Methanogen community composition and activity in Canadian high Arctic soils in response to global warming

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Montreal, Quebec, Canada

Dec. 15, 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

DEDICATION

This document is dedicated to my cat Milo, my parents and family, my Shmoo, and baby Aubree.

"Laughter is timeless. Imagination has no age. And dreams are forever." -- W.D.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Lyle Whyte for allowing me the opportunity to conduct Arctic research and attend conferences in Microbial Ecology. I would also like to thank Dr. Charles Greer for providing the opportunity to be a part of the Biotechnology Research Institute (BRI) research team and use the gassing station and gas chromatographer. I would also like to thank the entire Department of Energy (DOE) Research team for their oversight. I would like to thank my main advisors, Dr. Nadia Mykytczuk and Dr. Christine Martineau for their indispensable training, assistance, problem-solving and general wisdom. I would also like to thank my lab mates, specifically: Roland Wilhelm for his initial training, Guillaume Lamarche-Gagnon for his help with the anaerobic chamber, Jackie Goordial for the immunochip assays, and the BRI team, Christine Martineau, Terrence Bell, Louis Jugnia, and Claude Masson for their training and troubleshooting. Finally, I would like to thank the following organizations for their funding: the DOE, Northern Science Training Program (NSTP), Polar Continental Shelf, Walter M. Stewart Postgraduate Scholarship in Agriculture, Provost's Graduate Fellowship Award, and McGill University travel grants.

LIST OF ABBREVIATIONS

AH-P1, AH-P2, AH-P3, AH-P4	Axel Heiberg Island cores from polygon sites 1 through 4 obtained in July 2010
AH-H4	Axel Heiberg Island core from polygon site 4 obtained in 2009
BLAST	Basic local alignment search tool
CARD-FISH	Catalysed reporter deposition fluorescence *in situ* hybridization
CH_4	Methane
CO_2	Carbon dioxide
CsCl	Cesium chloride
DAPI	4',6-diamidino-2-phenylindole
DFA	Discriminate function analysis
DNA	Deoxyribonucleic acid
DOE	Department of energy of the United States
DW	Dry weight
Er-H1	Eureka sampling site hole 1
FW	Fresh weight
GC	Gas chromatography
GHG	Greenhouse gases

H_2	Hydrogen gas
LOD	Limit of detection
LOI	Loss on ignition
mcrA	Methyl coenzyme-M reductase alpha-subunit
MIQE	minimum information for publication of quantitative real-time PCR experiments
OD	Optical density
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
pMMO	Particulate methane monooxygenase
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SIP	Stable isotope probing
sMMO	Soluble methane monooxygenase

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Recent modeling indicates that up to 90 % of the top 3 m of Arctic permafrost will melt by 2090 as a result of climate change. This thawing is predicted to expand anaerobic environments and provide previously frozen carbon stores for microbial decomposition. This may favour methanogen production of methane (CH_4), a greenhouse gas (GHG) 25 times more potent than carbon dioxide (CO_2). This study examined the effects of increased temperatures and substrates on Archaeal and methanogenic abundance, activity and diversity in active layer and permafrost soils from Axel Heiberg Island in the Canadian high Arctic. This was completed through *in situ* measurements and two permafrost microcosms experiments. The first microcosm experiment was unamended at 4 and 15° C, and the second was amended with the ^{13}C-labelled substrates: acetate, methanol and CO_2, at 4 and 22° C. The activity of methanogens was determined by the anaerobic production of CO_2 and CH_4, and their diversity and abundance were determined by 454 pyrosequencing of the Archaeal 16S rRNA gene and qPCR of the methanogen *mcrA* gene. In addition, DNA stable isotope probing (SIP) determined the active community in the 70 cm 22°C CO_2 microcosm sample.

The production of CO_2 *in situ* and in unamended microcosms correlated with organic matter and temperature but only increased with temperature in acetate-amended microcosms. The production of CH_4 correlated with the combined effects of substrate and temperature, being highest with CO_2 amendment. The Nitrososphaerales were the most abundant Archaeal order, averaging 88 % of total 454 pyrosequencing reads. The abundance and diversity of methanogens increased with depth, from 1 % of total Archaeal reads at 30 cm to 11 % at 70 cm, likely due to the increased anaerobicity. All of the methanogen orders were detected, but the most abundant were Methanomicrobiales and Methanococcales. The Methanomicrobiales also accounted for the highest increase in methanogens, from 9.5 % in the unamended core to 39.5 % in the 70 cm 4°C CO_2 sample. However, this was not associated with an increase in CH_4 production. The Methanopyrales were the only active order detected by DNA-SIP in the 70 cm 22°C CO_2 sample, and correlated with the CH_4 production in all 70 cm samples. Therefore, despite their low abundance, the Methanopyrales may play an important functional role in the production of CH_4 in these high arctic polygon soils. As permafrost thaws, microbial decomposition of carbon stores is expected to increase

the release CO_2. Our results indicate that this increase in CO_2 may promote the production of CH_4 from Canadian high Arctic polygon soils.

ABRÉGÉ

Des prévisions récent indiquent qu'avant 2090, jusqu'à 90% des 3 premiers mètres du pergélisol va s'effondre à cause du réchauffement climatique. Ceci augmente les environnements anaérobiques et expose de grandes réserves de carbone à la dégradation microbienne. Ces conditions sont favorables pour la production de méthane (CH_4) par les méthanogènes, un gaz ayant un potentiel de réchauffement global 25 fois plus élevé que le dioxyde de carbone (CO_2).

L'impact de l'augmentation de la température et les substrats sur l'activité et la diversité des bactéries méthanogènes a été étudié dans les sols provenant de l'île d'Axel Heiberg, dans le haut Arctique canadien. La diversité et la production anaérobique de CH_4 à partir des substrats : acétate, CO_2 et méthanol à été déterminé à 4 et 22° C, et comparé aux productions *in situ.* La diversité à été déterminer par la « pyrosequencing » du gène de l'ARN ribosomal 16S des Archaeabactéries, et l'abondance par le PCR quantitatif du gène *mcrA* des méthanogènes. En plus, l'identification des méthanogènes impliquées dans la dégradation du CO_2 a été déterminé par la technique appelée « stable isotope probing » (SIP) de l'ADN.

La production de CO_2 *in situ* et dans les microcosmes incubés sans substrats était positivement corrélé avec la quantité de matière organique et la température, mais a seulement augmenter avec l'aétate. La production de CH_4 était positivement corrélé avec le niveau anaérobique, la température et les substrats, et a été le plus haut avec le CO_2. Les Nitrosophaerales étaient l'ordre le plus abundant, constituant 88 % des séquences Archaeabactériens. L'abondance et la diversité des méthanogènes a augmenté avec la profondeur, constituant 1 % des séquences à 30 cm, et 11 % des séquences à 70 cm à cause de l'anaerobicité dans ce dernier. Tous les ordres de méthanogènes ont été détectés, mais les plus communs étaient les Methanomicrobiales et Methanococcales. Les Methanomicrobiales ont aussi augmentés le plus, justqu'à 39.5 % dans le microcosm de 70 cm incubé à 4°C avec le CO_2. Cependant, cette augmentation n'a pas été associée avec un augmentation de la production de CH_4. Les Methanopyrales ont été le seul ordre impliqué dans l'utilisation du CO_2 par le SIP dans le microcosm de 70 cm incubé à 22°C avec le CO_2, et donc pourrait avoir un rôle fonctionnel important dans la production de CH_4.

Comme les prévisions indiquent le CO_2 va augmenter considérablement avec le fonds du pergélisol et relâcher du carbone

stocké, nos résultats indiquent que ceci pourrait augmenter la production de CH_4 provenant des sols de type polygone dans le haut Arctique canadien.

Chapter 1 Introduction

1.1 Introduction

This project was part of a much larger project by the U.S. Department of Energy (DOE) research group, whose goal was to determine the impact of climate change on the carbon cycle of Arctic permafrost through experimental and field-based studies. Permafrost currently underlies ~24 % of the Earth's surface, and contains up to 50 % of the world's soil organic carbon, equivalent to 1,672 Pg of carbon (Schuur *et al.*, 2008; Tarnocai *et al.*, 2009). Recent simulations of permafrost modeling indicate that up to 90 % of the top ~3 m of Arctic permafrost will melt by 2090 as a result of climate change (IPCC, 2007). This thawing is predicted to expand anaerobic environments and expose these previously frozen carbon stores for microbial degradation. This favours methanogen production of methane (CH_4), a greenhouse gas (GHG) 25 times more potent than carbon dioxide (CO_2) (Conrad *et al.*, 2002b; IPCC, 2007). Therefore, it has become imperative to increase our understanding of the complex interactions of the methanogen community and their reactions to climate change, in order to properly predict the GHG feedback effect of thawing permafrost on climate change.

1.2 Research Objectives

The main objective of my part of the DOE project was to determine the distribution and community structure of the methanogenic Archaea in the Canadian high Arctic, and their links with the soil characteristics in order to better understand the effects of climate change on CH_4 production. The specific objectives of my project were divided into two groups. The first included to characterize the abundance, GHG production, diversity and community structure of the *in situ* methanogen population. Specific objectives were defined for each process:

1) To determine the abundance of methanogens by qPCR of the *mcrA* gene
2) To determine the relative abundance and diversity of methanogens within the Archaeal community by pyrosequencing of the 16S rRNA gene
3) To determine the activity of methanogens based on the *in situ* CH_4 and CO_2 flux, and the flux from unamended anaerobic microcosms

The second group of objectives included the assessment of soil characteristics and changes associated with climate change, and their

potential effects on the methanogenic community abundance, GHG production, diversity and community structure.

1) To determine changes in soil characteristics in the Canadian high Arctic as climate change progresses
2) To determine which soil characteristics (water content, organic content, active layer depth etc.) may account for an increase in CH_4 and CO_2 production by statistical analyses
3) To determine the effects of increased temperatures and substrates on the abundance, diversity and GHG production by methanogens through microcosms amendments followed by qPCR, pyrosequencing and gas flux measurements
4) To determine the active methanogen community in the utilization of methanogenic substrates as temperatures increase by stable isotope probing

Chapter 2 Literature review

2-1. The Arctic environment with climate change

2-1-1. Arctic Warming

Arctic ecosystems, which are very vulnerable to environmental changes, are experiencing an accelerated rate of climate change. To date, the arctic has warmed two times more than the global average, and is expected to increase another 6-8° C by 2100 (Graversen *et al.*, 2008; Hoj *et al.*, 2008; IPCC, 2007). In comparison, the global mean annual temperature is expected to increase by 2.0-4.5° C (IPCC). This polar amplification of climate change is due to the northward transport of heat and moisture, positive ice-albedo feedback, and changes in cloud cover and sea-ice thickness (Graversen *et al.*, 2008; Lacis *et al.*, 2010; Min *et al.*, 2008). Despite the fairly uniform distribution of greenhouse gases (GHG) around the globe, the stably stratified troposphere of polar regions traps the long-wave radiation absorbed by increased atmospheric GHG closer to the surface. In addition, the retreat of snow and ice cover causes higher surface absorption and increased heat and water vapour flux from the ocean to the atmosphere, which again acts to trap long-wave radiation (Graversen *et al.*, 2008; Lacis *et al.*, 2010; Min *et al.*, 2008).

Along with warming, an increase in precipitation and river discharge, as well as ocean freshening (decrease in salinity) has also been observed in arctic regions. Over the past 50 years, precipitation has increased from 0.1mm to 0.4 mm per day (Min *et al.*, 2008). As climate change continues, precipitation is projected to increase by 5-19 % in summer, and by 13-36 % in winter by 2090, with an annual median change of 18 % (IPCC, 2007). Therefore, climate change in the arctic has already caused both amplified warming and increased moisture content, which is expected to worsen in the future, causing profound impacts on the vulnerable environment of the arctic.

2-1-2. Permafrost Thaw

The main concern for the large temperature increases in the Arctic is due to the vast expanses of frozen ground that are vulnerable to changes in climate. This ground consists of the surface ground layer called the active layer that undergoes seasonal freeze-thaw cycles, and may extend from 10 cm to 2 m in depth (Schuur *et al.*, 2008). Below this is the permafrost, defined as the subsurface earth material that remains frozen for at least two consecutive years (Permafrost Subcommittee, 1988). The northern permafrost region spans 18,782 x 10^3 km^2 which totals 16 % of the total global soil area (Tarnocai *et al.*, 2009). In addition,

permafrost extends up to 50 m deep in discontinuous zones, where there is a patchy distribution of permafrost and non-permafrost soil, and up to 1000 m deep in continuous zones (Coolen *et al.*, 2011; Steven *et al.*, 2007). The presence of frozen ground greatly relies on the climate, as it is controlled by surface temperature, moisture content, vegetation, snow cover, altitude and the physical and thermal properties of the soil (Coolen *et al.*, 2011). As temperature and precipitation in the arctic are increasing due to climate change, the permafrost is thawing. This thawing can occurs in two main ways, either gradually or abruptly (Grosse *et al.*, 2011).

The gradual thawing occurs through deepening of the active layer and the formation of taliks. Taliks refer to horizontal layers of permanently unfrozen soil found between the active layer and the permafrost in discontinuous permafrost zones. With climate change, taliks become progressively thicker, and allow for moisture and soil microbial activity during the winter (O'Connor *et al.*, 2010). The deepening of the active layer occurs as a result of increased air temperatures and precipitation, but also due to the indirect effects caused by these factors (O'Connor *et al.*, 2010; Schuur *et al.*, 2008). The indirect effects of increased precipitation are increased soil moisture, vegetation, moisture retention and snow cover. This will in turn increase the heat capacity, making it

slower to refreeze. In addition, increased snow cover insulates and also acts to reduce refreezing (O'Connor *et al.*, 2010). These effects will cause the overall deepening of the active layer through reduction of refreezing, or if confined to a small area, will also form taliks. The indirect effects of increased temperature include the thawing of the active layer, and increase in duration of the summer peak in microbial activity. Thawing has already increased the active layer thickness by 21 cm from 1956 to 1990, and is expected continue increasing as climate change continues (IPCC, 2007). The deepening of the active layer and formation of taliks are of importance as the active layer hosts most of the ecologic, hydrological, biochemical and soil-forming (pedogenic) activity in the arctic (IPCC, 2007).

The second main type of permafrost thaw occurs more abruptly, especially in ice-rich regions where the thawing of ice causes surrounding permafrost soil to collapse and form thermokarst terrain (IPCC, 2007; Schuur *et al.*, 2008). This thermokarst creates a chaotic surface composed of over-dry hills and water logged depressions forming pools, which expand laterally and trigger major changes in surface topography, hydrology and the dynamics of the system (Schuur *et al.*, 2008). There is continuous erosion and thawing of the permafrost edges surrounding the

pools, causing expansion of thermakarst pools into lakes (IPCC, 2007; Schuur *et al.*, 2008). This expansion of thaw lakes has been well documented in Russia and West Siberia, where 14.7 % and 12 % increases in lake area were seen between 1974-2000 (Walter *et al.*, 2007; Walter *et al.*, 2006). This is expected to continue and even worsen in the future (IPCC, 2007).

2-1-3. Arctic Permafrost Carbon Stores

The thawing of permafrost is of particular importance due large amount of carbon it contains. This vast expanse of permafrost has been recently re-estimated to contain up to 50 % of the global soil organic carbon pool (Tarnocai *et al.*, 2009). The arctic carbon pool is currently estimated at 1,672 Pg, with 61 % in the top 300 cm of frozen peatland and mineral soils, and the remainder in deeper yedoma and deltaic deposits (Schuur *et al.*, 2008; Tarnocai *et al.*, 2009). Frozen yedoma is an organic rich soil consisting of plant remains and animal matter deposited during the Pleistocene glacial age (Walter *et al.*, 2007; Walter *et al.*, 2006; Zimov *et al.*, 2006). This soil is of particular concern as it contains more labile carbon and has a higher organic content than mineral soils, an average of 2.6 % compared to 0.5 % (Zimov *et al.*, 2006). To grasp the immense 1,672 Pg carbon store found in the arctic, comparison are made to the

mere 6.5 Pg of carbon released annually by fossil fuels (Zimov *et al.*, 2006). Recent modeling has indicated that as much as 90% of the top ~3 m of Arctic permafrost will thaw by 2090 (IPCC, 2007). The importance of the permafrost thaw and release of organic carbon is that the carbon will become available for microbial decomposition and respiration. It is estimated that 47-61 % of the top 3 m of thawed permafrost will undergo microbial degradation by 2100, releasing 232-380 billion tonnes of CO_2 equivalents into the atmosphere (Schuur and Abbott, 2011). Therefore, the culmination of arctic warming and moistening along with the release of previously frozen carbon stores is expected to cause a large impact on the main drivers of arctic ecosystems, the microorganisms.

2-1-4. Fate of Thawed Permafrost Carbon

Microbial decomposition of previously frozen permafrost carbon stores may result in one of the most significant positive feedback loops from terrestrial systems to the atmosphere. The rate and form of GHG emitted from microbial decomposition depend on several interconnected environmental factors. The main factors are temperature, moisture availability, nutrient availability, and electron acceptor availability (Schuur et al., 2008). Warming is known to exponentially increase microbial activity based on the Q10 effect; however the increase in available substrates

(organic carbon stores) may be even more important than the direct effects of temperature on the decomposition of the newly thawed organic matter (Schuur et al., 2008). As the carbon pools thaw, they may either be decomposed under aerobic (presence of oxygen) or anaerobic conditions. Whether anaerobic or aerobic conditions have a greater impact on permafrost decomposition with climate change depends on whether the formation of a wet arctic of thermokarst lakes and taliks will dominate (anaerobic), or increased temperatures will cause increased evaporation to dry up the arctic (aerobic).

As depicted in Figure 2-1, the newly introduced organic matter often thaws at the transition zone, or boundary between the active layer and permafrost layer (Graham et al. 2011). The deeper zones are mostly anaerobic, whereas the surface soils of the active layer are aerobic. The level of water saturation is one of the key determinants of the boundary between aerobic and anaerobic zones as well as the rate and form of carbon emitted to the atmosphere (Schuur *et al.*, 2008). As seen during the last deglaciation in Greenland, a period of increased temperature and precipitation was followed by a rise in atmospheric methane (CH_4) for 100-300 years (Rigby *et al.*, 2008; Sowers, 2010; Walter *et al.*, 2007). Based on the isotopic carbon and hydrogen (H_2) concentrations of the released

CH_4, it originated from Arctic wetlands and lakes that formed from thawed permafrost (Sowers, 2010; Walter *et al.*, 2007). Currently, a similar rise in temperature and precipitation has expanded thermokarst wetlands in Canada, Alaska, Mongolia, China and Russia (Walter *et al.*, 2007). Walter and colleagues have attributed a 58 % increase in lake CH_4 emissions to a 14.7 % increase in lake area between 1974 and 2000. Of the CH_4 released, 60 % originated from Pleistocene-aged organic carbon, whereas 40 % was produced by decomposition of contemporary organic detritus (Walter *et al.*, 2007; Walter *et al.*, 2006). If this expansion of anaerobic wetlands continues, 49,000 Tg of CH_4 will be released from the Siberian ice complex alone (Walter *et al.*, 2007). Further studies confirm these predictions, estimating terrestrial ecosystems north of 60° to shift from a sink of 68 Pg carbon dioxide (CO_2) to a source of up to 62 Pg CO_2, and for CH_4 emissions to increase from 34 to 41-70 Tg per year by 2100 (Koven *et al.*, 2011). In addition to the large release of carbon as CO_2 and CH_4, thawing permafrost is also linked to the release the nitrous oxide, also a GHG, however this relationship remains uncertain and will not be elaborated further (Jungkunst, 2010). There also exist models that contradict the increasing release of GHG from thawing permafrost, predicting that an attenuation response will occur (Allison *et al.*, 2010).

This attenuation response will decline GHG emissions back to pre-warming levels, however may be counteracted by microbial adaptation and community changes (Allison *et al.*, 2010). If microbial community changes and adaptations increase the efficiency of carbon use, then it will accelerate soil-carbon loss, as predicted by other models (Allison *et al.*, 2010).

Figure 2-1. Key biological processes of the carbon cycle in permafrost.

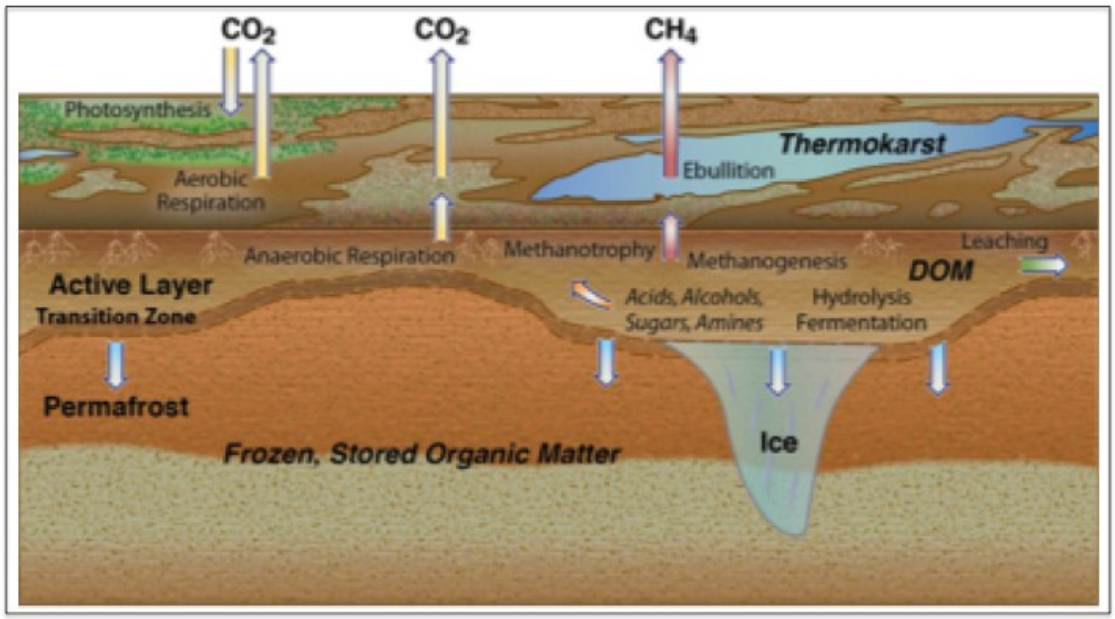


Figure 2-1: As climate change progresses, the active layer expands and previously frozen permafrost carbon stores become available for decomposition. The released complex organic matter will first undergo enzymatic hydrolysis into smaller fermentable substrates, creating an environment of organic acids, alcohols and microbial biomass. Methanogens then convert acetate, methylated compounds or H_2 and CO_2 into methane which is released into the atmosphere. Methanotrophs will oxidize some of the released methane into CO_2. Source: Graham et al. 2011

2-2. Arctic microbial CH_4 cycle with climate change

2-2-1. The global methane budget

Atmospheric CH_4 is of significant concern for climate change feedback as it is 25 times more potent than CO_2 as a GHG (Conrad *et al.*, 2002a; IPCC, 2007). This force is due to its radiative efficiency in the ozone layer (O'Connor *et al.*, 2010). The atmospheric concentration of CH_4 has increased from 380 ppb at the last glacial maximum, to 715 ppb in 1750, and 1787 ppb in 2008 (O'Connor *et al.*, 2010). This drastic increase is mostly due to anthropogenic (human-derived) sources, but a recent increase is being attributed to expansion of northern wetlands in response to accelerated temperature increases (Rigby *et al.*, 2008). The total concentration of CH_4 in the atmosphere is a balance between sources and sinks. The sources of CH_4 are either anthropogenic sources or natural sources. Anthropogenic sources account for 60-70 % of current emissions, and include energy, mining, landfills and waste treatment, ruminants, rice agriculture and biomass burning (O'Connor *et al.*, 2010). Natural sources account for the remaining 30-40 % and include wetlands, terminates, oceans, marine hydrates, geological sources, wild animals and wildfires, as depicted in Figure 2-2 (Conrad *et al.*, 2009; O'Connor *et al.*, 2010). It is imperative to note that of these sources, microbial production

of CH_4 (methanogenesis) is responsible for about 70-85 % of total emissions (Knittel and Boetius, 2009; O'Connor *et al.*, 2010). To balance these sources, there are several sinks of CH_4, including hydroxyl radicals (OH^-) in the troposphere, biological oxidation in drier soils (methanotrophy), reactions with chlorine or oxygen atoms in the stratosphere, and oxidation with chlorine atoms in the marine boundary layer (O'Connor *et al.*, 2010). The tropospheric hydroxyl radicals are the major sink, removing 85-90 % of CH_4 in the atmosphere (O'Connor *et al.*, 2010).

In terms of climate change, unlike anthropogenic sources and sinks of CH_4, natural sources are not well understood. Despite the increasing sophistication of climate models, the predictions for GHG flux from northern wetlands and permafrost regions remain poorly parameterized (Riley et al., 2011). Production of CH_4 from these natural sources is expected to occur sooner and at a higher rate than estimated by current models, creating significant positive feedback loops on climate change (Coolen *et al.*, 2011; Schuur and Abbott, 2011).

Figure 2-2. Global methane sources.

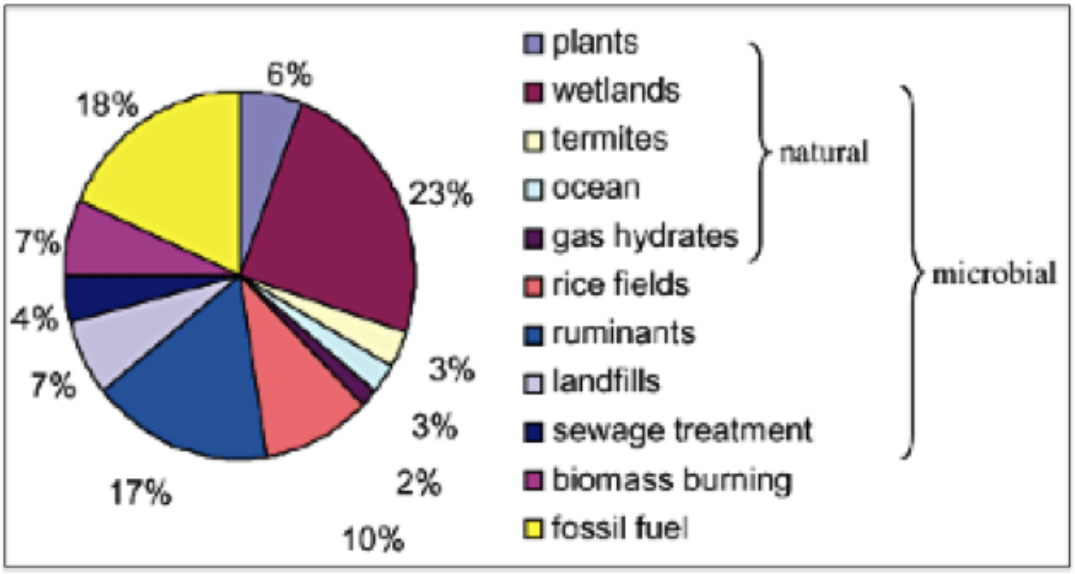


Figure 2-2: The total global methane sources are shown as a percentage of the total annual budget of 500-600 Tg. Microbial production of methane corresponds to 69% of total sources. Source: Conrad et al. 2009b

2-2-2. Microbial controls over CH_4 emissions

Microorganisms are responsible for up to 85 % of the annual global CH_4 production as well as 60 % of its consumption (Knittel and Boetius, 2009). Therefore, to understand and predict CH_4 emissions due to climate change one must first understand the effect of a changing climate on the responsible microorganisms. The microorganisms responsible for the regulation of CH_4 belong to only three functional groups of limited diversity, each of which will be discussed below (Knittel and Boetius, 2009). The production of CH_4 begins with the primary fermentation of organic carbon macromolecules to acetic acid, other carboxylic acids, alcohols, and CO_2 with H_2. A secondary fermentation will transform the carboxylic acids and alcohols to acetate, H_2 and CO_2 (IPCC, 2007). Finally, these are used to produce CH_4 through anaerobic respiration in water-saturated soils by microorganisms called methanogens. However, before reaching the atmosphere, this CH_4 produced by methanogens may be consumed. If the water table is below the surface, the produced CH_4 is partly consumed in the upper dry layers of soil by aerobic CH_4-oxidizing microbes called methanotrophs (Knittel and Boetius, 2009; Schuur *et al.*, 2009). However, this CH_4 may also be consumed in anaerobic zones if anaerobic methanotrophic Archaea (ANME) are present. Therefore, the

amount of CH_4 that reaches the atmosphere is dependent on both the production by methanogens and the consumption by methanotrophs and ANME (Knittel and Boetius, 2009; Niederberger *et al.*, 2010; Schuur *et al.*, 2009). However, the response of microbial communities to climate change and permafrost thaw remains poorly understood (Mackelprang *et al.*, 2011; Schuur and Abbott, 2011).

2-2-3. Methanotrophs

Methanotrophs are responsible for the aerobic oxidation of CH_4, and represent the major sink for CH_4 in terrestrial habitats (Liebner and Wagner, 2007). Methanotrophs consume 13-38 % of CH_4 produced in subarctic peat, and up to 90 % in wetlands and flooded rice fields (Liebner and Wagner, 2007). The oxidation of CH_4 begins with the oxidation of CH_4 to methanol by the key enzyme methane monooxygenase (Rigby *et al.*, 2008). This enzyme exists in two forms, the particulate form (pMMO) found in membranes, and the cytoplasmic soluble form (sMMO). All methanotrophs have the pMMO enzyme but a subset also have the sMMO enzyme. The regulation of these enzymes is based on the availability of copper, a cofactor of pMMO but not sMMO (Murrell and Jetten, 2009). The exception to this is *Methylocella* species, which only have the sMMO enzyme, and thus also lack the intracytoplasmic

membrane system to which pMMO is bound (Dedysh *et al.*, 2007). After the oxidation of CH_4 by an MMO enzyme, the produced methanol is oxidized to formaldehyde, which can either be assimilated into organic carbon or be further oxidized to CO_2. The formation of organic carbon from formaldehyde occurs by two pathways, which separate methanotrophs into two major groups. Type I methanotrophs will use the ribulose phosphate pathway, and type II methanotrophs will use the serine pathway (Angel and Conrad, 2009). Until recently, all methanotrophs belonged to the phylum *Proteobacteria*, with type I as *Gammaproteobacteria* and type II as *Alphaproteobacteria* (Murrell and Jetten, 2009). However, methanotrophs in the phylum *Verrucomicrobia* have recently been found (Dunfield *et al.*, 2007; Pol *et al.*, 2007). In addition to the two major types of methanotrophs, they can also be separated by their affinity for CH_4. Low-affinity methanotrophs oxidize CH_4 at high concentrations, whereas high-affinity methanotrophs oxidize CH_4 at low or atmospheric concentrations. However, the methanotroph *Methylocystis* was shown to produce both isoforms of pMMO, one with low-affinity and one with high-affinity oxidation kinetics, being expressed based on the CH_4 concentration available (Baani and Liesack, 2008). Recently, high affinity methanotrophs capable of high rates of CH_4

oxidation at low concentrations (15 ppm) were detected in upland tundra topsoil from the Canadian high Arctic (Martineau *et al.*, 2011). These methanotrophs have the potential to act as a sink for atmospheric CH_4 and mitigate the release of CH_4 expected with climate change (Martineau *et al.*, 2011).

2-2-4. ANME

ANME were only discovered in the late 1990's. They are a special lineage within the *Euryarchaeota* that seem to derive energy solely from the anaerobic oxidation of CH_4 (AOM) with sulfate as the most common final electron acceptor (Knittel and Boetius, 2009). As a result of this dependence on sulfate, they are often found in syntrophic relationships with sulfate reducing bacteria (SRB). ANME are most common in ocean floors, but have also been found in gas-laden sediments and cold CH_4 seep ecosystems with temperatures down to -1.5°C (Knittel and Boetius, 2009). The optimal ANME niche is the zone where sulfate from above and CH_4 from below meet, known as the sulfate-methane transition zone (SMTZ). There, ANME currently consume over 90 % of the total 85-300 Tg of CH_4 produced by ocean seafloors, which is equivalent to 7-25 % of the total global CH_4 production (Knittel and Boetius, 2009). ANME are very closely related to methanogens and share a coevolved biochemistry. AOM

is believed to proceed through reverse methanogenesis using the same (or very similar) enzyme from the methyl coenzyme M reductase gene (MCR) that also catalyzes the last step in the production of CH_4 by methanogens. The net reaction of AOM is: $CH_4 + SO_4^{2-} \rightarrow HCO^-_3 + HS^- + H_2O$ (Holler *et al.*, 2011). ANME have been recently seen to also use manganese and iron as electron acceptors, which may expand their distribution and importance in the global CH_4 cycle (Beal *et al.*, 2009). Sequences related to ANME have been detected in a subzero (-5°C), hypersaline (24 %), and perennial springs in the Canadian high Arctic (Niederberger *et al.*, 2010).

2-2-5. Methanogens

2-2-5-1. Methanogenic Archaea

Archaea are the third domain of life, and incorporate most of the extremophiles, such as hyperthermophiles and halophiles. However, with the recent advancements in culture-independent methods, Archaea have also been found to be ubiquitous in soils, sediments and oceans (Chaban *et al.*, 2006). There are currently separated in two phyla, the Euryarchaeota and Crenarchaeota. The Crenarchaeota are (hyper)thermophilic organisms originating from high temperature environments. The Euryarchaeota are more diverse, and include

methanogens, sulfate-reducers, halophiles and some (hyper)thermophiles (Chaban *et al.*, 2006). The major trait that distinguishes Archaea from other domains is that they incorporate ether-linked isoprenoid lipids with a glycerol-1-phosphate backbone (Cavicchioli, 2011). Otherwise, they share traits with both domains, having bacterial-like metabolism, lack of nucleus and organelles, but eukarya-like information processing systems (DNA replication, transcription apparatus, and translation elongation factors). In addition, Archaea are the only life forms capable of producing biological CH_4 through methanogenesis (Cavicchioli, 2011). Most Archaea are not culturable on traditional media, especially as agar doesn't withstand their extreme growth temperatures. In addition, Archaea lack the cellular selectable markers used to identify bacteria (such as antibiotics). Thus, Archaeal small-subunit rRNA phylogenetic trees consist mainly of proposed clades and uncultivated environmental sequences (Cavicchioli, 2011).

2-2-5-2. Methanogen phylogeny

Methanogenic Archaea are an extremely ancient taxon, as evidence for methanogenesis dates back 2.8 billion years (Bapteste *et al.*, 2005). There are currently six methanogen orders of the domain Archaea (phylum Euryarchaeota), which are the Methanobacteriales,

Methanopyrales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanocellales (Sakai *et al.*, 2011). Methanogens are morphologically and physiologically diverse, with less than 82 % sequence similarity between orders (Cavicchioli, 2011; Liu and Whitman, 2008). These orders are further divided into 12 families and over 30 genera, most of which are shown in Table 2-1 (Liu and Whitman, 2008). This table lacks the novel hydrogenotrophic order Methanocellales, that represents the previously uncultured Rice Cluster I (RC-I) group (Sakai *et al.*, 2011). However, a large fraction of methanogens remain uncultured, such as those found in rumens (Bapteste *et al.*, 2005; Conrad *et al.*, 2006; Liu and Whitman, 2008; Nicholson *et al.*, 2007). Nonetheless, all share an anaerobic lifestyle and the ability to produce CH_4 metabolically (Bapteste *et al.*, 2005).

The order Methanobacteriales generally use CO_2 as the substrate and H_2 as the electron donor for methanogenesis. The different pathways of methanogensis will be described in the following section. Some Methanobacteriales can also use formate, carbon monoxide or secondary alcohols as electron donors. However, the genus *Methanosphaera* can only use methanol as both substrate and electron donor. The cell walls of methanogens are known to be sturdy and difficult to penetrate as they

contain specific protein or murein layers. The cell wall of Methanobacteriales contain pseudomurein (Liu and Whitman, 2008). The order Methanococcales produce CH_4 with CO_2 and either H_2 or formate, and have a cell wall made of S-layer proteins. The Methanomicrobiales use CO_2 and H_2, but can also use formate and secondary alcohols. Most have protein cell walls that may be surrounded by a glycoprotein sheath. The Methanosarcinales have the widest range of substrates, using methyl-containing compounds, acetate, and some CO_2. They also have a wide range of morphologies, and a protein cell wall that may be surrounded by a sheath or heteropolysaccharides. The order Methanopyrales are represented by only one species that reduces CO_2 with H_2, and has a pseudomurein cell wall (Liu and Whitman, 2008). Finally, the newest order Methanocellales are obligate hydrogenotrophic methanogens only capable of using CO_2 and H_2 (Sakai *et al.*, 2011).

Table 2-1. Properties of methanogen taxonomic groups.

Table 2-1: Source: Liu and Whitman 2008.

Order	Family	Genus	Methanogenesis Substrates[a]	Temperature Optimum (°C)	Typical Habitats
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	H_2, (formate)	37–45	Anaerobic digestors, freshwater sediments, marshy soils, rumen
		Methanobrevibacter	H_2, formate	37–40	Animal gastrointestinal tracts, anaerobic digestors, rice paddies, decaying woody tissues
		Methanosphaera[b]	H_2 + methanol	37	Animal gastrointestinal tracts
		Methanothermobacter	H_2, (formate)	55–65	Anaerobic digestors
	Methanothermaceae	*Methanothermus*	H_2	80–88	Hot springs
Methanococcales	Methanococcaceae	*Methanococcus*	H_2, formate	35–40	Marine sediments
		Methanothermococcus	H_2, formate	60–65	Marine geothermal sediments
	Methanocaldococcaceae	*Methanocaldococcus*	H_2	80–85	Marine geothermal sediments
		Methanotorris	H_2	88	Marine geothermal sediments
Methanomicrobiales	Methanomicrobiaceae	*Methanomicrobium*	H_2, formate	40	Anaerobic digestors, groundwater, rumen
		Methanoculleus	H_2, formate	20–55	Anaerobic digestors, marine sediments, freshwater sediments, rice paddies, oil fields, hot springs
		Methanofollis	H_2, formate	37–40	Anaerobic digestors
		Methanogenium	H_2, formate	15–57	Marine sediments, freshwater sediments, rice paddies, animal gastrointestinal tracts
		Methanolacinia	H_2	40	Marine sediments
		Methanoplanus	H_2, formate	32–40	Oil fields
	Methanospirillaceae	*Methanospirillum*	H_2, formate	30–37	Anaerobic digestors, marine sediments
	Methanocorpusculaceae	*Methanocorpusculum*	H_2, formate	30–40	Anaerobic digestors, freshwater sediments
		Methanocalculus[b]	H_2, formate	30–40	Oil fields
Methanosarcinales	Methanosarcinaceae	*Methanosarcina*	(H_2), $MeNH_2$, Acetate	35–60	Anaerobic digestors, marine sediments, freshwater sediments, rumen
		Methanococcoides	$MeNH_2$	23–35	Marine sediments
		Methanohalobium	$MeNH_2$	40–55	Hypersaline sediments
		Methanohalophilus	$MeNH_2$	35–40	Hypersaline sediments
		Methanolobus	$MeNH_2$	37	Hypersaline sediments
		Methanomethylovorans	$MeNH_2$	20–50	Freshwater sediments, anaerobic digestors
		Methanimicrococcus	H_2 + $MeNH_2$	39	Animal gastrointestinal tracts
		Methanosalsum	$MeNH_2$	35–45	Hypersaline sediments
	Methanosaetaceae	*Methanosaeta*	Acetate	35–60	Anaerobic digestors, freshwater sediments
Methanopyrales	Methanopyraceae	*Methanopyrus*	H_2	98	Marine geothermal sediments

[a]Major substrates utilized for methanogenesis. $MeNH_2$ is methylamine. Parentheses means utilized by some, but not all species or strains.
[b]Placement in higher taxon is tentative.

2-2-5-3. Methanogenic pathways

The methanogenesis pathway is complex, and requires a large number of unique coenzymes (Liu and Whitman, 2008). Methanogens first require the breakdown of complex organic carbon by bacteria to simple sugars and then to acetate, formate, H_2, and CO_2 by syntrophs. There are three ways to produce CH_4 based on the substrate, each of which may have several reactions based on the electron donor. The hydrogenotrophic pathway utilizes CO_2 as a substrate; the acetoclastic pathway utilizes acetate, while the methylotrophic pathway utilizes methyl-group containing compounds. The reactions and typical organisms are shown in Table 2-2 (Liu and Whitman, 2008). Most methanogens are hydrogenotrophs that use H_2 as the electron donor, but some can also use formate, ethanol or secondary alcohols such as 2-propanol, 2-butanol and cyclopentanol. However, two species have been found that use carbon monoxide as an electron donor for methanogenesis from CO_2 (Daniels et al., 1977; Liu and Whitman, 2008; O'Brien et al., 1984). Secondly, methyl-group containing compounds include methanol, methylated amines (monomethylamine, dimethylamine, trimethylamine, and tetramethylamine), and methylated sulfides (methanethiol and

dimethylsulfide). Methyltrophic methanogens reside mostly in the Methanosarcinales, except for Methanosphaera species in the Methanobacteriales (Bapteste et al., 2005; Liu and Whitman, 2008). This reaction occurs through disproportionation, where the oxidation of some of the CO_2 substrate is used to reduce the remainder of the substrate into CH_4. Interestingly, the activation and transfer of the methyl group requires methylamine methyltransferases that encode an L-pyrrolysine, the twenty-second genetically encoded amino acid (Cavicchioli, 2011; Liu and Whitman, 2008). Some methylotrophs, namely *Methanomicrococcus blatticola* and Methanosphaera, are dependent on H_2 as the electron donor. Finally, despite acetate being a major biochemical intermediate, only the genera Methanosarcina and Methanosaeta utilize this substrate. In addition, most Methanosarcina are generalists and prefer methylotrophic or hydrogenotrophic methanogenesis to acetoclastic (Bapteste et al., 2005; Cavicchioli, 2011). Methanogens are currently the most thermally diverse organism, as they have been found to grow from subzero temperatures up to 122° C. This makes them ideal candidates for the study of thermal adaptation to both low and high temperatures (Cavicchioli, 2011).

Table 2-2. Methanogenesis reactions with associated free energies.

Table 2-2: Source: Liu and Whitman, 2008.

Reaction	$\Delta G^{\circ\prime}$ [a] (kJ/mol CH_4)	Organisms
I. CO_2-type		
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	−135	Most methanogens
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	−130	Many hydrogenotrophic methanogens
$CO_2 + 4$ isopropanol $\rightarrow CH_4 + 4$ acetone $+ 2\ H_2O$	−37	Some hydrogenotrophic methanogens
$4\ CO + 2H_2O \rightarrow CH_4 + 3\ CO_2$	−196	*Methanothermobacter* and *Methanosarcina*
II. Methylated C1 compounds		
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	−105	*Methanosarcina* and other methylotrophic methanogens
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−113	*Methanomicrococcus blatticola* and *Methanosphaera*
$2\ (CH_3)_2\text{-}S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	−49	Some methylotrophic methanogens
$4\ CH_3\text{-}NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$	−75	Some methylotrophic methanogens
$2\ (CH_3)_2\text{-}NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$	−73	Some methylotrophic methanogens
$4\ (CH_3)_3\text{-}N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$	−74	Some methylotrophic methanogens
$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	−74	Some methylotrophic methanogens
III. Acetate		
$CH_3COOH \rightarrow CH_4 + CO_2$	−33	*Methanosarcina* and *Methanosaeta*

SOURCE: Modified from Hedderich and Whitman[1] and Zinder.[43]

[a]The standard changes in free energies were calculated from the free energy of formation of the most abundant ionic species at pH 7. For instance, CO_2 is $HCO_3^- + H^+$ and HCOOH is $HCOO^- + H^+$.

2-2-6. Methanogens in cold terrestrial environments

Approximately 85 % of the earth′s biosphere is permanently below 5° C (Margesin and Miteva, 2011). Nonetheless, these cold environments contain bacteria, Archaea, yeasts, fungi and algae (Margesin and Miteva, 2011). Archaea have been found in cold environments such as marine waters, sea ice, deep-sea waters, oceans, alpine lakes, glaciers, permafrost as well as arctic streams and wetlands (Allen *et al.*, 2009; Cavicchioli, 2006). In permafrost environments, microorganisms must withstand subzero temperatures, freeze-thaw cycles, oligotrophic conditions, constant gamma radiation as well as complete darkness (Coolen *et al.*, 2011). To date, the majority of methanogens detected are related to the Methanosarcinales, as they are known to be the most versatile order in terms of growth capabilities as they have the widest substrate range (Kotsyurbenko, 2010). There are a limited number of isolated Archaeal sequences as most are not isolatable or culturable, especially those from polar areas (Allen *et al.*, 2009; Jansson, 2011). Nonetheless, psychrophilic methanogens have been isolated from Antarctic lakes, Alaskan sediments and Arctic permafrost (Allen *et al.*, 2009; Franzmann *et al.*, 1992; Franzmann *et al.*, 1997; Krivushin *et al.*, 2010; Morozova and Wagner, 2007; Simankova *et al.*, 2003; Singh *et al.*,

2005). In addition, with the advent of 454 sequencing, metaproteomes, metatranscriptomes and metagenomes, the identification of novel methanogens and their functions in polar environments has increased drastically (Burg *et al.*, 2011; Jansson, 2011; Williams *et al.*, 2011).

2-2-6-1. Methanogens in cold environments: Antarctica

Terrestrial Antarctic ecosystems are colder, drier, lower in available nutrients, and often more alkaline than their Arctic counterparts (Coolen *et al.*, 2011). Archaea are not abundant, and the majority are putative nitrifiers belonging to *Crenarchaeota* (Coolen *et al.*, 2011). Archaeal sequences have been found to decrease with increasing latitude (from 51°S to 72°S), where there are none detectable in the most southern soils, such as the McMurdo dry Valleys (Pointing *et al.*, 2009; Yergeau *et al.*, 2009b). In a wide spread survey of 51 Antarctic terrestrial sites, only 18 were found to contain Archaea. These positives sites were mainly coastal areas, and were dominated (>99%) by marine group 1 lineage, with Euryarchaeotal Marine group III (<1%) found at only one site (Ayton *et al.*, 2010). However, in a sampling from the McMurdo dry Valleys, methanogens were detected by epifluorescence microscopy in most samples, but at low levels of 2-22 cells per gram dry soil (Gilichinsky *et al.*, 2007). However, Antarctic aquatic environments have greater Archaeal

presence, and several methanogen strains have been isolated from Ace Lake. These methanogens include *Methanogenium frigidum* (Franzmann *et al.*, 1997), and *Methanococcoides burtonii*, an obligate methylotrophic methanogen capable of growth down to -2°C (Franzmann *et al.*, 1992). Since their isolation, the complete genome *M. burtonii* has been obtained (Allen *et al.*, 2009). This methanogen has high genome plasticity, with over 500 genes being differentially expressed between 4°C and 23°C (Campanaro *et al.*, 2011). In addition, a change from 1°C to -2°C causes *M. burtonii* to shift from cold adaptation to cold stress, where rescue mechanisms of chaperones to increasing numbers of damaged and misfolded proteins become insufficient and protein degradation must ensue (Williams *et al.*, 2011).

2-2-6-2. Methanogens in cold environments: Arctic

In the Arctic, there have been more studies of Archaea, focusing mostly on methanogens. Studies to date include permafrost soils in the Lena Delta, Kolyma lowlands of Siberia and the Canadian Arctic, as well as peat soils from Russia, Norway, Siberia and Spitsbergen. These studies have proven that Archaea and methanogens are adapted and active in cold environments up to 3 million years in age (Gilichinsky *et al.*, 2003; Hoj *et al.*, 2008; Metje and Frenzel, 2007; Rivkina *et al.*, 2007;

Rivkina *et al.*, 2002; Wagner *et al.*, 2005). However, only a few psychrophilic and psychrotolerant strains of methanogenic Archaea have been isolated. To date, the lowest recorded temperature for growth is -10.7°C by the methylotroph *Methanococcoides alaskense* isolated from marine sediments in Alaska (Singh *et al.*, 2005). However, evidence for methanogenesis has been detected down to -16.5°C in permafrost (Rivkina *et al.*, 2004). Another isolate, *Methanosarcina* SMA-21 isolated from Siberian permafrost has been found to have extreme tolerances of temperatures down to -78.5°C, salinity up to 6M, starvation, dessication and oxygen exposure. It has therefore been characterized as a possible candidate for surviving in Martian environments. (Morozova and Wagner, 2007).

2-2-6-3. Methanogens in cold environments: Siberian arctic

The highest abundance of methanogenic Archaea was counted through FISH from Siberian permafrost, at $3x10^8$ cells per gram of active layer soil. This study found that methanogens mostly favoured horizons above water level, despite the presence of higher oxygen concentrations. This was presumably due to higher organic carbon content and abundance of syntrophic bacteria to breakdown complex macromolecules into methanogenic substrates (Kobabe *et al.*, 2004). Similarly, other

studies of Siberian permafrost have also found methanogenic activity to be higher in upper horizons due to higher quality of organic carbon despite the presence of oxygen (Ganzert *et al.*, 2007). Anaerobic slurries of Siberian arctic and sub-arctic peat were often dominated by Methanosarcinales, but also contained Methanobacteriales and undefined crenarchaeotal phylotypes (Metje and Frenzel, 2007). A recent methanogen, *Methanobacterium veterum*, was isolated from a 28 m deep ancient permafrost sample (Krivushin *et al.*, 2010).

Russian peat soils contained a few methanogens of the orders Methanobacteriales, Methanomicrobiales and Methanosarcinales (Ochsenreiter *et al.*, 2003). Upon an increase in temperature of Spitsbergen peat slurries, an increase in CH_4 production correlated with an increase in methanogen abundance (Hoj *et al.*, 2008). In a survey of Lena Delta peaty and sandy loam, CH_4-containing layers were found sandwiched between layers free of CH_4, which were often icy complexes. In addition, two methanogenic strains, *Methanosarcina mazei* and *Methanobacterium sp.*, were isolated from enrichment cultures (Rivkina *et al.*, 2007). In a Tibetan wetland, an enrichment culture increased the abundance of a novel methanogen from 17 % to 42 % of total Archaeal sequences. Subsequent isolation of this methanogen, *Methanolobus*

psychrophilus, uncovered its high affinity for methanol and growth down to 0°C (Zhang *et al.*, 2008).

2-2-6-4. Methanogens in cold environments: Canadian arctic and Alaska

In Canadian high-Arctic, the Eureka site clone libraries were dominated by Crenarchaeota in the upper layers, below 2 m was dominated by halophilic euryarchaeotal sequences, and at 7 m a few *Methanobrevibacter* sequences were found (Steven *et al.*, 2008; Steven *et al.*, 2007). Subsequent metagenomic analyses found bacteria dominated 3 to 4-fold in active layer and 2 m permafrost samples respectively. Although no 16S rRNA Archaeal sequences were retrieved, functional genes related to *Methanomicrobia* (30-38% total Archaea)*,* as well as *Methanobacteria, Methanococci* and *Methanopyri* (<3%) were detected (Yergeau *et al.*, 2010). At the Axel Heiberg Island site, Crenarchaeota related to *Thermoprotei* dominated the 16S rRNA signatures, accounting for 71% in active layer and increased to 95% in permafrost. The closest relative to the *Thermoprotei*-related crenarchaeota was *Candidatus Nitrososphaera* of MGI 1.1b, a moderately thermophilic ammonia-oxidizer (Wilhelm *et al.*, 2011). In a recent study, an immunochip assay found positive reactions for an antibody to *Methanobacterium formicicum* from both Eureka core samples and an Axel Heiberg core sample (Goordial,

unpublished). However, a positive reaction does not confirm the presence of this species, but simply a species with a similar enough antigen.

In Alaska, several methylotrophic methanogens were isolated from the sulfate-reducing zone in sediments of Skan Bay. These included *Methanosarcina baltica* and a novel species, *Methanococcoides alaskense,* which grew down to -10.7°C (Singh et al., 2005). The abundance of methanogens was found to be relatively high, between 0.2-4.0% of total 16S rRNA sequences, but with low heterogeneity (Mackelprang *et al.*, 2011). Using metagenomics, the genome of a novel methanogen most closely related to the order *Methanocellales* was constructed (Mackelprang *et al.*, 2011).

2-3. Constraints on methanogens and effects of climate change

All organisms in permafrost environments must adapt to several constraints brought on by their frozen environment, including low temperature, lack of liquid water and lack of nutrients. However, methanogens have been found to be well adapted to extreme ranges of temperature, pH and salinity. Nonetheless, as many methanogens are dependent on other anaerobic microorganisms to decompose complex organic matter into useable substrates, community-level constraints will also affect them (Hoehler *et al.*, 2010; Kotsyurbenko, 2005). In frozen

environments, methanogens are most affected by energy availability, oxygen concentration, and competition for substrates (Hoehler *et al.*, 2010).

Global climate change is expected to relieve several of these constraints. Temperatures will increase overall reaction rates and production of methanogenic substrates. In addition, increased thaw depth and precipitation will augment water content and nutrient flow. Several studies have tested the reaction of methanogens in permafrost soils to the effects of climate change. However, as environmental systems are diverse in their distribution, vegetation, geochemistry and physiology, their rates of microbial decomposition of organic matter as well as the impact of permafrost thaw on microbial communities may vary greatly (Kotsyurbenko, 2010). Nonetheless, useful information can be found in each study about the current diversity, abundance and activity of methanogens, as well as their reactions to the various effects of climate change.

2-3-1. Constraints on methanogens: temperature

Temperature is proportional to the energy of motion of atoms or molecules of a substance (Bakermans, 2008). As such, reaction rates decrease exponentially with decreasing temperature, as exemplified by

the Arrhenius equation and the Q10 law. In addition, decrease in thermal energy also causes increased rigidity of membrane lipids, nucleic acids, and proteins, causing them to function less effectively (Bakermans, 2008; Feller, 2007). A third effect of cold temperatures is the lack of liquid water (Bakermans, 2008; Margesin and Miteva, 2011). However, the formation of salty brines has been documented within permafrost environments due to the exclusion of solutes as pure water crystallizes (Bakermans, 2008; Gilichinsky *et al.*, 2003). This creates hypersaline conditions, and forces organisms to cope with the difference in osmotic pressure between the interior of the cell and their surroundings. Methanogens will actively transport salt out of the cell and produce intracellular osmolites, such as glycine betaine and β-glutamate (Hoehler *et al.*, 2010). This requires additional energy, and thus methylotrophic methanogens, with their highest free energy yield, are most tolerant to hypersaline environments. In addition, their substrates are the breakdown products of osmoregulants, and thus, are more abundant due to the osmoregulation required in frozen soils (Hoehler *et al.*, 2010). However, as climate change continues, temperatures in arctic environments are expected to increase by 6-8° C, and permafrost thaw depth will increase up to 3 m by 2090. Increased seasonal thawing will promote water saturation of the soils and anaerobic

decomposition of organic matter into methanogenic substrates (Ganzert *et al.*, 2007).

Several recent studies have measured the activity of methanogens with increasing temperatures. Hoj and colleagues found that in unamended Spitsbergen permafrost slurries, non-methanogenic archea dominated below 10°C, but higher temperatures increased the diversity and relative abundance of methanogens. Within the methanogenic community, *Methanosaeta* populations showed the largest increase in abundance with temperature, while *Methanomicrobiales* showed little or no response, seemingly having a competitive advantage at lower temperatures (Hoj *et al.*, 2008). Even small changes in temperature, such as from -6.0 to -3.0°C, have been seen to cause substantial increases in methanogenic activity in several studies of Siberian arctic and sub-arctic permafrost (Metje and Frenzel, 2007; Wagner *et al.*, 2007).

2-3-2. Constraints on methanogens: substrate availability and competition

The main substrates for methanogens are H_2 and CO_2, acetate, and methyl-containing compounds. Acetate is the most abundant intermediate of anaerobic degradation of organic matter. However, it is mostly available in enrivonments rich in polysaccharides, which currently isn't the case in most Arctic environments (Hori *et al.*, 2007; Kotsyurbenko

et al., 2004). Methanol is derived from pectin or lignin and has been found to play a significant role in Siberian permafrost environments (Ganzert *et al.*, 2007). H_2 and CO_2 are used by the broadest range of methanogens, and as CO_2 is a product of fermentation, it is seldom limiting in anaerobic environments (Kotsyurbenko, 2010; Liu *et al.*, 2008).

Methanogens are subject to fierce competition, as the free energy yields of their reactions are low. They are outcompeted by bacteria for acetate and H_2 in environments with oxygen, nitrate, iron, sulfate, and manganese, as reactions using these compounds as electron acceptors are energetically favourable to the use of CO_2 by methanogens (Hori *et al.*, 2007; Liu *et al.*, 2008; Thauer *et al.*, 2008). In permafrost environments, the main competitors for methanogens are often sulfate reducing bacteria (SRB). However, complete inhibition is often not achieved in heterogeneous environmental systems (Hoehler *et al.*, 2010). In addition, methylotrophic methanogens are not in direct competition for substrates, and therefore, often dominate in permafrost zones with elevated sulfate (Ganzert *et al.*, 2007; Koch *et al.*, 2009). Homoacetogens also reduce CO_2 in anaerobic environments using H_2 or other substances such as sugars, alcohols, methylated compounds, carbon monoxide or other organic acids. However, acetogenesis with H_2 is energetically less favourable than

methanogenesis (Liu and Whitman, 2008). Despite this, one study has found that homoacetogens may be better adapted to cold environments than methanogens (Kotsyurbenko *et al.*, 2001). However, with temperatures increasing in permafrost environments, substrates are expected to increase due to the thawing of organic carbon stores as well as increased vegetation.

It must be kept in mind that methanogens are dependent on syntrophic interactions as their substrates are produced through the decomposition of organic matter by primary and secondary fermenters (Hoehler *et al.*, 2010; Kotsyurbenko, 2005). Therefore, environmental constraints that influence other microorganisms will alter the function of the entire trophic chain, thereby indirectly affecting methanogens (Kotsyurbenko, 2005). In addition, the total amount of organic carbon available will determine the quantity of methanogenic substrates. However, several studies have shown that it is not the total organic carbon content, but the quality of organic carbon that is limiting to microbial metabolic processes. The quality is a more accurate determinant of the availability of the organic carbon compounds as sources of both energy and carbon for the microbial community (Wagner *et al.*, 2007).

2-3-3. Constraints on methanogens: water table and precipitation

The content of water in soils and the water table are measures of the depth of oxygen penetration in soils. Methanogens are obligate anaerobes and cannot grow or produce CH_4 when trace amounts of oxygen are present (Hoehler *et al.*, 2010). However, methanogens have been found to tolerate oxygen without being irreversibly damaged. Therefore, methanogens are present and active in areas that fluctuate between aerobic and anaerobic seasons, or in microenvironments within otherwise aerobic zones (Hoehler *et al.*, 2010).

Climate change is triggering erosion, increased water temperatures and evaporation in some areas, causing overall drying, whereas other areas permafrost thawing and increased precipitation are expanding wetlands and thermokarst lakes (Vincent, 2010). Both are expected to cause positive feedback on global warming, through increased CO_2 and combustion in drier sites, or increased CH_4 production in flooded sites (Fan *et al.*, 2011). Precipitation increases have already been documented in the Arctic due to climate change. The total precipitation at the Eureka weather station in 2010 was 108.3 mm, which has been increasing over the last few years from 69.8 mm in 2009, 57.5 mm in 2008, and 42 mm in 2007 (http://climate.weatheroffice.gc.ca). Precipitation is known to have profound effects on the microbial community. For example, Archaea had

large community differences along a precipitation gradient of <100 mm up to 900 mm annually (Angel *et al.*, 2010). The community differences correlated with the increased precipitation and water content, as well as with the vegetative cover, but did not correlate to pH, salinity, or other nutrients such as phosphorus, nitrogen, carbon, magnesium or potassium (Angel *et al.*, 2010). In addition, the dynamics of heat transport and conduction at wetter sites are more vulnerable to warming and permafrost thaw than at drier sites, and thus experience earlier and larger increases in soil temperature (Fan *et al.*, 2011). Therefore, an increase in soil water content will amplify the increase in soil temperature caused by warming.

2-3-4. Constraints on methanogens: pH

Changes in environmental pH require cellular organisms to regulate their intracellular pH through the active regulation of ionic species such as H^+, OH^- and CO_3^{2-}, thereby increasing their energy expenditure. In addition, under alkaline environments methanogenic substrates, including acetic acid, carbonic acid and potentially methylamines, convert into ionic forms. These ionic forms require active transport into the cell, consuming more energy (Hoehler *et al.*, 2010). In northern Finland, acidic peat (pH 4.1) was dominated by hydrogenotrophic methanogens, whereas mildly acidic peat (pH 5-6) was dominated by acetoclastic methanogens, and

weakly acidic peat (pH 6.0) in Spitsbergen hosted all types of methanogens, such as Methanomicrobiales, Methanobacteriales, and Methanosarcinales, including *Methanosaeta* (Hoj *et al.*, 2008; Metje and Frenzel, 2007). These studies prove that most methanogens are adapted to near-neutral pH, despite being shown to tolerate ranges from 3-10 (Hoehler *et al.*, 2010). With climate change, the pH of arctic soils may become more neutral, as precipitation, soil water content and vegetation become more abundant; however, this remains uncertain.

2-3-5. Constraints on methanogens: vegetative cover

Several studies hypothesize that vegetation determines the microbial community, while others indicate the pH and available carbon are more important (Campbell *et al.*, 2010). With climate change, vegetation in the arctic is expected to increase, as temperatures and nutrients increase. Over the past 27 years, a study conducted on Ellesmere Island in the Canadian high Arctic found that the tundra community is under transition, and becoming more productive (Hudson and Henry, 2009). Although this increased vegetation provides nutrients and nitrogen for microorganisms, as well as aids the release of CH_4 in wetlands via their roots, it also uptakes CO_2, providing a sink for released carbon (Laanbroek, 2010; Schuur *et al.*, 2009). A study looked at the net

carbon release or uptake between newly and older thawed permafrost environments in Alaska. They show that increased vegetation will cause an initial sink in carbon, but after decadal timescales, release of carbon from thawing permafrost will overwhelm plant uptake, making permafrost a large potential carbon source as thawing continues (Schuur *et al.*, 2009).

2-4. Summary

The main factors controlling the GHG production by methanogens in the Arctic include water content, organic matter content and temperature (Ganzert *et al.*, 2007; Hoehler *et al.*, 2010; Hoj *et al.*, 2008; Hoj *et al.*, 2006; Wagner and Liebner, 2010; Wagner *et al.*, 2003; Wagner *et al.*, 2005). Climate change has already been shown to increase temperatures and precipitation, as well as thaw previously frozen carbon stores, all of which are expected to escalate in the coming years (IPCC, 2007; Schuur and Abbott, 2011; Schuur *et al.*, 2009; Walter *et al.*, 2007) (http://climate.weatheroffice.gc.ca). However, the effects of these changes on methanogen abundance, diversity and GHG production remain poorly understood (Wagner and Liebner, 2010). Therefore, it has become imperative to study the response of the methanogenic community to climate change in various environments order to help elucidate the magnitude of their positive feedback on climate change.

Chapter 3 – Materials and Methods

3-1. Site Sampling and characterization

The sampling was done at several sites in order to obtain various soil types representative of the Canadian high Arctic. The first site contained one core from Eureka, Ellesmere Island, Nunavut (80° N, 85° W) obtained in mid-July 2007 and kept frozen at -20°C until use. This core is called Er-H1 for Eureka (Er) and Hole 1 (H1). The second site included four cores obtained from the McGill Arctic Research Station (MARS), located on Axel Heiberg Island, Nunavut (79°433 N, 90°766 W) in mid-July 2009 and mid-July 2010, and were frozen at -20°C until use. Three cores were taken at sites across a polygon, and are called AH-P1, AH-P2 and AH-P3, for Axel Heiberg (AH) and polygon (P) cores 1 through 3 (Figure 3-1). A polygon is a representative terrain of the Canadian high Arctic. Polygons are formed by contraction cracks in permafrost formed during extreme cold. These cracks are typically long lines which will fill with snow and melt-water, forming ice wedges that then undergo repeated cracking during winter. As these long lines often cross each other almost perpendicularly, the result is a series of rectangles and polygons that resemble a honeycomb from above (Billings and Peterson, 1980). The fourth core was taken from a nearby wetland meadow site in order to

determine the effects of increasing water content and vegetation on the polygon soils, and is called AH-P4. The AH-P4 site was also sampled in 2009, and this sample is referred herein as AH-H4, for Hole 4. The AH-H4 sample has been previously studied for methanotrophs (Martineau *et al.*, 2010).

We measured several soil parameters in order to investigate the factors we hypothesized might influence the microbial community and GHG production, and to track any changes occurring *in situ* as climate change progresses at our sampling sites in the Canadian high Arctic. Active layer depth was measured by the depth of penetration of the permafrost probe stick through the unfrozen soil, until it reached the frozen soil. Water table depth was not measured properly in 2010, as it was measured in the hole post-coring, which gave an overly low value (water closer to the surface) due to hydrostatic effects. The technique was improved for the 2011 sampling, where the water level was measured both in the hole created by a hand-auger (1.5 inches diameter) as well as by measuring the wet portion of the permafrost probe stick. The water content was measured by heating 10 g of the soil sample in a crucible at 105° C until dry, and cooled to ambient temperature before weighing. The organic matter content was measured by loss on ignition (LOI) based on

the European Standard by project Horizontal (2004). Briefly, empty crucibles were heated at 550° C for 30 min and cooled to ambient temperature before weighing. Then 2 g of soil sample in triplicates were added to crucibles and heated at 550° C for 1 hr and cooled to ambient temperature before weighing. Crucibles with samples are then further ignited at 550° C for 30 min to ensure the obtained weight differs by less than 0.5 %, therby indicating it is the true mass of the residue. Soil pH was determined using the Hellige-Truog soil pH kit (Orbeco-Hellige Inc., FL, USA).

The concentrations of CH_4 and CO_2 in soil were determined using the protocol described by Wagner and colleagues to extract gas from the soil pore water, but with a few changes (Wagner *et al.*, 2003). Briefly, 9 g of the soil sample was homogenized and triplicates were made with 3 g of soil plus 6 mL of saturdated NaCl solution in 20mL vials. Vials were vortexed and incubated at 80°C for 1 hour. The gases, now transferred to the headspace, were measured by gas chromatography (GC).

GC was used to measure the headspaces concentration of CH_4 and CO_2 for both their concentrations in soil and for microcosm experiments (see below). The CH_4 headspace concentration was measured by injecting a 250µl sample into a Chromosorb 102 packed

column (Supelco, Bellefonte, PA, USA) on a Varian 3800 gas chromatograph with a flame ionization detector. The column was heated to 40°C for 2.5 minutes while the detector was at 150°C, with Argon as the carrier gas. A CH_4 calibration curve was made from dilutions of 99% pure CH_4 in nitrogen (from 1 to 80 ppm). The CO_2 headspace concentration was measured by injecting a 500µl sample into a HaysepQ packed column (Supelco, Bellefonte, PA, USA) on a SRI 8610C gas chromatograph with a thermal conductivity detector. The column was heated at 60°C for 1.5 minutes while the detector was at 100°C, with Helium as the carrier gas.

Figure 3-1. Aerial view of sample location on Axel Heiberg Island, Nunavut.

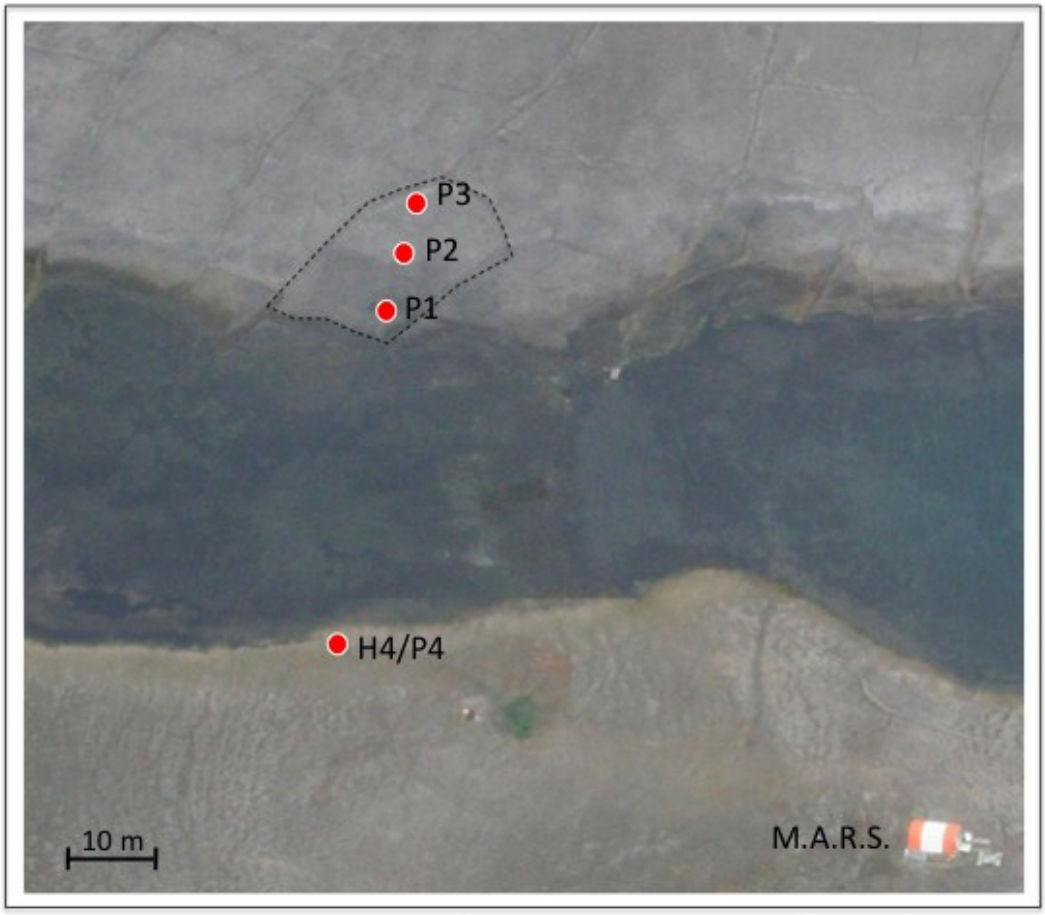


Figure 3-1: Location of sites AH-P1 (N 79° 24.917 W 90° 45.430), AH-P2 (N 79° 24.922 W 90° 45.453) and AH-P3 (N 79° 24.925 W 90° 45.473), which are found within a polygon on Axel Heiberg Island. Polygon terrain is typical to Arctic permafrost environments. The location of the H4/P4 site is within a wetland (N 79º 24.881, W 90° 45.503). The McGill Arctic Research Station (M.A.R.S.) can also be seen (N 79° 25.887, W 90° 45.952).

3-2. Field gas flux of Canadian high Arctic polygon and wetland sites

Field gas flux was measured *in situ* in order to determine the production and release of CH_4 and CO_2 from the study sites, as well as to track future changes as climate change progresses. Several different methods were used. In 2009 and 2010 the flux of CO_2 was measured at each site using the LiCor Li-8100 Automated Soil CO_2 Flux System (Li-Cor Environmetnal, Lincoln, NE, USA). The measurement of CH_4 flux in 2009 and 2010 was not successful, as it was attempted using a closed chamber system, where subsamples of the gas collected in the chamber were extracted into vials and analysed by GC. In 2011, CO_2 flux was again measured using the LiCor system, and due to the purchase of new equipment, both CO_2 and CH_4 flux could be measured with the Picarro soil CH_4 analyser (Santa Clara, CA, USA). The Picarro flux measurements were collected using the flux chamber set up as open flux systems with a 60ml/min flow rate, for a total of 480 mL of gas collected per site. The open flux system provides a continual flow of the gas sample to be analysed in order to provide an accurate measurement. Both atmospheric values and total flux values were measured for CO_2 and CH_4. The net flux from soil was calculated as the difference between the total flux and standard atmospheric values, taken as 393 ppmv CO_2 and 1.82 ppmv

CH_4. For example, the CO_2 flux from soils at the AH-P1 site was found to be 14213 mg/m^2/day whereas the flux of CO_2 based on its atmospheric concentration is 13809 mg/m^2/day, yielding a net flux of 404 mg/m^2/day. These *in situ* flux values were also converted from mg/m^2/day into nmol/gFW/day in order to compare with the microcosm flux values. The conversion of these values requires the estimation of the grams of soil per m^2 . This estimation was based on volume of soil per m^2, taken from the surface area and depth of the active layer, as well as the approximate density of these mineral soils, taken as approximately 1.5-2 g/cm^3 (Stackhouse *et al.* in progress).

3-3. Microcosm slurry and substrate-enriched incubations

Two sets of microcosm incubations were tested in order to determine the production of CH_4 and CO_2 from soils upon an increase in temperature, as well as with or without the addition of a variety of substrates. The changes in Archaeal and methanogen community composition were also determined in order to see their relation to the production of CH_4 and CO_2, the temperature increase and the specific substrate added.

The first microcosm set was an unamended slurry, similar to previous Arctic studies (Hoj *et al.*, 2008; Martineau *et al.*, 2010). This type

of microcosm experiment is useful in order to understand the effect of increases in temperature alone. In addition, it is often closer to the *in situ* conditions than microcosm experiments which add substrates, as the concentrations and forms of substrates found *in situ* or expected with climate change are difficult to replicate. In the unamended microcosm experiment, soil samples were taken from the Er-H1 core and the AH-H4 core. All the soil required for the experiment were homogenized, and 10 g fresh weight (FW) soil was added to each 120 mL serum vial along with 10 mL sterile dH_2O. The FW is the weight of the natural soil including water content, whereas dry weight (DW) is the weight excluding water. Soil was added to microcosms while frozen under sterile aerobic conditions but immediately crimp-sealed with a butyl rubber stopper and flushed with N_2 to create an anaerobic environment. The flushing of N_2 was done using a gassing station where vials were alternately vacuumed for 15 minutes then over-pressurized with N_2 for 1 minute, until the final step where the vials were filled with N_2 to a pressure of 1 atm. Triplicates of each sample were incubated at 4 °C and 15 °C for 40-50 days to simulate the average soil temperature (4°C) in summer and the length of summer season, as well as to simulate increased soil temperature expected as a result of climate change (15°C). Headspace concentration of CO_2 and CH_4 were

followed by GC as described above. Negative controls were made using microcosms prepared with the exact same soil, but autoclaved twice. Soil CH_4 and CO_2 production rates were calculated from the linear regression of the increase in headspace gas concentration over time, taking into account the available volume of headspace, the density of each gas, and the total grams per microcosm.

The second set of microcosms was amended with the three main methanogenic substrates, and used for stable isotope probing (SIP). This type of microcosm experiment is useful in order to target specific groups of interest that are often found in small numbers, in this case methanogens. In addition, it helps to elucidate potential changes in the methanogen community if climate change were to increase both temperature and available substrates. For the amended microcosms, the soil was added under sterile anaerobic conditions in an N_2 glove bag (Cole-Parmer Canada Inc., Montreal, Canada). In these microcosms, due to limited sample volume, 8 g of soil from two depths of the P1 core was mixed with 5 mL of sterile dH_2O. These were then amended with either 30 mM ^{13}C-sodium acetate, 30 mM ^{13}C-methanol or 80:20 H_2:$^{13}CO_2$ (99% pure, Cambridge Isotope Laboratories, USA). The substrate concentrations were taken as the average from several Arctic studies (Ganzert *et al.*,

2007; Jiang *et al.*, 2009; Morozova and Wagner, 2007; Rivkina *et al.*, 2007; Wagner *et al.*, 2005; Wagner *et al.*, 2007; Zhang *et al.*, 2008). In addition to these substrates, molybdate was added to a set of each treatment at 70 mM in order to inhibit sulfate reducers, the main competitors of methanogens for acetate and CO_2 (Gilichinsky *et al.*, 2003; Perreault *et al.*, 2008; Purdy *et al.*, 2003; Sorensen *et al.*, 2004). All of the microcosms amended with acetate or methanol (both with and without molybdate) were then crimp-sealed and flushed with N_2 as described above to create an anaerobic environment. The microcosms that were to be amended with H_2:CO_2 were crimp-sealed and vacuumed, and appropriate volumes of H_2 and CO_2 were added to a final pressure of 1 atm. Triplicates were made for each treatment and incubated at both 4° C and 22° C for 40-50 days, corresponding to average Arctic soil temperatures (4° C), and to simulate increased temperatures as are expected with climate change (22° C). Negative controls for SIP were prepared from the same treatments as above except with ^{12}C-labelled substrates (light carbon) instead of ^{13}C (heavy carbon). Negative controls for the overall experiment were prepared using microcosms containing soils that have been autoclaved twice. Positive controls for each treatment were made identically but with the addition of 200 uL of a turbid culture of

Methanosarcina barkeri (optical density not known). Headspace concentrations of CO_2 and CH_4 were monitored over time as described above.

3-4. RNA and DNA extraction from cores and soils post-incubation

Extractions of RNA and DNA were performed in order to determine the abundance of methanogens by quantitative polymerase chain reaction (qPCR), as well as the shifts in Archaeal community composition by pyrosequencing. Extractions were performed on intact cores that were frozen at -20°C, as well as from microcosm soils after substrate amendment and incubation. Extraction methods were optimized on a culture of *Methanosarcina barkeri* added to soils as traditional methods, such as heat-induced cell lysis, did not obtain DNA even from the pure culture broth. The best method found was via the RNA Powersoil total RNA isolation kit with the DNA elution accessory kit (Mobio Laboratories, Carlsbad, CA, USA) as per manufacturer's instructions but with a few changes. The changes include adding 2 g of glass beads to extraction tubes prior to the soil. Sonication was also done on samples prior to mechanical lysing for 4 intervals of 20 seconds on a direct horn W-185D sonicator at a nominal power of 150 W and probe amplitude of 96 μm (knob level 4) (model #4418, QSonica LLC, CT, USA). In addition, all of

the mechanical vortex times were increased by 5 minutes. Four replicates of the same sample were used for extraction, which were combined onto the same column. The nucleic acid precipitation step was extended overnight at -20° C, and final resuspension was done in 25µl. Protein was also extracted from the interphase after phenol chloroform separation and stored in 5 mL solution of 0.0286 g/ml guanidine hydrochloride in ethanol and saved for potential future research by the DOE project team. Concentration of RNA and DNA were measured using a NanoDrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) or PicoGreen dsDNA quantitation assay (Invitrogen, Carlsbad, CA, USA). Total RNA extracts were then synthesized into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) as per the manufacturer's′ instructions.

3-5. Methanogen abundance using qPCR of the *mcrA* gene

Quantitative polymerase chain reaction (qPCR) was performed in order to determine the abundance of methanogens *in situ* and the changes in methanogen abundance as a result of increased temperatures and amendment with various substrates. Initially, regular PCR was used as a positive control for successful DNA extraction of bacteria and Archaea as well as for the detection of methanogens. However, the limit of

detection (LOD) for methanogenic sequences by regular PCR was not sufficient for the low abundances found in high Arctic soils (see Annexe 2). Therefore, qPCR was performed due to its increased sensitivity and accuracy. Reactions of qPCR targetted the methanogen gene for the methyl coenzyme-M reductase alpha-subunit (*mcrA)* using the *mlas/mcrA-rev* primer set (Table 3-1). Several studies have used these primers for qPCR with varying reaction mixtures and protocols (Steinberg and Regan, 2008, 2009; Yergeau, 2009). The LOD was determined for the qPCR assay using 2-fold dilutions of the linearilized plasmid, from 100 down to 1 gene copy per reaction. The plasmid was made from the *mcrA* gene isolated from *Methanosarcina barkeri* using the EZ-10 Spin Column PCR purification kit followed by the EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic Inc., Markham, Ontario, Canada). Manufacturer's instructions were used but with several variations of ligation and transformation times. The variation that yielded the highest concentration by Picogreen dsDNA quantitation assay was a 1.5-2 hrs ligation with an overnight transformation. This was diluted to form a standard curve from 10^6 to 10^2 copies/µl. The limit of detection for qPCR was found using a cut-off value of 30 cycles and a level of confidence of 95 % and was found to be approximately 50 copies /µl (see Annexe 2). Reactions for qPCR

were performed in 15 µl volumes using the iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). Reactions contained 1-2 ng of DNA extract from soil samples. The protocol was 5 minutes of initial denaturation at 95°C followed by 40 cycles of 30 s at 95°C, 45 s at 55°C, 30 s at 72°C, and 15 s at 81°C reading step to avoid fluorescence from primer dimers. Melt curve analysis was performed from 50°C to 95°C for 10 s at each degree of change. Standards were made from 10-fold dilutions of linearilized *Methanosarcina barkeri* plasmids. The calculation of the copies of *mcrA* per gFW was done by taking into account both the original 8 gFW that was used for total DNA extraction. This 8 gFW was extracted into 25 µl, which was subsequently diluted depending on the resulting concentration for each sample to form a qPCR reaction mix of 2 ng / µl. Therefore, the copies / µl obtained from the qPCR reaction was multiplied by the dilution and by 25 µl, and divided by 8 gFW to yield the total copies / gFW.

Several other primer sets were tested, but did not yield successful results. The *METH* primer set was tested, which also targets the *mcrA* gene of methanogens. This primer set was made to enhance qPCR reactions, as the amplicon is shorter (287 bp vs. 500 bp) than those

generated by other *mcrA* primers (Colwell et al., 2008). However, these primers yielded poor results with our samples, and many non-specific amplicons. The best results for qPCR, with fewest non-specific amplicons, were obtained using the *mlas/mcrA-rev* primer set. Nonetheless, troubleshooting of the *METH* primer set with environmental samples should continue as these primers are expected to be more efficient and reliable for qPCR based on MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) (Bustin *et al.*, 2009). In addition, the addition of cloned bacteriophage lambda amplicon to soil samples (10^4 copies per µl) prior to RNA and DNA extraction was attempted in order to correct for potential PCR inhibitors present within soil samples. The DNA extract then undergoes qPCR targeting the bacteriophage lambda amplicon. If the recovery of lambda was below 100% (less than 10^4 copies per µl) the subsequent values for DNA extracts were corrected to account for the differences in DNA extraction and PCR inhibition as previously described (Yergeau *et al.*, 2009a). However, the bacteriophage lambda qPCR reactions were not efficient, and thus were could not be reliably used to measure PCR inhibition levels. Further troubleshooting of this method is required.

Table 3-1. Primers used in this study for PCR, qPCR and pyrosequencing.

Table 3-1

Target gene	Primer name	Sequence[a]	Reference
Bacterial 16S rRNA	U785F	5'-GGATTAGATACCCTGGTAG-3'	(Baker *et al.* 2003)
	U803R	5'-CTACCAGGGTATCTAATCC-3'	(Baker *et al.* 2003)
Archaeal 16S rRNA	newARCHf	5'-GYGCASCAGKCGMGAAW-3'	(Takai and Horikoshi 2000)
	newARCHRev	5'-GGACTACVSGGGTATCTAAT-3'	
	340F	5'- CCCTAYGGGGYGCASCAG-3'	(Gantner *et al.* 2010)
	1000R	5'-GGCCATGCACYWCYTCTC-3'	
mcrA	mlas	5'-GGTGGTGTMGGDTTCACMCARTA-3'	(Steinberg and Regan 2008)
	mcrA-rev	5'-CGTTCATBGCGTAGTTVGGRTAGT-3'	(Steinberg and Regan 2008)
	METH-f	5'-RTRYTMTWYGACCARATMTG-3'	(Colwell *et al.* 2008)
	METH-r	5'-YTGDGAWCCWCCRAAGTG-3'	(Colwell *et al.* 2008)

Primers were used for the detection of bacterial and archaeal 16S rRNA by PCR. Archaeal 16S rRNA primers were also used for pyrosequencing. Primers targetting the methanogen *mcrA* gene were used for the quantification of methanogen abundance by qPCR.

[a] Y = C,T; R = A,G; M = A,C; S = C,G; K = G,T; V = G,C,A; W = A,T; D = G,A,T; B = G,T,C;

3-6. Pyrosequencing of DNA from cores and soils post-incubation

The total DNA extracts from the AH-P1 core and post-incubated samples was completed in order to determine the Archaeal community composition present *in situ* and the changes that occurred after incubation at higher temperatures with substrate amendment. Pyrosequencing was done using the Roche 454 FLX and TITANIUM Genome Sequencer by the Research and Testing Laboratories LLC (Lubbock, TX, USA). Pyrosequencing targetted the Archaeal 16S rRNA gene using the *NewArchf/NewArchRev* primer set (Table 3-1). The Archaeal sequences of the resulting data set were extracted for downstream bioinformatic analyses. Analysis was completed using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu), but with a few changes to the default settings as suggested for the analysis of short pyrosequencing reads (Claesson *et al.*, 2009; Huse *et al.*, 2010). Changes included increasing the complete linkage clustering step to 2.0, increasing the dereplicate step maximum distance to 10, and decreasing the RDP Classifier confidence threshold to 50 %. The resulting clusters were used to form both total Archaeal and methanogen community profiles. RDP pipeline analyses were also used in order to give the rarefaction curves. The rarefaction curve shows the richness or diversity of OTUs for a given number of sequences obtained

from pyrosequencing. It allows a measure of the level of saturation of the pyrosequencing, as the curve should plateau when no more OTUs are found despite an increase in total number of sequences. The RDP pipleline was also used in order to calculate the Chao1 and Shannon indices, which both measure the diversity and richness of the pyrosequencing data obtained from each sample.

3-7. Stable isotope probing of CO_2-amended microcosms

Stable isotope probing was done in conjunction with pyrosequencing in order to determine the methanogenic community that is active in the utilization of CO_2 at higher temperatures, such as those expected as climate change progresses. The pyrosequencing of resulting fractions is recommended to detect the activity of less-abundant species and enrichments even in the presence of high levels of background DNA (Chen and Murrell, 2010). Microcosms were incubated with both ^{13}C and ^{12}C-labelled CO_2 as described previously. Between 1300-1500 ng of the total DNA extracts were separated by weight by cesium chloride (CsCl) gradient ultracentrifugation for 42-48 hrs at 46,000 rpm (168,500 x g maximum) in a Vti80 rotor (L8-70M ultracentrifuge, Beckman, Fullerton, CA, USA) based on several studies (Buckley *et al.*, 2007; Martineau, 2009; Martineau *et al.*, 2008). DNA was collected with a syringe into 36-40

fractions of 250 µl from the bottom of the tube. The buoyant density of each fraction was measured from 15 µl subsamples using a Reichart AR200 refractometer (Depew, NY, USA) and their DNA concentration was measured using the PicoGreen quantitation assay.

Based on the DNA-density profile constructed, equal density ranges from 1.6884 to 1.7037g/ml (fraction A) and from 1.7059 to 1.7211g/ml (fraction B) were pooled for both the ^{13}C-amended and ^{12}C-amended samples (Figure 3-2). Pooled fractions were purified and concentrated with Millipore Amicon Ultra centrifugal filters for DNA (Billerica, MA, USA). Samples were then sent for pyrosequencing at the Research and Testing Laboratories LLC (Lubbock, TX, USA) for Archaeal 16S rRNA using the *NewArchf/NewArchRev* primer set. Using SIP, the active organisms that utilize the substrate will incorporate the heavy carbon (^{13}C) into their DNA upon division and will be enriched in the heavy fraction, with a decrease in the light fraction (Bell *et al.*, 2011; Martineau *et al.*, 2008, 2010). However, as microorganisms are known to have a large range G-C contents, the weight of their DNA will vary. To ensure that the micoorganisms are indeed active, they must be absent from the heavy fraction naturally, when incubated with ^{12}C-labelled substrate (thus absent from fraction B of ^{12}C-amended samples). To determine the extent of the

enrichment, it is compared to the amount found in the light fraction when incubated with the heavy ^{13}C carbon. This may indicate only a small portion of the order is actively utilizing the substrate, or all of this order is actively incorporating the substrate and dividing. The overall percent change is calculated by subtracting the percentage of reads from both the light fraction of ^{13}C (^{13}C fraction A) and the heavy fraction of ^{12}C (^{12}C fraction B) from the enriched heavy ^{13}C fraction (^{13}C fraction B) (Figure 3-2). Those which have increased are enriched, and are thus actively incorporating the substrate and dividing.

Figure 3-2. Distribution of DNA in a caesium chloride density gradient based on buoyant density.

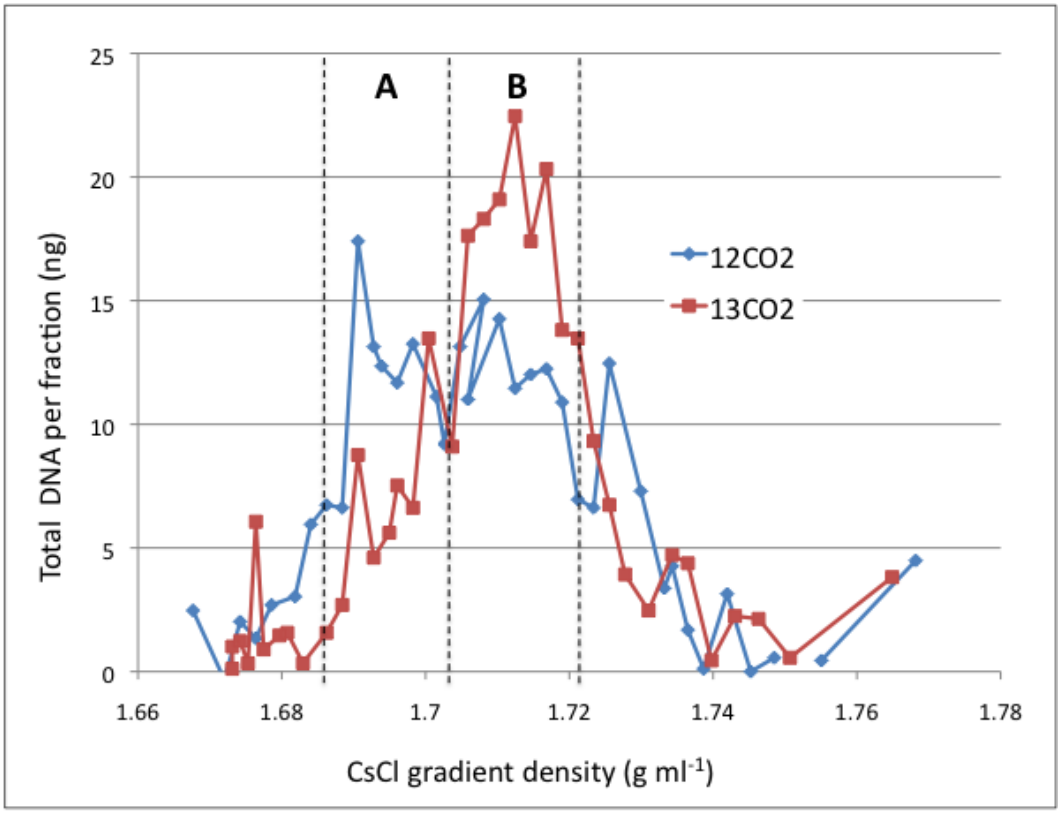


Figure 3-2: Stable isotope probing was performed on the AH-P1 70 cm sample by incubating microcosms with either ^{12}C or ^{13}C-labelled CO_2:H_2 (20:80) for 50-60 days. Total DNA was extracted and separated by caesium chloride (CsCl) density gradient ultracentrifugation. Fractions of 250 µl were collected from the bottom of the tube and measured for buoyant density by refractometry. This was plotted against the total DNA per fraction, measured by the PicoGreen quantitation assay. The shift of the peak to a greater density for the ^{13}C-labelled microcosms indicates the DNA is heavier due to the incorporation of ^{13}C (heavy carbon). Fractions within each set of dotted lines were pooled for both the ^{12}C and ^{13}C samples respectively, and sent for pyrosequencing.

3-8. Statistical Analyses

Statistical analysis for the soil parameters and production rates were done using Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). Statistical relationships between soil parameters and gas production rates, both in microcosms and *in situ,* were compared with the results of methanogen phylogeny and abundance using a one-way analysis of variance (ANOVA). Forward stepwise discriminate function analysis (DFA) was used to determine the correlation between substrate and CH_4 production (Statistica 6.0, StatSoft Inc., Tulsa, OK, USA) using the three substrate additions as groups (acetate, methanol, CO_2:H_2). Finally, principal component analysis (PCA) was done in order to show the principal factor explaining the distribution of each methanogen order (Statistica 6.0, StatSoft Inc., Tulsa, OK, USA). The factors included temperature, substrate, and the production rates of both CH_4 and CO_2.

3-9. Phylogenetic Tree of methanogen sequences

The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). A maximum likelihood phylogenetic tree was created based on the Tamura-Nei model (Tamura and Nei, 1993). The tree was formed using the methanogen sequences found at greater than 0.5 % abundance from all cores and amended microcosm samples, including the SIP

fractions. The primer and tag used for pyrosequencing were trimmed from the representative sequences from samples, and landmark sequences were selected from NCBI using the closest known cultured and uncultured relatives. In addition to the methanogenic sequences, two Nitrososphaerales (Thaumarchaeota) sequences from samples were added as a root, as well as two rare biosphere Methanopyrales sequences from samples in order to show their relation to the enriched SIP sequences.

The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985). Branches found in less than 50 % of replicate trees are collapsed. Next to each branch, the percentage of replicate trees in which the taxa are clustered together are shown (Felsenstein, 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The total analysis involved 41 sequences, in which all codon positions (1^{st}, 2^{nd}, 3^{rd}, noncoding) were used. There were a total of 265 positions in the final dataset.

Chapter 4 - Results

4-1. Characteristics of Canadian high Arctic soil samples

Sampling was done at two sites, Eureka in mid-July 2007, and on Axel Heiberg Island in mid-July 2009-2011, which are shown in Figure 3-1. There was a significant increase ($p= 0.0427$) in the depth of the soil active layer from 2009-2011 in the AH-H4 and AH-P4 sample (same site), and from 2010 to 2011 for the AH-P1, AH-P2 and AH-P3 samples. This indicates that the area of soil thawing each summer is increasing at our sites, which is associated with the release of previously frozen carbon stores and an increase the microbial activity. Despite this overall significant increase, the AH-P3 polygon sample furthest from the lake did not have an increase in active layer depth. As for the water table, the center of the polygon is relatively dry, an area that is known to be elevated with respect to the edges. The edge closest to the water has a higher water table, at 51 cm, than the edge furthest from the water at 73 cm (Table 4-1). The contents of the cores showed that the water content varied from 17 to 75 % in the soils, being highest in upper layers with proximity to the lake or in wetland areas (Table 4-2). Organic matter was higher in the wetland area (5.5 to 27 %) compared to the polygon area (4.2 to 6.9 %). The wetland sample had a statistically significant negative

correlation between organic content and depth (p= -0.9415). This sample had the highest values, with 27.0 % organic matter at 10 cm and 11.3 % at 25 cm, but reached approximately the same values as the polygon at 40 cm, at 5.5 % in 2010 and 9.0 % in 2011. This indicates that mostly the upper layers of the wetland site have higher organic matter contents, which is likely due to the large vegetative cover at this site. The CO_2 content was significantly higher for the wetland sample than the rest, yet all CO_2 contents were 10-50 times greater than the CH_4 content. The CH_4 content ranged from 22.43 nmol/g to 132.84 nmol/g. The soil pH in the polygon area was slightly acidic, between 5.5 and 6.5, whereas the wetland samples AH-H4 and AH-P4 as well as the Eureka Er-H1 samples were near neutral. Therefore, at all sites the active layer increased over our study period. In addition, the wetland site is characterised by an overall higher water content and CO_2 content, as well as a higher organic matter content in upper layers when compared to the polygon site.

Table 4-1. Soil characteristics of sampling sites taken in mid-July from Axel Heiberg Island and Eureka, in the Canadian high Arctic.

Table 4-1

Core	Type	Vegetation	Year	Active layer depth 2009[a]	Active layer depth 2010	Active layer depth 2011	Water table depth 2011
				cm	cm	cm	cm
AH-P1	Polygon wet edge	Low	2010	na	70.0	73.5	51
AH-P2	Polygon center	Low	2010	60	68.5	71	Dry
AH-P3	Polygon dry edge	Low	2010	na	73.0	73	73
AH-P4 AH-H4	Wetland site	High	2010 2009	40	≈45	59	4-10
Er-H1[b]	Upland tundra	Low	2007	≈60 [b]	na	na	na

The soil site characteristics were measured in mid-July at sites in the Canadian high Arctic on Axel Heiberg Island (AH) both across a polygon terrain (P1, P2 and P3) as well in a wetland site (P4/H4). Active layer depth was measured by the depth of penetration of the permafrost probe stick. Water table depth was measured by the water level in a hole created by a hand-auger as well as by the wet portion of the permafrost probe stick.

[a] Active layer depth was measured in July 2009 by previous studies. The polygon area was measured by Wilhelm *et al.* 2011 and the H4 site by Martineau *et al.* 2011.

[b] Er-H1 active layer depth was measured in 2007 by Steven *et al.* 2008.

na = not available

Table 4-2. Soil sample contents from cores taken in mid-July from Axel Heiberg Island and Eureka, in the Canadian high Arctic.

Table 4-2

Sample	Depth	H_2O content	Organic Matter	CO_2 content	CH_4 content	pH
	cm	%	%	nmol/gFW	nmol/gFW	
AH-P1	30	25.0	4.2	1064.1 ±153.8	130.1 ±18.5	5.5
	70	18.9	5.5	1224.2 ±46.6	81.5 ±9.1	5.5
AH-P2	30	22.1	5.5	1046.3 ±139.7	66.0 ±10.5	6.5
	70	22.6	6.9	939.8 ±84.7	61.5 ±6.3	6.5
AH-P3	30	17.9	4.5	846.6 ±43.6	83.9 ±0.1	6.5
	70	23.0	5.0	1028.0 ±0.1	118.2 ±0.0	6.5
AH-P4	25	75.0	11.3	4261.0 ±81.0	66.6 ±3.7	7.5
	40	49.8	9.0	5465.2 ±186.5	132.8 ±31.6	7.5
AH-H4*	10	72.8	27.0	1142.4	27.42	6.0
	40	22.3	5.5	2851.6	65.04	7.0
Er-H1	60	19.5	6.1	1469.9 ±18.6	27.3 ±5.6	7.8
	105	22.9	6.5	2447.4 ±632.6	22.4 ±8.4	7.8

Soil contents were measured from soil samples taken from two sites in the Canadian high Arctic. The first site was on Axel Heiberg Island (AH) in mid-July 2009 and 2010, where samples were taken from across a polygon terrain (P1, P2 and P3) as well as from a wetland site covered with vegetation (P4/H4). The second site was at Eureka (Er) in July 2007, where soil samples were taken from an upland tundra. Water content was measured by heat drying. Organic matter content was measured by loss on ignition at 550° C for over 1 hr. The contents of CO_2 and CH_4 in soil were measured in duplicates by gas extraction from soil pore water (Wagner *et al.* 2003). The soil pH was measured using the Hellige-Truog soil pH meter.

* H4 soil sample characteristics were measured in July 2009 by Martineau *et al.* 2011, except for the soil pH

FW = Fresh weight (includes water content)

4-2. Field Gas Flux from polygon and wetland high Arctic sites

Field gas flux was measured *in situ* in order to determine the production and release of CH_4 and CO_2 from the study sites, as well as to track future changes as climate change progresses. The *in situ* CO_2 flux measured by the LiCor detector in July 2010 found there to be 266 mg/m^2/day from the AH-P2 and AH-P3 sites, and 418 mg/m^2/day from the AH-P1 site (Table 4-3). The AH-P4 wetland core had a CO_2 flux of over 10 times greater, at 5244 mg/m^2/day. In 2011, all of the flux measurements increased compared to the 2010 measurements. The AH-P2, AH-P3 and AH-P4 cores increased slightly, whereas the AH-P1 core more than doubled. In 2011, the net flux was also measured by taking into account the atmospheric values of each gas measured. The net flux was found to be around 200-1300 mg/m^2/day for the polygon sites, and 11809 mg/m^2/day for the wetland site.

The *in situ* CH_4 flux in 2010 could not be detected using the closed chamber system. In 2011, the Picarro was used, and the CH_4 flux was found to be around 21-22 mg/m^2/day. The CH_4 flux is 15-300 times less than the CO_2 flux for the same soil, similar to the soil contents of each gas. The net flux measured in 2011 using the Picarro was found to be slightly negative for all sites, but closest to zero for the AH-P1 and AH-P4 sites.

This indicates that these Arctic soils are currently a net source of CO_2, but a slight sink of CH_4. Correlations were found between two datasets using the correlation coefficient based on the covariance and standard deviation. Significant correlations were found between the CO_2 flux from soils and several soil characteristics including the CO_2 content (p= 0.9806), organic matter content (p= 0.8931), pH (p= 0.8312) and active layer depth (p= -0.9853). Therefore, it was found that the wetland site had a much higher *in situ* flux of CO_2 than the polygon sites, which correlated with the higher organic matter content, CO_2 content, and near-neutral pH of the wetland site.

Table 4-3. *In situ* CO_2 and CH_4 flux from across a polygon and from a wetland site on Axel Heiberg Island, in the Canadian high Arctic.

Table 4-3

Sample	Year	CO_2 Flux LiCor 2010	CO_2 Flux LiCor 2011	Net CO_2 flux from soil 2011		Net CH_4 flux from soil 2011	
		$mg/m^2/day$	$mg/m^2/day$	$mg/m^2/day$	nmol/gFW/day	$mg/m^2/day$	nmol/gFW/day
P1	2010	418	1102	404	7.3 ± 1.0	-5.62	-0.28 ± 0.04
P2	2010	266	342	235	4.2 ± 0.6	-6.68	-0.33 ± 0.05
P3	2010	266	418	1297	23.3 ± 3.3	-8.78	-0.43 ± 0.06
P4	2010	5244	6995	11809	212.0 ± 30.5	-6.32	-0.31 ± 0.04

The *in situ* flux of CO_2 and CH_4 was measured for polygon sites (P1, P2 and P3) and one wetland site (P4) on Axel Heiberg Island (AH). In 2010, CO_2 flux was measured by the LiCor soil CO_2 flux detector. In 2011, CO_2 and CH_4 flux from soil was determined by both the LiCor CO_2 flux detector and the Picarro soil analyser. The net flux from soil was calculated as the difference between the total flux and standard atmospheric values, taken as 393 ppmv CO_2 and 1.82 ppmv CH_4. The net flux is the best measure of the production of each gas and their release from soils. Positive values indicate the soil is a net source of the gas, and negative values a net sink. The conversion of the net flux values from $mg/m^2/day$ into nmol/gFW/day was based on the estimation of the volume of soil per m^2. This estimation was based on the surface area and depth of the active layer, as well as the approximate density of these mineral soils, taken as the average and standard deviation between 1.5-2 g/cm^3.

4-3. CO_2 and CH_4 production rates in slurry and substrate-amended microcosms

Two sets of microcosm incubations were tested in order to determine the production of CH_4 and CO_2 from soils upon an increase in temperature, as well as with or without the addition of a variety of substrates. The CO_2 production rates were similar between the two experiments, but within the unamended slurries were found to correlate with organic matter content and temperature. The CH_4 production was higher with the addition of substrates; however this increase was dependent on the combined effects of temperature and the specific substrate used. It is important to note that the direct comparison between the two experiments is difficult as different soil types were used. The overall effect seen was that increasing substrates, temperature and anaerobicity in microcosms caused an increased ratio of CH_4 to CO_2 production rates.

The first set of microcosms were slurries made using the Er-H1 and AH-H4 samples incubated at 4°C and 15°C, and were not amended with any substrate. Slurry microcosms are useful in order to determine the effect of increasing temperatures alone, without substrate amendment. The CO_2 production rates in the slurry microcosms were higher in the AH-

H4 sample than the Er-H1, where AH-H4 ranged from 2.66 to 8.44 nmol/gFW/day and Er-H1ranged from 0.94 to 3.50 nmol/gFW/day (Figure 4-1). Both the CO_2 and CH_4 production rates were higher at 15°C than 4°C for almost all samples, but the differences were not significant. The exception was the Er-H1 60 cm sample that had an overall negative CH_4 flux at 15°C, despite a positive flux at 4°C. Overall, the CO_2 production rates were 3.4 to 32 times higher than CH_4 production rates. The CH_4 production rates for the AH-P4 sample ranged from 0.32 to 1.04 nmol/gFW/day and for Er-H1 from -0.97 to 0.28 nmol/gFW/day. This negative flux rate was due to a rapid increase in headspace CH_4 followed by a constant decrease in CH_4 concentration, making the overall production rate negative.

The second set of microcosms were all made from the AH-P1 core at two depths (30 cm and 70 cm) and were amended with various ^{13}C-labelled substrates and incubated at 4°C or 22°C. The amended microcosms are useful to determine the effect of both the increase of temperature and substrates, which is expected with climate change as permafrost thaws to release the previously frozen carbon stores. The CH_4 production rates for the AH-P1 30 cm samples ranged from 0.81 to 2.25 nmol/gFW/day, and the AH-P1 70 cm samples ranged from 0.55 to 2.80

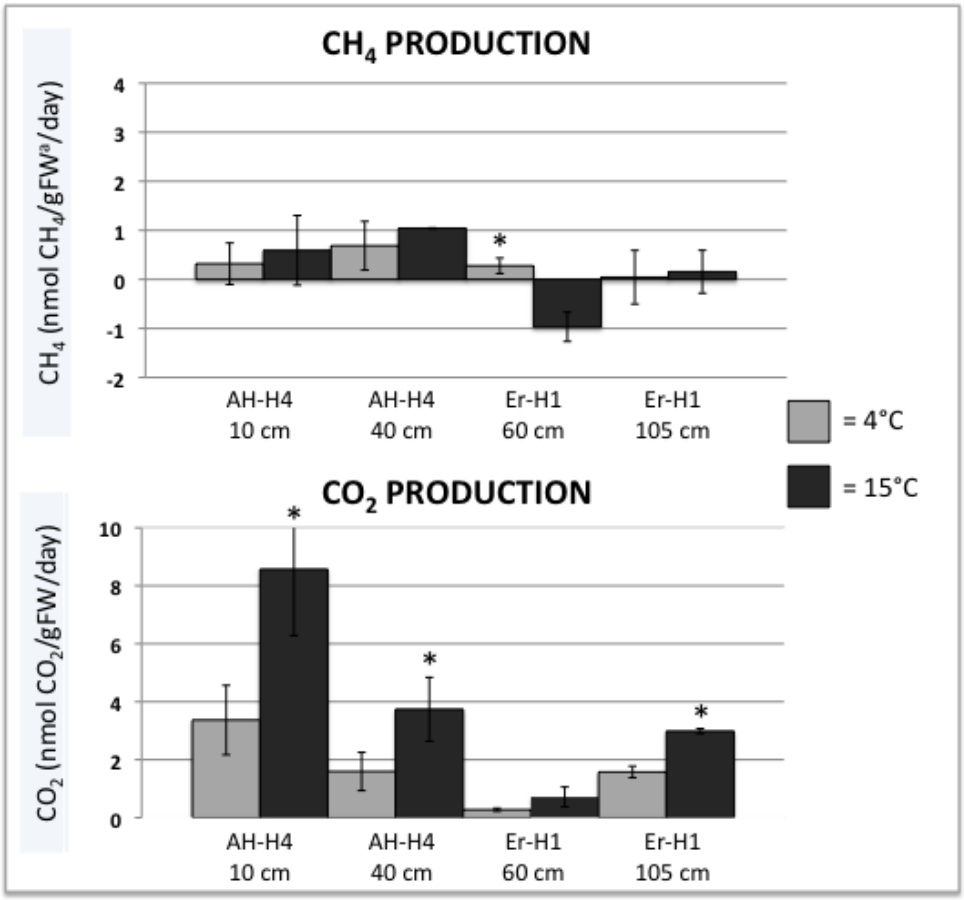
nmol/gFW/day (Figure 4-2). These production rates are higher than those for the unamended slurries for the first microcosm experiment. For all samples, CH_4 production rates were higher at 22°C than at 4°C, except for those incubated with acetate. The CH_4 production rates with acetate for both depths decreased from approximately 1.8 to 0.9 nmol/gFW/day. The microcosms amended with CO_2 had generally higher production rates than the other substrates. However, the very highest production rate of 2.80 nmol/gFW/day was seen with the AH-P1 70 cm sample incubated at 22°C with methanol.

The overall CO_2 production rates in the amended microcosms were 0.8 to 3.8 times higher than CH_4 production rates (Figure 4-2). The CO_2 production rates for the AH-P1 30 cm samples ranged from 3.03 to 3.47 nmol/gFW/day, and the AH-P1 70 cm samples from 0.43 to 3.01 nmol/gFW/day. Production of CO_2 was constant or higher at 22°C than at 4°C, and similar regardless of substrate (acetate or methanol). The exception was the low CO_2 production for the methanol-amended 70 cm sample incubated at 4°C. These production rates are similar to the unamended slurries from the first microcosms experiment.

Correlations using ANOVA and post-hoc Tukey HSD tests indicated that the production of CH_4 in amended microcosms is significantly

correlated with the combined variation of both substrate and temperature ($p= 0.0273$). Therefore, within a given substrate, the overall trend showed higher temperatures caused an increase in CH_4 production. Individually, this increase in production was statistically significant for all the methanol-amended 70 cm sample and CO_2-amended 30 cm sample. However, the acetate-amended samples at both depths showed a decrease in CH_4 production with increased temperature, despite an increase in CO_2 production. Depth, organic matter content, and other factors did not have significant correlations with the production of CH_4 or CO_2, as temperature and substrate had an overshadowing impact. For the production of CO_2 in the amended microcosms, there was no overall significant correlation between substrate and temperature. However, there was a significant increase in CO_2 production at higher temperatures for the methanol-amended 70 cm sample, and the acetate-amended 30 cm sample. The overall comparison between the two experiments is difficult as different soil types were used. Nonetheless, the CO_2 production rates were similar between the two experiments, whereas the CH_4 production was higher with the addition of substrates.

Figure 4-1. CH_4 and CO_2 production rates in unamended microcosms.

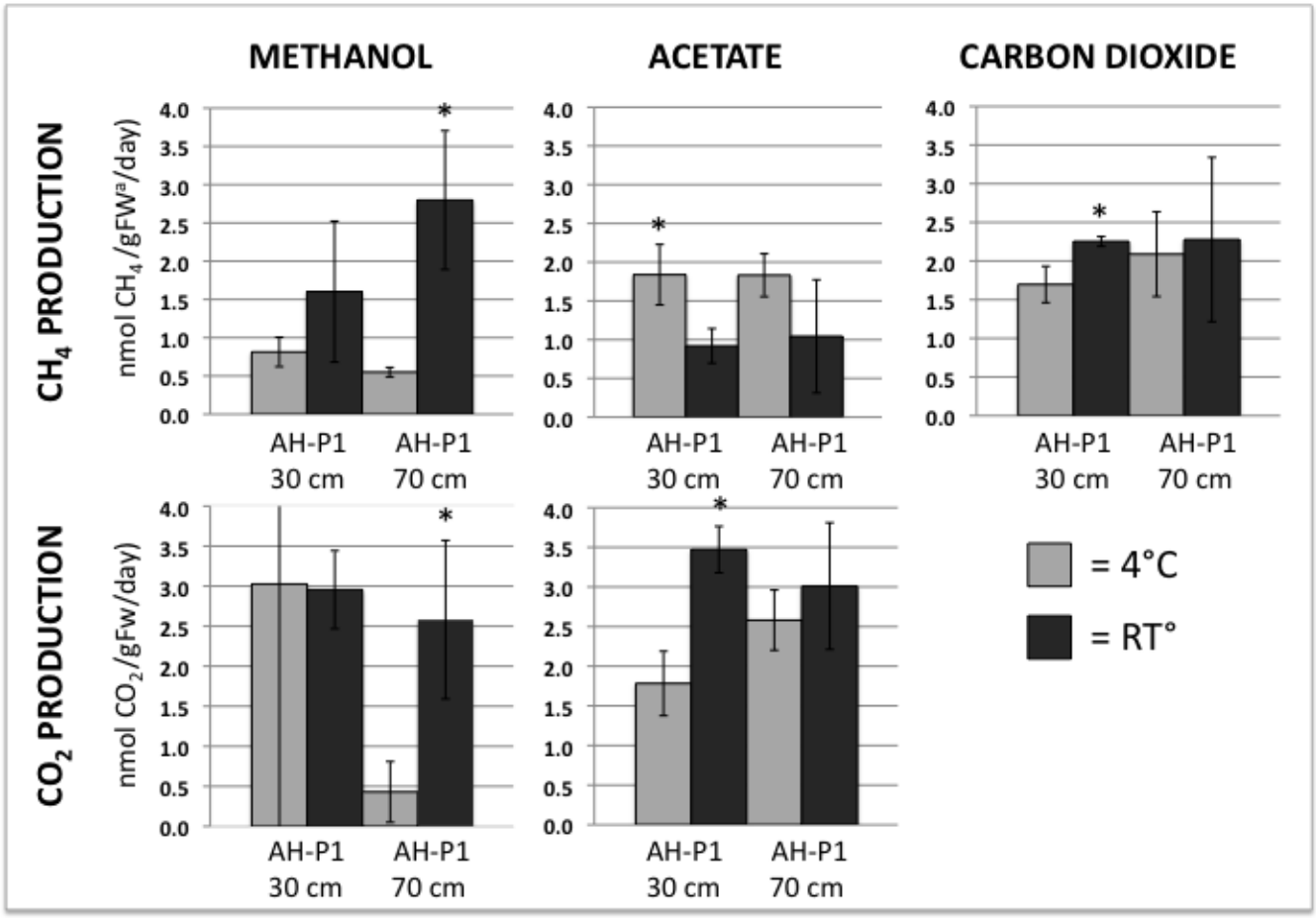


The AH-H4 core was sampled at 10 cm and 40 cm from a wetland site on Axel Heiberg Island in July 2009. The Er-H1 core was sampled at 60 cm and 105 cm from an upland tundra in Eureka in July 2007. All samples were incubated in triplicate slurry microcosms formed from 10 g soil plus 10 mL sterile H_2O without substrate amendment. Microcosms were incubated at 4°C and 15°C for 50-60 days. Gas production rates were calculated from the linear increase of headspace gas concentration measured bi-weekly by gas chromatography.

* Indicates difference in gas production between temperatures for that sample is statistically significant

[a] FW = fresh weight of soil (with water content)

Figure 4-2. CH_4 and CO_2 production rates in microcosms enriched with methanogenic substrates.



All soil samples were taken from the AH-P1 core from Axel Heiberg Island at one of two depths (30 cm and 70 cm). Microcosms were made with 8 g soil plus 5 mL sterile water and incubated with one of the ^{13}C labelled substrates: methanol (30mM), acetate (30mM) or CO_2:H_2 (20:80). Each sample with each substrate was incubated in triplicates at both 4° C and 22° C for 50-60 days. Gas production rates were calculated from the linear increase in headspace gas concentration measured bi-weekly by gas chromatography.

* Indicates the difference between temperatures for that sample is statistically significant

[a]FW = fresh weight of soil (includes water content)

4-4. Methanogen abundance using qPCR of the methanogen *mcrA* gene

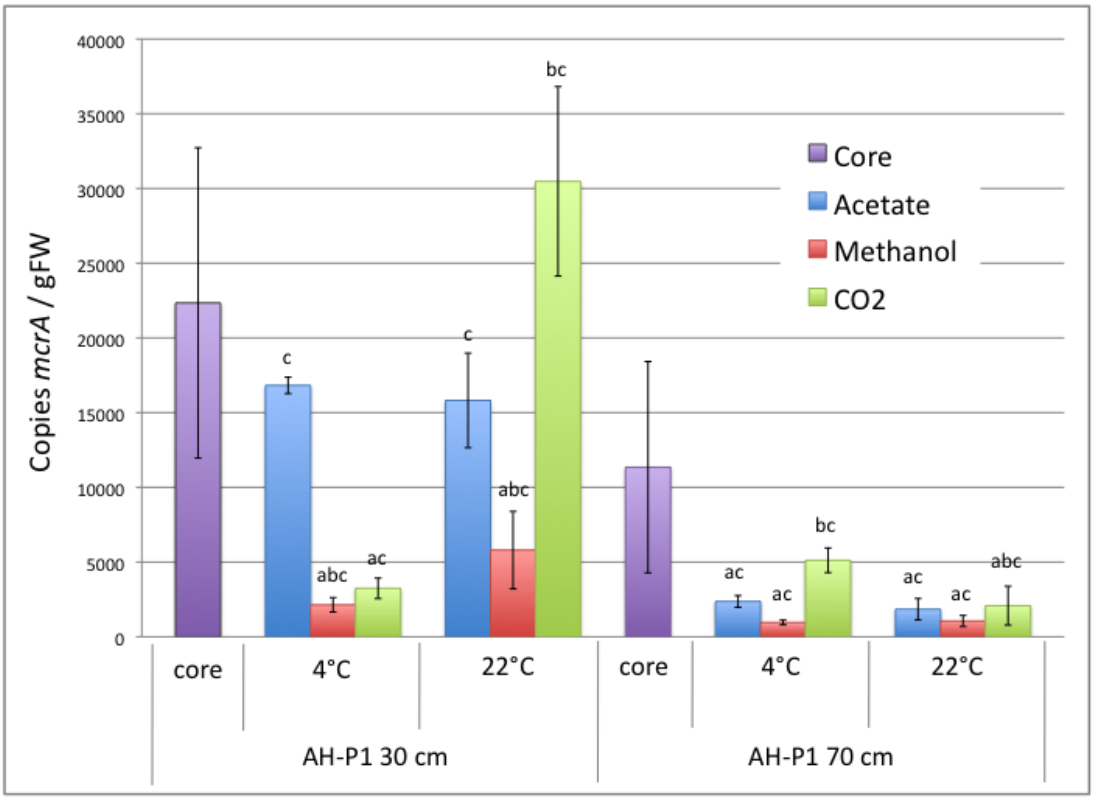
Methanogens from *in situ* soil samples and amended soil microcosms were quantified by qPCR targeting the *mcrA* gene, found in only one copy per methanogen. This was done in order to determine the levels of methanogens *in situ* in Arctic soils, as well as to determine changes in abundance upon increased temperatures and substrate amendment. It was found that the abundance of methanogens decreased upon increased temperatures and amendment when compared to the unincubated core. This was expected as Arctic environments are good preservers of DNA. Upon the incubated of microcosms at higher temperatures, this increase in temperature is expected to both destabilize DNA, and active DNAses within the soil. As such, the unincubated core is expected to have high concentrations of preserved DNA, while the incubated samples are expected to be lower due to the destruction of this DNA.

The *in situ* abundance in the 30 cm core was relatively high, having $2.2x10^4$ copies of *mcrA* per gFW (Figure 4-3). This was only surpassed by the 30 cm sample amended with CO_2 and incubated at 22°C, which had $3.0x10^4$ copies of *mcrA* per gFW. The abundance in the 30 cm samples

incubated with acetate were the highest of the 30cm samples, at both temperatures (4°C and 22°C).

The AH-P1 70 cm core had an average of $1.1x10^4$ copies of *mcrA* per gram of fresh weight soil. This is lower than the 30 cm core, but both cores had high standard deviations thus there was no statistically significant difference. Most of the microcosm incubations of samples from 70 cm depth were lower in *mcrA* abundance than those at 30cm. The only sample where the *mcrA* abundance was higher at 70cm than at 30cm was the 70 cm CO_2 sample incubated at 4°C. Interestingly, there was no significant correlation between the abundance of the *mcrA* gene and the production of CH_4 in microcosms. This incongruity has been seen previously in qPCR reactions targeting the *mcrA* gene (Parkes *et al.*, 2010; Webster *et al.*, 2009).

Figure 4-3. Abundance of methanogens by qPCR of the *mcrA* gene.



The qPCR reaction used *mlas/mcrA-rev* primers to determine copies of the *mcrA* gene per gram of fresh weight (FW) soil. The *mcrA* gene is found in one copy per methanogen, and thus is used to determine methanogen abundance. Samples were from the AH-P1 core site on Axel Heiberg Island in the Canadian high Arctic. The core represents the sample without enrichment and without incubation, and is thus a measure of the *in situ* abundance at time zero. All other samples are post-incubation with a substrate (acetate, methanol or CO_2) for 50-60 days, at either 4°C or 22°C. Averages and standard deviations were obtained from 3-6 replicate runs against a standard curve of *Methanosarcina barkeri*.
a = significant difference compared to the core
b = significant difference between temperatures
c = significant difference between depths

4-5. Archaeal diversity from cores, samples post substrate-amendedment, and SIP fractions

Pyrosequencing of total DNA extracts from cores and from soil samples post substrate-amendment was completed in order to determine the *in situ* Archaeal diversity as well as shifts in diversity as a result of increased temperatures and substrates. The pyrosequencing yielded a decent coverage and diversity, seeing as Archeae are notably difficult to sequence compared to bacteria, and we were the only group successful as of yet in our DOE project group. The most abundant Archaeal order in all samples was the Nitrososphaerales. No correlations of Archaeal orders could be found with substrate, temperature or depth.

The pyrosequencing of SIP fractions was also completed in order to determine the active Archaeal community. The pyrosequencing of core and amended microcosm DNA samples yielded an average of 4146 Archaeal 16S rRNA reads per sample after initial processing. The analysis of pyrosequencing data into operational taxonomic units (OTUs) and identification could only be done reliably at the order level. For each sample, the total number of OTUs at the 90 % similarity level can be seen in the rarefaction curves (Figure 4-4). The rarefaction curve shows the richness or diversity of OTUs for a given number of sequences obtained

from pyrosequencing. Most samples reached their plateau, indicating that the majority of abundant OTUs existant in the sample were found. However, several samples did not reach their plateau, indicating that not all of the abundant OTUs may have been detected by pyrosequencing. These samples include the AH-P1 30 cm sample incubated with acetate at 22° C, as well as the AH-P1 70 cm *in situ* core, and its 22° C counterparts incubated with acetate and methanol.

Figure 4-4 also indicates the total number of sequences (N), the Chao1 diversity index (measures the richness of the OTU set), and the Shannon diversity index (H′ ; measures the diversity by quantifying the entropy, a measure of the proportional abundance of each OTU). The diversity indices indicate that the AH-P1 70 cm core had the highest diversity, with a Chao1 value of 109.5 and Shannon index of 1.52 (Figure 4-4). The AH-P1 30 cm core had lower than expected diversity, having a Chao1 value of 12.0 and Shannon index of 0.40. All of the samples post-incubation had a lower number of OTUs and lower diversity indices than the corresponding cores. The average diversity indices were higher for the microcosms incubated at 22°C (Chao1= 41.5 and H'= 0.72) than at 4°C (Chao1 = 9.7 and H'= 0.33). The diversity was also seen to increase with depth, where the 30 cm samples had lower diversity indices (Chao1 =

20.3, and H'= 0.42) than the 70 cm samples (Chao1 = 40.9, and H'= 0.75). Together, these indicate that diversity increases in our soils with increased temperature and depth.

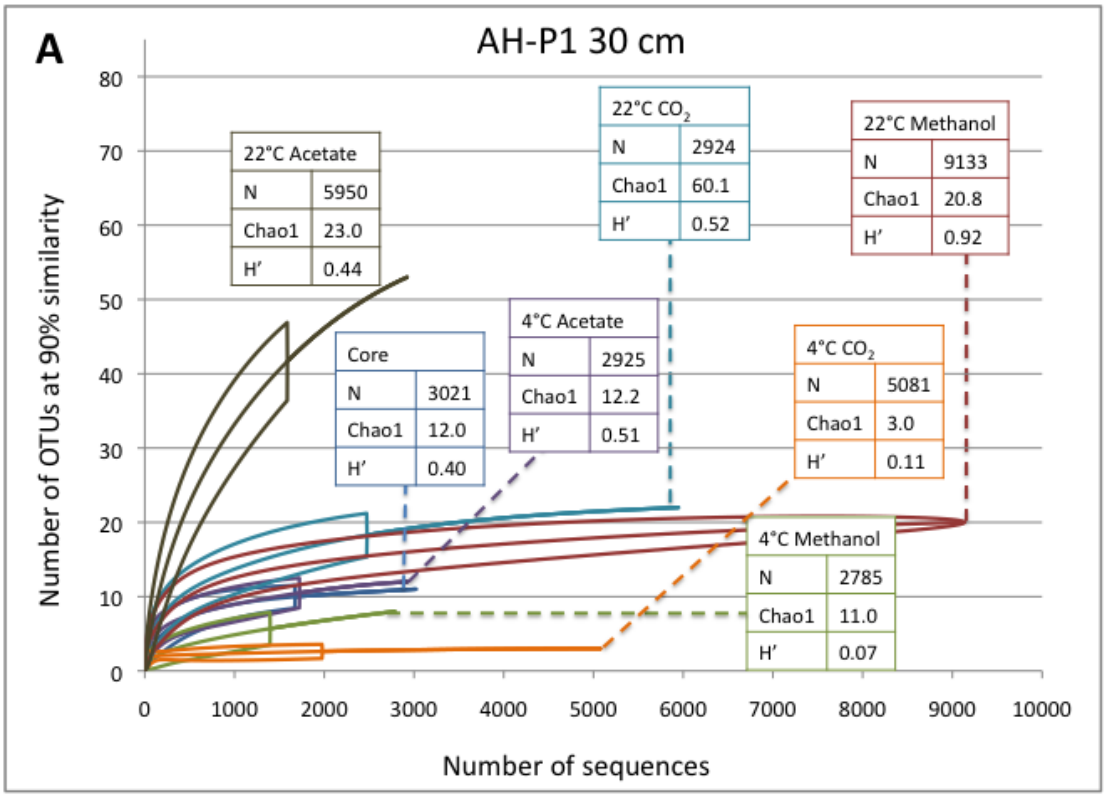
The pyrosequencing of the SIP DNA fractions A and B for both the heavy (^{13}C) and light (^{12}C) samples yielded an average of 2292 Archaeal 16S rRNA reads per sample after initial processing. This is approximately half of that obtained from the total DNA samples, which was expected as the total DNA was separated into approximately two fractions. For each fraction, rarefaction curves show the number of OTUs at the 90 % similarity level, as well as the total number of sequences and diversity indices (Figure 4-4). The diversity indices for the SIP data show a relatively low diversity, with an average Chao1 of 3.3 and Shannon index of 0.23.

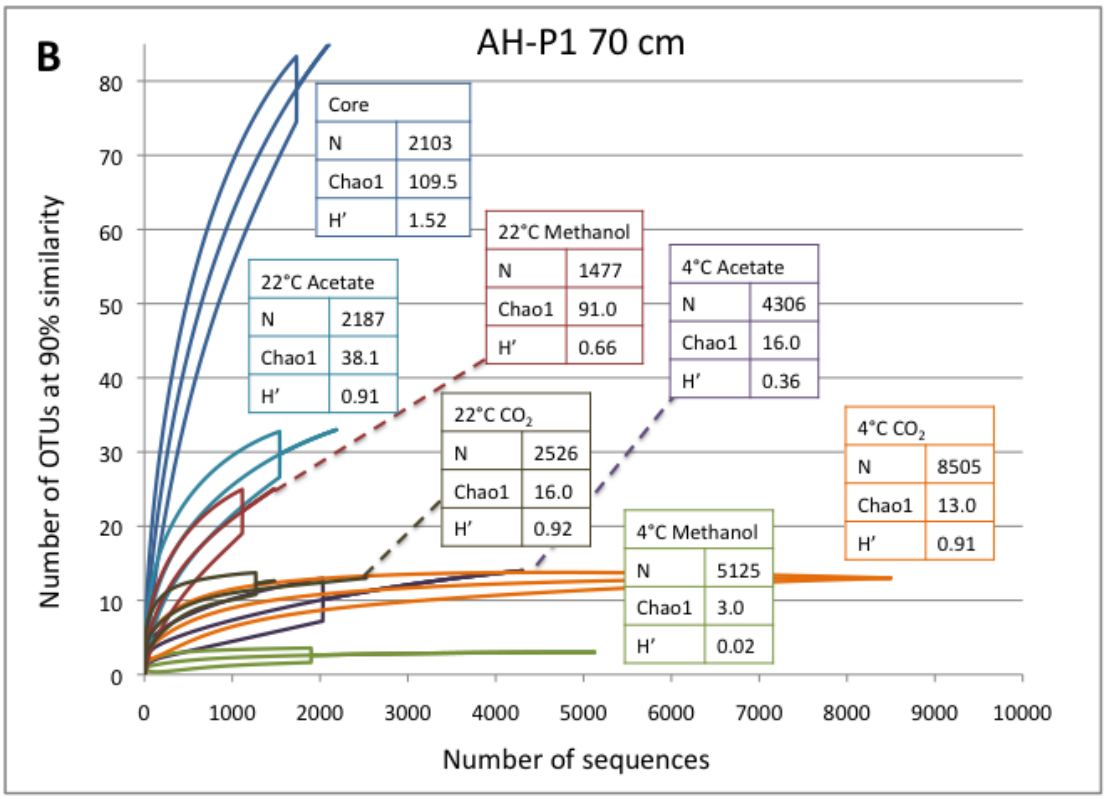
The dominant Archaeal order for all samples was found to be Nitrososphaerales, including the cores, post-incubated samples and SIP fractions (Figures 4-5 and 4-6). The Nitrososphaerales ranged from 60-100 % of total Archaeal sequences. The remainder of orders varied between samples. The order Thermoplasmatales was found to increase from the core (7.8 %) to the AH-P1 30 cm 22°C methanol sample (33 %) in total Archaeal reads. However, Thermoplasmatales decreased in most

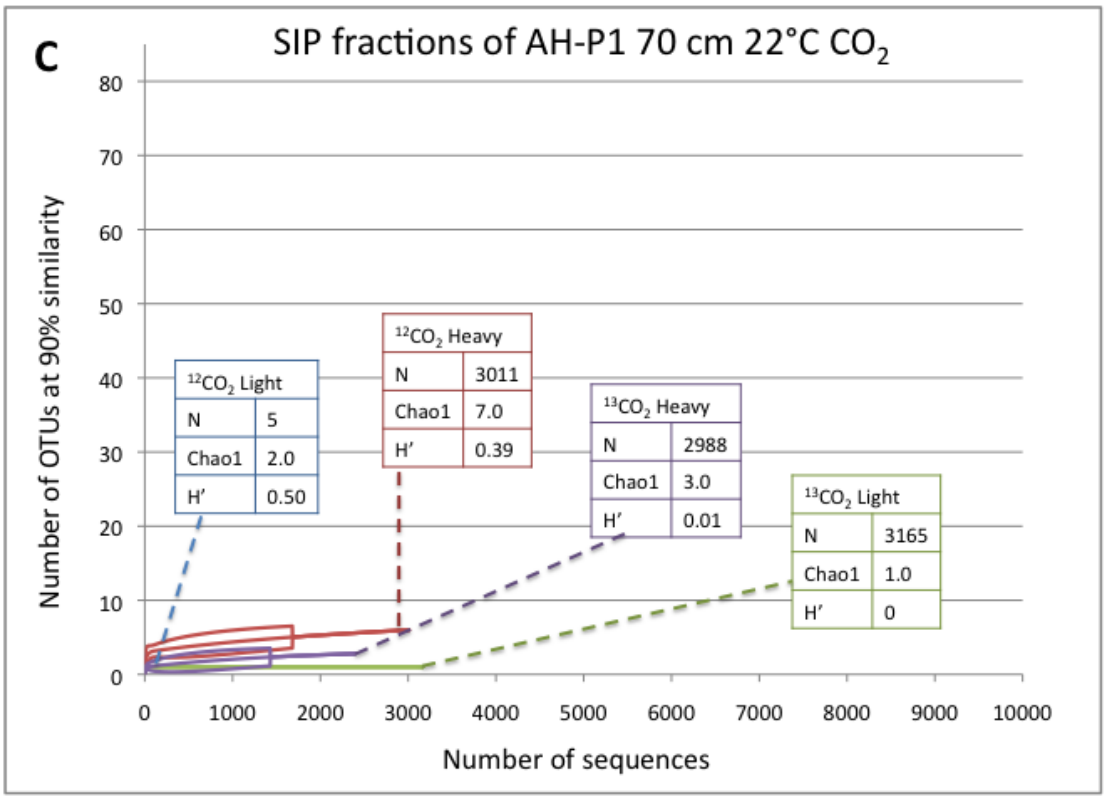
other samples. Halobacteriales were slightly more common at 70 cm than at 30 cm. Other orders found include Archaeoglobales, Sulfolobales, Desulfurococcales, and Thermoproteales. These orders were found at relatively low levels, with no significant correlation to substrate, depth or temperature. All of the methanogen orders were found, but each order was found at relatively low levels, and distribution varied per sample. Methanogen diversity between the amendments and SIP samples will be discussed in detail in the next two sections.

It is important to note that even at the order level, the identification of representative sequences within a cluster differs slightly, depending on the database used. Therefore, a comparison of the identification for each of the OTUs found at >1 % abundance was made between the output from the Research and Testing Laboratories LLC pyrosequencing company, BLAST, and the RDP classifier (refer to Annexe 3). In addition, the number of 16S rRNA environmental sequences in BLAST is increasing so quickly that the top hits of a BLAST search will change every few months. Nonetheless, the overall trend found that our sequences cluster with other environmental sequences from Arctic environments, hyper thermal regions, or marine regions.

Figure 4-4. Rarefaction curves and diversity indices for pyrosequencing data of AH-P1 core, substrate amended microcosms and SIP fractions.

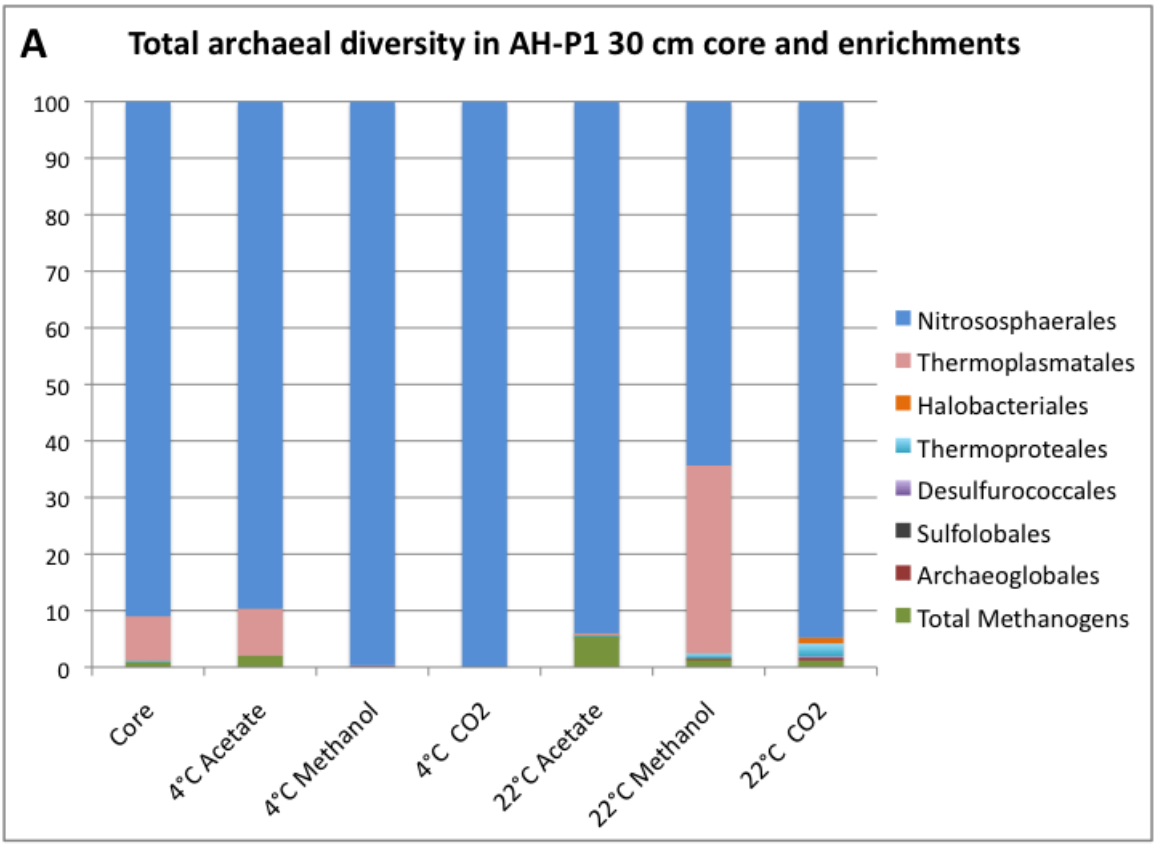


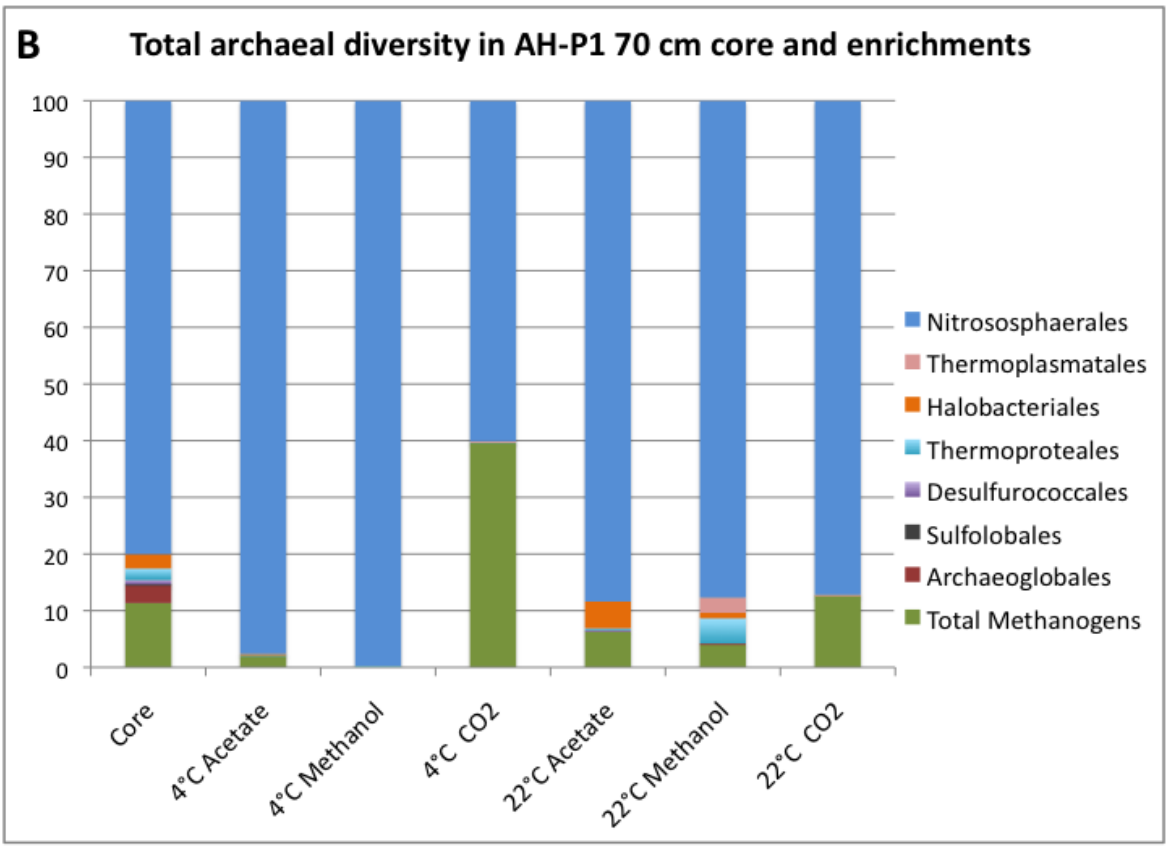

B
AH-P1 70 cm
Number of OTUs at 90% similarity
Number of sequences
0
10
20
30
40
50
60
70
80
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Core
N 2103
Chao1 109.5
H' 1.52
22°C Acetate
N 2187
Chao1 38.1
H' 0.91
22°C Methanol
N 1477
Chao1 91.0
H' 0.66
4°C Acetate
N 4306
Chao1 16.0
H' 0.36
22°C CO2
N 2526
Chao1 16.0
H' 0.92
4°C CO2
N 8505
Chao1 13.0
H' 0.91
4°C Methanol
N 5125
Chao1 3.0
H' 0.02



Rarefaction curves were taken from the number of OTUs at 90% similarity, versus the total number of sequences. The curve shows both upper and lower limits as well as the average for each sample. In addition, the total number of sequences (N), and the diversity indices Chao1 and the Shannon index (H') are shown. Chao1 is a measure of the richness of the OTU set, and H' quantifies the OTU diversity entropy through a measure of the proportional abundance of each OTU.
A) The AH-P1 30 cm core and enrichment samples post incubation with a substrate (acetate, methanol or CO_2) for 50-60 days at 4° C or 22° C.
B) The AH-P1 70 cm core and enrichment samples post incubation with a substrate (acetate, methanol or CO_2) for 50-60 days at 4° C or 22° C.
C) Stable isotope probing was completed on the AH-P1 70 cm core incubated at 22° C with either $^{12}CO_2$ or $^{13}CO_2$ for 50-60 days. Total DNA extracts from both the ^{12}C and ^{13}C incubated samples were separated by caesium chloride density gradient ultracentrifugation into a light fractions (within the lower density range; fraction A*) and a heavy fractions (within the higher density range; fraction B*). Rarefaction curves were taken from the number of OTUs at 90% similarity, versus the total number of sequences.
OTUs = operational taxonomic units
*see Figure 3-2 for exact densities of the light (fraction A) and heavy (fraction B) fractions

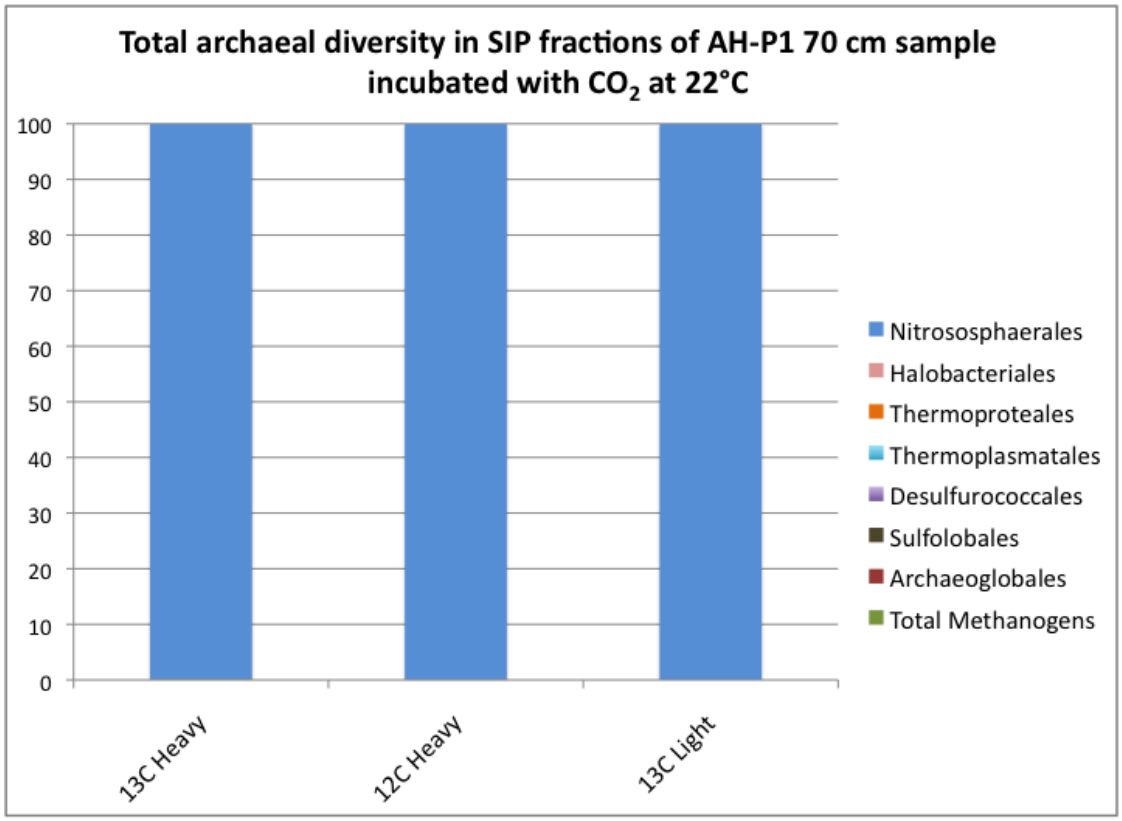
Figure 4-5. Total Archaeal diversity and relative abundance in microcosms enriched with methanogenic substrates.





Total archaeal diversity and relative abundance are based on total reads from pyrosequencing targeting the Archaeal 16S rRNA gene. Core samples were extracted in July 2010 and remained at -20 until DNA extraction. Microcosms were made from the AH-P1 core at either 30 cm or 70 cm depth. Samples of each depth were amended with 30 mM acetate, 30 mM methanol, or CO_2/H_2 (20:80), and incubated in triplicate either 4° C or 22° C for 50-60 days . In order to show the relative abundance of methanogens in each sample, the methanogenic orders have been grouped together. In all samples, the Nitrososphaerales were the most abundant Archaeal order, accounting for 60-100 % of total Archaeal reads. A) At 30 cm depth, the relative abundance of Thermoplasmatales increased in the methanol sample at 22° C. Total methanogen abundance was low, with the highest abundance in the sample incubated at 22° C with acetate (5.4 %). B) At 70 cm depth, Halobacteriales were more common. The total methanogen abundance was greater compared to 30 cm, with the highest in the samples incubated at 4° C with CO_2 (39.7 %).

Figure 4-6. Total Archaeal diversity and abundance in SIP fractions from the P1-70 cm incubated at RT° with CO_2.



Stable isotope probing was completed on the AH-P1 70 cm core incubated at 22° C with either $^{12}CO_2$ or $^{13}CO_2$ (as 20:80 CO_2/H_2) for 50-60 days. Total DNA extracts from both the ^{12}C and ^{13}C incubated samples were separated by cesium chloride density gradient ultracentrifugation into a light fractions (within the lower density range; fraction A*) and a heavy fractions (within the higher density range; fraction B*). Total Archaeal diversity and relative abundance are based on total reads from pyrosequencing targeting the Archaeal 16S rRNA gene. It can be seen that the Nitrososphaerales account for 99-100 % of total Archaeal reads. However, there were small differences between samples. In the ^{13}C heavy fraction, there was a lower relative abundance of Nitrososphaerales, Desulfurococcales and Sulfolobales, but a higher relative abundance of Methanopyrales.
* See Figure 3-2 for exact densities of light and heavy fractions (fractions A and B, respectively)

4-6. Methanogen diversity between the substrate-amended microcosm incubations

Methanogen abundance and diversity were found to be much greater at 70 cm than at 30 cm, and correlated with increasing temperatures, depth and CO_2 amendment. The most common orders in our samples were the Methanococcales and Methanomicrobiales; however the largest increases in abundance upon substrate amendment were seen in the Methanomicrobiales.

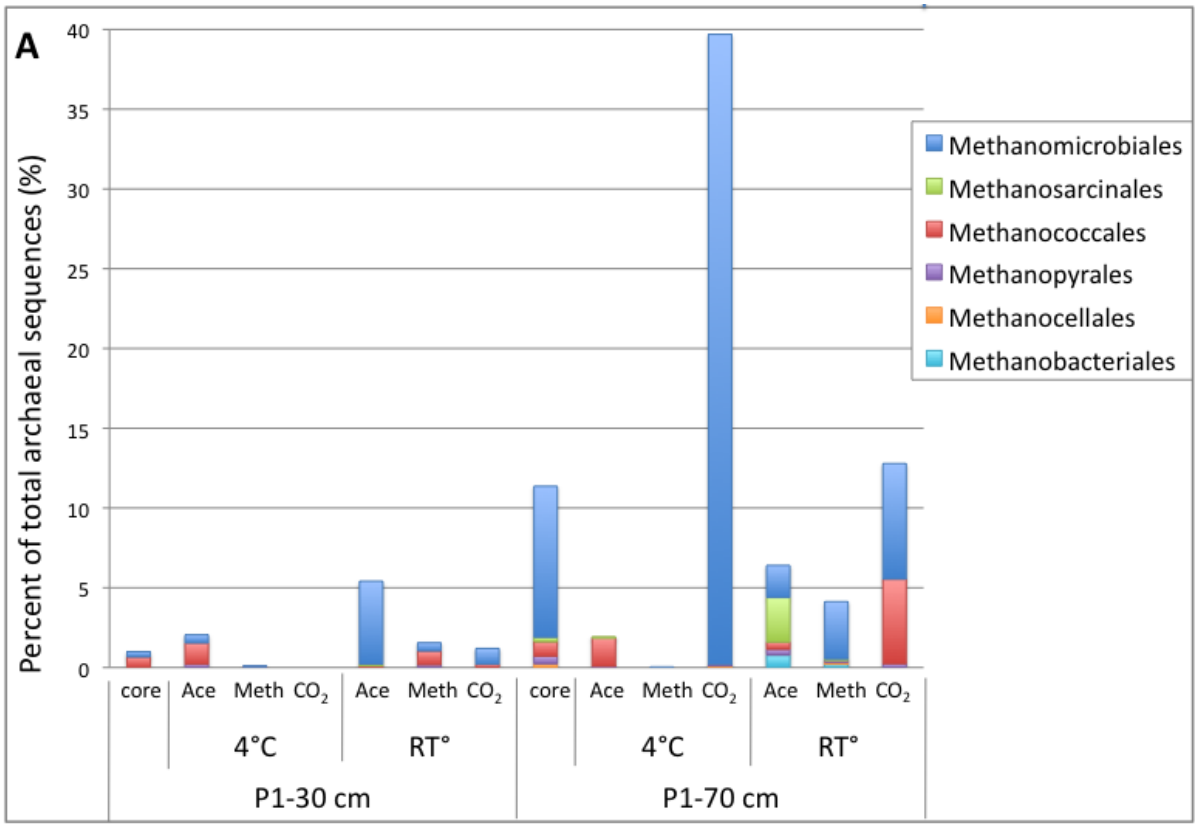
In the AH-P1 30 cm core, there was a relatively low abundance of methanogens (1.0 %) as a percent of total Archaeal sequences (Figure 4-7). The diversity of methanogens in the 30 cm core included Methanococcales (0.7 %) and Methanomicrobiales (0.3 %). The overall percentage of these methanogen orders relative to the total Archaeal sequences was similar for the 30 cm amendments. In addition, a similar distribution of methanogen orders was seen, with Methanococcales being the most abundant, followed by Methanomicrobiales. However, in the 4°C CO_2 sample there were no methanogen sequences detected, whereas in the 22°C acetate sample there was a large increase in relative abundance of methanogen orders, up 5.4 % (compared to 1.0 % in the core). This increase in abundance is due to the increase in Methanomicrobiales to 5.2

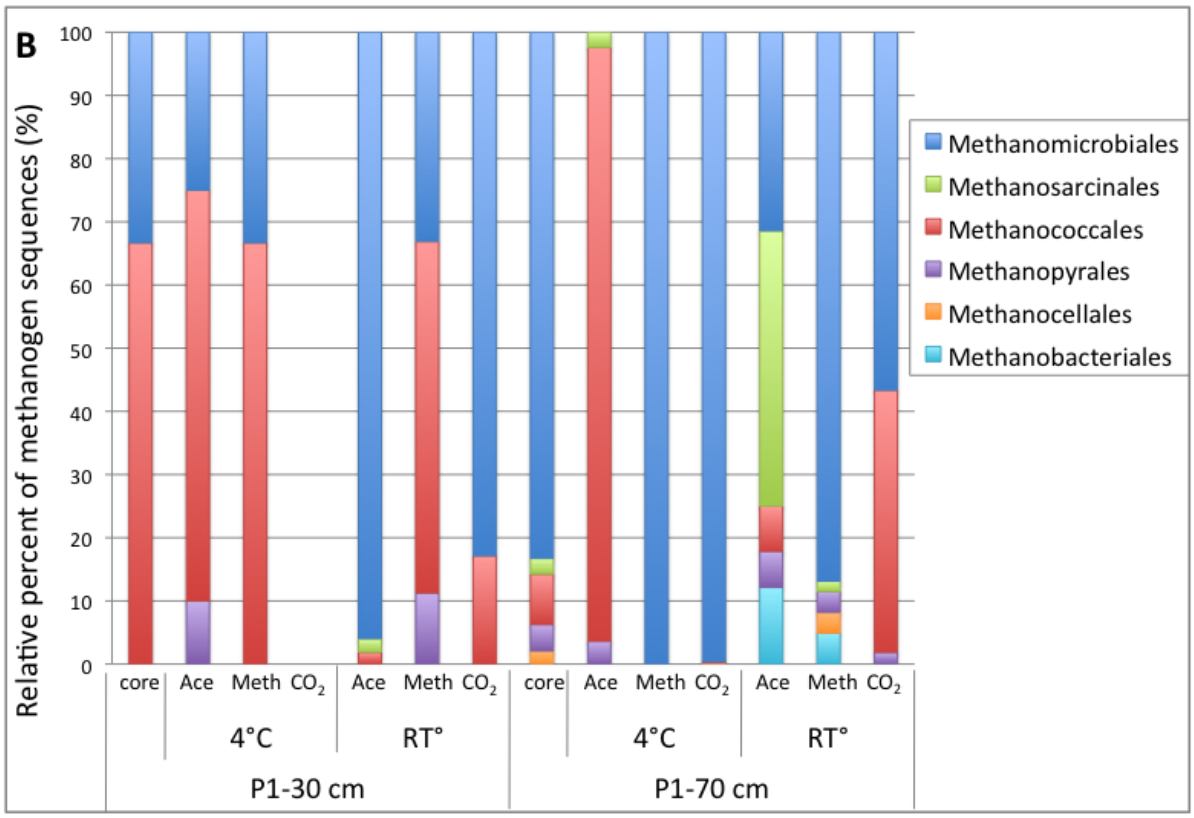
% (compared to 0.3 % in the core). Methanomicrobiales are strictly CO_2 reducers, and, therefore, this increase may be a by-product of increased CO_2 production within the microcosm environment. In addition, the order Methanosarcinales were detected in this sample, which was not detected in the core. Two other incubations also detected the order Methanopyrales, which was not found in the 30 cm core. These include methanol-amended 22°C amendment and the acetate-amended 4°C amendment.

The relative abundance of methanogens in the 70 cm core were much higher (11.4 %) than the 30 cm core (1.0 %) in terms of total Archaeal sequences. The methanogen diversity was also greater in the 70 cm core, and included the orders Methanomicrobiales (9.5 %), Methanocellales (0.2 %), Methanopyrales (0.5 %), Methanococcales (0.9 %) and Methanosarcinales (0.3 %). As in the 30 cm core, the most abundant order in the 70 cm core was Methanococcales. The 70 cm amended microcosm samples had greater differences in relative abundance. There were decreases in relative abundance in the acetate and methanol amendments incubated at both 4°C and 22°C. Only the CO_2 amended microcosms were found to have an increase in relative abundance of methanogens up to 39.7% in the 4°C sample, and to 12.8 %

in the 22°C sample, as a percent of total Archaeal sequences. This large increase in the percentage of methanogen sequences in the 4°C CO_2 core is attributed to the increase in the order Methanomicrobiales up to 39.5 % (compared to 9.5 % in the core). The Methanomicrobiales are the order that also caused a large increase in abundance in the 30 cm 22°C acetate core. These changes in the 4°C CO_2 sample were not seen with the increase in abundance of the 22°C CO_2 sample. At 22°C, the Methanomicrobiales decreased compared to the core, whereas the relative abundance of Methanococcales increased to 5.3 % (compared to 0.9 % in the core). Other differences between substrate amendments include the appearance of the order Methanobacteriales in the 22°C incubations of both acetate (0.8 %) and methanol (0.2 %), which coincided with a decrease in the other orders. Finally, in the 22°C acetate sample, there was a relatively large increase in Methanosarcinales compared to the core, from 0.3 % to 2.8 %.

Figure 4-7. Methanogen diversity and relative abundance between substrate amended microcosms.





Core samples were extracted in July 2010 and remained at -20 until DNA extraction. Microcosms were made from the AH-P1 core at either 30 cm or 70 cm. Samples of each depth were amended with 30 mM acetate, 30 mM methanol, or CO_2/H_2 (20:80), and incubated in triplicate either 4° C or 22° C for 50-60 days. A) The relative abundance of methanogens is shown as a percent of total reads obtained from pyrosequencing targeting the Archaeal 16S rRNA gene. It can be seen that both the overall relative abundance and the diversity of methanogens was higher at 70 cm depth compared to 30 cm depth. The single highest increase in methanogen relative abundance was seen in the 70 cm sample incubated with CO_2, where Methanomicrobiales increased to 39.5 % compared to 9.5 % in the core. B) The relative abundance of each methanogen order is shown as as a percent of the total methanogen sequences to elucidate the most common orders and shifts post-incubation. It can be seen that the Methanomicrobiales and Methanococcales were the most common orders. However, there were shifts in other orders, such as the Methanosarcinales and Methanobacteriales which increased in the 70 cm samples incubated at 22° C.

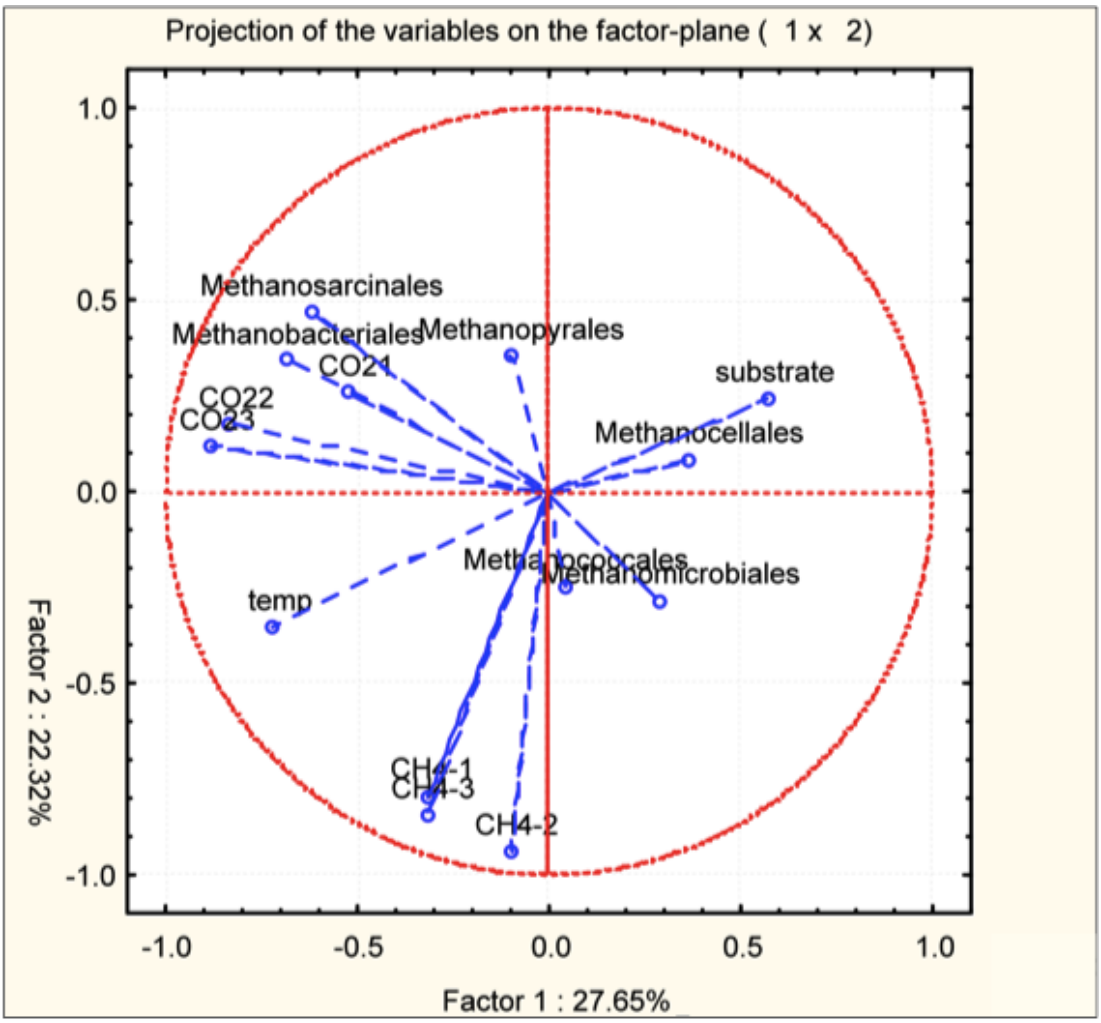
4-7. Statistical analyses of methanogen distribution and CH_4 production

Statistical analyses were done in order to determine the factors that play an important role in the distribution of methanogen orders, as well as in the production of CH_4. Principal component analysis of the data revealed the distribution of methanogen orders according to substrate, CO_2 production, CH_4 production, and temperature (Figure 4-8). It was seen that CH_4 and CO_2 production grouped closer to temperature than they did to substrate, indicating temperature had a greater effect. The orders Methanococcales and Methanomicrobiales were the two most abundant methanogen orders and were found in almost all samples. The orders Methanosarcinales, Methanobacteriales, and Methanopyrales acted similarly, grouping closest to CO_2 production. These orders are all capable of hydrogenotrophic methanogenesis, therefore may require a certain quantity of CO_2 produced before they are able to grow. The orders Methanococcales and Methanomicrobiales acted similarly, grouping closest to CH_4 production. This indicates that they may play a role in the production of CH_4. However, neither of these individual orders showed a significant correlation with the CH_4 production. It was only the presence of the order Methanopyrales that was found to correlate with CH_4 production; however, as shown by discriminate function analysis (DFA), the

Methanopyrales grouped closer to the production of CO_2. The order Methanocellales acted distinctly from other orders, grouping closest to substrate. The Methanocellales were only detected in microcosms amended with CO_2 or methanol. The Methanocellales would have also coupled with depth, as they only appeared in the 70 cm samples.

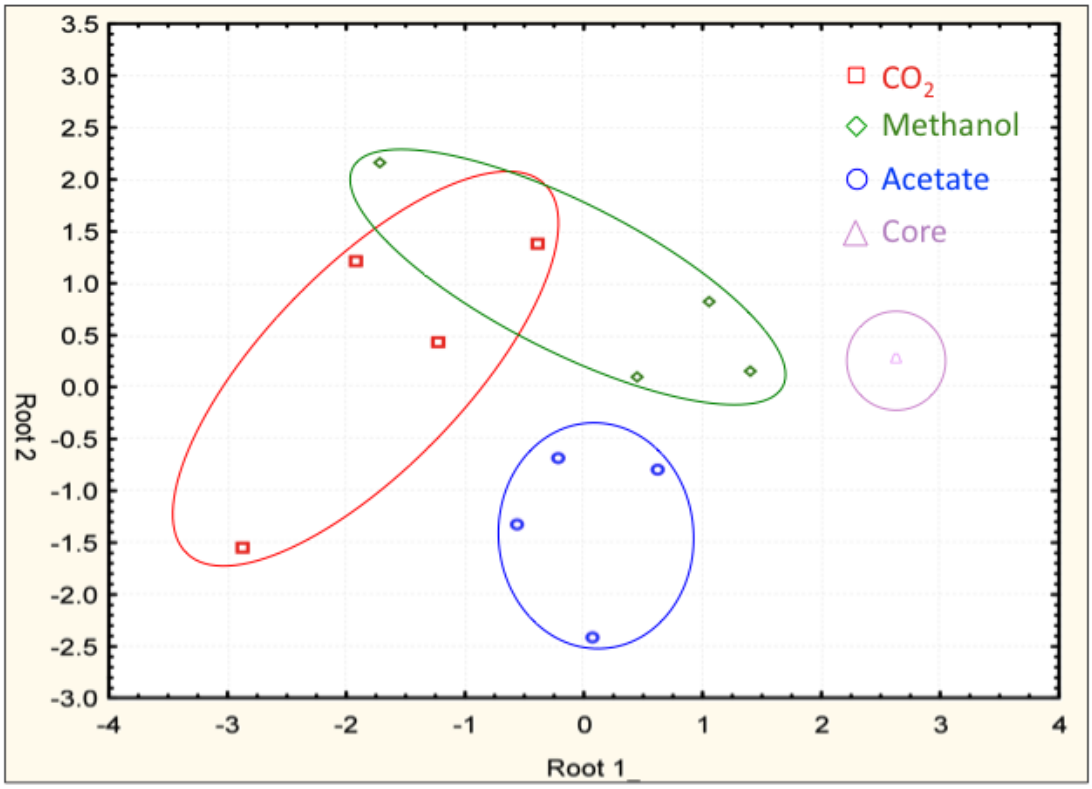
DFA was also done to show the pattern between the CH_4 production profile and the substrate added to microcosms (Figure 4-9). It can be seen that CH_4 production groups differently depending on the substrate added, and all substrates grouped differently from the *in situ* core samples. This indicates that substrate addition causes distinct shifts in the CH_4 production from these polygon soils. With an addition of CO_2, the overall CH_4 production was the highest, but overlapped with the methanol amended microcosms. This correlates with the DFA pattern shown. The acetate amended microcosms showed a distinct grouping, as the acetate-amended microcosms had the lowest CH_4 production of the three substrates.

Figure 4-8. Principal component analysis between methanogen order abundance, CH_4 production, CO_2 production, temperature, and substrate.



Principal component analysis was used to determine the which components could explain the different groupings between methanogen orders detected in our microcosm samples. The microcosms were incubated for 50-60 days with the additions of 30 mM acetate, 30 mM methanol, or CO_2/H_2 (20:80). Microcosms were also incubated at two temperatures (4°C and RT°). The components analysed include substrate, temperature, CO_2 and CH_4 production (three replicates each, denoted 1,2,3). This principal component analysis shows a correlation between the methanogen orders Methanomicrobiales and Methanococcales with CH_4 production, Methanocellales with substrate, and Methanosarcinales, Methanobacteriales, and Methanopyrales with CO_2 production.

Figure 4-9. Discriminate function analysis of anaerobic microcosm CH_4 production using substrate as the grouping variable.



	Eigenvalue	% Variance	Wilks Λ	Canonical *R*	*df*	*P*-level
Function 1	2.44	68.5	0.129	0.842	9	0.022
Function 2	1.00	28.3	0.447	0.708	4	0.105
Function 3	0.11	3.2	0.897	0.319	1	0.311

Discriminate function analysis (DFA) was done to determine the pattern of the CH_4 production rates from anaerobic microcosms based on the substrate added, either 30 mM acetate, 30 mM methanol, or CO_2/H_2 (20:80). The profiles of CH_4 production can be distinguished based on the substrates added. The acetate amended microcosms show a completely separate grouping, as they had significantly lower CH_4 production rates than those with CO_2 or methanol. In addition, all substrates grouped apart from the *in situ* core samples. This shows that distinct shifts in the CH_4 production profile will be produced depending on the particular substrate added to these polygon soils.

4-8. Active methanogen orders found by stable isotope probing

SIP was used in order to determine the active Archaeal community in the utilization of various methanogenic substrates. Only one sample that was used for SIP was successful, the AH-P1 70 cm microcosm incubated with CO_2 at 22° C. This was the only sample that had yielded enough DNA upon post-centrifugation recovery. There was a low recovery of the DNA that was added to the high CsCl salt medium after centrifugation, thus the protocol requires future optimization. The only methanogen order found to be enriched in the heavy fraction, and therefore considered part of the active community, was the Methanopyrales.

In the profile of buoyant density versus DNA concentration of the AH-P1 70 cm CO_2 microcosm incubated at 22° C, two peaks of DNA could be seen. These peaks were presumed to coincide with the light (fraction A) and heavy (fraction B) DNA fractions (Figure 3-2). These peaks were seen around the buoyant densities 1.700 and 1.715 g/ml. The expected separation for 100% ^{13}C-labelled DNA centrifuged for 65 hours in a CsCl gradient is 0.036 g/ml (Buckley *et al.*, 2007). However, as the incorporation of ^{13}C carbon into our DNA is likely incomplete, and samples were centrifuged for only 48 hours, a smaller separation was expected.

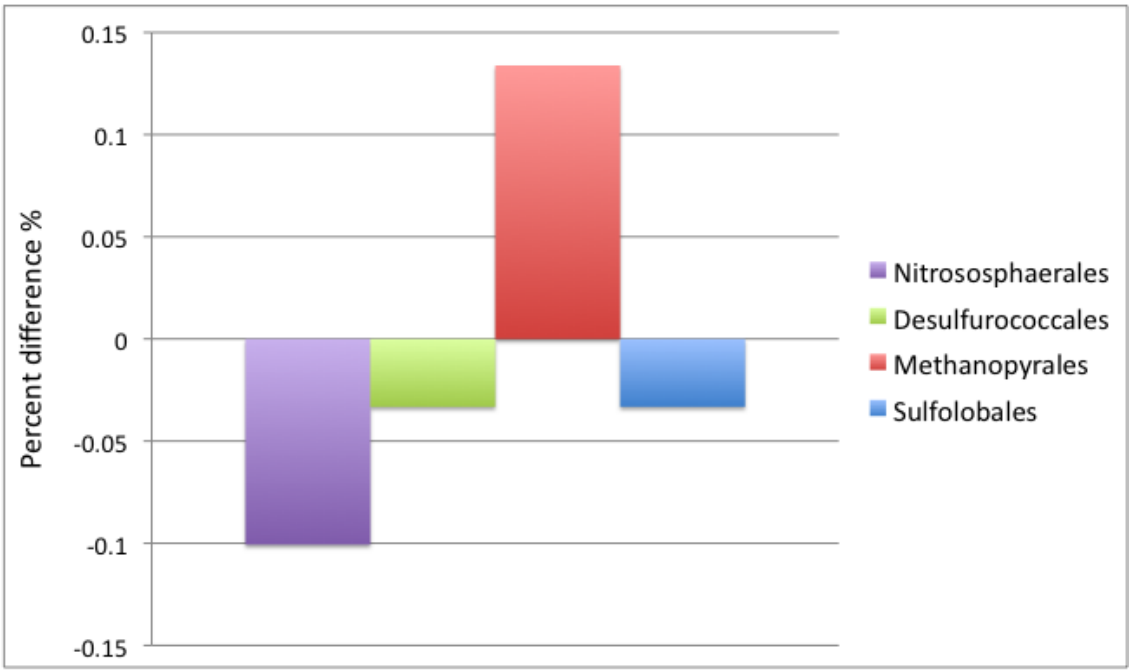
The percent difference of total Archaeal reads was calculated from the difference between heavy and light fractions (Figure 4-10). Several orders showed a slight decrease in the heavy fraction incubated with ^{13}C-labelled CO_2, including Nitrososphaerales, Desulfurococcales, and Sulfolobales. This does not mean that these orders are not active, but rather their DNA naturally has a high G-C content and is always found in the heavy fraction, or that these orders may utilize other substrates or may utilize CO_2 but are not dividing.

Those which have increased in the heavy fraction versus the light fraction are enriched, and are thus actively incorporating the substrate and dividing. It can be seen that the only order that was enriched was Methanopyrales. The percent enrichment of Methanopyrales was only of 0.13 % of total Archaeal reads. Despite this values seeming small, this is significant because Methanopyrales are normally found at these low levels in Arctic soils. In addition, the enrichment was 100 %, meaning that all of the Methanopyrales were active. There were no Methanopyrales remaining in the light fraction when incubated with heavy ^{13}C carbon. This indicates that despite their low abundance, they may play an important functional role in the production of CH_4. Normally, the enrichment of an order is indicated by the fold difference between the heavy (active) and

light (inactive) fractions; however, as all of the order was enriched and none (zero) remained in the light fraction, this could not be done.

When entering the Methanopyrales sequences from the SIP fractions into nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/) within Archaeal 16S rRNA genes, the closest relatives show between 83-100 % similarity; however, the 100 % similarities coincide with a low query coverage (5-10 %). The closest relatives include Archaeal Halobacteriales (*Halogeometricum borinquense, Halosimplex carlsbadense, Halorhabdus tiamatea),* Methanosarcinales at 80-100 % similarity (*Methanosaeta thermophila, Methermicoccus shengliensis, Methanolobus zinderi)*, Thermoproteales (*Thermofilum pendens*), and Methanobacteriales (*Methanothermoavter thermoautotrophicus*). The halophilic *H.tiamata* originates from a deep-sea hypersaline anoxic basin of the Red Sea. *M.shengliensis* is a thermophilic methylotrophic methanogen isolated from oil-production water. The methylotrophic methanogen *M.zinderi* was isolated from a deep subsurface coal seam. Anotherr close relative for our 16S rRNA genes was found to be a bacterial genus, Thermovibrio (*Thermovibrio ammonificans).* The thermophilic, chemolithotrophic, nitrate-ammonifying bacteria *T.ammonificans* originates from deep-sea hydrothermal vents.

Figure 4-10. Percent difference of total Archaeal reads using SIP on AH-P1 70 cm microcosms incubated with $^{13}CO_2$ at 22° C.



Stable isotope probing was completed on the AH-P1 70 cm core incubated at 22° C with either $^{12}CO_2$ or $^{13}CO_2$ for 50-60 days. Total DNA was extracted from both the ^{12}C and ^{13}C incubated samples and separated by cesium chloride density gradient ultracentrifugation. The separated samples were then pooled into light (lower density range; fraction A*) and heavy (higher density range; fraction B*) fractions respectively. The light and heavy fractions for both the ^{12}C and ^{13}C samples were sent for pyrosequencing of the Archaeal 16S rRNA gene. The percent difference shown is calculated by the percent abundance of each order in the ^{13}C heavy fraction minus that in the ^{13}C light (to show shift) and that in the ^{12}C heavy fractions (to show order is not normally found in heavy fraction). A positive percent difference indicates the orders are enriched in the ^{13}C-heavy fraction because they has shifted from the light fraction by an increase of their DNA weight through the incorporation of the $^{13}CO_2$. Therefore, these orders are deemed active in the utilization of CO_2. The only active order found was the methanogen order Methanopyrales, which increased by 0.13 % of total Archaeal reads.

4-9. Phylogenetic analysis of methanogen sequences from Canadian high Arctic polygon soil samples from *in situ* cores, amended microcosms, and SIP fractions

A phylogenetic tree was done in order to determine where the core and amended microcosm sequences lie in relation to known methanogenic sequences and other environmental sequences. It was also done in order to determine the closest relatives to the active SIP sequences. The maximum likelihood phylogenetic tree shows the clustering of the methanogen sequences from the samples closest to the known methanogen orders Methanosarcinales, Methanocellales and Methanomicrobiales. The enriched SIP sequences do not cluster with other sample sequences, but still cluster with known methanogens of the orders Methanobacteriales, Methanococcales and Methanopyrales. However, these enriched SIP sequences were found to cluster with low-abundance sequences found in other 70 cm samples.

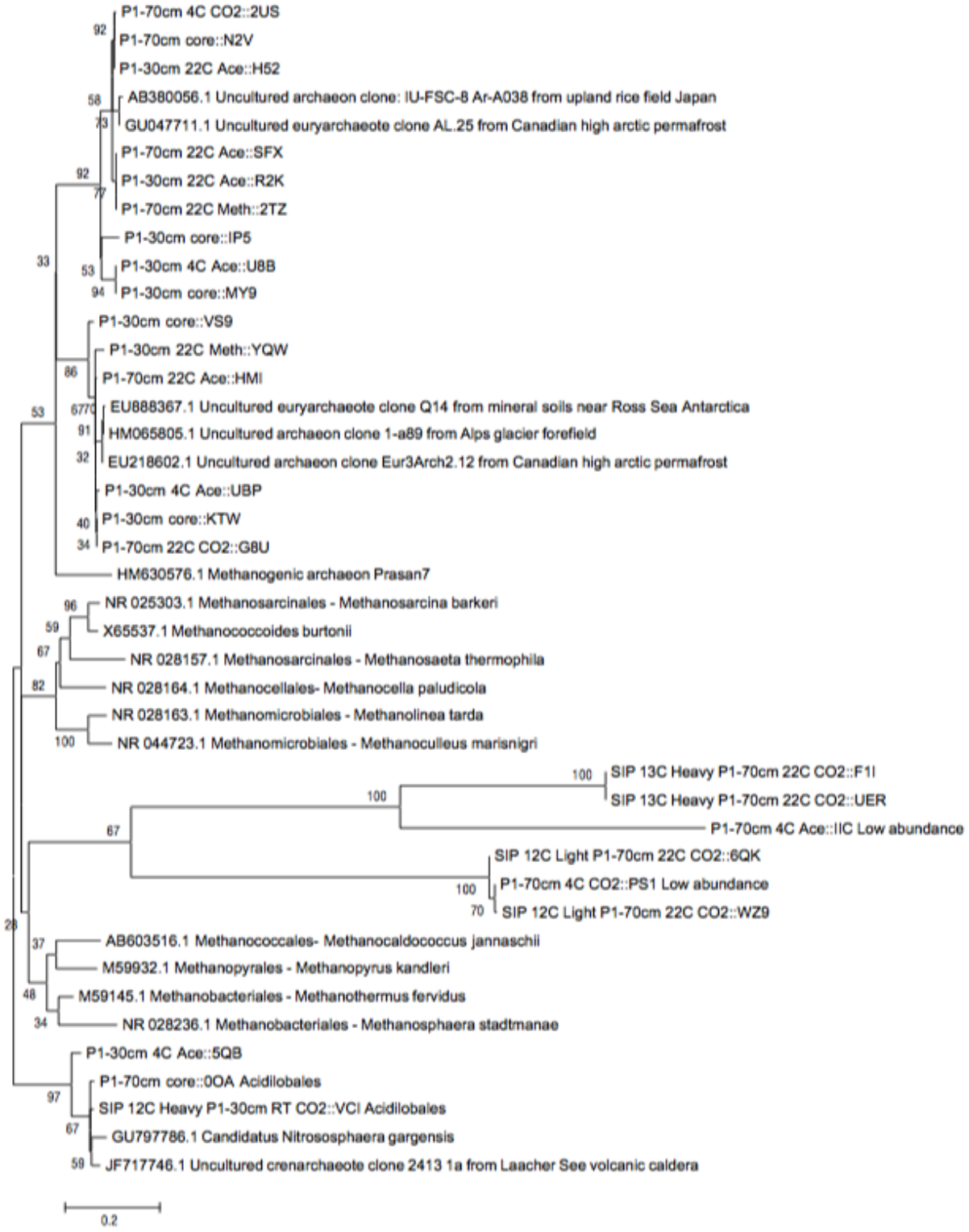
The most abundant methanogen sequences from the amended microcosm experiment cluster closer to the known methanogens than to the more distant Nitrososphaerales (Thaumarchaeota) and crenarchaeota sequences (Figure 4-11). The closest relatives include environmental samples from the high Arctic, Antarctic, glaciers, and Japanese rice field

soils. The closest cultured relative is the methanogen Prasan 7, which is an unclassified organism in the order Methanomicrobiales, found by the faculty of marine science of Annamalai University in India. The closest known methanogens are the Methanosarcinales, Methanocellales and Methanomicrobiales. One of the sequences from the AH-P1 30 cm sample incubated 4° C with acetate does not cluster with the remainder of the methanogen sequences from the samples, but rather with the Nitrososphaerales and crenarchaeotes. As such, it is probable that its classification as a methanogen sequence is inaccurate.

The sequences enriched from the SIP samples can also be seen on this phylogenetic tree. These sequences were identified by RDP as belonging to the methanogenic order Methanopyrales. They are quite distant from any of the other >0.5 % methanogen sequences from the samples. However, they do also cluster with the known methanogens, closest to sequences from the orders Methanobacteriales, Methanococcales and Methanopyrales. Upon closer analysis, it was found that the enriched sequences from SIP cluster with representative sequences found in other AH-P1 70 cm amendment samples that are found at 0.047 % and 0.023 % of total Archaeal reads. These are rare biosphere sequences that are also identified by RDP as belonging to the

order Methanopyrales. This shows that the methanogens that were most active in incorporating the heavy ^{13}C carbon are actually part of the rare biosphere. This coincides with the growing notion that important biological functions are not always completed by abundant microbial species (Arumugam *et al.*, 2011).

Figure 4-11. Phylogenetic tree of methanogen sequences from AH-P1 core, amended microcosms, and SIP fractions.



A bootstrap consensus maximum likelihood phylogenetic tree was created from 500 replicates in MEGA5, using a total of 41 sequences and 265 positions. The tree was formed using the methanogen sequences found at a relative abundance greater than 0.5 % from the AH-P1 core, amended microcosms, and SIP fractions. Landmark sequences of the nearest environmental samples and cultured methanogens were taken from NCBI. In addition, several Nitrososphaerales sequences from samples were added to act as a root, and two rare biosphere methanogen sequences from our samples were added. It can be seen that the most abundant methanogen sequences from the amended microcosms clustered with other environmental samples from the high Arctic, Antarctic, glaciers, and Japanese rice field soils. The closest cultured relative was an unclassified Methanomicrobiales called methanogen Prasan 7. The active SIP sequences from the 70 cm sample incubated at 22° C with CO_2, classified by RDP as Methanopyrales, did not cluster with the other methanogen sequences from the microcosms. Instead, they clustered with several rare biosphere sequences (less than 0.05 % relative abundance) found in the 70 cm samples.

Chapter 5 – Discussion

This thesis determined the abundance, diversity and activity (anaerobic production of CH_4 and CO_2) of methanogens in Canadian high Arctic soils, and related these results to differences in soil contents and characteristics. In addition, it determined the changes in the abundance, diversity and activity of methanogens in a polygon soil sample at two depths when incubated at increased temperatures, and when amended with various methanogenic substrates. This was done in order to help understand changes that may occur as climate change continues to increase temperatures and provide increased nutrients through the thaw of permafrost carbon stores.

5-1. Significant differences are detected in soil characteristics as climate change progresses in the Canadian high Arctic

Environmental factors such as soil characteristics and temperature are major factors controlling and limiting microbial abundance and GHG production (CH_4 and CO_2) in high Arctic soils (Hoehler *et al.*, 2010; Schuur *et al.*, 2008). The continuous measurement of soil characteristics as climate change progresses will provide vital insights into the effects of climate change on the environmental system dynamics, in terms of their consequences on microbial abundance and GHG production.

Previous studies have already found environmental factors such as temperature and precipitation to be increasing in the Arctic environments (Graversen *et al.*, 2008; Hoj *et al.*, 2008; IPCC, 2007; Min *et al.*, 2008). Our study has also found that the depth of the active layer has significantly increased at both our polygon site and wetland site on Axel Heiberg Island over a period of three years. In addition, our sites show signs of increased water content, organic matter content, CO_2 content, and CH_4 content. However, it will require a few more years of measurement at our site for this trend to be reliable.

The mean annual temperature at the high Arctic Eureka weather station (79°59'00 N, 85°56'00 W) has been steadily increasing. The 1971-2000 average temperature was -19.7°C, which is expected to increase by 6-8°C by 2100 (IPCC, 2007). In the sampling year of 2010, the average temperature was -15.9°C, already showing an increase of almost 2°C (http://climate.weatheroffice.gc.ca). The total precipitation has also increased at the high Arctic Eureka weather station. In 2010 total precipitation was 108.3 mm, which has increased over the last four years from 69.8 mm in 2009, 57.5 mm in 2008, and 42 mm in 2007 (http://climate.weatheroffice.gc.ca). However, the temperature and

precipitation was not measured at our exact site, and differences between regions are possible.

Our study has shown that during the study period of 2010-2011, the depth of the active layer was found to have a statistically significant increase ($p = 0.0213$). In addition, previous studies on Axel Heiberg Island have recorded the active layer depth at 40 cm in the same wetland site and 60 cm in the polygon area (Martineau *et al.*, 2011; Wilhelm *et al.*, 2011). Together, this makes the three-year increase (2009-2011) a total of 19 cm at the wetland site and 12.5 cm in the polygon area of Axel Heiberg Island. This increase, although taken over a short period of time, shows a deepening of the active layer at our sites on Axel Heiberg Island. This may be the result of climate change in the Canadian high Arctic, however, it may also be a result of differences in temperature at the time of sampling between the sampling years. Therefore, future measurements at these sites will be required before this trend is reliable. Nonetheless, an increase in active layer depth exposes more soil and a larger microbial community to above zero temperatures and increased substrates through the thaw of carbon stores. Together, these factors would be expected to increase microbial GHG production, and thus it is important to continue measurement of parameters such as the active layer depth.

The remainder of soil content characteristics can only be compared between the wet meadow site in 2010 (AH-P4) and 2009 (AH-H4), as soil core samples from the polygon site were only taken in 2010. At the wet meadow site, there were signs of increase in the water content, CO_2 content, CH_4 content and organic matter content. However, further years of study will be required before these changes are statistically significant. The water content increased from 22.3 % to 49.8 % in the 40 cm sample ($p = 0.1027$). Both the CO_2 and CH_4 contents were nearly significant, with an increase in CO_2 from 2851.6 to 5465.2 nmol/gFW at 40 cm ($p = 0.0545$), and CH_4 from 65.04 to 132.8 nmol/gFW at 40 cm ($p = 0.0855$). The organic matter content increased between 2009-2010 from 5.5 to 9.0 % at 40 cm. The overall organic matter content varied greatly between the AH-H4 10 cm (27 %) and 40 cm (5.5 %) samples, but are similar to those found in other Arctic sites, and depend on type of soil and depth (Ganzert *et al.*, 2007; Rivkina *et al.*, 2007; Vishnivetskaya *et al.*, 2006; Wagner *et al.*, 2005). The organic matter content in the wet meadow site was higher than at the polygon site at a similar depth (11 % compared to 5 %). This difference is likely due the large presence of a larger vegetative cover at the wet meadow site. However, the vegetative cover is increasing throughout the Arctic as climate change progresses, which will increase

the organic matter contents of soils (Hudson and Henry, 2009; Laanbroek, 2010; Schuur *et al.*, 2009).

Our study points towards an increase in the active layer depth, and may show increases in water content, organic matter content, CO_2 content, and CH_4 content. The changes in these factors may be a result of climate change, but may also be due to other differences between sampling years. As such, these trends will require several more years of measurement in order to be reliable. In addition, these measurements were taken at only a few distinct sites on Axel Heiberg Island, and therefore are not necessarily representative of the entire Canadian high Arctic. Nonetheless, climate change has been shown previously to have increased temperature and precipitation in Arctic environments. These changes are argued to be occurring faster than originally predicted (Schuur and Abbott, 2011; Schuur *et al.*, 2008; Tarnocai *et al.*, 2009). As such, it is imperative to measure and document these changes at many sites in order to better understand the effects of climate change on the high Arctic.

5-2. *In situ* and anaerobic microcosm production of CO_2

5-2-1. Interpretation of microcosm CO_2 and CH_4 production rates

The lack of studies of anaerobic Archaea such as methanogens stems from the difficulty of studying anaerobic environments due to their sensitivity to oxygen, as well as the fact that many other microorganisms within soil use methanogenic substrates, and therefore it is difficult to isolate and enrich for these organisms (Kotsyurbenko, 2010; Parkes *et al.*, 2010). The temperature of the active layer soil on Axel Heiberg Island in the Canadian high at a depth of 40 cm was found to vary between -16°C in May (early spring) to 4°C in July (midsummer) (Wilhelm *et al.*, 2011). As such, the temperatures used for microcosm experiments were 4°C, to approximate the *in situ* summer temperature, and either 15°C or 22°C, in order to determine the effects of warming. The rates of CH_4 and CO_2 production in microcosms were calculated over a span of 50-60 days. Many studies calculate gas production rates from the initial linear increase in gas concentration; however, these values are abnormally high due to a primary burst originating from both the release of gases from the frozen soil and the initial effect of an increase in substrate and temperature on microbial communities (Mackelprang *et al.*, 2011; Rivkina *et al.*, 2007). Conditions more similar to those *in situ* would include a longer calculated time period to incorporate changes in system dynamics associated with changes in microbial community, carbon-use efficiency and potential

acclimation (Allison *et al.*, 2010; Eliasson *et al.*, 2005). As such, to better estimate the occurrences *in situ*, the production rates of CO_2 and CH_4 in the microcosms were taken as the average across the entire incubation period. Therefore, while several of the microcosms in this study were found to have a higher rate at the initial incorporation of substrate, this was not used as the reported rate as it does not reflect the occurrences *in situ.*

5-2-2. *In situ* CO_2 flux correlates with soil organic matter content, CO_2 content, and pH in polygon and wetland soils on Axel Heiberg Island

The *in situ* gas flux measures the changes in total gas concentration for a surface of soil per unit time. The total flux of CO_2 increased by 29-164 % at all sites (average of 71 %) between 2010 and 2011. However, due to the short period of study, this increase was not statistically significant ($p = 0.0916$). Taking into account the atmospheric values for CO_2, the net CO_2 flux was found to be positive for all sites, indicating they are a source for CO_2. The net flux was found to vary across the polygon terrain, with the highest flux nearest to the water (+1297 $mg/m^2/day$), and the lowest flux in the raised center (+235 $mg/m^2/day$). The net CO_2 flux at the wet meadow site (+11809 $mg/m^2/day$) was an average of 18 times greater than at the polygon sites. This large difference

in net CO_2 flux between sites shows significant positive correlations with several soil characteristics: the water content (r = 0.9338), pH (r= 0.8313), CO_2 content (r= 0.9920), and organic matter content (r= 0.9552).

A higher water content, and a pH that was weakly alkaline (7.5), as seen in the wet meadow, correlated with the higher CO_2 flux values. The CO_2 flux decreased as soils became more acidic (from 6.5 to 5.5) across the polygon site. The correlation with pH may have originated from the increase in water content and vegetation found at the wet meadow site, which caused a shift in microbial community and increase in activity. This has been shown by a previous study where there was a shift in Archaeal community that correlated with the increased water content and vegetative cover (Angel *et al.*, 2010). It is also known that most microbes, including methanogens, are more adapted to near-neutral pH values (Hoehler *et al.*, 2010).

The CO_2 content within the soil was 11 times greater in the wet meadow than across the polygon sites, a similar ratio to net flux of CO_2 which was 18 times greater. The ratio between the wet meadow and polygon sites was slightly larger the net flux of CO_2 than for the CO_2 content in soils. This difference may be attributable to the increased

release of CO_2 from the wet meadow to the atmosphere via the roots of vegetation (Laanbroek, 2010; Schuur *et al.*, 2009).

The organic matter content in the wet meadow was only 2 times than in the polygon, but was associated with an average CO_2 flux of 18 times greater. The organic matter content is an important factor in controlling Arctic community activity and production of GHG, as seen in many studies (Ganzert *et al.*, 2007; Martineau *et al.*, 2010; Thomson *et al.*, 2010; Wagner *et al.*, 2005; Yergeau *et al.*, 2007). It is predicted that climate change will cause an increase in water content, vegetation and organic matter in the Arctic through warming, precipitation and permafrost thaw (IPCC, 2007; Laanbroek, 2010; Min *et al.*, 2008; Schuur *et al.*, 2009). This study indicates that there may be a correlation between these factors and an increase in net CO_2 flux into the atmosphere from soils in the Canadian high Arctic.

5-2-3. Anaerobic CO_2 flux in unamended microcosms correlate with temperature and organic matter content

The CO_2 production in the anaerobic unamended microcosms was significantly higher at 15°C than at 4°C for all samples ($p = 0.0487$). In the AH-H4 sample, CO_2 production was 2.3 times higher at 15°C, and in the Er-H1 sample it was 1.8 times higher. This indicates a strong effect of

temperature even without substrate addition. In addition, the CO_2 production was 2-3 times higher in the wet meadow (AH-H4) sample than the Eureka (Er-H1) sample. This difference correlates with the higher organic matter content of the wet meadow soil (r = 0.9607), as seen with the *in situ* CO_2 flux.

5-2-4. CO_2 flux in acetate-amended microcosms correlate with temperature

The production of CO_2 could only be calculated in microcosms amended with acetate and methanol. The overall production for both substrates showed no significant correlations with substrate or any soil characteristic, and only a nearly significant correlation with temperature (p = 0.0687). However, when only looking at the acetate-amended samples, there was a significant correlation between temperature and production of CO_2 (r = 0.8516). In the acetate-amended samples, as the CO_2 production increased, the production of CH_4 decreased. This may show that acetoclastic methanogens were outcompeted by CO_2-producing microorganisms at higher temperatures, as they are known to be favoured by low temperatures (Casper *et al.*, 2003; Hoj *et al.*, 2008; Kotsyurbenko, 2005). There was also a significant increase in CO_2 production with temperature in the 70 cm sample incubated with methanol. This large

increase in CO_2 production was paralleled by a significant increase in CH_4 production. However, as methylotrophic methanogens produce a 3:1 ratio of CH_4:CO_2, the 1:1 increase in CO_2 production seen here cannot be solely explained by methanogenesis, and thus indicates an increase in activity of other organisms (Liu and Whitman, 2008). Therefore, there was a significant increase in CO_2 production with temperature when amended with acetate, but only a small increase when amended with methanol. These results indicate that as temperatures increase, the magnitude of the increase in CO_2 production will depend on the specific substrates present.

5-3. *In situ* and anaerobic microcosm production of CH_4

5-3-1. Both the *in situ* and the unamended microcosm CH_4 fluxes correlate with soil water content and anaerobicity

The *in situ* flux of CH_4 or CO_2 measures the changes in total gas concentration for a surface of soil per unit time. The net CH_4 flux was calculated using the difference in the CH_4 flux and the CH_4 concentration in the air (atmospheric values). The net flux of CH_4 in 2011was found to be similar between the wet meadow and polygon sites, between -5.62 to -8.78 mg/m^2/day. These fluxes are all small negative values, indicating these sites are currently slight CH_4 sinks. Across the polygon site, the CH_4 flux significantly correlated with the water content of the soil (r= 0.9966).

The greater the water content of the soil, the greater was the release of CH_4. This was expected as soil hydrology is known to be an important factor controlling methanogenic communities and CH_4 production (Hoehler *et al.*, 2010; Hoj *et al.*, 2006; Wagner *et al.*, 2003).

The anaerobic CH_4 production rates in the unamended set of microcosms were measured at 4°C and 15°C, and ranged from -0.97 to 1.04 nmol/gFW/day. Despite this large range, the majority of samples had positive average fluxes, indicating the soils were slight sources of CH_4. However, many samples had a standard deviation that passed over the zero line demonstrating that within the replicates there existed both a sink and source of CH_4. This was expected, as the Arctic is currently a slight net sink of CH_4, as seen by our *in situ* flux measurements. The wet meadow soil (AH-H4) had a CH_4 production rate approximately 7 times higher than the Eureka soil (Er-H1); however, the difference was only significant between the wet meadow 40 cm sample incubated at 15°C and the Eureka samples. The 60 cm Eureka sample incubated at 15°C was the only sample with a negative flux. This negative flux could be attributed to an increase in activity of ANME, or methanotrophs in microoxic zones. It has been previously shown that methanotrophs increase activity in aerobic microcosms as both temperature and CH_4 concentration increase

(Martineau *et al.*, 2010; Martineau *et al.*, 2011). Martineau and colleagues showed that in similar soils from Eureka, methanotroph methane oxidation increased from 2 to 15 nmol CH_4/gDW/day, with an increase in temperature of 4° C to 22° C (Martineau *et al.*, 2010). This is enough to override the CH_4 production shown in our microcosms to create a negative flux. However, these microcosms were amended with 1 mL of CH_4 under aerobic conditions, thus the rates are likely much higher than those found in our microcosms. This shows that a complex balance exists between the increase in production of CH_4 by methanogens and the increased oxidation by methanotrophs and likely AMNE as well. However, despite the detection of ANME sequences in the high Arctic, their activity remains poorly characterized (Niederberger *et al.*, 2010).

The overall fluxes obtained in the unamended microcosms were slight sources of CH_4, whereas our *in situ* soil fluxes were all slight sinks. This difference was not seen to result from an increase in temperature. Instead, it may result from the increased water content and anaerobic conditions found in the microcosms.

Several models of climate change predict an increase in the water content in many Arctic areas, through the combined effects of precipitation and thaw (IPCC, 2007). Our results indicate that an increase in soil water

content and anaerobicity may cause a shift in soils from a slight sink to a slight source of CH_4. However, our results were determined at only one site, and therefore requires repetition with other soil samples in order to be reliable and representative. If our trend is repeated in future studies, it indicates that the water content of soils may be an important factor in determining the reaction of microbial communities to climate change and the release of GHG from high Arctic soils.

5-3-2. Production of CH_4 in amended microcosms significantly correlates with the combined effects of substrate and temperature

In our study, the combined variation of temperature and substrate type was seen to have a significant effect on the CH_4 flux ($p= 0.0273$) by ANOVA and post-hoc Tukey-HSD tests. Many Arctic studies have found CH_4 production rates increased with temperature and substrate amendment (Ganzert *et al.*, 2007; Hoj *et al.*, 2008; Rivkina *et al.*, 2007). When analysed by DFA, there was a distinct shift in the CH_4 production profile between the amended samples compared to the core, and different groupings between the substrates. These findings correlated with the actual fluxes seen, which found CO_2-amended microcosms to have the highest CH_4 production, followed by methanol, and the lowest production was acetate. The CH_4 flux values for methanol and CO_2 at 22°C reached

an average of 2.2 nmol/gFW/day. This is similar to a few Siberian Arctic amended microcosms studies which produced between 0.05-10 nmol/g/day of CH_4 (Rivkina *et al.*, 2007; Wagner *et al.*, 2005). However, these flux values are much lower than described by many Arctic studies, where fluxes ranged between 160 and 924 nmol/gFW/day (or 38.5 nmol/g/h) (Barbier *et al.*, 2012; Metje and Frenzel, 2007). This large discrepancy may result from either the region sampled (sub-Arctic soils) or the significantly higher organic matter content in their soils, which ranged from 23-80 %, whereas our soils ranged from 4-11 % (Barbier *et al.*, 2012; Metje and Frenzel, 2007).

Interestingly, whereas the CH_4 flux values increased with temperature for methanol incubated samples, it decreased for acetate-amended samples, and remained the same for CO_2 samples. This may be attributed to differences in the methanogen community and their adaptation to cold. This has been seen in soils from the Spitzbergen Arctic, where individual populations of methanogens showed differences in their temperature response (Hoj *et al.*, 2008). Our results indicate a high abundance of hydrogenotrophic methanogens throughout the active layer and permafrost table that are well adapted to *in situ* temperatures as well as warmer temperatures. It would also indicate that methylotrophic

methanogens reside preferentially in deeper zones near the permafrost table but are less adapted to *in situ* temperatures, becoming 5 times more active at 22°C. In contrast, despite acetoclastic methanogens being most abundant in the active layer of our samples, they seemed to have been favoured by low temperatures, as seen in several studies (Barbier *et al.*, 2012; Casper *et al.*, 2003; Kotsyurbenko, 2005).

Surprinsingly, molybdate addition did not affect the production of CO_2 or CH_4 in these microcosms. This was not expected, as many previous studies found that the inhibition of sulfate reducers increased CH_4 production (Purdy *et al.*, 2003; Sorensen *et al.*, 2004). This incongruence may result from the substrates in our study being present in high enough concentrations for the competition to be minimal, the low abundance of sulfate reducers in these soils, methanogens using other sources of carbon as substrates, or the production of H_2 and CO_2 through other means and their subsequent use for methanogenesis (Finke *et al.*, 2007; Hoehler *et al.*, 2010). A previous study on Axel Heiberg Island found that there were few sulfate reducing bacteria in these soils (Wilhelm *et al.*, 2011).

Our results indicate that it is not only the increase in temperature, but also the type of available substrate that will effect the production of

CH_4. This was further confirmed by discriminate function analysis, which showed the CH_4 production profile correlated with the specific substrate added. The CO_2-amended microcosms were found to have the highest CH_4 production and acetate the lowest. However, our results were obtained from only one site in the Canadian high Arctic in one experiment, and will require repetition with other soil samples in order to be reliable and representative. As climate change continues, concentrations of CO_2 are expected to increase, as it is a major product of microbial decomposition of thawed carbon stores (Hoehler *et al.*, 2010; Kotsyurbenko, 2010). If the trend in our results is repeated at other sites, it indicates that the production of CH_4 may increase significantly as climate change continues in the Canadian high Arctic.

5-4. Increasing substrates, temperature and anaerobicity in microcosms causes an increased ratio of CH_4 to CO_2 flux rates

The ratio of CH_4 to CO_2 flux rates is a good measure of the relative activities of methanogens, methanotrophs and other heterotrophs. The *in situ* ratio found CO_2 production to be 15-300 times higher than CH_4. The unamended microcosms found CO_2 production 3.4-32 times greater than CH_4. The amended microcosms showed CO_2 production only 0.8-3.8 times higher than CH_4. This last ratio shows CH_4 production actually

surpassed the production of CO_2 in the case of the amended samples: the 70 cm methanol at 22° C and the 30 cm acetate at 4° C. These ratios indicate that as environments increase in anaerobicity and methanogenic substrates, the ratio of CO_2 to CH_4 diminishes. This was seen by Hoj and colleagues, where the CO_2 production was 348 times greater than CH_4 than at 1° C, but shifted to 1:1 at 20° C (Hoj *et al.*, 2008). This increase in the ratio of CH_4 to CO_2 production is expected for the anaerobic decomposition of organic matter (Schuur *et al.*, 2008). The anaerobic decomposition of organic matter in our soils is mostly driven by the water content as seen by the *in situ* flux. The *in situ* CO_2 flux across the polygon site shows a significant negative correlation with the water content of the soil (r= -0.8436), whereas the *in situ* CH_4 flux shows a strong positive correlation (r= 0.9966). Therefore, as water content in our soils increased, the ratio of CH_4 to CO_2 increased. This shift was further amplified by the increase in temperature and methanogenic substrates in the amended microcosms.

Interestingly, the acetate-amended samples showed a decrease in CH_4 production as temperatures increased. This decrease in CH_4 production was negatively correlated with CO_2 production, which increased with temperature (r = -0.8769). This was also seen by Hoj and

colleagues, where the production of CO_2 to CH_4 increased in acetate-amended samples from 67 to 236 times between 1° C and 20° C (Hoj *et al.*, 2008). This further indicates either the adaptation of acetoclastic methanogens to lower temperatures, and/or their out-competition at higher temperatures even under anaerobic conditions (Casper *et al.*, 2003; Hoj *et al.*, 2008; Kotsyurbenko, 2005).

Our results indicate the increase in ratio of CH_4 to CO_2 seems to be driven by the water content of the soil, but further amplified by the increase in methanogenic substrates, and by the increase in temperature with methanol and CO_2 amendment. However, our results were obtained from only one site, and in one experiment. Nonetheless, if our trends are seen at other sites, then they will indicate an important increase in GHG production as climate change increases wetlands, temperatures and substrates through thawing carbon stores in the Canadian high Arctic.

5-4-1. Anaerobic conditions increase greenhouse gas feedback effect on climate change in polygon and acidic Canadian high Arctic soils

Theoretical mathematical calculations indicate that despite the greenhouse effect of CH_4 being 25 times greater than CO_2, the anaerobic decomposition of carbon stores will result in a smaller greenhouse effect than under aerobic conditions (Conrad *et al.*, 2002b; IPCC, 2007; Schuur

et al., 2008). This was not seen with our microcosm amendments, as the increase in CH_4 was greater than the compensatory decrease in CO_2. Our anaerobic microcosm experiments indicated that an increase from 4° C to 22° C with an increase in CO_2 or methanol substrates caused production of CO_2 to increase by up to 4 times, and CH_4 production to shift from a net sink to a net source, increasing by up to 45 times. From these results, the average greenhouse effect in our samples was determined, where greenhouse effect was taken from the respective radiative efficiencies of each gas in the ozone layer, and thus their feedback on climate change (where the greenhouse effect of CH_4 = 25 times that of CO_2). Together, the greenhouse effect created by an increase in temperature and CO_2 substrate under anaerobic conditions in our samples was found to potentially be over 1000 times greater.

The greenhouse effect caused by CH_4 and CO_2 production increased in our AH-P1 amended microcosms by 18 times compared to the unamended microcosms, and over 200 times compared to the *in situ* fluxes. However, when these amended fluxes are compared to the *in situ* AH-P4 flux values, the greenhouse effect is 4 times smaller due to the very large CO_2 production at this site. This indicates that the polygon soils may become greater producers of CH_4 and CO_2 under anaerobic

conditions, whereas the wetland soils produce more CH_4 and CO_2 under their current aerobic conditions, as predicted by Schuur and colleagues (Schuur *et al.*, 2008).

A similar phenomenon is seen when our flux values were compared to those obtained by Martineau and colleagues for the aerobic microcosm incubation of similar Canadian high Arctic soils (Martineau *et al.*, 2010; Martineau *et al.*, 2011). Our anaerobic CH_4 and CO_2 flux produced a greenhouse effect 1.7 times greater than the aerobic incubation of acidic soils at 10° C, and much greater than the Eureka soils incubated at 22° C, as the latter were a sink of CH_4 (Martineau, 2011; Martineau *et al.*, 2010). However, when our flux results are compared to the aerobic microcosm fluxes for the AH-P4 and upland tundra sites on Axel Heiberg Island, the aerobic incubation produced a greenhouse effect is 2 times greater (Martineau *et al.*, 2011). This shows that within upland and wet meadow soils the greenhouse effect may be greater under aerobic conditions, whereas within acidic and polygon soils the greenhouse effect may be greater under anaerobic conditions. Therefore, the soil type (acidic, upland tundra, wet meadow or polygon) may be an important factor controlling whether the soil will produce a greater greenhouse effect under aerobic or anaerobic conditions. This may be the direct result of their intrinsic soil

characteristics and microbial communities. Therefore, it is imperative to continue studies on various soil types in order to understand the microbial communities within each soil type, and to track shifts that occur as climate change continues in the high Arctic.

5-5. Archaeal and methanogen abundance and diversity

5-5-1. Methanogen abundance by qPCR of the *mcrA* gene does not correlate with production of CH_4

The abundance of methanogens by qPCR of the *mcrA* gene was not found to correlate to the production of CH_4 in this study. After incubation with methanogenic substrates at higher temperatures, the abundance of methanogens was equal or smaller compared to the unincubated core. This was expected as Arctic environments are good preservers of DNA (Coolen *et al.*, 2011; Graham *et al.*, 2011). Upon the incubated of microcosms at higher temperatures, this increase in temperature is expected to both destabilize DNA, and active DNAses within the soil. As such, the unincubated core is expected to have high concentrations of preserved DNA, while the incubated samples are expected to be lower due to the destruction of this DNA. It is also possible that there may have been an increase in activity with little increase in abundance, or that the increases in methanogens were small in

comparison to the increases in other microorganisms, causing a smaller relative abundance upon DNA extraction. Upon incubation at higher temperatures, this DNA may have no longer been stable. Overall, the abundance of methanogens based on the *mcrA* gene was not a good indicator of methane production and GHG feedback on climate change, as seen previously (Webster *et al.*, 2009).

5-5-2. Interpretation of pyrosequencing data

The interpretation of the pyrosequencing data must be done with care, as the presence of DNA sequences do not prove viability or activity (Willerslev *et al.*, 2004). This is especially important in frozen environments such as permafrost, as they are known to preserve biological material such as DNA (Coolen *et al.*, 2011; Graham *et al.*, 2011). As a result, SIP was also done in order to determine which of the communities present were active in one our amended microcosms.

The taxonomic classification of sequences depends highly on the reference sequences and taxonomic hierarchy used to train a model. Most taxonomic classifications use a naïve Bayesian classifier (RDP II) due to its favourable automation, speed and accuracy (Werner *et al.*, 2011). A study found that using the largest, most diverse training set they constructed from the Greengenes database, they could reduce their

unclassified reads by 50 % at the phylum level (Werner *et al.*, 2011). However, for mouse gut and anaerobic digester samples, there were still a greater number of unclassified sequences at the genus level than there were classified taxonomic units due to a limited diversity in existing reference databases (Werner *et al.*, 2011). This limited diversity of existing references applies even more to Archaeal sequences originating from permafrost environments. As a result, the taxonomic classification of sequences varies greatly between databases, and barely extends reliably to the order level, as seen with our results.

5-5-3. The novel Thaumarchaeota order Nitrososphaerales is the most abundant order in high Arctic polygon soils

The overall Archaeal diversity was similar in all of our samples, with Nitrososphaerales being the most abundant order, accounting for an average of 88 % of total Archaeal reads. The other abundant orders were seen in unique samples, and included Thermoplasmatales at 33 % in the AH-P1 30 cm 22° C methanol sample, and Methanomicrobiales at 39.5 % in the AH-P1 70 cm 4° C CO_2 sample. The closest cultured organism to our sequences is *Nitrososphaera gargensis*, one of the few representatives of the Thaumarchaeota (Tourna *et al.*, 2011). The Thaumarchaeota are autotrophic, ammonia-oxidizing Archaea (AOA), and

are among the most abundant Archaea on Earth (Pester *et al.*, 2011; Schleper *et al.*, 2010). Despite the majority of Thaumarchaeota being charactierized as mesophilic and thermophilic organisms, they have been found in almost every environment on Earth, including Antarctic sediments, Canadian high Arctic hypersaline springs and permafrost soils (de la Torre *et al.*, 2008; Gillan and Danis, 2007; Lay *et al.*, 2012; Wilhelm *et al.*, 2011; Zhang *et al.*, 2010). Due to their recent detection at high abundance in soils, they have been proposed to also act as heterotrophs with a diverse organic substrate range including methane, short-chained alkanes, chlorinated ethenes and aromatic hydrocarbons (Pester *et al.*, 2011). Therefore, their detection as the most abundant order in high Arctic soils may implicate the Thaumarchaeota in an important functional role in the emission of GHG related to both the nitrogen and carbon cycles, however these roles remain to be elucidated.

5-5-4. Methanomicrobiales and Methanococcales are the most abundant methanogen orders in Canadian high Arctic polygon soils

All six of the methanogenic orders were detected, although the Methanomicrobiales and Methanococcales were the only orders found in most samples. Our methanogenic sequences detected clustered closest to uncultured environmental clones from Antarctic mineral soils, Canadian

high Arctic permafrost, Alp glaciers and Japan rice field soils. The closest cultured relative was the methanogenic Archaeaon Prasan 7, whereas the known cultured methanogens were approximately equidistant from our sequences.

At 30 cm depth, the methanogenic sequences averaged 1 % of total Archaeal reads, with Methanomicrobiales averaging 43 % and Methanococcales averaging 39 % of these methanogenic sequences. At 70 cm depth, the methanogenic sequences averaged 11 % of total Archaeal reads, and Methanomicrobiales made up approximately 65 % and Methanococcales 22 %. The Methanomicrobiales have been previously found as one of the most abundant methanogen orders in soild from the Siberian Arctic and Antarctica, constituting up to 57-58 % of the methanogenic sequences, along with the other abundant orders Methanosarcinales and Methaonbacteriales (Ganzert *et al.*, 2007; Hoj *et al.*, 2008; Koch *et al.*, 2009; Purdy *et al.*, 2003). However, in the Canadian Arctic, the most abundant orders were found to be the Methanosarcinales and Methanobacteriales, which does not correlate with our data (Barbier *et al.*, 2012). In our data, the Methanosarcinales were detected at an average of only 2 % of the methanogen sequences, and almost exclusively in acetate-amended samples. The Methanobacteriales were

found at an even lower abundance. The Methanococcales were the second most abundant order in our samples, but have not been detected in other Arctic environments. However, they are one of the most abundant methanogen orders in marine sediments, along with Methanomicrobiales (Liu and Whitman, 2008), as was found in our study. This may be a result of the marine origins of surface deposits on Axel Heiberg Island (Pollard, 2005). The remainder of the orders (Methanopyrales, Methanocellales and Methanobacteriales) were almost exclusively found at 70 cm depth at relatively low abundances.

There were two samples in which the relative abundance of methanogens increased significantly. The 70 cm 4° C CO_2 sample had the highest relative abundance of methanogens, at 39.7 % of total archeal reads. This increase in abundance was due to an increase in Methanomicrobiales up to 39.5 % (compared to 9.5 % in the core). The second case was the 30 cm 22°C acetate sample where, again, the Methanomicrobiales increased to 5.2 % of total Archaeal reads (compared to 0.33 % in the core). This may indicate that the Methanomicrobiales are an important methanogen order in these Canadian high Arctic polygon soils. However, these increases in Methanomicrobiales were not associated with similar increases in CH_4 production.

Other notable increases compared to the core include an increase in Methanococcales by 6 times in the 70 cm 22°C CO_2 sample, which may be a result of their hydrogenotrophic nature. The 10 times increase in Methanosarcinales in the 70 cm 22°C acetate sample, likely a result of acetoclastic methanogenesis. And finally the appearance of Methanobacteriales exclusively in the 70 cm 22°C acetate and methanol samples for unknown reasons as they are hydrogenotrophic methanogens.

5-5-5. Methanogen diversity and relative abundance correlates with increasing temperatures, depth and CO_2 amendment

The average diversity indices for the Archaeal pyrosequencing data are similar to those found in other Arctic studies, which range from 0.63-2.0 (Hoj *et al.*, 2008; Liebner *et al.*, 2009; Martineau, 2011; Niederberger *et al.*, 2010; Perreault *et al.*, 2007; Wilhelm *et al.*, 2011). Our results show the growth and proliferation of a larger diversity of Archaea to detectable levels as both depth and temperature increase. The average diversity was found to increase with temperature, being higher for the microcosms incubated at 22°C (H'= 0.73, Chao1 = 41.5) than at 4°C (H'= 0.33, Chao1 = 9.7). This has been seen with Siberian Arctic soils, where diversity was higher at 20°C than 5°C (Hoj *et al.*, 2008). In addition, the diversity was

found to increase with depth, being higher at 70 cm (H'= 0.76, Chao1 = 40.9) than at 30 cm (H'= 0.42, Chao1 = 20.3). However, a similar region of the Canadian high Arctic has been previously shown to decrease in diversity with depth (Wilhelm *et al.*, 2011). In our study, the increase in overall Archaeal diversity with depth is mostly attributable to the increase in methanogenic diversity and abundance at 70 cm compared to 30 cm, as seen in other studies (Ganzert *et al.*, 2007; Hoj *et al.*, 2008; Koch *et al.*, 2009). The increase in methanogens may be a result of the increased anaerobicity found at this depth, based on the porewater manganese and an increased C/N ratio in our soils (Stackhouse *et al.* in progress).

Overall, the largest diversity and abundance of methanogens in our soils were detected at the 70 cm depth when incubated with CO_2 or at 22°C. The 70 cm depth currently exists at the permafrost table, in the deepest part of the active layer. This is of importance as this large diversity of methanogen orders are now being exposed to newly thawed carbon stores and above zero temperatures, which is expected to increase their production of CH_4.

5-6. Potential important functional role of Methanoyprales in Canadian high Arctic polygon soils

5-6-1. Presence of Methanopyrales, but not relative abundance, correlates with CH_4 production

When comparing the relative abundance and diversity detected with the methane production rates, neither the total relative abundance of methanogens nor a particular methanogen order accounted for the microcosm CH_4 production rates. This is consistent with previous Arctic studies, indicating the CH_4 cycle is quite complex (Ganzert *et al.*, 2007; Hoj *et al.*, 2005, 2008). In terms of the distribution of methanogen orders by principal component analysis, the orders Methanococcales and Methanomicrobiales grouped closest to CH_4 production. This is likely not a causal correlation, as these orders were the only two found in most samples. Indeed, the Methanomicrobiales were the order that accounted for the two significant increases in abundance, however those samples had relatively low CH_4 production rates. The orders Methanosarcinales, Methanobacteriales and Methanopyrales grouped closest to the production of CO_2. As they are all capable of hydrogenotrophic methanogenesis, this may indicate that the quantity of CO_2 produced in the microcosm may be an important factor determining their abundance.

In addition, as the CH_4 production was highest in the CO_2 amended samples, these orders may actually be a better marker for CH_4 production. In fact, the presence of Methanopyrales, but not their abundance, showed a moderate correlation with CH_4 production in 70 cm samples (r = 0.7168). This may be of importance, as the relative abundance determined by the percentage of total Archaeal reads between samples is not necessarily indicative of their total abundance or activity in soils.

5-6-2. SIP shows Methanopyrales are the only active methanogen order in the AH-P1 70 cm sample incubated with CO_2 at 22° C

The Methanopyrales were the only methanogenic order to show enrichment when AH-P1 70 cm sample was incubated at 22° C with ^{13}C-labelled CO_2. This enrichment was equivalent to an increase of 0.13 % of total reads between the light and heavy fractions. This enrichment was not a large increase in overall abundance, as Methanopyrales were also found in the other microcosm enrichments at a similar level of 0.12 % of total reads. However, the enrichment was significant because 100 % of the Methanopyrales detected were active. The Methanopyrales sequences clustered between the other methanogenic sequences from the samples and the known methanogens within the orders Methanopyrales, Methanococcales and Methanobacteriales. Although these sequences

seem quite distant from both groups, they were found to cluster with low abundance Methanopyrales sequences from our other 70 cm amended microcosm samples, found at less than 0.05 % of total sequences. As they were the only methanogenic order to show activity, this indicates that despite their low abundance, they may play an important functional role in the production of CH_4 in this sample (Arumugam *et al.*, 2011).

The production and content of CO_2 is expected to increase as climate change develops in the high Arctic. In our sample, the abundance of Methanopyrales correlated with the production of CO_2 as seen by PCA, and their presence correlates with the production of CH_4 in all 70 cm samples. This depth is where the majority of CH_4 is produced by methanogens due to its increased anaerobicity, and CO_2 was the substrate which produced the highest flux of CH_4. Finally, the Methanopyrales were the only methanogenic order found in our sample by SIP to be active in incorporating CO_2 in order to produce CH_4. Therefore, our results indicate that the Methanopyrales, despite their low abundance, may play an important functional role in the production of CH_4 from CO_2 in our soils. However, our results are based on only one sample and one incubation condition. Therefore, these results will need to be repeated using other soils and conditions before they become reliable.

5-7. Conclusions

- Statistically significant increases were found in wetland and polygon soil *in situ* active layer depth, water content, organic matter content, CO_2 content, and CH_4 content. However, these increases were only taken over a period of 2-3 years, and thus require further years of study before they become reliable. In addition, similar trends will need to be seen in other areas before these can be representative of the Canadian high Arctic.
- The production of CO_2 *in situ* was found to correlate with organic matter content, CO_2 content, water content and pH. In the unamended microcosms, CO_2 flux was dependent on organic matter and temperature. In amended microcosms, the CO_2 flux was highest with acetate amendment, where it increased with temperature. This may be a result of the increase in activity of other acetate-utilizing bacteria.
- The CH_4 production rates were found to be dependent on the anaerobicity and combined effects of temperature and substrate, being highest in the incubations at high temperatures or with CO_2 as a substrate. This is likely a result of the majority of methanogens using the hydrogenotrophic pathway. The CH_4 production rates in microcosms did not show an effect of anaerobicity or depth, likely due

to the similar conditions used by the flushing with N_2 and addition of water.

- The Thaumarchaeota order Nitrososphaerales were the most abundant Archaeal order in all samples, accounting for an average of 88 % of total Archaeal reads.
- The abundance and diversity of methanogens was found to increase with depth, temperature and CO_2 amendment. At 30 cm depth, methanogens averaged 1 % of total Archaeal reads, whereas at 70 cm depth, methanogens averaged 11 %. This increased abundance is likely due to the increased water content and anaerobicity associated with depth. The level of anaerobicity based on soil hydrology has been shown to be a main factor in limiting methanogenesis (Hoehler *et al.*, 2010; Hoj *et al.*, 2006; Wagner *et al.*, 2003)
- The two most abundant methanogen orders were the Methanomicrobiales and the Methanococcales. The Methanomicrobiales were also responsible for the highest increase in methanogen abundance up to 39.5 % of total Archaeal reads in the 70 cm 4° C CO_2 sample (compared to 9.5 % in the core). However, this increase did not correlate with an increase in CH_4 production.

- The Methanopyrales were the only active methanogen order in the 70 cm 22° C CO_2 sample detected by SIP. Their presence also correlated with CH_4 production in the 70 cm soil samples, and therefore, the Methanopyrales may play an important functional role in the production of CH_4 from CO_2 in our polygon soils.
- Together, our results indicate that the most important drivers for the production of CH_4 may be the level of anaerobicity, the type of soil (polygon vs. wetland), the temperature and the concentration of CO_2 as a substrate for methanogenesis.

As climate change continues in the high Arctic, temperatures are expected to increase, causing the thaw of carbon stores and increase in microbial activity, thus increasing the concentrations of CO_2 as a product of microbial degradation. An increase in substrate and temperature in our soils has been shown here to increase methanogen diversity, abundance and production of CH_4.

Whether aerobic or anaerobic conditions predominate as climate change progresses is dependent on whether the expansion of wetlands through thawing of permafrost and increase in precipitation will predominate over the drying up of Arctic soils through increased

evaporation. An increase in thermokarst area and precipitation has already been documented in the Siberian Arctic, Alaska, and Canada (Walter *et al.*, 2007); http://climate.weatheroffice.gc.ca). If this trend continues in polygon and acidic soils, the results of this study indicate the conditions in the Canadian high Arctic may increase methanogen abundance and diversity as well as increase the ratio of production of CH_4 to CO_2, thereby amplifying the greenhouse feedback effect on climate change. However, as our study was based on a limited number of soil samples and types and was completed over a limited number of years. As such, the trends and results presented are not necessarily reliable and representative of the entire Canadian high Arctic. Therefore, further studies are recommended.

Our study had several weaknesses. Firstly, the soils used for the unamended microcosms were not the same as those used for the amended microcosms due to limited sample quantities. Therefore, the results between the unamended and amended microcosms could only be roughly compared. In addition, the amended microcosm experiment was only done on one core sample at two depths. Therefore, similar experiments should be repeated using other soil samples, with both amended and unamended microcosms for the same samples in order for

the trends seen to be reliable. In addition, the SIP experiment in our study was only successful for one of the samples, and therefore only one temperature and substrate were analysed for the active community. As such, the results will need to be reproduced with other soils and incubation conditions in order to confirm the active orders found in this study. Finally, the detection of Thaumarchaeota as the most abundant Archaeal order in Canadian high Arctic polygon soils, and the hypothesis of their potential activity as heterotrophs utilizing methane as a substrate, could implicate them in an interesting role in carbon cycle that would be interesting to elucidate.

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Appendix 1 : CARD-FISH

Materials and Methods

Catalysed reported deposition fluorescence *in situ* hybridization (CARD-FISH) was performed on samples from the AH-H4 40 cm site, and the AH-P1 30 cm sample after microcosm incubation with CO_2 at 22° C as described (Pernthaler and Pernthaler, 2007; Pernthaler *et al.*, 2002; Wendeberg, 2010). The probes for CARD-FISH targeted both Archaea (probe Arch 915) and methanogens of the family *Methanobacteriaceae* (probe MB1174) (Raskin *et al.*, 1994). Several change and specifications were made to the protocol by Pernthaler, and will be desribed. In brief, soil solutions were fixed for 1 hr at 22°C in a 2 % formaldehyde solution, made from a stock stored in an N_2 atmosphere in order to keep the solution stable. Samples were then lightly centrifuged and resuspended in phosphate buffered saline (PBS) several times. They were then either sonicated for 20 seconds at minimum speed or non-sonicated, or order to determine which technique worked best. Aliquots of the solution were diluted in PBS and added in 1:1 ratio with a PBS solution containing 0.1% agarose and 0.001% SDS for 4 minutes at 55°C. Aliquots of 10µl were added into the wells of a diagnostic slide, air-dried, then dehydrated in increasing concentrations of ethanol in PBS (50%, 80%, 96%). Slides

were air dried and stored overnight at 4°C. Endogenous peroxidases were inactivated through incubation with 0.1 % hydrogen peroxide in methanol for 2 minutes, followed by washes in water. Cells were permeabilized using both 75 and 750 µg/ml proteinase K (40 U activity) for 30 minutes at 37°C in a humid container. The humid container consisted of water-soaked kimwipes lining a horizontal falcon tube containing the slide. Slides were then washed several times in water, dehydrated in 50 % ethanol in PBS, and air-dried to be stored at -20°C overnight. Probes were quality checked using the Nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) in order to adjust the probe concentration to be added according to its efficiency. The efficiency of the probe is calculated by the optical density (OD) at 260 nm, or OD_{260}, divided by the OD_{440}, and should be approximately 3 (Pernthaler and Pernthaler, 2007; Wendeberg, 2010). The probes were then diluted 300:1 in hybridization buffer using 35 % formamide, to form the hybridization-probe mix. Aliquots of 30 µl of the hybridization-probe mix were added to the well and hybridized in a humid container for 15 hrs at 46°C. Remaining liquid was aspirated and the slide was washed with sterile water. Wells were then incubated with amplification buffer containing diluted hydrogen peroxide and 2 µl of fluorescent tyramide for 15 minutes in the dark. Wells

were then washed, and counter-stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature. Wells were washed, dehydrated in 100% ethanol, and air dried for microscopic observation.

Results

Both samples yielded positive results for the presence of Archaea and methanogens of the *Methanobacteriaceae* family (Figure 4.2). However, as each step in CARD-FISH is highly dependent on specific concentrations and types, such as the target sample, enzyme, probes etc. clear results were often difficult. It was difficult to distinguish the number of cells from background staining, perhaps as a result of cell bursting. As a result, the staining could only be used to determine the presence of the targeted microorganism, *Methanbacteriaceae*. However, it was not clear enough to be quantifyable in order to yield reliable cell counts. Therefore, further optimization is required.

Figure A1-1: CARD-FISH staining on Arctic soils with DAPI counterstain.

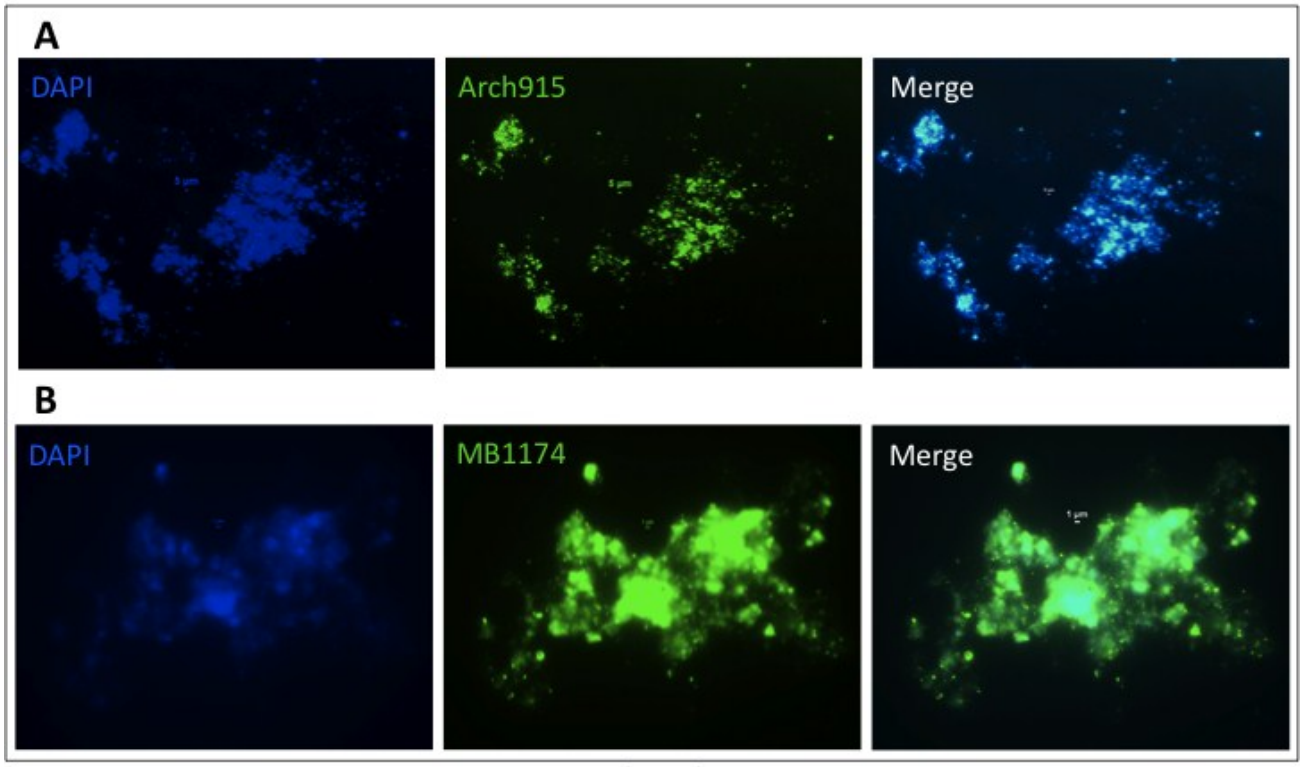

Figure 4.2: CARD-FISH stanining on Arctic soils with DAPI counterstain.
A) Presence of archaea in P4-40cm sample using Arch915 probe. B) Presence of methanogens of the family *Methanobacteriaceae* in P1-30cm sample post-incubation with CO_2 at RT° using the MB1174 probe.

Appendix 2 : PCR and qPCR limits of detection for the methanogen *mcrA* gene

Materials and Methods

PCR of the both Archaeal and bacterial 16S rRNA genes were conducted as positive controls for successful DNA extraction using the primers seen in Table 4.1. The *mcrA* gene was also targeted, using both the *mlas*/*mcrA-rev* primer set (Steinberg and Regan, 2008) and the METH-f/METH-r primer set (Colwell *et al.*, 2008). These reactions underwent many stages of optimization as non-specific amplification was often seen. A limit of detection was also conducted for the more successful set of primers, *mlas*/*mcrA-rev* using 10-fold dilutions of the mcrA gene isolated from *Methanosarcina barkeri*. The mcrA gene of *M.barkeri* was isolated using the EZ-10 Spin Column PCR purification kit followed by the EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic Inc., Markham, Ontario, Canada). Manufacturers instructions were used but with several variations of ligation and transformation times. The variation that yielded the highest concentration by Picogreen dsDNA quantitation assay was a 1.5-2 hrs ligation with an overnight transformation. This was diluted to form a standard curve from 10^6 to 10^2 copies/µl.

Results

The limit of detection for PCR of the methanogen *mcrA* gene was found to be 10^3 or 1000 copies per reaction (Figure A2-1). This limit of detection is too high for the majority of Arctic samples, as one reaction will contain the equivalent of approximately 0.002-0.01 grams of soil, requiring methanogens be found at 10^5 to 10^6 copies per gram of soil (1 *mcrA* gene per methanogen). As a result, qPCR was performed. The limit of detection of qPCR for the methanogen *mcrA* gene was determined to be 50 copies/µl (Figure A2-2). Samples down to 50 copies/µl had appropriate melt curves, whereas the negative was shifted left, indicating the signal was a result of primer dimmers (Figure A2-3). The lower limit of detection of qPCR only requires methanogens to be found at 10^3 to 10^4 copies per gram of soil, a more realistic expectation for high Arctic soils.

Figure A2-1: PCR limit of detection of the methanogen *mcrA* gene.

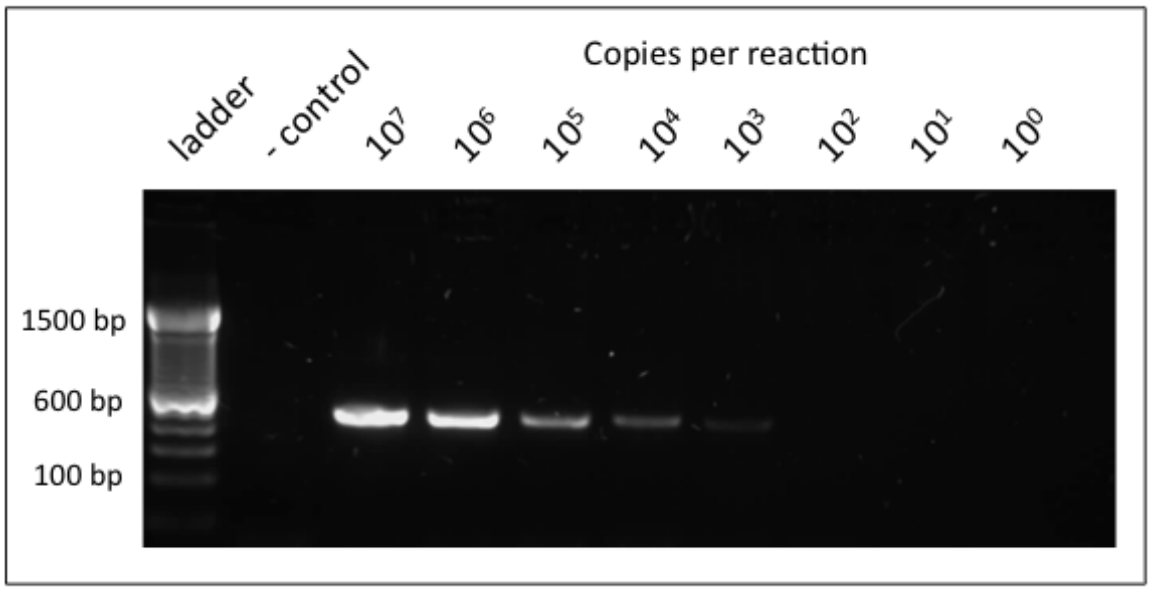

Figure A2-1: The *mlas/mcrA-rev* primers were used on decreasing concentrations of *Methanosarcina barkeri* linearalized plasmid, in copies per reaction, in order to determine the limit of detection. Detection was done using 1% agarose gel containing 2 µl SYBR-safe and 8 µl PCR product.

Figure A2-2: qPCR limit of detection of the methanogen *mcrA* gene.

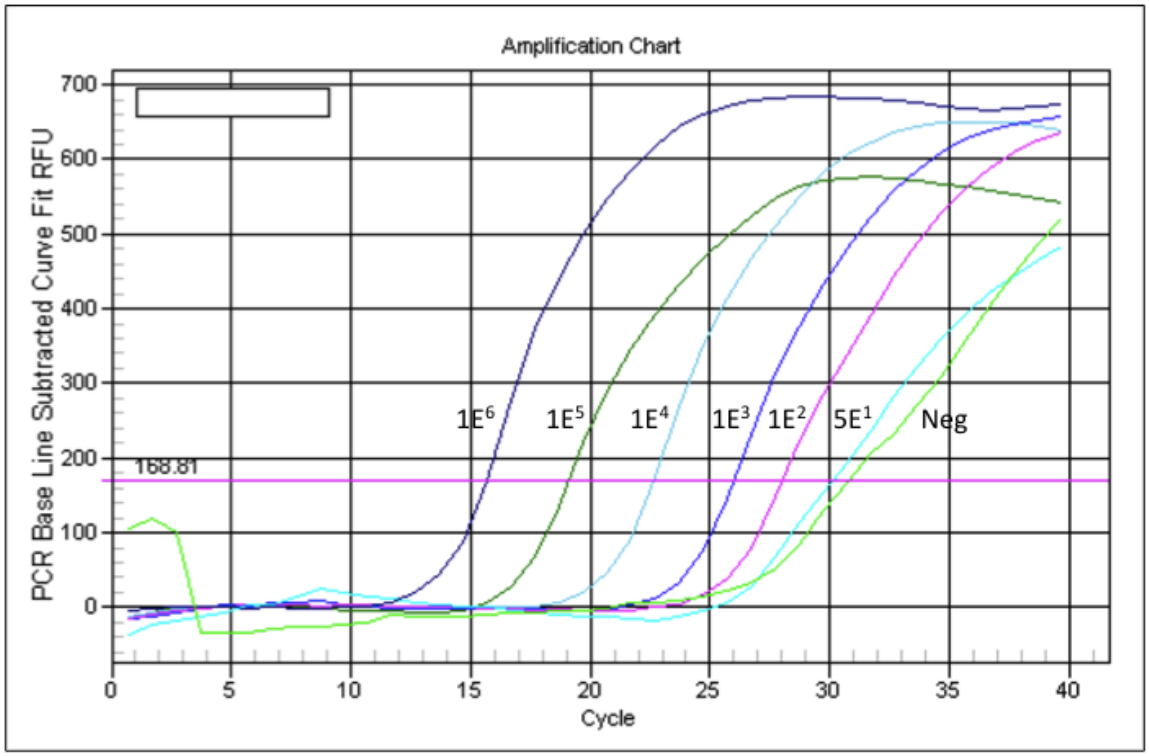

Figure A2-2: The *mlas/mcrA-rev* primers targetting the methanogen *mcrA* gene were used on decreasing concentrations of *Methanosarcina barkeri* linearalized plasmid in order to determine the limit of detection by quantitative PCR. The negative control was sterilized water (Neg). The limit of detection was found at $5^{E}1$ copies per reaction.

Figure A2-3: Melt curve analysis of qPCR methanogen *mcrA* gene for the limit of detection reaction

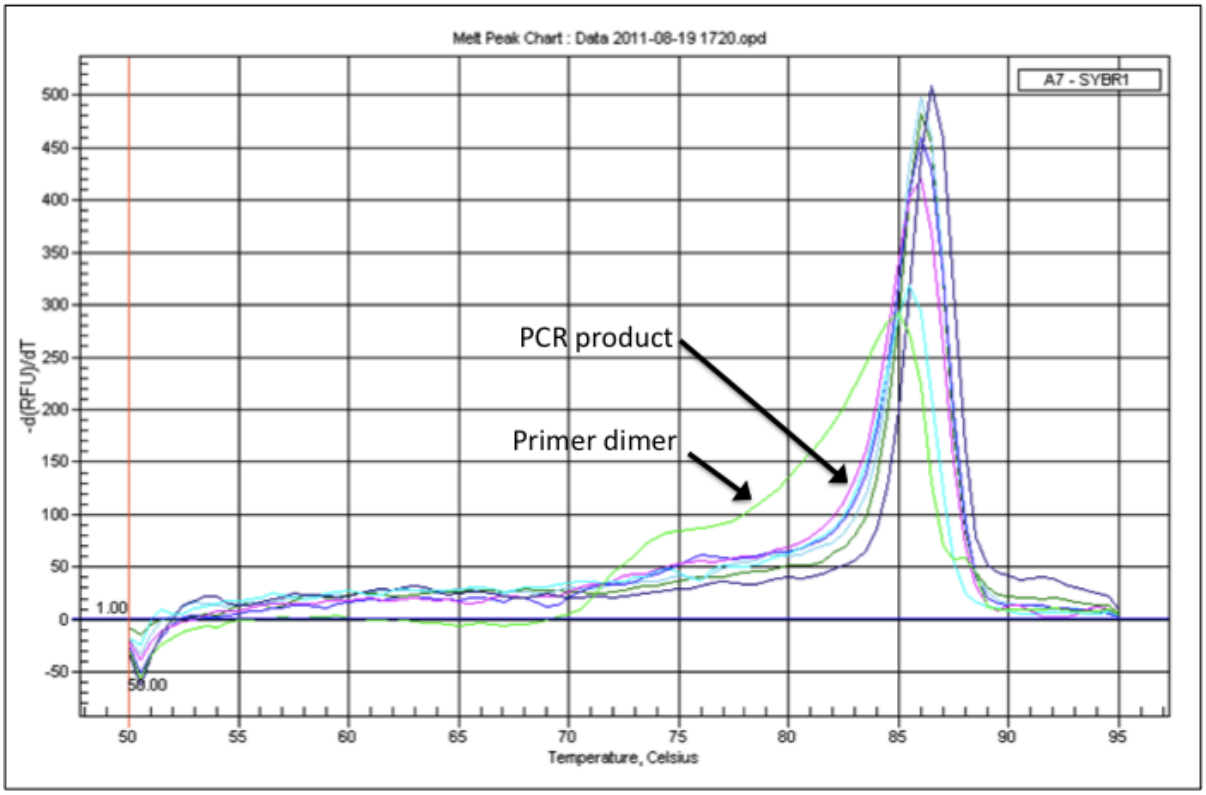

Figure A2-3: Melt curve analysis was done on the qPCR limit of detection reaction using the *mlas/mcrA-rev* primers targetting the methanogen *mcrA* gene. Melt curve analysis shows that the PCR product is found between 83 and 90 degrees, whereas the primer dimmers found in the negative control (green line) are shifted to the left, starting at 73 degrees.

Appendix 3 : Comparison of sequence identification between databases

Materials and Methods

A comparison was made between the identification of pyrosequencing results between the Research and Testing Laboratories LLC pyrosequencing company, the RDP classifier, and the closest relatives from the nucleotide BLAST. The Research and Testing Laboratories LLC pyrosequencing company taxonomic identification involves sorting sequences by decreasing size and clustering them into OTUs with 96.5 % identity using USEARCH (Edgar, 2010). The OTUs are then searched against a database of high quality sequences from NCBI using a distributed .NET algorithm that utilizes BLASTN+ (KrakenBLAST www.krakenblast.com). The outputs are then compiled and data reduction analysis is performed. The RDP pipeline taxa assignment is based on the RDP naïve Bayesian rRNA classifier (Wang *et al.*, 2007). The nucleotide BLAST was used to search the closest Archaeal relatives of the top >0.5 % OTUs.

Results

The resulting orders and percentages were similar for both the Research and Testing Laboratories LLC pyrosequencing company and

the RDP classifier. However, the one large difference found was that the most abundant member was identified as Acidilobales by the RDP classifier, whereas it was found to be Nitrososphaerales by the Research and Testing Laboratories LLC pyrosequencing company, the nucleotide BLAST search, as well as the phylogenetic tree constructed in this study. This discrepancy was because the RDP classifier does not yet contain the Thaumarchaeota, including Nitrososphaerales. In addition, some of the samples were sequenced earlier, and thus were not identified by the Research and Testing Laboratories LLC pyrosequencing company as Nitrososphaerales, but instead as Thermoproteales. However, all of these sequences cluster together by phylogenetic tree and the closest BLAST hits are *Nitrososphaera species.* Therefore, as the majority of the databases and the phylogenetic tree confirm these sequences cluster with the Nitrososphaerales, this identification was used. Other than this exception, the remainder of the RDP classifier results were used. The nucleotide BLAST results indicate that the sequences found in this study cluster closest to other unidentified environmental sequences. The closest relatives originate from a wide variety of environments, from cold environments such as the Arctic or Antarctic, to rice field and garden soils,

to hyperthermal environments such as deep-sea chimneys and volcanic calderas.

Table A3-1: Comparison of sequence identification and classification between databases (Texas pyrosequencing company, RDP pipeline, and BLAST closest relatives).

	RDP Pipeline		Texas Output		NCBI Closest Relatives		
Sample		**% Total**		**% Total**		**Accession**	**Origin**
AH-P1 30cm Core	Acidilobales	91.00	Nitrososphaerales	91.24	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Thermoplasmatales	7.85	Thermoplasmatales	4.92	Uncultured archaeon clone 1-a83	HM065809	Receding glacier Switzerland
	Methanococcales	0.66	Methanobacteriales	2.28	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Methanomicrobiales	0.33	Methanosarcinales	0.65	Uncultured Thermoplasmata LL41	JQ071780	India desert soils
	Thermoproteales	0.17	Thermoproteales	0.45	Uncultured euryarchaeote W18	HQ271202	Sulfur biofilms petroleum water pipelines
			Methanomicrobiales	0.15	Archaeon clone Eur3Arch2	EU218602	Permafrost ground ice Canadian high Arctic
			Thermococcales	0.15			
AH-P1 70cm Core	Acidilobales	97.61	Thermoproteales	83.1	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Methanococcales	1.81	Nitrososphaerales	11.97	Unidentified clone A16S13	AF298080.1	California Sand DNA
	Thermoproteales	0.44	Unclassified	3.52	Haloferax volcanii (Halobacteriaceae)	CP001956.1	
	Methanopyrales	0.07	Methanobacteriales	1.41			
	Methanosarcinales	0.05					
	Halobacteriales	0.02					
AH-P1 30cm 4°C Acetate	Acidilobales	89.68	Nitrososphaerales	90.07	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Thermoplasmatales	8.27	Thermoplasmatales	5.99	Uncultured crenarchaeote 2413_1a	JF717746.1	CO2 affected Laacher See volcanic caldera
	Methanococcales	1.33	Methanobacteriales	2.41	Methanoculleus marisnigri JR!	CP000562	Black Sea anoxic sediments
	Methanomicrobiales	0.51	Thermoproteales	0.92	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Methanopyrales	0.21	Thermococcales	0.24	Uncultured Thermoplasmata LL41	JQ071780	India desert soils
			Methanosarcinales	0.21			
			Methanopyrales	0.09			

Sample	RDP Pipeline		Texas Output		NCBI Closest relatives (limit Archaea)		
		% Total		% Total		Accession	Origin
AH-P1 30cm 4°C Methanol	Acidilobales	99.64	Nitrososphaerales	99.74	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Desulfurococcales	0.25	Thermoproteales	0.19	Haloferax volcanii (Halobacteriaceae)	CP001956.1	
	Methanococcales	0.07	Methanobacteriales	0.06	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Methanomicrobiales	0.04			Uncultured archaeon AVAglg16	HQ268010	Aerated soils under wet anoxic conditions
AH-P1 30cm 4°C CO_2	Acidilobales	100	Nitrososphaerales	99.30	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
			Thermoproteales	0.70	Uncultured crenarchaeote F160cmFL255	JN002694	Low temperature ultramafic rocks Norway
AH-P1 70cm 4°C Acetate	Acidilobales	97.61	Nitrososphaerales	94.14	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Methanococcales	1.81	Thermoproteales	3.98	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Thermoproteales	0.44	Methanobacteriales	1.78	Uncultured archaeon ARCu-140	GQ127384	Tibetan plateau meadow soil
	Methanopyrales	0.07	Halobacteriales	0.06	Uncultured archaeon GDIC2IK01CAZ21	JF528546	Anaerobic digestion of carrot waste
	Methanosarcinales	0.05	Methanosarcinales	0.04	Methanosarcina maziu Go1	JQ346757	
	Halobacteriales	0.02			Uncultured archaeon A0610D002_F23	AB655643.1	Japan rice paddy soil
AH-P1 70cm 4°C Methanol	Acidilobales	99.73	Nitrososphaerales	99.96	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Thermoproteales	0.23	Methanomicrobiales	0.04	Uncultured Thermoprotei PE.5.246523458_T1-H11	GU047760	Canadian high Arctic acidic wetland
	Methanomicrobiales	0.04			Uncultured archaeon Arch8	JQ822093.1	Anaerobic digester
					Uncultured archaeon 46	JN219984.1	Canadian high Arctic ice wedge

Sample	RDP Pipeline		Texas Output		NCBI Closest relatives (limit Archaea)		
		% Total		% Total		Accession	Origin
AH-P1 70cm 4°C CO_2	Acidilobales	60.08	Nitrososphaerales	60.55	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Methanomicrobiales	39.49	Thermoplasmatales	24.96	Uncultured archaeon IU-FSC-8_Ar-A038	AB380056	Japan upland rice field soils
	Thermoproteales	0.13	Methanosarcinales	7.86	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Methanocellales	0.13	Methanobacteriales	3.74	Uncultured archaeon ARCu-154	GQ127606.1	Tibetan plateau meadow soil
	Thermoplasmatales	0.09	Methanomicrobiales	1.33	Uncultred archaeon Amb_16S_arch_6651	EF021612	CO2 affected soil of a trembling aspen
	Methanopyrales	0.07	Thermoproteales	1.21	Uncultred Thermoprotei AL.61	Gu047747	Canadian high Arctic acidic wetland
			Thermococcales	0.23			
			Cenarchaeales	0.08			
			Halobacteriales	0.03			
			Methanocellales	0.01			
AH-P1 30cm 22°C Acetate	Acidilobales	92.08	Thermoproteales	96.04	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Methanomicrobiales	4.59	Nitrososphaerales	1.52	Methanogenic archaeon Prasan7	HM630576.1	
			Unclassified	1.52	Archaeon LL37A35	AJ745148.1	Anoxic riverbank riparian soil
			Unclassified Thermoprotei	0.91			
AH-P1 30cm 22°C Methanol	Acidilobales	64.33	Nitrososphaerales	64.33	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienne garden soil
	Thermoplasmatales	33.24	Thermoplasmatales	25.74	Uncultured archaeon CHRA E6	GU366921	Oregon Douglas-fir and Red Alder soils
	Methanococcales	0.86	Methanobacteriales	7.21	Uncultured Thermoprotei AL.58	GU047744	Canadian high Arctic acidic wetland
	Thermoproteales	0.86	Thermoproteales	1.97	Uncultured archaeon 1-a82	HM065810	Switzerland recending glacier
	Methanomicrobiales	0.51	Methanomicrobiales	0.41	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Methanopyrales	0.18	Thermococcales	0.29	Methanopyrus kandleri AV19	AE009439.1	2000m deep black smoker chimney
	Archaeoglobales	0.01	Methanosarcinales	0.05	Uncultured crenarchaeote 2413_1a	JF717746.1	CO2 affected Laacher See volcanic caldera

Sample	RDP Pipeline	% Total	Texas Output	% Total	NCBI Closest relatives (limit Archaea)	Accession	Origin
AH-P1 30cm 22°C CO_2	Acidilobales	94.77	Thermoproteales	96.74	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Thermoproteales	2.29	Unclassified Thermoprotei	2.17	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Halobacteriales	1.03	Unclassified	1.09			
	Methanomicrobiales	0.99					
	Archaeoglobales	0.44					
	Desulfurococcales	0.27					
	Methanococcales	0.21					
AH-P1 70cm 22°C Acetate	Acidilobales	87.20	Thermoproteales	80.15	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Halobacteriales	4.21	Nitrososphaerales	11.45	Thermococcus sp. 4557	CP002920.1	Deep-Sea hydrothermal vent area
	Methanosarcinales	3.57	Unclassified	6.1	Methanogenic archaeon Prasan7	HM630576.1	
	Methanomicrobiales	1.51	Unclassified Thermoprotei	1.53			
			Halobacteriales	0.76			
AH-P1 70cm 22°C Methanol	Acidilobales	87.14	Thermoproteales	76.92	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Thermoproteales	3.39	Unclassified	9.23	Uncultured Thermoplasmata W50mCGB760	JN002515	Low temperature ultramafic rocks Norway
	Thermoplasmatales	2.64	Nitrososphaerales	7.69	Uncultured archaeon AS.A60	Gu29876.1	Antarctic and Arctic glacial cryoconite holes
	Methanomicrobiales	3.39	Unclassified Thermoprotei	3.08	Uncultured archaeon 1-a82	HM065810	Switzerland recending glacier
			Methanobacteriales	1.54	Uncultured euryarchaeote Q14	EU888367	Antarctica Ross Sea mineral soils
			Methanosarcinales	1.54			

Sample	RDP Pipeline	% Total	Texas Output	% Total	NCBI Closest relatives (limit Archaea)	Accession	Origin
AH-P1 70cm 22°C CO_2	Acidilobales	87.17	Nitrososphaerales	83.51	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Methanomicrobiales	7.24	Methanobacteriales	6.03	Methanogenic archaeon Prasan7	HM630576.1	
	Methanococcales	5.30	Thermoproteales	5.15	Euryarchaeote D4.75-4	AF199374.1	Egypt Solar lake hypersaline
	Thermoplasmatales	0.04	Thermoplasmatales	4.40			
	Methanopyrales	0.24	Methanosarcinales	0.44			
			Methanomicrobiales	0.24			
			Methanopyrales	0.10			
			Thermococcales	0.07			
			Halobacteriales	0.07			
SIP 12-CO_2 Light	Desulfurococcales	80.00	NaN	NaN	Uncultured archaeon CH2a80	DQ228596	Sulfide-microbial incubator
	Methanopyrales	20.00			Uncultured close F99a13	DQ228530	Hydrothermal chimney
					Haloferax volcanii (Halobacteriaceae)	CP001956.1	
					Methanoculleus marisnigri JR1	CP001956.1	Black Sea anoxic sediments
SIP 12-CO_2 Heavy	Acidilobales	99.93	Nitrososphaerales	96.27	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Sulfolobales	0.03	Thermoproteales	3.73	Uncultured crenarchaeote 2413_1a	JF717746.1	CO2 affected Laacher See volcanic caldera
	Desulfurococcales	0.03			Haloferax volcanii (Halobacteriaceae)	CP001956.1	
SIP 13-CO_2 Light	Acidilobales	100	Nitrososphaerales	100	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
					Uncultured Thermoprotei PE.5.246523458_T1-H11	GU047760	Canadian high Arctic acidic wetland
SIP 13-CO_2 Heavy	Acidilobales	99.87	Nitrososphaerales	100	Candidatus Nitrososphaera sp. EN123	FR773158.1	Vienna garden soil
	Methanopyrales	0.13			Uncultured archaeon CHRA E6	GU366921	Oregon Douglas-fir and Red Alder soils
					Uncultured Thermoprotei PE.5.246523458_T1-H11	GU047760	Canadian high Arctic acidic wetland
					Haloterrigena turkmenica DSM 5511	CP001860.1	

Figure A3-1: Comparison between the identification of pyrosequencing results between the Research and Testing Laboratories LLC pyrosequencing company, the RDP classifier, and the nucleotide BLAST closest relatives. The resulting orders and percentages given from the Research and Testing Laboratories LLC and the RDP classifier are presented in descending order for each sample. The BLAST closest relative was used solely to confirms the identity of the OTUs found at >0.5 %. The resulting orders and percentages were similar for all databases, with one large difference. As the RDP classifier does not yet include the novel order Nitrososphaerales, the most abundant order in most samples was identified instead as Acidilobales. However, as both the RDP classifier and the closest BLAST relative both confirm these sequences as members of the Nitrososphaerales, this identification was used.