The subzero microbiome: Microbial activity in frozen and thawing soils

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Title: The subzero microbiome: Microbial activity in frozen and thawing soils

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One sentence summary: This review highlights studies that focus on elucidating microbial activity and functions in frozen soils and relating these to microbial responses to climate warming in polar regions.

Abstract

Most of the Earth's biosphere is characterized by low temperatures (<5 °C) and cold-adapted microorganisms are widespread. These psychrophiles have evolved a complex range of adaptations of all cellular constituents to counteract the potentially deleterious effects of low kinetic energy environments and the freezing of water. Microbial life continues into the subzero temperature range, and this activity contributes to carbon and nitrogen flux in and out of ecosystems, ultimately affecting global processes. Microbial responses to climate warming and in particular, thawing of frozen soils are not yet well understood although the threat of microbial contribution to positive feedback of carbon flux is substantial. To date, several studies have examined microbial community dynamics in frozen soils and permafrost due to changing environmental conditions, and some have undertaken the complicated task of characterizing microbial functional groups and how their activity changes with changing conditions, either *in situ* or by isolating and characterizing macromolecules. With increasing temperature and wetter conditions microbial activity of key microbes and subsequent efflux of greenhouse gases also increase. In this review, we aim to provide an overview of microbial activity in seasonally frozen soils and permafrost. With a more detailed understanding of the microbiological activities in these vulnerable soil ecosystems, we can begin to predict and model future expectations for carbon release and climate change.

Introduction

"Beware of little expenses. A small leak can sink a great ship"- Benjamin Franklin

Benjamin Franklin may have been discussing economics in this famous quote but it also applies to the role of microbes in greenhouse gas flux. Microbes certainly emit only small amounts of carbon into the atmosphere individually, but the global abundance of microbes that mineralize carbon and nitrogen compounds into greenhouse gases gives them the power to geo-engineer the climate. We are just beginning to understand the roles of microbes in carbon and nitrogen flux in aquatic, ice and soil ecosystems, and while progress is being made, efforts need to be focused on ecosystems most vulnerable to climate change. Permafrost, defined as soils frozen for two or more years, and seasonally frozen soils are particularly vulnerable ecosystems. For example, frozen tundra covers 20% of the Earth's surface and store approximately 40% of the global soil carbon pool (Schuur *et al.* 2015). Currently, Arctic and the Antarctic permafrost harbors ~25% of the world's total soil organic material (Tarnocai *et al.* 2009). Especially susceptible to climate change is the Yedoma region, an expanse of carbon-rich permafrost spanning the Arctic from Siberia to Alaska; an area which harbors approximately 400 gigatonnes of carbon (Khvorostyanov *et al.* 2008; Strauss *et al.* 2013). Climate change is predicted to impact microbial communities, which include bacteria, archaea, and eukaryotes (in particular fungi), in these frozen soils the most. This warming may lead to changes in microbial metabolic activity and potentially create a positive feedback loop promoting accelerated thawing conditions (Graham *et al.* 2012; Koven *et al.* 2011; Schuur *et al.* 2008; Schuur *et al.* 2009; Vincent 2010; Zimov *et al.* 2006). In order to accurately model greenhouse gas release from microbial activity in frozen soils, a multi-dimensional approach that links microbial community dynamics to mineralization of carbon and nitrogen from ecologically representative subzero temperatures to warmer temperatures is needed. Perhaps then, we can begin to appreciate how small "leaks" of

greenhouse gases from microbial activity could lead to the tipping point for Earth's frozen ecosystems.

As with other ecosystems, frozen soils harbor both inactive and active microbial cells and their response to a changing environment will be important to biogeochemical cycling in the near-future (Fig. 1). Activity/incorporation studies under controlled conditions offer an attractive approach for determining the composition of the active community. By following the incorporation of labeled compounds containing carbon and nitrogen, we can determine the extent of activity under various physical conditions such as: stages of thaw, decreasing snow cover, increasing nutrients and changing liquid water availability. Although not all-inclusive, these environmental variables are expected to impact microbial activity in frozen soils as they are increasingly exposed to climate warming. Activity-incorporation studies such as the ones discussed in this review allow for a more bottom-up approach, tracking the carbon and nitrogen use through the specific phylotypes of microbes actively responding to changing environmental conditions. It is those microbial groups active in sub-zero soils and soon-to-be-active members which are the most ecologically relevant types for the question of greenhouse gas release and the fate of frozen soils susceptible to climate change.

In this review, we present a wide range of studies that directly examine microbial activity in permafrost and seasonally frozen soils in response to warming and other environmental changes. In particular, we highlight studies that measure soil carbon respiration, RNA-based approaches, exoenzyme activity, microbial growth, and substrate incorporation, and summarize how these address the overall question of how microbes contribute to greenhouse gas flux from frozen soils.

Detecting and measuring subzero activity

Although microbial diversity and ecology in permafrost have been summarized in the recent reviews (Jansson and Tas 2014; Steven *et al.* 2006), research specifically documenting microbial activity and changes in a variety of frozen ecosystems in response to climate warming is still limited. The concept that microbial metabolic activity ceases when soil temperature falls below 0°C is changing with the realization that microbial activity continues through wintertime during the non-growing season (Drotz *et al.* 2010; Fahnestock *et al.* 1999; Öquist *et al.* 2009). In fact, microbial activity and specifically the heterotrophic bacterial activity, will be the driving force behind carbon remineralization from frozen soils (Graham *et al.* 2012). Preliminary evidence indicates that cryoturbation contributes to lability and flux of organic carbon by microbes in Arctic permafrost (Ernakovich *et al.* 2015). Historically, studies of microbial activity in permafrost have focused on cultivating and isolating microorganisms at low temperatures (Ayala-del-Rio *et al.* 2010; Panikov and Sizova 2007; Vishnivetskaya *et al.* 2000), establishing growth optima of isolates (Bakermans *et al.* 2003; Mykytczuk *et al.* 2013), recording enzyme activity (Waldrop *et al.* 2010; Wallenstein *et al.* 2009), RNA-based measurements involving ribosomal profiling and transcriptomics (Coolen and Orsi 2015; Männistö *et al.* 2013), or characterizing changes in microbial community in response to variable conditions (Dedysh *et al.* 2006; Pankratov *et al.* 2011; Steven *et al.* 2008). Low temperature activity of microbes has also been examined by ^{14}C-substrate respiration (Rivkina *et al.* 2000; Schimel and Mikan 2005; Steven *et al.* 2007; Steven *et al.* 2008), and even stable isotope probing at subzero temperatures to detect specific active groups (Tuorto *et al.* 2014). Table 1 summarizes studies that have

measured microbial activity at cold or sub-zero temperatures in both soils and cultured isolates from soils and other cold environments.

Seasonally frozen soils are even more common than permafrost both in polar and temperate environments and their dynamic nature also has the potential to contribute to greenhouse gas flux. Soils at temperate latitudes freeze seasonally in winter and may undergo multiple freeze-thaw cycles which are known to drastically affect the composition of microbial communities and possibly activity (Öquist *et al.* 2009; Schimel and Mikan 2005). In mountainous regions, such as the Himalayas and Colorado Rocky Mountains, microbial community composition and respiration fluctuate greatly with increases in temperature and availability of labile carbon in wintertime (Brooks *et al.* 2005; Stres *et al.* 2010). In light of the large area of soils that are potentially vulnerable to thawing or warming due to climate change, we must clearly document and predict microbial contribution to carbon and nitrogen cycling. Even in their frozen state a significant amount of greenhouse gas is generated in temperate and polar soils. With climate warming, these vast frozen landscapes are beginning to thaw at the surface, and a thicker active layer may form which is only frozen during winter. The increasing thickness of the active layer in the near future will no doubt have an amplifying effect on the greenhouse gases generated by microbial activity, which makes seasonally frozen soils a good analog for what we might expect from permafrost. Because of their importance in the global carbon cycle while frozen and their vulnerability to thawing in coming years, we must begin to understand how microbes in these soils will contribute to the global carbon feedback.

In 1960, Robert E. Hungate outlined some important questions we must answer in order to conduct a complete ecological analysis of microbial ecology in the rumen (Hungate 1960), and these same principles can be applied to the microbial ecology of frozen soils. The main

issues we must focus on are: 1) What kinds of organisms are present and in what abundance? This involves identification, classification, and enumeration. 2) What are their activities? Food and metabolic products must be identified, and habits of growth, reproduction, and death known. A complete determination of activities necessitates a complete knowledge of the environment. 3) To what extent are their activities performed? This involves quantitative measurement of the entire complex as well as its individual components. Our review highlights studies that aim to fully understand these aspects of microbial ecology in frozen and thawing soils.

Respiration in Frozen Soils

Perhaps the most common way for measuring aerobic and anaerobic biological activity in frozen soils is by monitoring gas release, such as CO_2 for aerobic respiration, and NO_2- for anaerobic respiration on nitrate (denitrification). The generation and release of CH_4 from anaerobic methanogenic decomposition has also been measured from frozen soils. Respiration measurements allow for a simple quantification of biological activity and release of greenhouse gases from a variety of frozen soil types ranging from permafrost to cold deserts (see Table 1). For example, in frozen soils from pasture and arable lands in Iceland, aerobic respiration by heterotrophic microbes was measured down to -10°C and as low as -18°C in tundra soils from Greenland (Elberling and Brandt 2003, Guicharnaud *et al.* 2010). Respiration increased by orders of magnitude when soil temperature increased just from -1 to 0°C, as measured by constant CO_2 monitoring from conifer forest soils in the Colorado Rocky Mountains (Monson *et al.* 2006), which highlights the importance of even a slight shift in the physical environment of frozen soils. Similar effects on microbial growth and respiration were measured by CO_2 flux

between -3°C and 0°C in both conifer and deciduous forest soils from the Rocky Mountains, and additions of simple organic carbon compounds indicated that respiration below freezing was only limited by carbon in these ecosystems (Brooks *et al.* 2005). These studies suggest that soil respiration at sub-zero temperatures is significant and correlates with flux in temperatures.

The level of ecosystem respiration in soils can be influenced by a host of factors, including, but not limited to, soil organic matter content (Knoblauch *et al.* 2013; Michaelson and Ping 2003), vegetation cover (Anderson 2012; Elberling 2007), water availability (Hicks Pries *et al.* 2013a; Jefferies *et al.* 2010; Öquist *et al.* 2009; Schimel and Mikan 2005), snow accumulation or cover (Elberling 2007), temperature (Bakermans *et al.* 2014; Brooks *et al.* 2005; Guicharnaud *et al.* 2010), and microbial biomass (Anderson 2014). Soil respiration has also been shown to have a linear relationship with heterotrophic bacterial numbers, at least in Alaskan tundra soils (Anderson 2014). Thus, any changes in growth and replication of heterotrophic bacteria are likely to have a significant impact on net respiration efflux from Arctic soils. In frozen soils specifically, temperature and water availability are important factors affecting heterotrophic bacterial activity (Karhu *et al.* 2014). For example, respiration increased in both pasture and arable sub-arctic soils as temperature increased from -10 to 10°C (Guicharnaud *et al.* 2010), and aerobic respiration increased in wintertime frozen tundra soils compared to thawed soils (>0.5°C) (Mikan *et al.* 2002). Additionally, temperature-dependent respiration rates can differ in soils with high organic carbon content versus soils higher in mineral content, and the relationship between soil organic horizons and temperature needs to be taken into consideration when measuring microbial respiration as changes in these also affect microbial activity (Michaelson and Ping 2003). Water content also contributes to different rates of respiration in soils with high versus low organic carbon content and affects how temperature-dependent

respiration proceeds (Michaelson and Ping 2003; Öquist *et al.* 2009). These results suggest that changes in physical environment of frozen soils can drastically affect respiration and CO_2 efflux, and should be measured in addition to respiration in future studies.

The conversion of the large store of organic carbon from tundra and permafrost soils to the atmosphere as CO_2 and CH_4 is of major concern due to rapid climate warming and the effects of carbon-climate feedback (MacDougall *et al.* 2012; Schuur *et al.* 2008). Therefore, many studies have focused on soil respiration occurring under freeze-thaw conditions in order to predict and model current and future carbon flux when frozen soils thaw. Freeze-thaw cycles are likely to become a common occurrence in permafrost soils as these become the active layer as a consequence of climate warming. In fact, numerous studies show a rapid increase in respiration when soils transition from frozen to thawing in Arctic taiga and tundra (Clein and Schimel 1995; Schimel and Clein 1996; Sharma *et al.* 2006), as well as in soils in more temperate climates (DeLuca *et al.* 1992). A similar increase in respiration was observed immediately after the first freeze-thawing in bacterial isolates cultured from frozen soils (Skogland *et al.* 1988). The sudden spike in respiration during initial thaw may partially be a stress response because the spikes in respiration are less pronounced with subsequent freeze-thaw cycles. More specifically, recent studies indicate that rate of CO_2 respiration is highest around 0°C in soils during the transition from frozen to thaw (Elberling and Brandt 2003; Larsen *et al.* 2002; Schimel and Mikan 2005). However, this same rapid response in respiration is not observed when soils are warmed from -5 to -2°C, indicating that the thaw itself and the availability of liquid solute in the substrate may be triggering microbial metabolic activity (Clein and Schimel 1995; Elberling and Brandt 2003).

In a study to improve current estimates of permafrost carbon vulnerability Knoblauch *et al.* (2013) demonstrated through multiyear measurements of CO_2 and CH_4 production (aerobic

and anaerobic incubations at a constant temperature of 4°C) that a significant amount of labile organic matter in the permafrost could be readily mineralized after thawing. The main predictor for carbon mineralization in the different permafrost samples was the absolute concentration of organic carbon. Thus, mineralization of organic matter in permafrost deposits may not be a function of age but instead depend on the quality and amount of organic matter formed under different past climatic conditions. In contrast, a study of CH_4 flux from the surface of Arctic tundra indicated that the majority of annual CH_4 release was during the Fall/Winter months from September to May (Zona *et al.* 2016). The largest releases correspond with the "zero curtain" when the groundwater temperature is at the freezing point, but remains in the liquid state. However, these researchers also found instances where significant daily CH_4 fluxes were measured during the middle of winter, well beyond the "zero curtain", particularly at the furthest inland study site (Ivotuk, AK).

In an effort to determine whether autotrophic or heterotrophic respiration is more susceptible to climate warming in Arctic tundra underlain by permafrost, Hicks Pries *et al.* (2013b) measured ecosystem level respiration by partitioning autotrophic respiration (incubations of plant structures) and heterotrophic respiration (by microbes in soil incubations) through three consecutive months in each of two summer seasons. Combining $\delta^{13}C$ and $\Delta^{14}C$ measurements of all samples plus field measurements taken between -1 to 0°C, they determined that heterotrophic respiration increased significantly in surface soil as well as in old soil with thawing conditions. Autotrophic respiration ranged from 40 to 70% of ecosystem respiration and was greatest at the height of the growing season, while old soil heterotrophic respiration ranged from 6 to 18% of ecosystem respiration and was greatest where permafrost thaw was deepest. Thus, when the active layer and permafrost are subject to thawing conditions, the ecosystem will

experience increased autotrophic and heterotrophic respiration when the surface plant structures become active and fix CO_2 into biomass. However, as this new plant biomass is decomposed and transformed into labile carbon, the heterotrophic microbial respiration in the active layer and permafrost will eventually outpace autotrophic carbon fixation activity, making the frozen soil ecosystem into a massive source of CO_2 (Schuur et al. 2009).

Nitrogen cycling in dynamic soils

With increasing depth of thaw in the active layer above permafrost and in permafrost where oxygen is minimal, the release of nitrous oxide can be measured to extrapolate the activity of nitrogen metabolizing microbes. Of interest is also freeze-thaw in agricultural soils at temperate latitudes, which are rich in fertilizers and provide soil microbes with fixed nitrogen leading to nitrous oxide production in both winter and during crop growth (Harder Nielsen *et al* 1998; Röver *et al* 1998). For example, Wertz *et al.* (2013) observed shifts in some nitrifier and denitrifier communities between frozen and unfrozen conditions and a stimulation of N_2O emissions at 1 °C possibly through a restriction of N_2O reductases and/or accumulation of NO_{2-} at this temperature (Wertz *et al.* 2013). In addition, bacterial community analysis highlights nitrogen-cycling functional groups as abundant and important players in the active layer of permafrost (Schostag *et al.* 2015).

In this section, we highlight some studies of nitrous oxide release from both permafrost and other frozen soils under natural conditions as well as fertilized conditions in agricultural soils, which show that nitrous oxide emissions measured from these soils are higher than previously estimated (Elberling *et al.* 2010; Marushchak *et al.* 2011). For example, rates of

nitrous oxide released to the atmosphere from a permafrost core reached up to 34 mg N $m^{-2}d^{-1}$, which is similar to the daily average in tropical forest soils (Elberling *et al.* 2010). High rates of N_2O release are not found consistently in soils across the Arctic and evidence indicates "hot spots" for large amounts of emissions, particularly in cryoturbated soils versus unturbated soils, or soils experiencing frost churning/frost heave (Marushchak *et al.* 2011; Palmer *et al.* 2012). For example, palsa peats (circular frost heaves) are strong to moderate sources or even temporary sinks for N_2O (Palmer and Horn 2012). The source and sink functions of palsa peat soils for N2O were associated with denitrification, with actinobacterial nitrate reducers and *nirS*-type and *nosZ*-harboring proteobacterial denitrifiers playing important roles in the N_2O flux. In boreal soils, sub-zero emissions of N_2O at -4°C due to denitrification were as high as emissions at higher temperatures of +10 and +15°C (Öquist *et al.* 2004), suggesting that changing temperature alone may not play as important a role in gaseous nitrogen release as it does in carbon respiration. During summer the nitrogen flux doubled compared with winter rates in a sub-Arctic peat bog in Sweden. This rate change was not attributed to the <1°C warming, but to the release of organic carbon and nitrogen by a seasonal die-off of soil microbes (Weedon *et al.* 2012). Additionally, the change in the wet and dry dynamics of permafrost and peatland are thought to control nitrous oxide greenhouse gas emissions from frozen soils and peatlands (Marushchak *et al* 2011; Schaeffer *et al* 2013). For example, thawing by itself did not have a stimulatory effect on nitrous oxide emission from permafrost. Rather, thawing and rewetting combined increased release of this greenhouse gas 15 times above average (Elberling *et al.* 2010). Finally, thermokarst morphology was also shown to interact with landscape characteristics to determine both displacement of organic matter and subsequent carbon and nitrogen cycling (Abbott and Jones 2015).

The release of N_2O from northern latitude soils to the atmosphere depends upon type of soil, the initial concentrations of nitrogenous compounds, as well as fertilization activity. Agricultural soils are most susceptible to microbial N_2O release under frozen conditions (Katayanagi and Hatano 2012; Miao *et al.* 2014; Uchida and Clough 2015). For example, a frozen agricultural soil in Germany emitted two times as much N_2O compared to fallow soil, and released up to 4 times as much N_2O compared to forest soil, attributed to availability of nutrients remaining from fertilizer applications (Teepe *et al.* 2000). Overall, N_2O release increases with thaw and availability of nutrients (Alm *et al.* 1999; Brooks *et al.* 2011; Papen and Butterbach-Bahl 1999; Risk *et al.* 2013), and we can now begin to model how microbial communities in agricultural soils may affect levels of this potent greenhouse gas during frozen and thawing conditions as this is likely to be a larger N_2O contributor than even tundra soils.

Genomic approaches and RNA-based studies

The increasing numbers of microbial genomes and metagenomes sequenced from frozen environments allow an elucidation of the microbial community and their metabolic potential, while transcriptomic and metatranscriptomic studies provide clues into the active functional groups of microbes within ecosystems. However, due to difficulty in accessing permafrost, few studies have been able to use "meta-omic" approaches to gauge microbial communities active in permafrost and frozen soil (Coolen and Orsi 2015; Hultman *et al.* 2015; Mackelprang *et al.* 2011; Schostag *et al.* 2015). More commonly, genomes of bacterial isolates cultured from permafrost and seasonally frozen tundra soils have provided insights into both survival strategies for cold-adapted organisms as well as a glimpse into metabolic potential and response to

environmental changes. Out of several studies addressing genomes of psychrophilic bacteria, which we broadly define as those capable of growth/activity below 1°C, a few examples are discussed below. The genomes of Siberian permafrost isolates, *Psychrobacter arcticus* 273-4 and *Psychrobacter cryohalolentis*, revealed several genes for cold-shock proteins which could enhance translation, as well as mechanisms for increased membrane fluidity common to psychrophilic bacteria (Ayala-del-Rio *et al.* 2010; Bakermans *et al.* 2006). The genome of the permafrost bacterium, *Planococcus halocryophilus* strain Or1, shows several copies of osmolyte uptake genes which may allow for better isozyme exchange to maintain growth under frozen conditions (Mykytczuk *et al.* 2013). These osmolyte regulation genes were observed to be upregulated in the transcriptome datasets as well (Mykytczuk *et al.* 2013). Unfortunately, most genomic studies of psychrophilic microbes do not yet have parallel transcriptomic information to elucidate active responses and adaptations to subzero temperatures in freezing soils.

Although isolates provide valuable insight into individual genomic potential and activity under controlled environmental conditions (see Table 1), there is a need for determining the active microbes in situ. One approach would be to directly compare RNA to DNA content of cells to assess the different microbial phylotypes which are active versus being merely present in frozen soils. Evidence to date indicates that RNA and rRNA within a cell increase with increased cell growth (Kerkhof and Kemp 1999), and the use of RNA-based methods to measure microbial activity and growth has been extensively reviewed (Blazewicz *et al.* 2013). In particular, the ratio of RNA and 16S rRNA copy number can be normalized to DNA and 16S rRNA gene copy number, respectively, for any particular clade of bacteria in order to measure activity, although this approach has not often been utilized in sub-zero environments. Recently, DNA versus RNA-derived bacterial community profiles of Arctic tundra were compared using terminal restriction

fragment length polymorphism (TRFLP) and *Acidobacteria* were found to be dominant in more oligotrophic, wind-swept soils (Männistö *et al.* 2013).

Now "meta-omics" studies are becoming more common in frozen soils and they add another dimension to predict ecosystem level responses of microbial communities to future climate warming (Chauhan *et al.* 2014; Coolen and Orsi 2015; Hultman *et al.* 2015; Krivushin *et al.* 2015; Mackelprang *et al.* 2011; Tas *et al.* 2014). A recent study examined transcriptional response of microbial communities in Alaskan permafrost under thawing conditions (Coolen and Orsi 2015). The most transcriptionally active microbial groups under frozen conditions included *Gamma-* and *Betaproteobacteria*, as well as *Firmicutes*, *Acidobacteria*, and *Actinobacteria*. In thawing permafrost, the transcriptional response of *Firmicutes*, *Bacteroidetes*, and the archaeal *Euryarchaeota*, increased relative to other groups, suggesting that these groups may have key functional roles when permafrost thaw continues to occur in coming years (Coolen and Orsi 2015). Transcripts of genes encoding for extracellular protein degradation, carbohydrate metabolism, and enzymes like hydrolase were also upregulated at several depths in thawed permafrost, indicating the potential for rapid carbon and nitrogen metabolism during Arctic warming (Coolen and Orsi 2015).

The insights from metatranscriptomic surveys can be further refined by a more targeted analysis of gene expression activity, such as in the case of genes involved in methanogenesis. The release of methane, as a highly potent greenhouse gas, from thawing permafrost and frozen soils is a major concern (Koven *et al.* 2011; McCalley *et al.* 2014; Wagner *et al.* 2013). Of particular concern is the steady release of CH_4 in frozen bog soils of Siberia even at -16°C and from Arctic active layer soils rich in organic carbon (Panikov and Dedysh 2000; Treat *et al.* 2015). Further evidence indicates that both hydrogenotrophic and acetoclastic methanogenesis

may be common in thawing permafrosts, and that acetoclastic methanogenesis increases in thawed permafrost (McCalley *et al.* 2014; Mondav *et al.* 2014). Both acetoclastic and hydrogenotrophic methanogenesis were shown to increase by two orders of magnitude when temperatures increased from -16°C to 0°C, in both warmer wet Arctic tundra soil and wet polygonal tundra on Herschel Island (Barbier *et al.* 2012; Rivkina *et al.* 2004). Barbier *et al.* (2012) also noted that acetoclastic methanogenesis as measured by the gene expression of methyl coenzyme M reductase subunit A (*mcrA*) and particulate methane monooxygenase subunit A (*pmoA*) was more prevalent deeper in tundra layers at 10°C, potentially leading to large movements of organic carbon anaerobically to the atmosphere. In addition, transcripts encoding for *mcrA*, which catalyzes the last step in methanogenesis, were markedly increased in thawing permafrost along with several other methanogenesis genes (Coolen and Orsi 2015). Similarly, increases in methanogenesis transcripts were observed in warming Arctic peat soils (Tveit *et al.* 2015). These recent studies, along with many others, provide evidence that the anaerobic production of methane in thawing permafrost will increase as Arctic permafrost turns into an active layer undergoing freeze-thaw. However, additional research remains to be done on methane release from frozen soils at other latitudes, as in Zona *et al.* (2016).

Microbial genes involved in nitrogen and carbon processing also shift in relation to climate change. Sharma *et al.* (2006) demonstrated a sharp increase in gene expression for periplasmic and cytochrome nitrate reductase genes (*napA* and *nirS*, respectively) immediately after thawing in farm and grasslands soils. This up-regulation of nitrogen processing genes was strongest after the initial thaw, suggesting that denitrifying bacteria responded rapidly to warming conditions in frozen soils (Sharma *et al.* 2006). Although the use of DNA and RNA microarrays has been limited for quantifying gene or transcript expression changes, microarrays

can elucidate the response of microbial communities to carbon availability and other changes in physical environment. In a microarray analysis examining over 10,000 genes in 150 functional groups, Yergeau *et al.* (2007) found the expression of cellulose degradation genes was correlated with temperature in Antarctic soils lacking vegetation cover. A functional gene array using cDNA prepared from mRNA of frozen soil microbial communities could provide deeper insight into the functional networks active in various environmental conditions.

Fluorescence in situ hybridization (FISH) has also been used in order to measure bacterial activity and is extensively reviewed (Amann and Fuchs 2008). While more of a microscopy-based method than an RNA-based method to measure activity, FISH probes do bind to 16S rRNA and thus the more ribosomes within a cell, the larger the FISH signal, which allows us to estimate activity (Odaa *et al.* 2000; Poulsen *et al.* 1993). For example, in the coastal waters of the West Antarctic Peninsula where seawater temperature fluctuates between 3°C in summer to -1.7°C in winter, summer FISH signal of two *Gammaproteobacteria* groups were larger than in the fall season (Nikrad *et al.* 2014). In contrast to sub-zero ocean ecosystems, the complex structure of frozen soils hamper microscopic analyses with FISH. At least one study detected 59% of microbial cells in the upper layer of tundra soil in Siberia by using FISH, although detection decreased with depth, which suggests higher microbial activity in tundra surface (Kobabe *et al.* 2004). In general, RNA and rRNA content can elucidate microbial activity in frozen soils, and can do so at the resolution of microbial phylotypes, or targeted functional genes.

Subzero growth and activity of isolates

Due to logistical difficulties related to directly studying microbes in frozen environments in situ, many studies have focused on isolating and culturing psychrophilic strains to study their activity under controlled conditions (see Table 1). Some guidelines exist for isolating microbes from frozen soils (Vishnivetskaya *et al.* 2000), however it remains a difficult task to culture psychrophilic microbes from soils under in situ frozen conditions in order to then study their psychrophilic metabolism and enzyme activity (Bakermans *et al.* 2003). The optimal growth temperatures of microbes isolated from frozen soils are typically not in fact sub-zero, however, if they are capable of growth in frozen soils and permafrost then they are relevant to the question of carbon and nitrogen flux from these ecosystems. Genomes of psychrophilic microbes show adaptations necessary for growth at low temperatures, such as a reduced fraction of saturated fatty acids for increased membrane flexibility, DNA repair mechanisms, and increased protein flexibility by reduced use of acidic amino acids (Ayala-del-Rio *et al.* 2010). These adaptations to cold temperatures allow bacteria to synthesize proteins and other macromolecules, as well as grow and divide at sub-zero temperatures without ice damage within the cells. For example, isolates from Siberian permafrost underwent significant morphological changes at -10°C compared to cultures grown at 4°C, including reduction in cell size, centralization of DNA, and appearance of intracellular membrane inclusions (Bakermans *et al.* 2003). Growth of the psychrophile *Planococcus halocryophilus* was reported down to -25°C, although the optimal temperature for this strain is -16 °C based on genome analysis of cold adapted strategies (Mykytczuk *et al.* 2013). *Rhodococcus* sp. JG3 is a novel isolate from the McMurdo Dry Valleys of Antarctica which can grow down to -5°C and has multiple stress and cold response adaptations in its genome, which are found in many psychrophiles (Goordial *et al.* 2016). Thus, it is likely that microbial strains isolated from permafrost and frozen soils are adapted to growth

at low temperatures and are similar in genetic makeup to psychrophiles isolated from other frozen environments (De Maayer *et al.* 2014; Raval *et al.* 2013).

Winter can also be the peak time for release of extracellular materials, such as hydrolytic enzymes in Arctic tundra soils (Wallenstein *et al.* 2009). Many psychrophilic bacterial strains also exude extracellular polysaccharides (EPS) under cold conditions, such as *Pseudoalteromonas arctica* and the aptly named *Mucilaginibacter* genus (Jiang *et al.* 2012; Kim and Yim 2007; Männistö *et al.* 2010; Pankratov *et al.* 2007). This ability to produce cryoprotective EPS demonstrates use of carbon compounds, active metabolism, and protein catalysis at temperatures below freezing. Generation of EPS may also play a big part in the flux of carbon through cold ecosystems because it requires a large intake of organic carbon by each cell, which is then excreted, providing labile organic carbon as a food source for enhanced respiration by other heterotrophic microbes (Junge *et al.* 2006). Estimating growth activity of EPS-generating microbes in general could allow us to model the process of how carbon can be recycled within a cold soil ecosystem (Boetius *et al.* 2015; Deming *et al.* 2011).

The documenting of differential activity by microbes under various sub-zero conditions is important for extrapolating how certain functional groups of microbes may contribute to biogeochemical cycling in permafrost and seasonally frozen soils. For example, determining the activity response of isolated methanogenic archaea is particularly critical when attempting to predict future greenhouse gas release (Dedysh *et al.* 1998; McCalley *et al.* 2014). While a few studies have examined stress response of methanogens to extreme environmental conditions (Schirmack *et al.* 2015) only recently has the activity of methanogenic isolates been examined under predicted climate change conditions in order to elucidate how methanogenesis might contribute to positive carbon feedback (Dedysh 2011). Several methanogenic archaea have

already been isolated from permafrost (Krivushin *et al.* 2010; Shcherbakova *et al.* 2011; Wagner *et al.* 2013) and optimal growth temperatures of these archaea is much higher than what they experience in frozen soils. Thus, overall methane production by these archaea will likely increase as permafrost thaws and ecosystems begin warming. Recent studies are examining the response of methanogenic archaea to warmer and wetter conditions in frozen soils (Barbier *et al.* 2012; McCalley *et al.* 2014; Tveit *et al.* 2015; Wagner *et al.* 2007). For example in Lena Delta permafrost, methane gas was generated by cold-adapted archaea up to a depth of four meters, suggesting that this functional group plays a large role in the climate feedback loop (Wagner *et al.* 2007). Methanogens are likely to be a main driver of greenhouse gas release from tundra and understanding their activity in frozen systems is paramount.

Enzyme activity in frozen soils

Microbial growth in both culture and soil conditions can be measured by the production and activity of enzymes. In particular, the bioprocessing of organic carbon requires the production of catabolic enzymes, including: glucosidases, cellulases, hydrolases, phosphatases, and numerous others. While the number of studies examining microbial activity even in frozen ecosystems is too great to summarize in this section, we highlight a few here in direct relation to carbon processing in changing permafrost and tundra (see Table 1). One group of the most commonly examined enzymes in frozen soils is glucosidases, which are involved in the breakdown of glucose. In warming environmental conditions such as thawing, glucosidase activity increased dramatically, suggesting an availability of simple organic carbon immediately after warming in Holocene permafrost soil (Coolen *et al.* 2011). Beta-glucosidase activity was

also higher in the active layer of Arctic tundra than in the permafrost below, along with phosphatase and N-acetyl glucosaminidase activity (Waldrop *et al.* 2010). Bacteria also seemed to increase the production of oxidative enzymes such as peroxidases in permafrost-affected topsoils, while deeper in wet fen soils enzymes associated with anaerobic fermentation were more common (Gittel *et al.* 2014). With Arctic permafrost poised to transform into active layer with climate warming, activity of these enzymes is likely to substantially increase and rates of carbon breakdown can be more easily measured through exoenzyme activity.

Carbon availability affects enzymatic activity in frozen soils, and carbon can become available by more factors than warming and thawing conditions. For example, a recent study conducted in Arctic tundra soils showed the increased activity of enzymes involved in carbon breakdown after fertilization of the soils with nitrogen and phosphorous, which suggests that increasing agricultural activity in the Arctic is likely to have a significant impact on labile soil carbon (Koyama *et al.* 2013). As an added affect, an increase in the availability in labile organic carbon and subsequent and breakdown of this carbon by abundant microorganisms may actually "kick start" the breakdown of more recalcitrant soil organic carbon as well (Coolen *et al.* 2011). In addition to increases in nitrogen availability, factors such as soil pH can also affect the activity of enzymes such as Beta-glucosidase, with higher pH limiting enzyme activity overall (Stark *et al.* 2014). Some links also exist between enzyme activity in the subarctic tundra due to the effect of light and heavy grazing by ungulates on the surface vegetation cover (Stark *et al.* 2015), which stresses the importance of examining enzyme activity under conditions beyond warming and thawing soils.

Incorporation studies

Some of the most informative methods for measuring active growth/assimilation by microbes in soils, as well as other ecosystems, are incorporation studies using isotopically labeled carbon and nitrogen compounds. However, incorporation studies for microbial activity in situ are not easy to conduct, often requiring long incubation times from months to years as well as long processing and analysis times. Evidence for incorporation of isotopic labels and 5-bromo-3-deoxyuridine (BrdU) into macromolecules has been demonstrated in cold ecosystems such as snow (Carpenter *et al.* 2000), ice (Christner 2002), and saline ice formations and sea ice brine (Junge *et al.* 2004; Junge *et al.* 2006). Few studies have examined microbial assimilation of labeled compounds in frozen soils (Table 1), which are more common environments globally than snow or ice but which do present some interesting experimental challenges, as frozen soils do not homogenize easily and thawing can occur (Drotz *et al.* 2010; McMahon *et al.* 2009; Schwartz *et al.* 2014; Tuorto *et al.* 2014).

In order to study macromolecule synthesis by microbial isolates, incorporation of ^{13}C-, ^{14}C- or ^{3}H-labeled substrates is commonly used. Using ^{3}H-thymidine incorporation, both *Psychrobacter cryohalolentis* and *Psychrobacter arcticus* were shown to synthesize DNA at -15°C, however the rate of synthesis by *P. arcticus* was up to 10-fold faster than *P. cryohalolentis* (Amato *et al.* 2010). Similarly, a strain of yeast isolated from Antarctica ice incorporated ^{3}H-leucine down to -15°C, indicating active metabolisms at sub-zero temperatures (Amato *et al.* 2009). Incorporation of ^{3}H-leucine and ^{3}H-thymidine was measured at 4°C and 10°C in soil from the Antarctic continent, with incorporation into heterotrophic bacteria occurring within a few hours of labeled substrate addition (Tibbles and Harris 1996). The bacterial growth rate in a forest and an agricultural soil from Sweden increased steadily with incubation temperatures from

0 to 30°C as measured using thymidine incorporation, and fungi also incorporated labeled acetate in a similar trend (Pietikäinen *et al.* 2005). Both these studies used incubation temperatures above freezing and microbial incorporation activity at these warmer temperatures provides a useful analog about the potential activity of microbes in soils with climate warming. However, a comparison to sub-zero temperatures would provide a more complete picture for the predictions of microbial roles in climate change, especially knowledge of which functional groups are most abundant and active now and in the near future.

In Siberian permafrost cores, Rivkina *et al.* (2000) measured bacterial incorporation of ^{14}C-labeled sodium acetate over a 550-day period at temperatures ranging from -20°C to 5°C. Total incorporation of radiolabeled substrates increased at higher temperatures, but was measurable down to -10°C. However, very little incorporation was observed at -15°C and -20°C and a doubling time for bacteria of 20 days at -10°C and 160 days at -20°C was estimated (Rivkina *et al.* 2000). Measuring incorporation of ^{14}C-labeled compounds is a quantitative method of examining microbial community activity as a whole, although it does not by itself provide information about the types of microbes that are active. McMahon *et al.* (2009) tested changes in the structure of the active microbial community growing in frozen Arctic soils by using both ^{13}C-glucose and BrDU incorporation. Gram negative bacteria in Arctic tundra surface soils incorporated more ^{13}C-glucose into their lipids than Gram positives, as assessed by phospholipid fatty acid analysis. Incorporation of ^{13}C-glucose into lipids indicates synthesis of new membranes down to -2°C, suggesting that growth activity of microbes in the Arctic continues through winter time. In the same study, fungi were found to be more active than bacteria. Overall BrdU incorporation, however, indicated that microbial DNA synthesis was also occurring in early and late winter (McMahon *et al.* 2009). Similarly, when tracer amounts of

substrate were added to measure DNA synthesis via BrdU incorporation in Arctic tundra, the microbial community shifted towards a greater diversity of phylotypes in the active fraction as measured by terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA sequence analysis. This increase in the diversity of active microbes was reported in soil microcosms incubated with multiple substrates at a wintertime temperature of -2°C and thawing temperature of 4°C (McMahon *et al.* 2011). One of the main obstacles for conducting incorporation studies in frozen soils is homogenizing the labeled compounds into the frozen soil without heating or thawing the soil. Most studies have achieved this homogenization through various combinations of hammering, grinding, and blending.

Recently, an stable isotope probing (SIP) incorporation study found differences in the microbial community active at various sub-zero temperatures when microcosms of Alaskan permafrost soil were incubated with ^{13}C-acetate (Tuorto *et al.* 2014). After 6-month incubations, 152 OTUs were identified in the active fraction of permafrost microcosms (representing 80% of all OTUs detected) which could incorporate ^{13}C-acetate into their genomic DNA between 0 to -20°C. Interestingly, while some OTUs showed active genome replication at all temperatures, a few only assimilated acetate within a narrow temperature range, suggesting adaptation to a narrow niche. Combining SIP with phylogenetic analysis of a clone library, Tuorto *et al.* (2014) were able to identify the active bacterial groups, namely *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes*, and *Proteobacteria* at the lowest temperatures, including -9 and -12°C. Overall, a greater diversity of OTUs was active at the lower temperature incubations than at 0°C (Tuorto *et al.* 2014: 139-49). In this way, SIP plus16S rRNA gene sequencing provides data about the microbial community structure and function in potentially any type of

frozen soil, including information on active genome replication, substrate preference, and identity of the metabolically active microbial groups.

Conclusions and Outlook

Bulk measurements of microbial activity are an efficient way to understand the roles of microbes at the ecosystem level, but there are limitations. Respiration measures the release of carbon from soil as a greenhouse gas flux, which is important information for climate modeling. However, respiration measurements alone do not provide information on the identity of the specific microbes that are active in metabolism in frozen soils, and does also not necessarily indicate active microbial growth and replication. Furthermore, release of CO_2 from frozen soils could be the result of a release in trapped CO_2, or caused by basal microbial metabolism of bacteria, archaea, and fungi. While knowing fine scale microbial community structure may not be important in understanding overall ecosystem function, community structure can explain process differences in intra-seasonal variation and in experimental microcosms (Bier *et al.* 2015; Graham *et al.* 2014). Examining gene expression changes of microbes in frozen soils via metatranscriptomics and more targeted gene analysis enables an understanding of their response under various physical conditions. While "meta-omics" studies provide clues to the active metabolic processes of microbial cells in sub-zero soils, the knowledge gleaned from these studies is still limited by poorly annotated or unannotated genes in the available databases. Microbial function and growth can be examined by more direct methods such as enzyme activity measurements and substrate incorporation.

The landscape of frozen ecosystems is changing rapidly. Unfortunately, our knowledge of microbial activity in frozen soils is advancing slower than the environmental change that is occurring. The studies discussed in this review provide examples of microbial activity measurements using multiple techniques, all of which provide valuable information towards understanding and predicting the role of microbes in a changing climate. Ecosystem level measurements, such as respiration of carbon dioxide, methane, and nitrous oxide, and meta-genomic and meta-transcriptomic approaches, provide a reference framework from which we can build hypotheses and expectations for more targeted studies. These broad approaches address ecosystem level carbon flux which makes sense on the global scale of modeling climate warming in the short term. However, in order to better predict and then project how soil microbial ecosystems will respond to environmental changes in the near and far-future, fitting in more pieces of the puzzle is imperative. Some important gaps that we have yet to fill include: 1) characterizing both the functional composition of microbial communities and how they respond to changing physical environment as a whole, 2) understanding how soil organic matter assimilation and cell growth will affect organic carbon flux into biomass, and 3) because current heterotrophic bacterial enzyme activity in frozen soils is likely limited by low temperatures, how does nutrient availability affect microbial functional groups and their enzyme activity.

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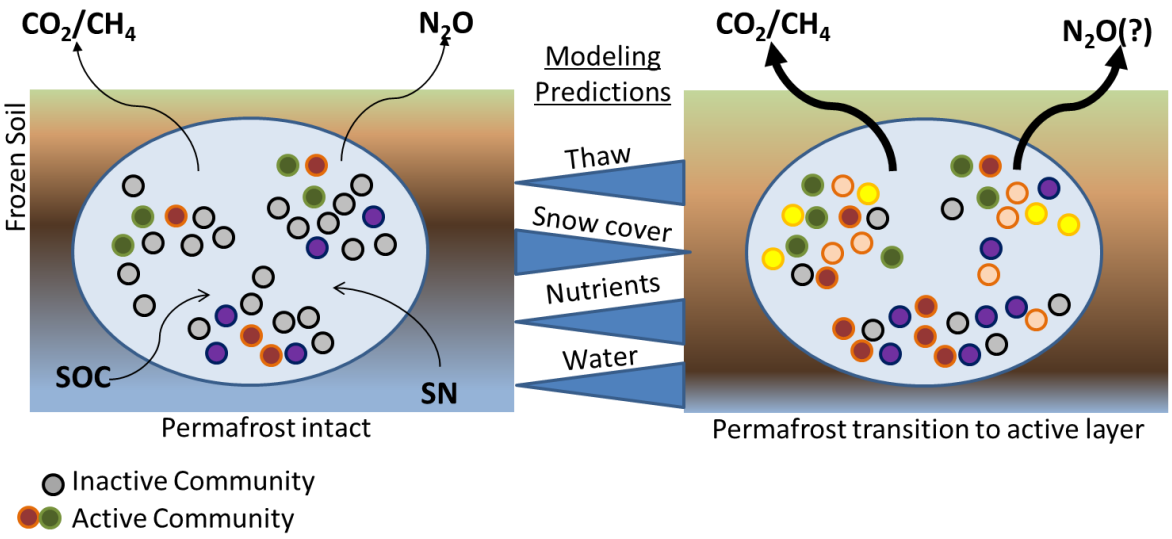

Figure 1: In frozen soil ecosystems there are both inactive and active microbial cells. We can determine the composition of the active community by doing incorporation studies. Incorporation of labeled compounds containing carbon and nitrogen can be followed to determine the extent of activity under various conditions, including stages of thaw, decreasing snow cover, increasing nutrients and water content. Although not all inclusive, these are some common environmental conditions we may expect in humic, carbon-rich frozen soils as they are exposed to climate warming. Release of greenhouse gases such as CO_2, CH_4, and N_2O can then be measured from these mesocosms, thicker black arrows indicate increased release of gases under changing conditions. Whether there will be an increase in the release of N_2O is not yet well understood and evidence is conflicting regarding these results.

Table 1: List of studies examining microbial activity in frozen soils and permafrost. Studies are organized by method used to measure activity, and include key results or findings. Many of the studies could be classified under two or more sections of the table because they use multiple methods to measure microbial activity; however they have been organized according to key results. FT = freeze-thaw.

Respiration			
Soil or Isolate	**Method for Measuring Activity**	**Key Result**	**Reference**
Soil and bacterial isolates	Plate counts and CO_2 respiration	Burst of cell death and respiration after first freeze-thaw, then effect of freeze-thaw cycling is reduced	Skogland *et al*. (1988)
Agricultural soils, Iowa	N-remineralization measured using N-release	Freeze-thaw treatment released significant nitrogen from soils	DeLuca *et al*. (1992)
Tundra soil, Alaska	CO_2 respiration in frozen soils	Soil warmed from -2 to 0°C releases more CO_2 than soil warmed from -5 to -2°C.	Clein and Schimel (1995)
Tundra soil, Alaska	CO_2 respiration during freeze-thaw cycles	High respiration during first freeze-thaw, low respiration in subsequent FT cycles	Schimel and Clein (1996)
Tundra soil, Arctic	CO_2 respiration in winter soils	Wintertime CO_2 efflux ~ 45 g CO_2 $m^{-2,}$ increases current annual CO_2 efflux estimate by 17%	Fahnestock *et al*. (1999)
Peat bog soil, Siberia	CO_2 respiration at -16°C	Steady respiration seen at -16°C, CO_2 and CH_4 released after thaw occurs	Panikov and Dedysh (2000)
Agricultural and other soils, Germany	N_2O emissions at sub-zero temperature	Agricultural soil released the most N_2O	Teepe *et al*. (2000)
Subarctic heath soil	CO_2 respiration and biomass during freeze-thaw	Wintertime CO_2 efflux ~ 25 g CO_2 m^{-2}, which is a significant source of CO_2	Larsen *et al*. (2002)
Tundra soil, Alaska	Calculated Q10 under various conditions	Q10 gives limited understanding of microbial carbon use at cold temperature	Mikan *et al*. (2002)
Tundra soil, Greenland	CO_2 respiration in frozen soils	Respiration measured to -18°C, increased in spring after frozen soil thaws	Elberling and Brandt (2003)

Various soil samples, Alaska	Respiration at -2°C in 88 soil samples	Mineral soil enriched with organic carbon had higher respiration rates than organic soils at sub-zero temperature range	Michaelson and Ping (2003)
Boreal forest soil, Sweden	Nitrous Oxide production in frozen-thaw	Nitrogen mineralization and N_2O production rates at -4°C similar to 15°C	Öquist *et al*. (2004)
Forest soil, Colorado Rockies	Glucose addition and CO_2 measured	Respiration measured from 0 to -3°C indicates microbes are carbon limited at sub-zero temperatures	Brooks *et al*. (2005)
Tundra soils, Arctic	^{14}C substrate and CO_2 respiration	High microbial activity occurred around 0°C during multiple freeze-thaw cycles	Schimel and Mikan (2005)
Temperate soil	Studying link between respiration and snow cover over many years	Less respiration in years with less winter snow cover. Driven by sub-zero microbial communities	Monson *et al*. (2006)
Farm soils, Germany	Transcript PCR for denitrifying functional genes, N_2O release	Denitrifying activity is high immediately after thaw begins	Sharma *et al*. (2006)
Tundra soil, Arctic	CO_2 respiration in frozen soils	Annual respiration above 100 g C m^{-2} and varied with types of vegetation cover	Elberling (2007)
Boreal forest soil, Sweden	CO_2 production in frozen soils	Respiration by soil microbes in frozen soil depends on water availability	Öquist *et al*. (2009)
Subarctic soils, Iceland	Respiration, biomass, enzyme activity during freeze-thaw	Respiration and enzymatic activity were temperature-dependent	Guicharnaud *et al*. (2010)
Soil cores, Himalayas	Degradation of aromatics and CO_2 respiration	Freeze-thaw cycles select for some microbial communities	Stres *et al*. (2010)
Tundra soil, Arctic	Autotrophic and heterotrophic respiration	Autotrophic and heterotrophic respiration both increased with permafrost thaw	Hicks Pries *et al*. (2013)
permafrost from Antarctic Dry Valleys	^{14}C-acetate incubations at varying temperatures	CO_2 release was measured down to -5°C in microcosms of Dry Valley permafrost	Bakermans *et al*. (2014)

RNA-Based			
Tundra soils, Canada	RNA/DNA ratio during incubations	RNA/DNA ratio highest when hydrocarbon degradation is highest	Eriksson *et al*. (2001)
Tundra soils, Siberia	FISH detection of active bacteria	59% of DTAP-labeled microbes detected by FISH	Kobabe *et al*. (2004)
Temperate and rock desert soils, Antarctica	Microarray for functional genes in carbon and nitrogen use	Carbon metabolism important in vegetation-poor soils, and nitrogen metabolism important with increased temperatures	Yergeau *et al*. (2007)
Arctic tundra soils from Finland	DNA and RNA TRFLP analysis	Acidobacteria dominate microbial community in oligotrophic winter soils	Männistö *et al*. (2013)
Permafrost soils Alaska	Metatranscriptomic analysis	Gene transcripts encoding for enzymes are upregulated with thaw	Coolen and Orsi (2015)
Thermokarst bog soils	Multi-dimensional meta-omics analysis of microbial processes.	Metagenomics, -transcriptomics, and -proteomics data is well correlated with process rates data for dominant microbial processes, such as methanogenesis and nitrogen metabolism.	Hultman *et al*. (2015)
Arctic peat soils	Metatranscriptomics and metabolic profiling	Warming causes high CH_4 release and shifts in microbial community. Syntrophic propionate oxidation may be rate limiting step for CH_4 production at lower temperatures	Tweit *et al*. (2015)
Arctic permafrost active layer	DNA and RNA based analysis	Distinct summer and winter bacterial communities	Schostag *et al*. (2015)

Permafrost thaw ponds	16S rRNA analysis	Sequences corresponding to methanotrophs were abundant indicating the importance of methane as energy source	Crevecouer *et al*. (2015)
Greenland ice sheet supraglacial samples	DNA and RNA based analyses	Differences between the total and potentially active community of supraglacial environments	Cameron *et al*. (2016)
		Enzyme Activity	
Coastal island soil, Antarctica	Enzyme activity and nitrogen processing genes	Freezing has greater effect on fungi and warming has greater effect on bacteria	Yergeau and Kowalchuk (2008)
Tundra soil, Alaska	Enzymatic activity in winter and summer soils	Relatively high enzyme activity in winter	Wallenstein *et al*. (2009)
Arctic permafrost soils and active layer soils	Measured exoenzyme activities in permafrost compared to active layer	ß-glucosidase, N-acetyl-glucosaminidase, phosphatase, and peroxidase activity were lower in permafrost than in active layer, but active layer enzymes depleted in activity over time	Waldrop *et al*. (2010)
Holocene permafrost soil	Measured exoenzyme activities in permafrost, in response to thaw	Phosphatase and ß-glucosidase depleted soil surface carbon rapidly in response to thaw, and exoenzymes in deeper layers may aid in breaking down recalcitrant carbon.	Coolen *et al*. (2011)
Tundra soil, Arctic	Enzymatic response to pH and nutrients	High pH lowered enzyme activity	Stark *et al*. (2014)
Upland Alaskan boreal forest permafrost	Enzyme activities and metagenomic analysis	Fire affect active layer and permafrost microbial communities	Tas *et al*. (2014)

Permafrost-affected soil	Hydrolytic and oxidative enzyme activities and microbial community structure	Actinobacteria may assume the role of fungi for degradation of phenolic and complex substrates	Gittel *et al*. (2014)
Subarctic tundra	Effects of grazing by ungulates on soil microbial activity	B-glucosidase activity higher in lightly-grazed soil than heavily-grazed soils	Stark *et al*. (2015)
Isolate Growth Studies			
Isolate from Siberian permafrost	^{14}C-acetate incorporation into lipids	Activity to -20°C observed after 160-day incubation	Rivkina *et al*. (2000)
Isolating microbes from permafrost	Isolation protocols	Enrichment cultures and direct isolation from permafrost	Vishnivetskaya *et al*. (2000)
Isolates from Siberian permafrost	Doubling time of isolates in culture	Isolate grew at -10°C with generation time of 39 days	Bakermans *et al*. (2003)
Psychrobacter cryopegella from Siberian permafrost	^{3}H-adenine DNA/RNA, ^{3}H-leucine	RNA and DNA synthesis rates as well as growth rate decreased significantly below the critical temperature of 4°C	Bakermans and Nealson (2004)
Carnobacterium pleistocenium from Alaskan permafrost	Optimal growth measurements of isolate	Growth optimum of the isolate was at 23°C. Facultative anaerobe which uses various sugars for carbon	Pikuta *et al*. (2005)
Isolates from Siberian permafrost	Growth and lipid measurements, as well as stress response to freezing	Decrease in fatty acid chain length in membranes of isolates at -2.5°C compared to 23°C. Long term freezing did not affect isolates	Ponder *et al*. (2005)
Clostridium algoriphilum from permafrost brine	Growth and other characterization	Anaerobic growth on xylan. Optimal growth temperature 5-6°C	Shcherbakova *et al*. (2005)
Seven EPS-producing strains from Antarctica	EPS generation and characterization	EPS P-21653 of *Pseudomonas arctica* is made from galactose and glucose and has cryoprotective properties	Kim and Yim (2007)

Isolates from Alaska cultured below freezing	Fungal and bacterial growth kinetics at low temperatures using ^{14}C-ethanol and $^{14}CO_2$	Growth of fungi and bacteria, and the incorporation of ^{14}C-ethanol was observed down to -17°C	Panikov and Sizova (2007)
Isolating yeasts from Antarctic ice	Sub-zero growth and ^{3}H-leucine incorporation of yeast	Growth was measured to -5°C, and ^{3}H-leucine incorporation was observed from 15 to -15°C	Amato *et al*. (2009)
Acidobacterial isolate from peat bog	Substrate addition and FISH growth on various types of amended media	Acidobacterial strains in subdivision I grew at pH 3.5-4.5, and all 26 subdivisions grew at low temperature	Pankratov *et al*. (2008)
Virgibacillus arcticus from Arctic permafrost	Growth on high-salt media from -5 to 37°C.	Halophilic isolates grew well from 0-30°C with optimal temperature at 25°C	Niederberger *et al*. (2009)
Psychrobacter cryohalolentis and *P. arcticus* growth	DNA synthesis and ^{3}H-thymidine incorporation after ionizing radiation at -15°C	Protein and DNA synthesis is slow in both strains at low temperature, but still occurring at -15°C after ionizing radiation. *P. arcticus* synthesized DNA faster than *P. cryohalolentis*	Amato *et al*. (2010)
Psychrobacter arcticus 273-4	Genome sequenced	2.65 Mb genome shows low temperature adaptation genes	Ayala-del-Rio *et al*. (2010)
Mucilaginibacter sp. from Arctic tundra	Growth and cellular characterization	3 novel species of *Mucilaginbacter* proposed, growth from 0-33°C	Männistö *et al*. (2010)
Planococcus halocryophilus Or1 from Arctic permafrost	Growth and characterization	New species capable of growth at -10 to 37°C, optimal growth at 25°C	Mykytczuk *et al*. (2012)
Planococcus halocryophilus Or1 from Arctic permafrost	Genome, cell physiology, and transcriptome compared at -15 and 25°C growth.	Isolate at -15°C has more saturated lipids in cell membranes, greater protein flexibility, and many upregulated genes	Mykytczuk *et al*. (2013)
Rhodococcus sp. isolate from Antarctic permafrost	Genome of cold-adapted isolate compared to mesophiles	Adaptations may allow for increased enzyme function at subzero temperatures	Goordial *et al*. (2016)

Incorporation Studies			
Bacterial cells frozen in ice	^{3}H-thymidine/-leucine for 100 days at -15°C	Bacteria synthesized DNA and protein at temperature of -15°C, but not at -70°C	Christner (2002)
Microbes in brines/cryopegs	^{14}C-glucose uptake	Glucose uptake by microbes in cryopegs down to -15°C	Gilichinsky *et al*. (2003)
Tundra soil, Arctic	^{13}C-glucose and BrDU incorporation	Microbial respiration detected down to -39°C. ^{14}C respiration declined steeply with depth	Panikov *et al*. (2006)
Tundra soil, Canada	$^{14}CO_2$ respiration using ^{14}C-acetic acid or ^{14}C-glucose	Activity detected at -15°C using a more sensitive method to detect ^{14}C respiration	Steven *et al*. (2007)
Permafrost and ground ice core, Arctic	$^{14}CO_2$ respiration using ^{14}C-acetic acid or ^{14}C-glucose	Activity at -15°C. *Proteobacteria* and *Euryarchaeota* dominant in permafrost, *Actinobacteria* and *Crenarchaeota* dominant in active layer	Steven *et al*. (2008)
Tundra soil, Arctic	^{13}C-glucose and BrDU incorporation	Fungi most active for carbon use and DNA synthesis, non-Gram(+) bacteria also active at -2°C	McMahon *et al*. (2009)
Boreal forest soil	^{13}C-glucose use by ^{13}C magic-angle spinning NMR	Heterotrophic activity detected at -4°C, but much less at -9°C. Between 9 and -4°C, the same level of microbial activity is detected	Drotz *et al*. (2010)
Tundra soil, Alaska	BrDU incorporation plus 16S RNA T-RFLP	TRFs in the active winter fraction of microbes may be the rare types as they are not detected in summer TRFs	McMahon *et al*. (2011)
Dry Valleys soil, Antarctica	ATP Metabolism	Less ATP activity is detected in frozen soils and with depth	Stomeo *et al*. (2012)

Permafrost cores, Alaska	Stable isotope probing and sequence analysis combined	High diversity of bacteria active at -20°C. Greater diversity of TRFs detected at sub-zero than warmer temperatures	Tuorto *et al*. (2013)
McMurdo Dry Valley soils	Stable isotope probing with ^{18}O water and 16S rRNA sequence analysis	Members of *Proteobacteria* as part of the active bacterial population	Schawartz *et al*. (2014)