbial viability or greater activity of a small fraction of live microbes.

Dramatic changes are induced by permafrost disturbances, with long-lasting impacts on plant community composition, plant physiognomy, and soil chemistry in both modern and historic sites (Forbes et al., 2001). Soil horizon removal is the most severe of these disturbances, requiring 20–75 years to recover plant community function (Forbes et al., 2001). As industrial activity becomes more commonplace in the subarctic, anthropogenic disturbances may disrupt plant communities and shift the source of this fluxed carbon to old, frozen, and recalcitrant deposits. Both natural and artificial disturbances can induce lake formation. In other permafrost affected environments outside of the Arctic, artificial disturbances – namely infrastructure development – are the most common causes of thermokarst lake formation in the Qinghai-Tibetan Plateau (Lin et al., 2016). The construction of oil wells, pipelines, gold mines, and other mineral extractions are also becoming more common in the Arctic (Elias, 2014). These industrial developments are conducive to the formation of thermokarst ponds across the Circumpolar North. It is unknown how the microbial community of active layer and permafrost will change in response to these direct industrial disturbances or if industrial disturbances differ from natural disturbances.

In this site-specific study, we assess the soil microbial community structure shifts and drivers at a locally disturbed site where soils and vegetation were stripped in preparation for mining at a permafrost-affected site near Dominion Creek, Yukon, Canada. The study site was disturbed by the installation of a drainage ditch a few weeks prior to our arrival at the site by a large excavator (Fig. 1). As microbial diversity has been closely linked to edaphic parameters, we hypothesized that microbial community shifts in disturbed permafrost are dependent on edaphic parameter changes. In addition, we hypothesized that thaw would induce the proliferation of viable microbial cells, increasing microbial viability ratios with disturbance, due to the exposure to atmospheric temperatures for six weeks. This information helps clarify how permafrost communities restructure upon thaw.

2 Methods and Materials

2.1 Field Site and Sampling Procedure

To assess the impacts of disturbance and thaw on permafrost-affected soils, we sampled after a disturbance along a gradient from undisturbed to a thawed thermokarst pond (Fig. 1). Surface samples and soil cores were collected in May 2016 at a gold mining site near Dominion Creek, Yukon Territory, Canada (NTS:116-B/3; DMS: 60°42′′59.5′ N, 138°33′′0.14′ W) . Cores were taken with a light portable gas-powered permafrost drill (EDR-260, ECHO Inc., Lake Zurich, IL, USA), (Saidi-Mehrabad et al., 2020a). Cores were collected using a system devised in Calmels et al. (2005) which takes individual ~ 40 cm cores which are then broken off from the surrounding permafrost and recovered. The study site was disturbed in preparation for placer gold mining a few weeks prior to our arrival at the site by a large excavator. The primary disturbance was the development of a drainage ditch (Fig. 1c) which removed the upper active layer at Core Site 2, and all of the active layer at Core Site 3 (Fig. 1b). Site 1 was located away from the disturbance site.

Quadruplicate surface samples (unfrozen soils) were taken at six locations including undisturbed permafrost-affected soils (site A; 474 cm from the thermokarst pond); disturbed active layer soils (sites B–D; 363, 288, and 213 cm from the thermokarst pond, respectively); and putatively disturbed permafrost sediments (sites E–F; 109 and 0 cm from the thermokarst pond, respectively). Site A represents the least disturbed reference sample along a spatially-linked position,

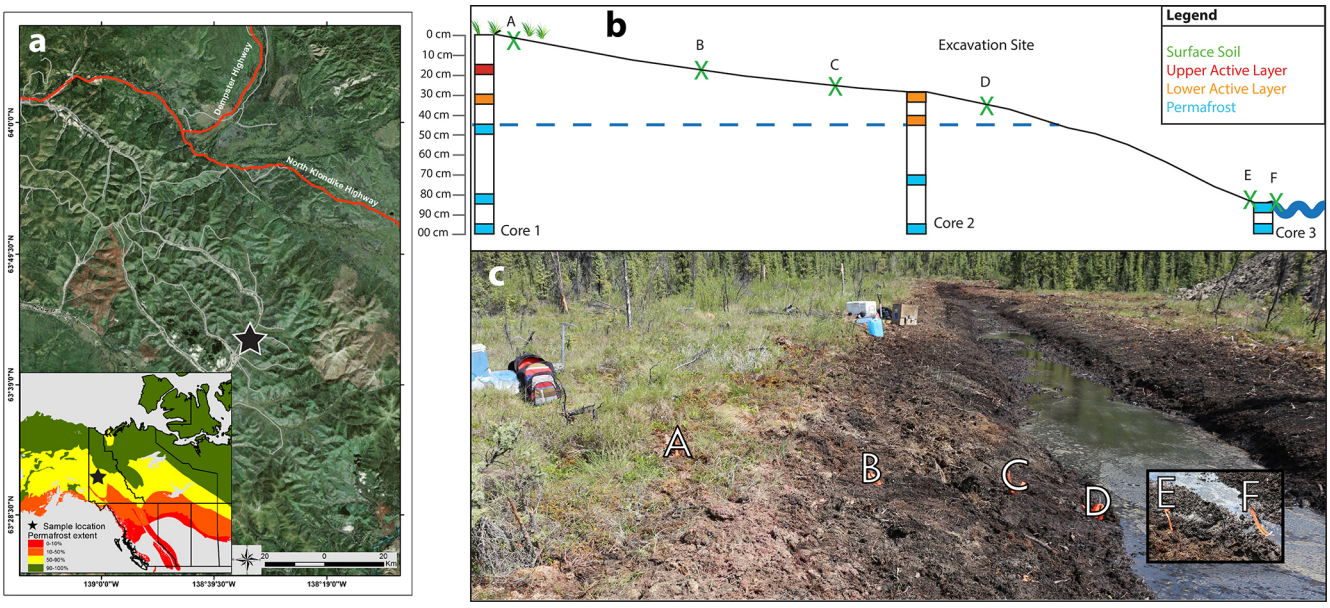

Figure 1. Dominion Creek core and surface sample locations. **(a)** Map of Dominion Creek area and sampling site within Yukon Territory (source: USGS and The Government of Canada). The map of the Dominion Creek region was assembled in ArcGIS 10.6.1. **(b)** Cross-section of disturbance gradient soils and permafrost cores. The horizontal line represents the surface soil profile as it had been disturbed from road construction preparation. The undulating line refers to the forming thermokarst pond. A scale depicts the depth of each permafrost sampling layer. The dashed blue line refers to the permafrost table. **(c)** The disturbance gradient soils including A (least disturbed), B–D (disturbed active layer), and E–F (disturbed permafrost). Disturbed permafrost samples are inset and were found 3 m away at a shallower location of the thermokarst pond.

leading to a single thaw feature, rather than as independent controls and experimental treatments. Organic layers and surface vegetation were removed with a spade before sampling. A separate spade was cleaned with 10 % bleach and 70 % ethanol before sampling at approximately 5 cm depth and stored in sterile Whirlpak® bags and homogenized by mixing both in the field and in the lab prior to chemical and biological analyses.

Soil cores, which were 1–3 m in depth, were collected at three sites along the same thaw gradient (from Core 1–Core 3). Core 1 was sampled in a nearby clearing analogous to the conditions at sample A (the "undisturbed" site). Core 2 was sampled 2.6 m from the thermokarst pond between surface sample sites C and D. The Core 2 site had all vegetation, the organic soil horizon, and the surface soil horizon of active layer removed; however, active layer soils were still present in Core 2. Core 3 was sampled directly adjacent to the thermokarst pond, with all soil layers above the permafrost table removed. The thaw depth was 14, 34, and 85 cm for Cores 1, 2, and 3, respectively (Fig. S1 in the Supplement). All thawed soil above 15 cm was removed prior to coring. Each core subsection had a 10 cm diameter and ranged in length from 14–36 cm long, the total core depth and reached at least 1 m for every core as measured from the soil surface. Loose material on the surface of soil cores was removed by scraping with razor blades and discarded. Frozen soil cores were placed in clear polypropylene bags (Uline, Pleasant Prairie, WI, USA), transferred to coolers with ice packs in the field, transported frozen to the laboratory, and stored at −20 °C until further analysis. Permafrost cores were collected frozen while surface samples were thawed at the time of collection. All samples were transported back to a freezer in Dawson City, frozen to ca. −20 °C and transported frozen to the University of Alberta Permafrost Archives Laboratory.

Surface samples along a disturbance gradient were collected to assess the impact of thaw as a result of the disturbance, allowing for direct comparison to undisturbed frozen soil cores beneath them. In this study we consider the following as active layer samples: Core 1 – 30 cm, Core 2 – 30 cm, and Core 2 – 45 cm. Active layer soil was distinguished from permafrost by measuring gravimetric water content at 1 cm intervals along each core. A pronounced increase in water content was observed at the transition zone at 45 cm as shown in Fig. S1: Core 1 – 45 cm, Core 1 – 75 cm, Core 1 – 95 cm, Core 2 – 75 cm, Core 2 – 95 cm, Core 3 – 75 cm, and Core 3 – 95 cm. Soil water accumulates at the bottom of the active layer, allowing for determination of the maximum active layer depth through the determination of gravimetric water content (Chang et al., 2024). Surface sample A was denoted as an undisturbed active layer soil, surface samples B–E were denoted as disturbed active layer soil, and sample F was denoted as a disturbed permafrost soil (Fig. 1). Adjacent undisturbed areas were used as controls to assess the impact of dis-

turbance on microbial community composition. These soils were collected as nearby as possible to minimize the impact of soil spatial heterogeneity, however, some underlying spatial variation cannot be fully removed.

2.2 Soil Chemistry Subsampling and Analysis

Physicochemical analysis was performed as in Saidi-Mehrabad et al. (2020b). Surface soils were subsampled as follows: 0.5 g for loss on ignition (LOI), 0.5 g for gravimetric water content; 0.5 g for pH; 5 g for nitrate analysis; 0.5 g for dissolved metal and TN/TC (Total Nitrogen/Total Carbon) analyses. Cores were longitudinally sectioned into $\sim 1/3$ (3 cm width) and $\sim 2/3$ (7 cm width) sections for chemical and biological analyses, respectively. An additional two longitudinal subsections were taken from the $1/3$ portions (chemical analysis section) of the cores. Subsections were scraped with a razor blade to remove all thawing and contaminating materials. The first section was cut into 1 cm^3 cubes and used for pH, SOM, and gravimetric water content throughout the entire profile of each core. Soil water content was determined by oven drying the samples at 105 °C for 24 h. Soil pH was determined using a soil to 0.01 M $CaCl_2$ ratio of 1 : 2 with an AB15 pH meter (Fisher Scientific, Waltham, MA, USA). Soil organic matter was measured using loss on ignition (LOI) procedures (Lim and Jackson 1982), using a Lindberg SB Muffle Furnace (Thermo Fisher Scientific Inc., USA). Subsamples from the second section were homogenized, and 0.5 g of material was taken for TN, TC and dissolved metals while the remainder was used for NO_3 and NH_4 measurements at the Natural Resources Analytical Laboratory (NRAL, Edmonton, AB, Canada). Total Nitrogen and TC were analyzed using standard dry combustion methods with a Costech Model EA 4010 Elemental Analyzer (Costech International Strumatzione, Florence, Italy, 2003) (Sparks et al., 2020). Extraction of NO_3 and NH_4 was performed using a 2 M KCl solution and measured using a Diazo Coupling method with a SmartChem Discrete Wet Chemistry Analyzer, Model 47 200 (Westco Scientific, Brookfield, CT, USA) (Maynard et al., 2007). Trace metal analysis was conducted after an HNO_3/HCl acid digest using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) which measured P, K, S, Mg, Ca, Fe, Cu, Mn, Zn, Na using standard protocols on a Thermo iCAP6300 Duo ICP-OES (ThermoFisher, Cambridge, UK) (Skoog et al., 2007).

2.3 Biological Subsampling and Contamination Detection

The $2/3$ portions of the cores ("biological sections") were cut into discrete 5 cm long sections and subsamples were taken at 15, 30, 45, 75, and 95 cm below the soil surface: Core 1 was subsampled at all horizons; Core 2 was subsampled at 30, 45, 75, and 95 cm; Core 3 was subsampled at 75, and 95 cm. Contamination detection was performed as in Saidi-Mehrabad et al. (2020a). To detect contamination, each subsample was painted with 10 mL of 1× PBS solution containing *E. coli*: DH10B transformed with pBAD *mNeonGreen* at 8.3×10^9 cells mL^{-1}; for a total of 8.3×10^{10} cells/core in a 4 °C walk-in refrigeration unit. All solutions (including 10 % bleach and water) were kept at 4 °C to maintain the integrity of the core. Any thawed soil was removed using an autoclaved and sterile razor blade. The remaining soil was reserved for DNA extraction. Following decontamination, the presence of PCR-amplifiable pBAD vector DNA would be interpreted as potential contamination. DNA extraction subsamples were prepared in a class 10 000 clean lab at the University of Alberta which has not been used previously for DNA amplification. To decontaminate cores, samples were: (1) immersed in 100 % bleach solution, (2) washed with DNA-free water, and (3) scraped approximately 4–7 g of material using a razor blade; this cycle was repeated twice. Subsamples were then homogenized within a sterile Whirlpak® bag. The resulting decontaminated soils were then stored at −20 °C until DNA extraction. Genomic DNA was extracted from 0.5 g samples using the MOBio PowerSoil kit according to the manufacturer's instructions. Replicate extractions were pooled before contamination checks and sequencing. A portion of each soil sample was reserved for DNA extraction using PMA to test assess the effects of free DNA removal (Methods in the Supplement).

Conditions for pBAD PCRs are outlined in Saidi-Mehrabad et al. (2020a). In brief, contamination was assessed using primers pBAD-forward and pBAD-reverse under standard PCR conditions with Platinum Taq DNA polymerase. No PCR products were observed in all DNA extractions, indicating no contamination. Blank extractions were performed on each of the three DNA extraction kits. To check for DNA contaminants, PCRs of blank exactions were performed using the primers 341F and 518R (Muyzer et al., 1993). No bands were observed following gel electrophoresis of blank extract PCR amplification.

2.4 16S rRNA Gene Amplicon Sequencing and Analysis

The microbial communities of soil samples were surveyed by small-subunit rRNA gene amplicon sequencing. DNA from the V4 region of the 16S rRNA gene was amplified with the primers 515F and 806R primers (Caporaso et al., 2012). Sequencing was performed commercially by Microbiome Insights (Microbiome Insights, Vancouver, BC, Canada), with an Illumina MiSeq using V2 chemistry (Illumina, San Diego, California, US). Before sequence processing, a total of 552 252 reads were recovered from 18 samples including surface samples, core samples, as well as 5 blank extractions, negatives. The read count ranged from 10 525 to 47 389 reads per sample, and most samples had high coverage (Fig. S3). Sequence processing was per-

formed in USEARCH v10 (Edgar, 2010). Overlapping reads were combined from forward and reverse reads with the following qualifiers: no unknown base pairs allowed (Ns), a maximum number of sequence mismatches of 10 nt in the alignment, a minimum merge length of 230 bp, a maximum merge length of 300 bp, and an ID cut-off of 80 %. Of the 552 252 reads recovered, 515 916 reads were aligned into overlapping reads (93.4 % of the total), at an average length of 253 bp, with an expected size of 291 bp. Quality filtering with a maximum expected error cut-off of 1.0 resulted in 510 757 (99.0 % of the total) reads passing (Edgar and Flyvbjerg, 2015). Dereplication identified 148 294 unique sequences and 105 589 singletons (71.2 % of the unique reads) for the total dataset. OTU clustering at 97 % using UPARSE identified a total of 5964 OTUs as well as identifying and removing 12 508 chimeras while removing all singletons and doubletons (Edgar and Flyvbjerg, 2015). After sequence processing 386 419 reads remained. Taxonomy was assigned using *de novo* picking as implemented in SINTAX using the RDP v16 database at a bootstrap cut-off of 80 (Ribosomal Database Project) (Edgar, 2016). Sequences were separated for bacterial (377 578 reads) and archaeal (8841 reads) analysis. Taxa identified as chloroplast/streptophyta using SINTAX were removed from the downstream analysis and no mitochondrial reads were observed, leaving 361 034 reads in the bacterial dataset (Edgar, 2016). Reads identified in extraction blank samples were removed from analysis. A NEWICK formatted 16S rRNA phylogenetic tree was constructed using USEARCH v10. For bacterial analysis, samples were rarefied to 10 000 reads.

2.5 Microbiological Diversity, Taxonomy and Assembly

$\alpha-$diversity metrics, including species richness, Chao 1 richness estimation, Shannon Diversity, Shannon Evenness, Simpson Diversity, Simpson Evenness, and Heip's Evenness were calculated using Mothur v 1.39.5 (Schloss et al., 2009). A Kruskal–Wallis test followed by posthoc testing with Kruskal–Wallis multiple comparisons as implemented in SigmaPlot v13.0 tested for significant differences between samples. β-diversity was calculated in USEARCH v10 using Bray-Curtis, Jaccard presence/absence, and weighted UniFrac distance metrics. Visualization of community dissimilarities and significance testing were carried out using R v3.4.1, in the RStudio IDE with the *vegan* package v2.4-4 (Dixon, 2003). Hierarchical clustering was visualized with the dendextend package v1.5.2 (Galili, 2015). Statistical differences in community composition between clusters were assessed using permutational multivariate analysis of variance in the *vegan* package v2.4-4. Environmental parameters were fitted to the NMDS based on a significant Spearman correlation ($p < 0.05$) plotted using a total of 999 permutations. The direction of vector indicates the direction of change while the length is proportional to the correlation between the communities and variable. Differences in soil chemistry were visualized in a redundancy analysis (RDA). Values used a z score standardization technique ("standardize") to create a standard normal distribution of 0 with a mean standard deviation of 1. A forward selection of the model using ordiR2step in vegan was used to determine the most appropriate variables for the RDA. In addition, we used variance partitioning to remove the possibility of collinearity of parameters included in the model. The parameters included in the model are: SOM (%), pH, Cu ($mg\,kg^{-1}$), TC ($mg\,kg^{-1}$), NO_3 ($mg\,kg^{-1}$), and NH_4 ($mg\,kg^{-1}$). Differences between permafrost – and permafrost layers – as well as active layer – and active layer – were determined using a PERMANOVA test in R. Phylum, class, and genus level tables were constructed using SINTAX (Edgar, 2016). A stacked bar chart of relative abundance for class-level taxonomic classification was constructed in MS Excel. Assessment and visualization of significant ($p < 0.05$) differential abundances were assessed using analysis of the composition of microbiomes (ANCOM) (Mandal et al., 2015). Significance testing was followed by post-hoc analysis using a false discovery rate to correct for multiple comparisons. Only significantly different groups were visualized. Indicator OTUs for either permafrost cores or active layer cores were determined using the *indicspecies* package in R (De Cáceres et al., 2011) using an $\alpha < 0.05$ and a minimum IndVal of 0.8. Active layer OTUs were defined from Sect. 3.1, where active layer samples included Core 1 – 30 cm, Core 2 – 30 cm, and Core 2 – 45 cm while permafrost samples included Core 1 – 45 cm, Core 1 – 75 cm, Core 1 – 95 cm, Core 2 – 75 cm, Core 2 – 95 cm, Core 3 – 75 cm, and Core 3 – 95 cm. The presence of either permafrost indicator OTUs or active layer OTUs was then determined in surface soils using Mothur v 1.39.5 using the shared richness function.

2.6 Cell Enumeration

Permafrost, active layer, and surface soils (0.5 g) were diluted 1 : 100 (*w/v*) in 10 mM tetrasodium pyrophosphate (Fisher Scientific, Hampton, NH, USA). The resulting soil slurry was hand shaken for 15 min with 5 mL of 2 mm glass beads then sonicated at 42 kHz for 2×40 s in an ultrasonic bath (Elma, Singen, Germany), and allowed to settle for 5 min. Slurry taken from the middle of the sample bottle was further diluted to a final 1 : 100 (or 1 : 500 for surface sample A) in 10 mM tetrasodium pyrophosphate (*v/v*) before pre-filtration through a 5 µm pore size polysulfone filter (Burlington, Massachusetts, US) to remove larger particles. The resulting filtrate was then passed through a black polycarbonate 0.22 µm filter (Burlington, Massachusetts, US). Bacterial cell counts were determined by epifluorescence microscopy using the LIVE/DEAD® BacLight™ staining solution with 6 µM Syto 9 and 30 µM propidium iodide (Carlsbad, California, US) (Boulos et al., 1999). Filters were stained in a 1 : 1 mixture (300 µL stain solution: 300 µL 0.85 % NaCl) for 1 h in the dark at 4 °C. Fifteen random fields of view were counted on

a Leica DMRXA fluorescent microscope. The resulting live and dead cell counts were averaged for each sample, and corrected for by dry weight, with the sum of both live and dead representing the total cell counts.

Surface samples were sampled in triplicate ($n = 3$; A–F). Permafrost samples included Core 1 (45–50, 75–80, 95–100 cm), Core 2 (75–80 and 95–100 cm) and Core 3 (75–80 and 95–100 cm) ($n = 7$). Active layer samples included Core 1 (15–20, 30–35 cm), and Core 2 (30–35, 45–50 cm) ($n = 4$).

3 Results

3.1 Edaphic Parameter Analysis of Dominion Creek Soils

Edaphic parameters separated active layer soils from permafrost soils in soil cores in an RDA (Fig. 2 and Tables S1 and S2 in the Supplement; $p = 0.035$). Disturbed soils did not form a separate grouping to the exclusion of permafrost and active layer core samples ($p = 0.084$). Of the surface soils, A–E grouped with active layer soils, while F grouped with permafrost soils (Fig. 2; $p = 0.022$), indicating that while A–E likely originated from active layer soils, F likely originated as a permafrost soil. Surface soil samples of C and D were distinct from other soil samples (Fig. 2); C and D had higher Mn, P, SOM, and water content than other samples (Tables S1 and S2).

3.2 Viability is negatively impacted by disturbance

To assess the impact of disturbance on active layer and permafrost microbial cell abundance and viability, counts of live and dead cells were determined across surface samples and core samples. The proportion of viable cells (Table 1) ranged from 18 %–86 %. Microbial cell counts ranged from 9.10×10^7 (in Core 1 95 cm) to 9.20×10^8 (in surface sample A) cells gdw^{-1}. Total microbial abundance in active layer cores was greater than in permafrost cores, with microbial abundance averages of $1.26 \times 10^8 \pm 6.30 \times 10^7$ cells gdw^{-1} in active layer cores and $2.30 \times 10^7 \pm 2.79 \times 10^7$ cells gdw^{-1} in permafrost cores (Table 1). The viability ratios of permafrost soils were not significantly different from active layer soils ($p = 0.07$). Surface soil cell abundance declined significantly in surface samples E and F ($\sim 1.8 \times 10^8$ cells gdw^{-1}) in comparison to other surface soils ($\sim 5.2 \times 10^8$ cells gdw^{-1}) (Table 1). Viability ratios did not change significantly with the degree of disturbance, although viability ratios in disturbed permafrost and active layer soils (B–F) were significantly lower than undisturbed active layer (A) (Table 1).

3.3 Diversity of disturbed soil resembles active layer soils

Observed OTU richness across all samples ranged from a low of 387 OTUs in the deepest Core 1 permafrost sample to a high of 1738 OTUs in surface sample F, the disturbed permafrost soil (Table S3). Higher Shannon diversity, Shannon evenness, and OTU richness in active layer indicate that active layer communities harboured a more diverse microbial community at the OTU level than in permafrost (Fig. 3 and Table S3). Lower evenness suggests the permafrost microbial communities were dominated by a small number of abundant OTUs.

Surface soils were not significantly different from active layer soils in any α-diversity metric; both had higher richness, diversity, and evenness than permafrost soils (Fig. 3 and Table S3). Microbial diversity of disturbance gradient soils most closely resembled undisturbed active layer soils and differed from permafrost.

3.4 Microbial community composition shifts with disturbance

There were three distinct bacterial community clusters in the studied soils: (1) upper active layer and undisturbed soils (surface sample A); (2) lower active layer and the disturbed soils (surface samples B–F); and (3) exclusively permafrost ($p < 0.05$) (Fig. 4). These clusters were observed with different community distance metrics, including Jaccard presence/absence (Fig. S4), OTU composition (Fig. 4), and phylogenetic dissimilarity (Fig. 6). All disturbed soil communities clustered with lower active layer soil communities (including Core 1 30–35 cm, Core 2 30–35 cm, and Core 2 45–50 cm). Sample groups separated across NMDS1, while NMDS2 separated within-cluster community differences. Active layer and disturbed soils group together to the exclusion of permafrost soils (Fig. 4).

Both Bray-Curtis and Jaccard presence/absence distances within clusters correlated significantly with differences in pH ($r^2 = 0.4316$ and 0.5000, respectively, $p < 0.05$), while differences across clusters correlated with Zn concentration ($r^2 = 0.3609$ and 0.3461, respectively, $p < 0.05$) (Fig. 4).

In an attempt to describe additional community variation, cohesion metrics (where positive cohesion represents a situation in which both taxa become more abundant with association while negative cohesion where the presence of one taxon negatively impacts another taxon) were compared against Bray-Curtis dissimilarity. Positive cohesion ($r^2 = 0.6760$) and negative cohesion ($r^2 = 0.8069$) explained 29.0 % of bacterial community variation and 40.2 % of the community variation when combined with pH and Zn (Fig. 4). Cohesion not only explained a greater fraction of bacterial community variation but also explained an additional 20.3 % of variation beyond environmental parameter differences, as Zn and pH explained only 19.9 % of the variation alone.

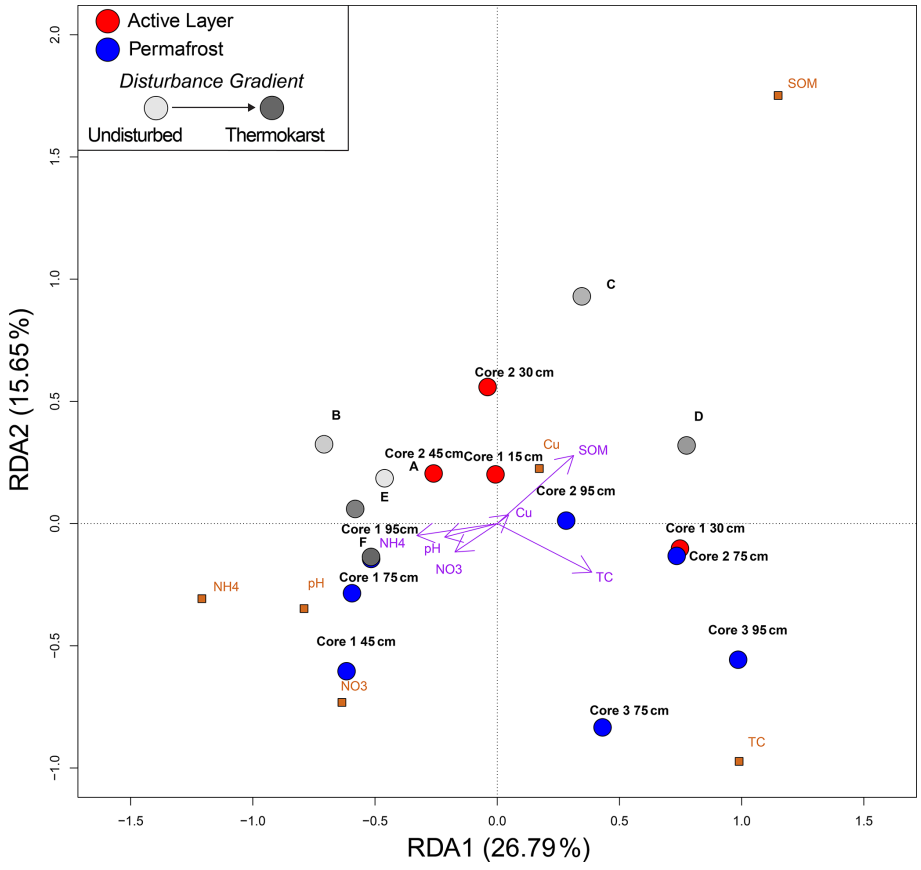

Figure 2. Thawed permafrost surface soils were similar to intact permafrost samples, while thawed active layer surface soils were similar to intact active layer samples. Redundancy analysis of soil physicochemical parameters in permafrost, active layer, and disturbance gradient soils ($R^2 = 0.5268742$). Soil chemical parameters are represented by arrows, and sites are distinguished by coloured circles. Darker circles represent greater disturbance across the disturbance gradient soils.

Table 1. Live-dead cell counts of viable and non-viable microbial cells across the disturbance (A–F) gradient, as compared to undisturbed permafrost and active layer core samples. Counts are grouped (like letters are the same group) based on a one-way ANOVA ($P < 0.05$) with a post-hoc Tukey HSD calculator. Error indicates standard deviation as calculated through propagation of error across each field of view as well as biological replicates.

	Viable (cells gdw^{-1})	Non-Viable (cells gdw^{-1})	Total (cells gdw^{-1})	Ratio (%)
A	$6.28 \times 10^8 \pm 1.27 \times 10^8$ (a)	$2.92 \times 10^8 \pm 1.46 \times 10^8$ (ab)	$9.20 \times 10^8 \pm 1.31 \times 10^8$(a)	26.79 ± 10.14 (a)
B	$1.15 \times 10^8 \pm 4.86 \times 10^7$ (bc)	$1.68 \times 10^8 \pm 1.41 \times 10^8$ (bc)	$2.83 \times 10^8 \pm 1.87 \times 10^8$ (bcd)	18.13 ± 3.87 (ab)
C	$8.65 \times 10^7 \pm 2.46 \times 10^7$ (bc)	$2.78 \times 10^8 \pm 7.37 \times 10^7$ (ab)	$3.64 \times 10^8 \pm 8.48 \times 10^7$ (bc)	21.29 ± 13.59 (b)
D	$1.29 \times 10^8 \pm 4.53 \times 10^7$ (bc)	$3.95 \times 10^8 \pm 9.24 \times 10^7$ (a)	$5.24 \times 10^8 \pm 1.88 \times 10^7$ (b)	26.93 ± 4.98 (b)
E	$8.57 \times 10^7 \pm 8.09 \times 10^7$ (bc)	$1.18 \times 10^8 \pm 5.1 \times 10^7$ (bc)	$2.03 \times 10^8 \pm 1.15 \times 10^7$ (cd)	49.08 ± 11.86 (b)
F	$4.20 \times 10^7 \pm 6.76 \times 10^6$ (bc)	$1.16 \times 10^8 \pm 4.25 \times 10^7$ (bc)	$1.58 \times 10^8 \pm 4.84 \times 10^7$ (cd)	36.74 ± 7.76 (b)
Permafrost	$2.30 \times 10^7 \pm 2.79 \times 10^7$ (b)	$6.77 \times 10^7 \pm 2.86 \times 10^7$ (c)	$9.07 \times 10^7 \pm 4.11 \times 10^7$ (d)	25.43 ± 14.41 (b)
Active Layer	$1.26 \times 10^8 \pm 6.3 \times 10^7$ (c)	$1.84 \times 10^8 \pm 7.27 \times 10^7$ (bc)	$3.10 \times 10^8 \pm 1.14 \times 10^7$ (bc)	39.92 ± 9.21 (b)

3.5 Persistence of permafrost indicator OTUs in disturbed permafrost soils

Indicator taxon analysis identified 94 active layer indicator OTUs (Table S4) and 60 bacterial permafrost indicator OTUs (Table S5). Despite disturbance and thaw, permafrost indicator OTUs persisted following permafrost thaw at relatively high proportions in sample F. The proportion of permafrost indicator OTUs present in surface samples increased in both disturbed active layer and disturbed permafrost soils, with less than 5 % of permafrost indicator OTUs present in the

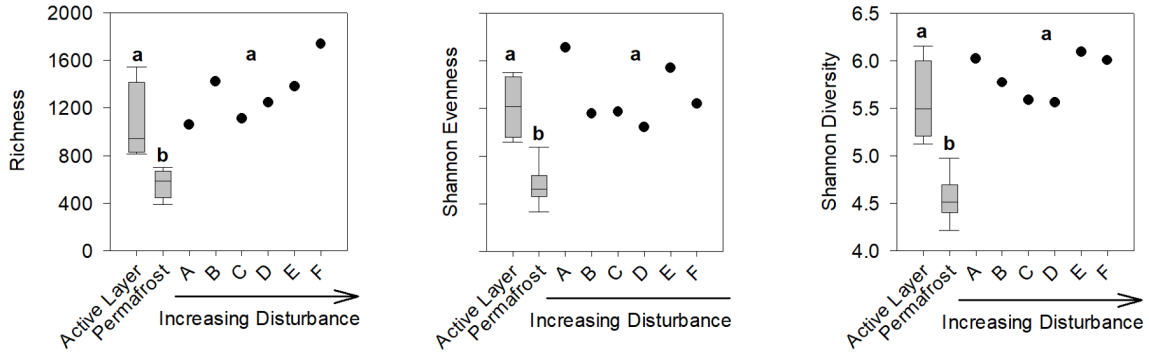

Figure 3. Microbial diversity in permafrost responds to abrupt thaw. Box and whisker plots of diversity metrics across active layer, permafrost, and disturbance gradient soils. Disturbance gradient soils were combined for statistical testing. Letters signify statistically different groupings ($p < 0.05$) as assessed using one-way ANOVA with a post-hoc Tukey test. Surface samples were assessed as a single statistical grouping ($n = 6$). Permafrost samples were grouped across Core 1, Core 2, and Core 3, including depths 75–80 and 95–100 cm as well as 45–50 cm in Core 1 ($n = 7$). Active layer samples were grouped together across Core 1 (15–20, 30–35 cm) and Core 2 (30–35, 45–50 cm) ($n = 4$).

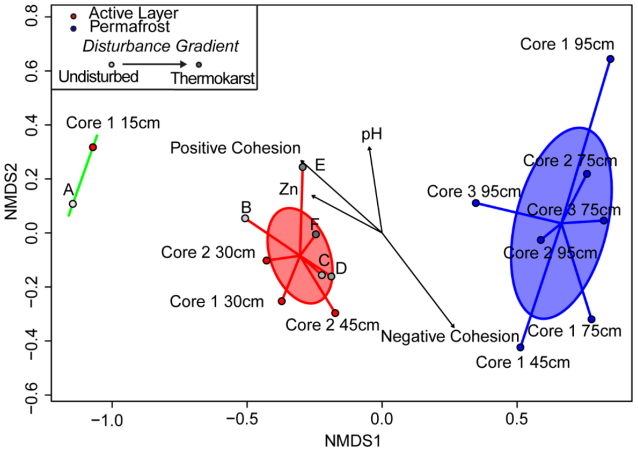

Figure 4. Dissimilarity of microbial communities visualized by nonmetric multidimensional scaling (NMDS) of total microbial assemblages. Groupings of soil samples are based on clusters recovered from a hierarchical clustering algorithm using Bray-Curtis community distances. Ellipses encompass clusters determined by weighted average algorithms: (1) upper active layer and undisturbed soils, (2) lower active layer and disturbed soils, and (3) permafrost. Stress = 0.06.

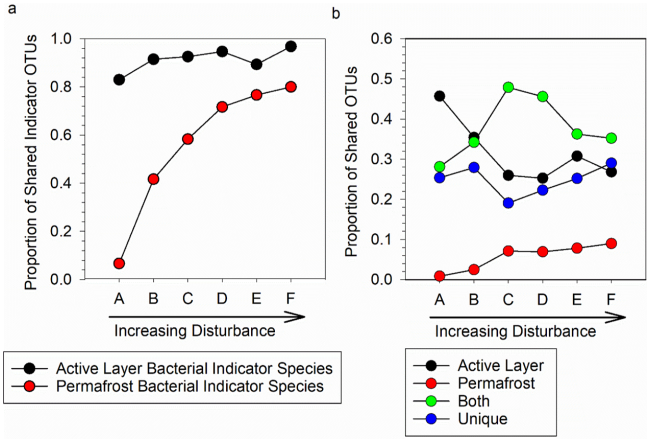

Figure 5. Indicator species analysis and shared richness assessed across the disturbance gradient as compared to combined active layer and permafrost soils. **(a)** The proportion of indicator OTUs assigned to permafrost or active layer soils that are shared within disturbance gradient soils. Indicator OTUs were defined as having indicator values above 0.80. Each indicator group is calculated independently. The proportion represents the fraction of indicator OTUs from either active layer or permafrost that are present within each surface soil. **(b)** Shared richness of disturbance gradient soils compared against the combined assemblages of permafrost or active layer. "Uniques" were unique to the disturbance gradient soil, and "Boths" were found in both active layer samples and permafrost samples.

undisturbed surface sample (sample A) increasing to 80 % of bacterial permafrost indicator OTUs present in sample F (Fig. 5a). By comparison, the presence of permafrost indicator OTUs ranged from 93 %–100 % in permafrost soils.

Across the surface soils, F shared the highest proportion of OTUs with permafrost soils (9.0 %) and the lowest with active layer soils (26.8 %) (Fig. 5b). Likewise, the undisturbed soil shared the lowest proportion of OTUs with permafrost soils (0.1 %), and the highest with active layer soils (45.7 %). Endemism was highest in soil F (29.0 %), but lowest in intermediately disturbed soils C and D (19.1 % and 22.3 % respectively). Endemic OTUs were lowest in disturbed active layer but increased within disturbed permafrost. OTUs shared with permafrost soil communities increased in prominence with greater levels of disturbance. Thus, although the overall community composition in the most disturbed soils was quite similar to active layer soils, indicator bacterial OTUs of relict permafrost were still present. We interpret

these findings to indicate that the permafrost microbial communities were still present but were being supplanted by a colonizing active layer community following thaw.

3.6 Community membership of disturbed soils resembles active layer soils

Phylogenetic distances of community composition (weighted UniFrac) indicated similar cluster structure as found in Bray-Curtis and Jaccard distances (Fig. 6). Community dissimilarity was greatest in permafrost soils, indicating that permafrost soil microbial communities were more heterogeneous than those of disturbed active layer and undisturbed active layer soils. A total of 26 phyla, 66 classes, and 235 genera were assigned across all soils, with 32.4 %, 46.1 %, and 62.3 % of all sequences unassigned at the phylum, class, and genus levels, respectively. At the phylum level, all bacterial communities combined were predominantly composed of Proteobacteria (21.1 %), Actinobacteria (8.6 %), Acidobacteria (7.9 %), Bacteroidetes (7.9 %), and Firmicutes (5.5 %), with substantial proportions of Verrucomicrobia (4.2 %), and Planctomycetes (4.0 %) and above a 3 % cut-off. Across class levels, Actinobacteria (8.5 %), Alphaproteobacteria (6.6 %), Deltaproteobacteria (4.2 %), Planctomycetia (4.0 %), Gammaproteobacteria (3.7 %), Clostridia (3.3 %), and Betaproteobacteria (3.2 %) were most abundant and above a 3 % cut-off (Fig. 6). Bacilli, Clostridia, Anaerolineae, and Bacteroidia were significantly more abundant in permafrost soils than in active layer soils, while Acidobacteria group 4, Acidobacteria group 17, and Nitrospira were all significantly more abundant in active layer soils than permafrost (Fig. S2). Class level taxonomic composition across the surface soils was relatively static with some exceptions: Clostridia (A = 0.04 %, B = 0.529 %, F = 1.96 %), Bacteroidia (A = 0.03 %, B = 0.49 %, F = 2.1 %), and Acidobacteria group 7 (A = 0.71 %, B = 1.81 %, F = 3.19 %) all increased with disturbance, while Planctomycetia decreased (A = 2.85 %, B = 1.63 %, F = 0.775 %). Bacteroidia and Clostridia were more abundant in disturbed and permafrost soils than in active layer soils.

4 Discussion

4.1 Analogues of natural disturbances

Discrete naturally occurring physical disturbances to active layer and permafrost soils (such as retrogressive thaw slumps, thermokarst thaw ponds, and active layer detachment slides) are becoming increasingly common. Removal of the active layer, as well as subsequent detachment slides of the newly forming active layer, results in a similar geographic formation as anthropogenic excavation. Indeed, anthropogenic disturbances can also result in active layer detachment slides and thermokarst formation (Lin et al., 2016). Both in natural retrogressive thaw slumps and anthropogenic excavation, physical soil displacement and thaw exposure are co-occurring processes. Our study was chosen to simulate these localized thermokarst disturbance conditions, where thaw and soil displacement occur simultaneously. The distinction between physical mixing and biological dispersal may be less meaningful under field conditions, since both processes occur simultaneously. Our data indicate that the edaphic parameters measured, including a variety of micronutrients, did not shift as a result of permafrost thaw or disturbance. The exposure of preserved organic acids, such as fulvic acids and humic acids, likely decreased the pH in our disturbed permafrost soils. Both excavated sites and naturally disturbed sites undergo slow re-vegetation, and we predict that although microbial community replacement occurs rapidly, plant community succession may occur over decadal time scales in anthropogenically and naturally disturbed sites (Forbes et al., 2001; Lantz et al., 2009). With time, additional nutrients may be released into the newly formed surface soils from permafrost soils, including NO_3^--N, NH_4^+-N, Na, and S found in disturbed permafrost samples in this study. Future studies should follow industrially disturbed sites over multiple years to investigate how evolving edaphic parameters change with increased mobilization.

4.2 Anthropogenic Disturbance does not reliably shift edaphic parameters

As water potential, nitrogen content, carbon content, and nutrient availability are amongst the most important drivers of microbial community composition in these soils, permafrost thaw is expected to lead to microbial community shifts. Anthropogenic disturbance alters soil pH, while thawing permafrost leaches micronutrients and macronutrients into downstream thaw waters (Forbes et al., 2001; Lantz et al., 2009). Organic matter in permafrost is generally more recalcitrant and holds higher concentrations of dissolved nitrogen than active layer soils above it (Keuper et al., 2012). The dissolved nitrogen preserved within permafrost can be rapidly released following permafrost thaw, releasing nutrients into the active layer and downstream aquatic systems (Reyes and Lougheed, 2015). Thus, we expected dramatic changes in soil edaphic parameters following disturbance in the Dominion Creek system, including a lowered pH, increased mineral nitrogen, increased carbon content, and increased micronutrient abundance. However, the measured edaphic parameters did not shift within six weeks of thaw, although higher nitrogen contents found in disturbed permafrost were similar to the high levels of nitrogen found in undisturbed permafrost. The lack of a shift may be due to the short timeline since thaw; environmental parameters may need more time to shift in these disturbed soils, particularly as many of our analyzed metals have a slow rate of change.

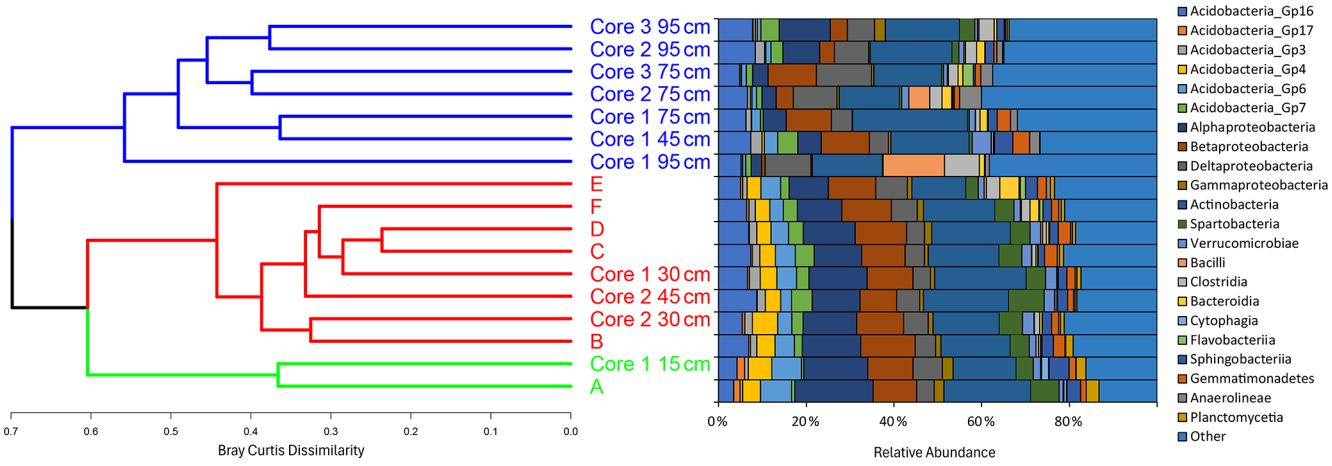

Figure 6. Hierarchical clustering of cores and surface sample communities alongside class level relative abundances. Classes $< 1\,\%$ in any sample was grouped into "other" alongside unassigned taxa. Samples which are not significantly different ($P < 0.05$) across weighted UniFrac distances have the same colour. Active layer samples are labelled in red, permafrost is labelled in blue, and disturbance gradient soils are labelled in black.

4.3 Permafrost bacterial communities respond to disturbance

Most experiments studying in situ permafrost thaw show either that microbial communities shift to resemble the active layer (Tas et al., 2014), or that new microbial communities develop (Mondav et al., 2017). Unlike in situ experiments, laboratory thaw experiments identify significant changes in microbial community structure, transcriptional activity, respiration, and genetic potential (Coolen and Orsi, 2015; Coolen et al., 2011; Oelbermann et al., 2008; Mackelprang et al., 2011). Interestingly, we observe rapid microbial community shifts in disturbed permafrost soils in response to thaw. Previous incubation experiments identify microbial community changes in both active layer and permafrost soils with rapid thaw and suggest that the rapid succession of microbial communities is related to carbon recalcitrance (Coolen et al., 2011). However, while we observed a significant shift in microbial composition in disturbed permafrost soils, we did not observe the same trend in disturbed active layer soils. Together, we suggest that microbial community dynamics with significant physical disturbance is likely to induce rapid microbial community shifts in permafrost soils, but not in active layer soils. This can be extended not only to anthropogenic disturbances, but to thermokarst formation as well. During this in situ thaw experiment, microbial community successional patterns were observed; however, interpretations of function from taxonomic assignment must be made with caution, requiring further study into the genetic potential and activity of the community. Thermokarst formation as found in this study can induce anaerobic conditions, increasing acetogenic fermentation and methanogenesis (Coolen and Orsi et al., 2015). Future study into methane cycling, such as through the use of stable isotope probing of methanotrophic communities, may be particularly beneficial to understanding functional shifts in anthropogenically disturbed permafrost soils such as the ones studies here.

Indeed, longer-term field warming experiments show a deepening of the ice table, allowing the colonization of plant roots into deeper soil horizons (Monteux et al., 2018). Microbial communities in these thawed permafrost layers shifted to resemble those of the active layer. The mechanism of this change is the colonization of active layer taxa into deeper soil horizons. Our results support these findings, as permafrost microbial communities shifted to resemble the community of the lower active layer. However, in the absence of plant roots, we suggest the perturbation of soil horizons by anthropogenic activities allowed the colonization of active layer microbial communities into thawed permafrost horizons. Physical mixing may also contribute to these community shifts, which has previously been shown in laboratory experiments (Doherty et al., 2025). The mechanical disruption of soil horizons during site preparation may have introduced active layer microorganisms into formerly permafrost horizons, meaning that the overlap between disturbed soil and active layer communities may reflect physical translocation rather than biological recruitment. However, if bulk redistribution of active layer microorganisms were caused by physical mixing we would expect to see an equivalent level of active layer indicator OTUs across all disturbed samples. Instead our study observed the progressive accumulation of active layer indicator OTUs along the disturbance gradient. These results are further supported by the presence of both active layer and permafrost indicator OTUs within disturbed permafrost soils, as a small abundance of permafrost microbiota may have persisted after disturbance. We expect that disturbances, such as thermokarst formation, in the Arctic and subarctic are likely to create homogenous microbial communities similar to the

active layer regardless of the soil source. Rather than observing indigenous microbial communities shifting in abundance in response to thaw, we observed dispersal of active layer microorganisms into exposed permafrost layers. Soil fauna, such as collembola, may be responsible for the transport of soil microorganisms into thawing permafrost layers (Monteux et al., 2022). Therefore, we suggest that discrepancies between in situ thaw experiments and laboratory thaw experiments may be due in part to the lack of surrounding active layer disturbance in the in situ experiments.

4.4 Drivers of Microbial Community Composition are Biotic

Resident microbiota must adapt to numerous shifting environmental variables in response to permafrost thaw or they will be supplanted by active layer microbiota. Physicochemical parameters are suggested to control the fate of microbial communities in response to permafrost thaw (Hayden et al. 2012; Rousk et al. 2012); however, rapid microbial community shifts have been observed under only seven days of warming, before any significant changes in chemistry or physical parameters in the system would be possible (Mackelprang et al., 2011). Therefore, it is unknown if: (1) physicochemical characteristics control microbial communities following permafrost thaw, and so microbial communities in samples with similar environmental parameters should resemble each other (environmental filtering) or (2) permafrost thaw induces microbial community shifts, and so environmental and microbial groupings do not resemble one another (biotic filtering). Soil microbial communities in thawing permafrost are generally driven by environmental factors, most significant of which is pH (Chen et al., 2017; Tripathi et al., 2018). We therefore expected that disturbance and thaw would result in rapid changes to pH, water content, micronutrient concentration, and exchangeable nitrogen content which would correlate to microbial community composition. These anticipated changes reflect documented biogeochemical responses to thaw (Scheel et al., 2022; Chen et al., 2017; Tripathi et al., 2018). However, in our study the variance in bacterial community composition was poorly explained by physicochemical parameters. One exception within our study, the correlation of Zn to bacterial community composition, may be due to the site-specific mobilization of Zn during permafrost thaw or due to other underlying processes (Burn et al., 2025). Previous studies have shown permafrost and active layer prokaryotic communities are controlled by distinct mechanisms (Chen et al., 2017; Tripathi et al., 2018). However, we observed no such correlation between microbial composition and these parameters in our system. Our results suggest that biotic filtering plays a dominant role in microbial community structuring under the current experimental parameters measured in this study.

Interestingly, while the short 6-week time frame since disturbance in our study did not lead to a significant shift in environmental parameters, the microbial community profiles found in permafrost underwent substantial changes. We suggest that the indigenous microbial community of permafrost cannot respond to the opportunities presented by permafrost thaw (e.g. increased nutrient availability, increased temperatures, and the availability of new niches) as rapidly as the overlying active layer community. Instead of a "blooming" viable permafrost microbial community, active layer taxa appear to invade the disturbed permafrost environment. While taxon membership in disturbed soils appears to be partly of permafrost origin, as indicated by the presence of numerous permafrost indicator taxa in disturbed soils, this signal is not strong enough to affect overall community membership or composition. The disturbed permafrost microbial community converges with the active layer community, is driven by biotic processes, and likely occurs through the invasion of active layer taxa. Cohesion metrics support the hypothesis that biotic filtering was impacting microbial community composition with disturbance.

4.5 Taxonomic profiles resemble other regional studies across the Arctic

Taxonomic profiles in our study were similar to other periglacial soils and differential abundance analysis identified numerous taxa that may be ecologically significant across active layer and permafrost habitats (Malard and Pearce, 2018). Previous studies have indicated that complex hydrocarbons preserved in permafrost are both aerobically and anaerobically degraded into greenhouse gases, including CO_2 and CH_4, as well as volatile fatty acids. While physiology cannot be directly inferred from taxonomy, trends in the membership of these communities are consistent with these previous findings. Classes containing aerobic heterotrophic bacteria (e.g. class Bacilli and most class Actinobacteria), anaerobic heterotrophic bacteria (e.g. class Clostridia), and volatile fatty acid oxidizers (e.g. *Smithella propionica*) were all prominent across Dominion Creek soils and are common globally in active layer and permafrost soils (Jansson and Taş, 2014; Malard and Pearce, 2018; Metje and Frenzel, 2007). Planctomycetes phylum sequences were more abundant in active layer than in permafrost, and disturbance also decreased the relative abundance of Planctomycetes-related sequences in these surface soils, as has been seen in previous studies (Taş et al., 2014). Relatives of spore-forming bacteria, including Clostridia and Bacilli, were more abundant in permafrost soils than in active layer soils. Increased abundance of these putative spore formers is likely due to niche partitioning and survival strategies precluding spore formation (Johnson et al., 2007). The distinct life strategies between permafrost and active layer classes suggest that community functionality may also differ across permafrost, active layer, and disturbed soil communities, similar to previous findings (Hultman et al., 2015).

4.6 Viability and survivability in permafrost

The impact of permafrost thaw on microbial survivability is unknown. We posit that shifts in microbial community structure could be accomplished by growth of active bacteria and/or death of poorly adapted bacteria. Viability did not differ between the active layer and permafrost soils at Dominion Creek; we propose this lack of difference is due to long-term adaptation to both seasonal thaw in the active layer (Schostag et al., 2015), as well as constant and perennial stressor adaptations in the permafrost horizons (Mackelprang et al., 2017). Our data suggests that abrupt thaw does not dramatically increase microbial cell abundance or viability of microorganisms in permafrost soils. However, previous studies have suggested increases in microbial activity, transcription, and genetic potential with permafrost thaw in the laboratory, and in the field (Coolen et al., 2011; Coolen and Orsi, 2015; Hultman et al., 2015; Mackelprang et al., 2011). Due to these increases in metabolic activity, transcriptional activity, and genetic potential under both rapid and long-term thaw, lower biomass and lower viability with discrete and substantial perturbation were unexpected. Future studies may investigate whether activity changes result from a small but viable community in thawing permafrost which becomes more abundant with thaw.

5 Conclusions

Six weeks of thaw following disturbance was sufficient to shift bacterial community composition, membership, and diversity in disturbed permafrost soils to become more similar to lower active layer soils at a single permafrost-affected site. These communities are driven primarily by the biotic filtering of an invading active layer microbial community in disturbed permafrost communities. Thaw does not increase the viability or abundance of bacteria, suggesting increasing activity, transcription, and genetic potential found in other thawing soils may be due to a more active, yet sparse, microbial community. This study should be expanded to determine if disturbance creates similar microbial community shifts in naturally occurring rapid permafrost thaw zones. Interpretation of activity measurements and characterization of important active microbial taxa may suggest future avenues of research to develop our understanding of microbial succession in response to permafrost thaw induced by direct anthropogenic disturbance.

Data availability. The data for the findings in this manuscript can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA999916 (last access: 28 July 2023).

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Author contributions. Supervision and resources were provided by BL and DF; conceptualization was undertaken by BL, DF, and ASM; methodology was designed and undertaken by ASM and PN; curation of data, formal analysis, and manuscript writing was performed by PN; reviewing and editing of the manuscript was performed by BL, DF, and ASM. All authors made substantial contributions to the publication of this work.

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