Investigating thermophilic methanotrophs in a Manitoba landfill biowindow

by

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Abstract

In Canada, landfills are responsible for 23% of all emissions of methane, a greenhouse gas with 28x the potency of carbon dioxide. Gas collection systems can be used to mitigate landfill emissions, but they are not feasible for many municipal solid waste facilities in Canada and around the world. This has led to the increased importance of exploring innovative biocover solutions which incorporate on-site compost materials to enhance methane mitigation by microorganisms known as methanotrophs. Methanotrophs consume methane gas as their carbon source, producing carbon dioxide and water as by-products. Recently the exploration of these bacteria in landfill biotic systems, such as biocovers and biowindows, has become a topic of interest. However, relatively little is known about methanotrophs' roles in thermophilic biowindows found in continental climates. This thesis examines the microbial community of biowindow soil alongside conventional landfill cover soil and on-site compost windrows, with a focus on aerobic methanotrophic bacteria. Initial methane oxidation potential of the different soil types were measured throughout various seasonal conditions, revealing high activity in the spring but substantially decreased levels of methane oxidation throughout drought-like conditions in the summer of 2021 and through into late fall. 16S rRNA analysis as well as amplicon sequencing of the methanotroph marker gene *pmoA* in the soil detected high proportions of methanotrophs, with a particular abundance of the cyst-forming *Methylocystis* and the thermophile *Methylocaldum*. Amplicon sequencing of *pmoA* using methanotroph enrichment culture DNA also identified a large proportion of unclassified organisms at the genus level, and phylogenetic analysis strongly suggested that these uncultured bacteria belong in the genus *Methylocaldum*. Enrichment cultures using soil inocula found that despite low initial oxidation potential methanotrophs responded positively given the optimum conditions, indicating their

resilience to desiccation and large temperature fluctuations occurring *in situ*. Attempted isolation of thermophilic methanotrophs resulted in the identification of *Meiothermus silvanus* via whole genome sequencing, the first documentation of this organism in a landfill environment and the first known record of a thermophilic organism being isolated from a continental climate landfill. *M. silvanus* belongs to a genus of heterotrophic thermophiles that has been previously suggested to display synergistic relationships with *Methylosinus*, a methanotroph genus that was also detected in this study by amplicon sequencing of both soil and enrichment cultures. This study demonstrated that even in a climate that faces weather extremes exceeding -30°C to +30°C, thermophilic methanotrophs persist throughout the seasons and presumably play a substantial role in methane oxidation within the biowindow. The research performed in this thesis helps to increase our understanding of landfill biotic systems and their application in a continental climate.

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Dedication

This work is for my niece and nephews,

in the hope that you grow old in a world better than ours is today.

And for Mario. For being everything I needed you to be.

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List of Abbreviations

BRRMF	Brady Road Resource Management Facility
BW	Biowindow
Ce	Cerium
CH_4	Methane
CO_2	Carbon dioxide
Cr	Chromium
CS	Cover soil
Cu	Copper
CW	Compost windrow
gDNA	Genomic DNA
GWP	Global warming potential
GHG	Greenhouse gas
LFG	Landfill gas
MOB	Methane oxidizing bacteria
MOP	Methane oxidizing potential
NMS	Nitrate mineral salts medium
O_2	Oxygen
OTU	Operational taxonomic unit
pMMO	Particulate methane monooxygenase
sMMO	Soluble methane monooxygenase

Chapter 1: Introduction

1.1 Methanotrophs

Methanotrophs, or methane oxidizing bacteria (MOB), are a group of aerobic Gram-negative bacteria that use methane (CH_4) as their sole source of carbon and energy, producing only carbon dioxide (CO_2) and water as by-products (Whittenbury et al. 1970). Methane is a potent greenhouse gas (GHG) with 28 times the global warming potential (GWP) of carbon dioxide over a period of 100 years, and is one of the six greenhouse gases outlined in the Kyoto protocol as requiring mitigation (Shukla et al. 2019; IPCC 2022a). Natural sources make up roughly half of global methane production and include wetlands, melting permafrost, oceans, wildfires, and termites, although it is also produced anthropogenically through oil and gas production, rice paddies, animal agriculture, and landfills (Kirschke et al. 2013). Methanotrophs have been identified in many of these methane-rich environments (Hanson and Hanson 1996; Guerrero-Cruz et al. 2021). These organisms belong to a larger subset of bacteria known as methylotrophs, which are classified by their ability to use single-carbon compounds as a carbon and energy source, including methane or methanol (Hanson and Hanson 1996).

1.1.1 Types of methanotrophs

Methanotrophs are found in three different phyla: Proteobacteria, Verrucomicrobia, and Methylomirabilaeota (Table 1) (Houghton et al. 2019). The Proteobacteria are further divided into the subgroups Type I (Gammaproteobacteria) and Type II (Alphaproteobacteria), both of which assimilate formaldehyde produced from the oxidation of methane into methanol (Hanson and Hanson 1996). Where these two groups differ principally is in their formaldehyde assimilation pathway, as Type I MOB use the Ribulose Monophosphate (RuMP) pathway, while

Type II MOB employ the Serine cycle (Hanson and Hanson 1996). A third subgroup encompasses the Verrucomicrobia, which utilize the Calvin-Benson-Bassham (CBB) cycle to assimilate carbon and produce biomass (Houghton et al. 2019). These organisms are unique in that they are obligate acidophiles and are found in acidic geothermal environments (Houghton et al. 2019). Verrucomicrobia methanotrophs are represented by only one family, *Methylacidiphilaceae,* and two known genera, *Methylacidiphilum* and *Methylacidimicrobium* (Table 1) (Houghton et al. 2019).

The various types of methanotrophs differ also in their preferred ratio of oxygen to methane, as Type I are typically found to outcompete Type II in areas with high oxygen:methane ratios, or methane-limiting conditions (Amaral and Knowles 1995; Hanson and Hanson 1996; Guerrero-Cruz et al. 2021). Conversely, Type II methanotrophs thrive and outcompete Type I in methane-rich and oxygen-limiting environments such as aquatic plant rhizospheres (Hanson and Hanson 1996). Type II MOB also have been shown to dominate under copper or nitrogen-limiting conditions (Hanson and Hanson 1996). Overall, it has been widely shown that methane, oxygen, and nitrogen concentrations are key determinants for which types of methanotrophs will occupy a niche (Hanson and Hanson 1996). Temperature likewise plays a role in differentiating these sub-groupings, where Type I methanotrophs have been more commonly found at lower temperatures as compared to Type II, and Verrucomicrobia are entirely thermophilic, thriving at temperatures of 50°C and above (Börjesson et al. 2004; Houghton et al. 2019).

Candidate phylum NC10 represents a recently identified unique type of methanotroph that includes the genus *Methylomirabilis* (He et al. 2016). These organisms are found in methane-rich anaerobic environments yet are able to perform a variation of aerobic methane oxidation by coupling it with denitrification (He et al. 2016). NC10 use oxygen produced from

nitric oxide to intra-aerobically oxidize methane under anoxic conditions (He et al. 2016). While this group technically performs methane oxidation aerobically, they are not found in aerobic environments and are therefore not a direct focus of this research since their presence is not expected, though their existence and unique metabolic pathway is worth noting.

Table 1. Classifications of aerobic methanotrophs.

MOB Type	Phylum	Class	Order	Family	Genus
Type I	Proteobacteria	Gammaproteobacteria	Methylococcales	*Methylococcaceae*	*Methylobacter* ***Methylococcus*** ***Methylocaldum*** *Methyloparacoccus* *Methylogaea* *Methylomagnum* *Methylomicrobium* *Methylomarinum* *Methylomonas* *Methylosoma* *Methylosphaera* *Methyloterricola* *Methylotetracoccus* *Methylovulum*
				Methylothermaceae	*Methylohalobius* ***Methylothermus*** ***Methylomarinovum***
Type II		Alphaproteobacteria	Hypomicrobiales	*Methylocystaceae*	*Methylosinus* *Methylocystis*
				Beijerinckiaceae	*Methylocella* *Methylocapsa* *Methyloferula*
	Verrucomicrobia	Verrucomicrobia	Methylacidiphilae	*Methylacidiphilaceae*	***Methylacidiphilum*** ***Methylacidimicrobium***
	Candidate Phylum NC10				*Methylomirabilis*

Genera for which thermophilic representatives have been reported are shown in bold.

NCBI Taxonomy Browser (Schoch et al. 2020)

1.1.2 Methane monooxygenase

While methanotrophs can differ in the biochemical pathway used to ultimately produce carbon dioxide, all of these organisms require the enzyme methane monooxygenase (MMO) to initiate the conversion of methane into methanol (CH_3OH) (Kolb et al. 2003). In most methanotrophs, methanol is next oxidized into formaldehyde by methanol dehydrogenase, and can then be changed into formate by the tetrahydrofolate or tetrahydromethanopterin pathway (Kolb et al. 2003). Following this, formate dehydrogenase is used to oxidize formate into carbon dioxide as the end product (Semrau et al. 2018). This pathway differs for members of the Verrucomicrobia group, which use the XoxF enzyme to generate formate directly from the oxidation of methanol, skipping the formaldehyde intermediate (Houghton et al. 2019). Verrucomicrobia use the CBB cycle for carbon uptake during this process, while other methanotrophs use either the RuMP or Serine pathway (Houghton et al. 2019). Several studies have shown that rare earth metals cerium and lanthanum are required for methanotrophy in the Verrucomicrobia family (Sharp et al. 2014; Pol et al. 2014).

MMO is found in two forms that share no genetic or structural homology. There is a membrane bound enzyme known as particulate MMO (pMMO), and a cytoplasmic, soluble MMO (sMMO) (Hanson and Hanson 1996). Methanotrophs can have either one or both of the enzyme types, although pMMO is found in nearly all species and only a few known MOB carry sMMO alone, including *Methylocella* and *Methyloferula* (Pandey et al. 2014). The gene *pmoA* of the *pmoCAB* operon encodes the large subunit of pMMO and is commonly used as a marker gene in molecular surveys as it is highly conserved in most methanotrophs (Koo and Rosenzweig 2021). For detecting the soluble version of this enzyme, the *mmoX* gene on the *mmoXYBZC*

operon of sMMO can be targeted (Koo and Rosenzweig 2021). Copper (Cu) plays an important role in the regulation of these enzymes, as pMMO is expressed in the presence of Cu, whereas sMMO activity is inhibited by it (Semrau et al. 2010). This is known as the "copper-switch". The copper-switch occurs due to differentiating metal compositions of the two enzymes forms, with copper being an essential metal-binding component of pMMO (Semrau et al. 2018). A recent cryo-EM study confirmed the presence of copper in the PmoA, PmoB, and PmoC subunits (Chang et al. 2021). On the other hand, sMMO is a soluble di-iron monooxygenase that has no confirmed copper molecules present (Semrau et al. 2018). Therefore, the addition or omission of copper in media plays a significant role in selecting for different types of methanotrophs when cultivating these organisms.

1.1.3 Thermophilic methanotrophs

The majority of known methanotrophs are mesophilic with an optimal growth temperature between 20°C and 40°C, however an increasing number of thermophilic or thermotolerant species have been identified in recent years (Houghton et al. 2019). Thermophilic bacteria are defined as having a temperature optima above 40°C, whereas thermotolerant refers to organisms with a maximum growth temperature between 42°C and 50°C (Houghton et al. 2019). Psychrophilic species which thrive in temperatures below 20°C have also been recognized (Börjesson et al. 2004). Consequently, this group of bacteria exist in an exceptionally wide range of known temperatures, despite the bulk of the characterized strains being mesophilic. As it stands mesophilic and psychrophilic methanotrophs are comprised of greater than 50 isolated species, while the number of thermophilic methanotrophs is less than half of that (Houghton et al. 2019; Islam et al. 2020; Hogendoorn et al. 2021; Picone et al. 2021). Thermophilic

methanotrophs are most commonly found in hot springs, volcanoes, and geothermal soils – areas of extreme heat and high concentration of methane emissions (Houghton et al. 2019; Islam et al. 2020; Picone et al. 2021). Interestingly, in addition to those previously well-known locales, thermophilic methane oxidizing activity was recently detected in landfill soil in a continental climate (Berenjkar et al. 2021). While several molecular based studies have detected sequences belonging to this group, thermophilic methanotrophs have not yet been isolated from landfills (Huang et al. 2005).

Despite these organisms' expansive scope of temperature capabilities, many reports show the highest rates of methane oxidation occur predominantly under mesophilic conditions (Raksha et al. 2020). That is to say, while MOB may grow in hot or cool environments, their ability to oxidize methane is consistently most efficient between 20°C - 40°C (Raksha et al. 2020). An exception to this was found in a recent landfill biowindow study, which revealed through laboratory batch experiments that soil methane oxidation rates were higher at thermophilic than mesophilic temperatures (Berenjkar et al. 2021).

1.2 Landfills

In Canada, municipal solid waste (MSW) landfills are the main system for waste disposal (Government of Canada 2021). Materials that end up at landfill facilities include garbage from households, businesses, institutions, and construction, as well as recyclable and compostable materials (Government of Canada 2021). The purpose of these sites is aimed at disposing of MSW safely while minimizing environmental and social impacts (Government of Canada 2021). While modern facilities use a variety of implementations to achieve that goal, including

recycling, composting, leachate collection, gas capture, and incinerating, pollutants are still continually released into the air and water (Government of Canada 2021).

1.2.1 Methane emissions

Methane is a potent greenhouse gas with 28 times the GWP of carbon dioxide and globally accounts for 13% of all GHG emissions (IPCC 2022b). Rising GHG emissions have led to a 1.09°C increase in global surface temperature since 1850 (IPCC 2022b). In their most recent report, the Intergovernmental Panel on Climate Change (IPCC) has declared the need to limit this temperature increase to less than 1.5°C by the year 2040 in order to reduce the risk of the severe and irreversible impacts of climate change (IPCC 2022b).

The top three anthropogenic sources of atmospheric methane are agriculture, oil and gas, and landfills (Climate Watch 2022). In 2019, global anthropogenic methane emissions totalled 3870 $MtCO_2e$ (Megatonnes of CO_2 equivalents), with emissions from waste contributing 665 $MtCO_2e$ of that (Climate Watch 2022). In Canada, MSW account for 23% of our national methane emissions (Government of Canada 2022). In 2019, Canadian landfills produced 1,420 kilotonnes (kt) of methane, or approximately 39 $MtCO_2e$ (Government of Canada 2022). Of that, 473 kt were recovered, leaving 851 kt of methane being emitted into the atmosphere, or 23 $MtCO_2e$ (Government of Canada 2022). This is referred to as fugitive emissions.

There are several different gases that are emitted from landfills, with methane accounting for 50% of these and the remainder primarily including carbon dioxide and volatile organic compounds (VOCs) (Scheutz et al. 2009). Methane is produced during the anaerobic biodegradation of waste, in a process called methanogenesis (Grillo 2014). There are 5 phases in the life span of a landfill, each governed by the roles of important microorganisms (Figure 1).

Phase 1, or the Initial Phase, describes the placing of the waste, an increase in moisture content and microbial population, and the initiation of decomposition under aerobic conditions (U.S. EPA 2006). Phase 2 is the Transition Phase, which is the brief period in which oxygen is quickly consumed by bacteria and conditions switch to anaerobic, resulting in carbon dioxide displacing oxygen (U.S. EPA 2006). Phase 3, or Acid Formation, encompasses the hydrolysis of biodegradable waste, a decrease in pH, and the rapid consumption of substrates and nutrients (U.S. EPA 2006). Phase 4 is Methane Fermentation, in which methanogens convert acidic intermediates from Phase 3 into methane and carbon dioxide (U.S. EPA 2006). It is during this phase that most of the waste decomposes and heat is produced. Phase 5 represents the Final Maturation and Stabilization of the landfill (U.S. EPA 2006). During this phase limiting nutrients such as Phosphorus leads to a decreased rate of biological activity, and negligible amounts of methane are produced (U.S. EPA 2006). Oxygen also beings to reappear as it seeps in from the atmosphere, along with the presence of oxidized organisms (U.S. EPA 2006).

To attempt to mitigate fugitive landfill gas (LFG) emissions during this process, collection systems are commonly installed in modern landfills above a certain capacity (Riham et al. 2019). Some of these gas collection systems may use LFG as energy to power the landfill site, or a neighbouring region, while others may simply flare the gas off (Riham et al. 2019). However even when gas collection systems are in place, uncaptured LFG still escapes as fugitive emissions with some literature citing as little as 50% efficiency (Barlaz et al. 2012). Unfortunately, these systems are also very costly to install, and are only practical for large scale waste management sites (Barlaz et al. 2012). Currently in Canada, just over 100 MSW landfills have gas collection systems instated out of a total of 3000 sites (Government of Canada 2022).

Around half of that total are active landfills, while the rest are closed (Government of Canada 2022).

As a standard for minimizing emissions as well as leachate, landfill covers typically are used to cap off cells that have reached capacity (Chiemchaisri et al. 2012). This generally involves a clay cap being placed on top of the completed landfill cell followed by a thin layer of topsoil to promote vegetation growth (Chiemchaisri et al. 2012). This method prevents some LFG from escaping, however fugitive emissions are still an ongoing concern due to erosion and cracking of the clay cover (Huber-Humer et al. 2008). For the gases that do escape through the cover, they are met with a very poor environment for methane oxidation. Clay has a very low porosity, low nutrient content, and does not provide suitable conditions for methanotrophy (Scheutz et al. 2009). Therefore, any gases that are able to evade the cover soil will not be in contact with sufficient methane oxidizing bacteria to convert methane into carbon dioxide, leading to increased GHG emissions.

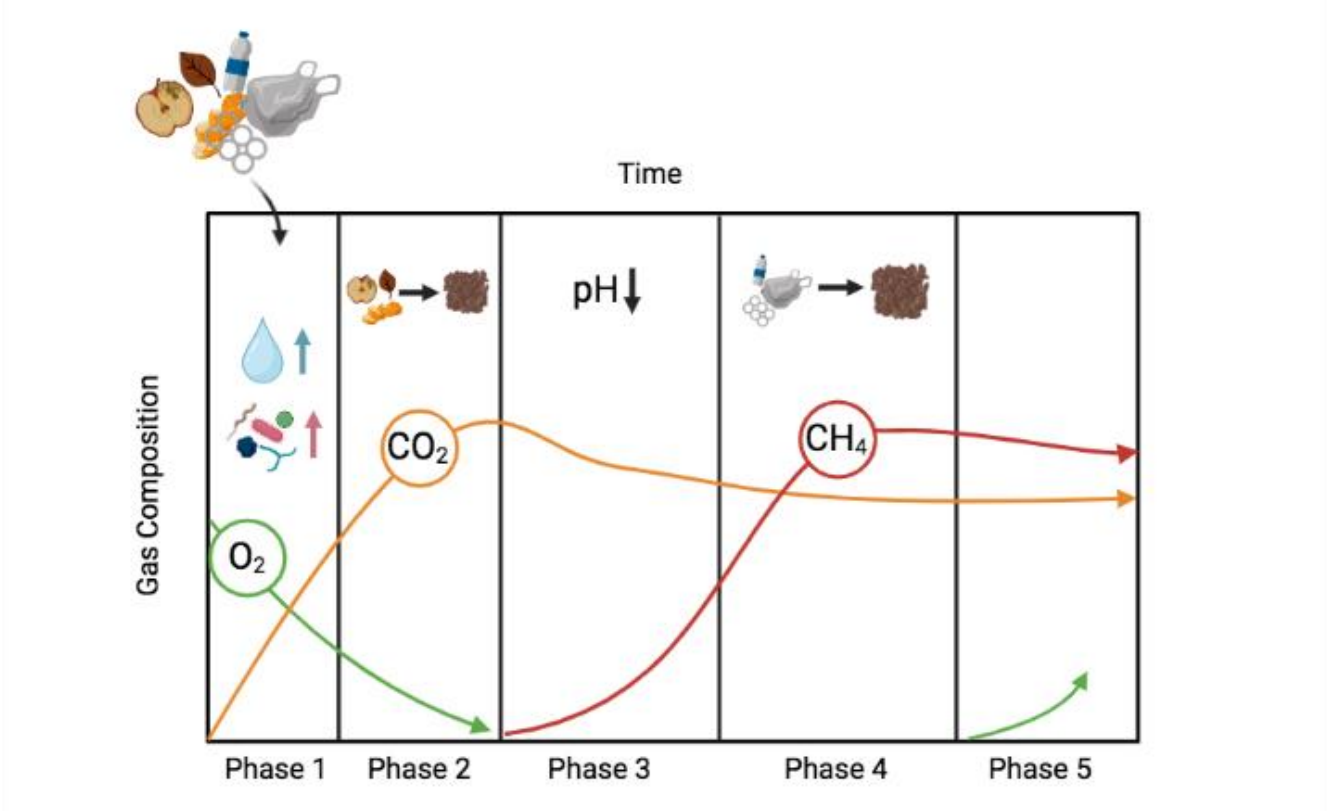


Figure 1. Phases of a municipal solid waste landfill. Relative gas composition shown on Y-axis. Diagram created with BioRender.com.

1.2.2 Landfill biotic systems

Over the last two decades a number of alternatives to conventional soil covers have been explored through the engineering of landfill biotic systems (Humer and Lechner 1999). The goal of these methods is to optimize the landfill cover materials for bacterial methane oxidation and subsequently reduce fugitive emissions (Humer and Lechner 1999). One example of this is a biocover, which uses compost materials of a specific porosity and moisture content and covers a large area of a finished landfill in place of a typical cover (Sadasivam and Reddy 2014). As

compared to conventional topsoil or clay covers, compost has been proven to have ideal porosity, moisture content, and nutrient availability for methane oxidizing bacteria (Sadasivam and Reddy 2014). It is essential that mature compost materials, with little easily available organic matter, are used for these approaches to limit competition with heterotrophic organisms (Huber-Humer et al. 2008). Mature compost is comprised primarily of humic acids and derivatives of cellulose and lignin, which are only slowly degradable and therefore less appealing to heterotrophs.

Biowindows are an example of another landfill biotic system and are highly similar to biocovers, with the key difference being a biowindow covers a relatively small, excavated area of the landfill versus an entire section (Huber-Humer et al. 2008). Biowindows are sometimes described as "pilot-scale biocovers". Biocovers and biowindows require a gas distribution layer (GDL) made up of course, highly permeable materials that allow LFG to be distributed evenly throughout the system (Huber-Humer et al. 2008). Common GDL constituents include gravel, tire shreds, and glass (Stern et al. 2007; Philopoulos et al. 2008; Berenjkar et al. 2021) The GDL is at the base of the system and precedes the "oxidation layer", which is designed to contain materials with appropriate qualities to promote growth of methane oxidizing bacteria (Sadasivam and Reddy 2014). Examples of these methanotroph-friendly materials include sewage sludge, peat, or compost. Materials that are readily available on-site at landfills are typically ideal for these biotic systems, such as yard waste and leaf compost (YWLC) from curbside collection programs, or biosolids compost (BSC), the nutrient-rich by-product of sewage treatment (Sadasivam and Reddy 2014; Berenjkar et al. 2021). Landfill biotic systems have potential for implementation in waste management facilities of small, rural populations, such as First Nations communities of Canada. The implementation of low maintenance, inexpensive biotic systems such as biocovers or biowindows would seek to address some of the environmental issues of

current waste management practices that stem from impacts of colonialism and lack of government funding (Oyegunle and Thompson 2018).

1.2.2.1 Brady landfill pilot bio-window

A recent pilot biowindow study at Brady Road Resource Management Facility (BRRMF) in Winnipeg, Manitoba is among a minority of research that exists on landfill biotic systems in continental climates, based on the Köppen-Geiger classification system (Kottek et al. 2006; Berenjkar et al. 2021). To construct this biowindow, a 3.5 m x 2.5 m area in the existing clay landfill cover was excavated from a cell that was filled and closed in 1993 (Figure 2) (Berenjkar et al. 2021). The GDL was made of 0.55 m limestone gravel, and the oxidation layer was comprised of 0.75 m of YWLC and BSC at a ratio of 1:4 (Berenjkar et al. 2021). Gas measurements were taken over a span of 2 years using probes installed at various depths in the window to evaluate its performance (Berenjkar et al. 2021). The study revealed thermophilic conditions present in the biowindow during summer, and laboratory batch tests confirmed the activity of thermophilic methanotrophs (Berenjkar et al. 2021). Determination of methane oxidation rates revealed that methane was oxidized at a higher rate at thermophilic temperatures, compared to mesophilic (Berenjkar et al. 2021). It is hypothesized that compost windrows are a potential source of thermophilic inoculum for the biowindow, as during the composting process high internal temperatures are reached for a sustained duration of time (Halet et al. 2006). Berenjkar's biowindow study was the first of its kind in analyzing wide seasonal fluctuations including frost-cover, in using BSC supplemented with YWLC, as well as in investigating the presence of thermophilic methane oxidizers in landfills. Further investigation of the

methanotrophs in the biowindow is necessary to better understand the community of organisms occupying this niche.

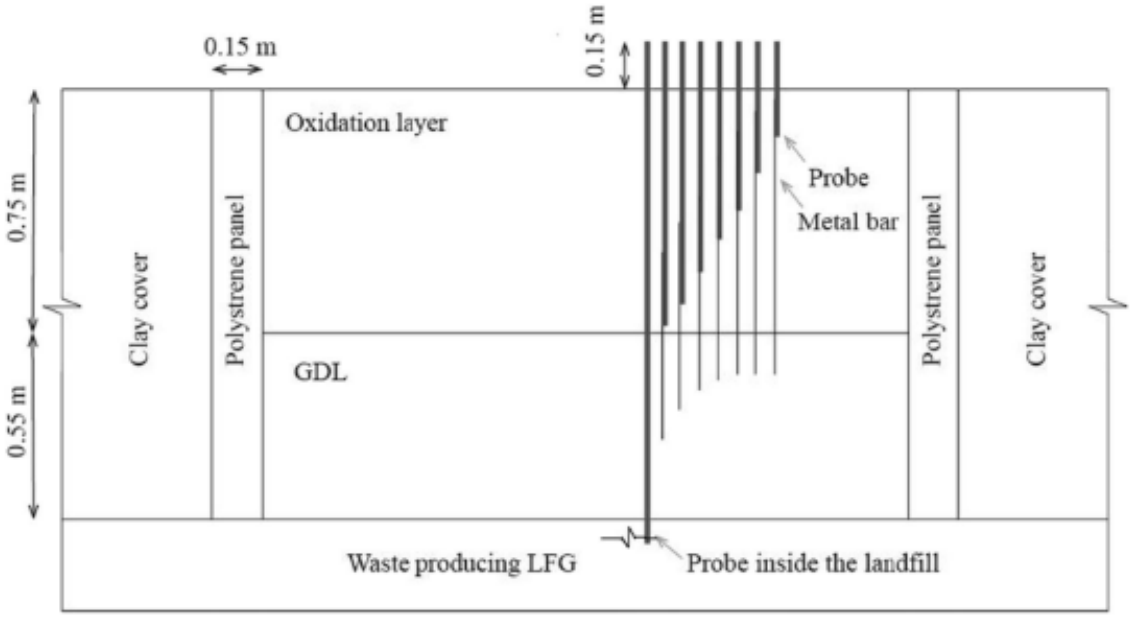


Figure 2. Cross-section of pilot biowindow at BRRMF. Extracted from Berenjkar et al. 2021.

1.2.2.2 Laboratory batch tests and column tests

While field-scale testing of landfill biotic systems offers many benefits, smaller laboratory scaled experiments may be used to optimize methane oxidizing conditions before moving to the field. Field tests are a substantial amount of work and are best used for longitudinal studies. Laboratory batch experiments performed in sealed bottles are commonly used as a rapid way of testing parameters for methanotrophy. These batch tests were implemented by Niemczyk et al. (2021) and Berenjkar et al. (2021) for determining ideal conditions to apply in further, larger scale analysis of methane oxidation at BRRMF. Major conclusions from these assays were that YWLC had a higher methane oxidation potential (MOP) than BSC, but YWLC mixed with BSC was more efficient than YWLC alone (Niemczyk et al. 2021). Optimal moisture content was reached between 50% - 65% (Berenjkar 2021; Niemczyk et al. 2021).

Humer and Lechner (1999) described an alternative method for analyzing methanotrophy, in which flow-through columns are filled with materials to optimize methane oxidation. Columns of this type can be a useful progression from batch tests, as they provide a continuously fed microcosm for experimental analysis. Such columns were used by Niemczyk et al. (2022) for analysis of materials for the BRRMF biowindow. They are constructed by filling a large PVC or Plexiglas tube with the desired mixture of materials for methane oxidation (Figure 3) (Niemczyk et al. 2022). Gas comprised of methane and carbon dioxide flows into the bottom of the column to simulate LFG, and oxygen flows across the topmost layer to oxygenate the system and simulate wind (Niemczyk et al. 2022). Gas sampling ports all along the column allow for in depth analysis of the activity at each level of the system (Niemczyk et al. 2022). These

microcosms can run for longer durations that batch scale tests, more accurately mimic landfill conditions, and allow for thorough analysis of the vertical profile of packing materials (Niemczyk et al. 2022). For optimization of the BRRMF pilot biowindow, column tests were performed to determine a desired ratio of compost to limestone gravel for ideal porosity and aeration (Berenjkar 2021; Niemczyk et al. 2022). The results of these experiments showed that maximum methane removal efficiency occurred in the columns with the addition of ¼" gravel and compost, at a ratio of 1:7 .

As the focus of both the column work and batch tests were on gas flow and methane oxidation, the microbial community present in these columns remained unexamined. Given the efficiency of the columns it was hypothesized that these microcosms are home to an abundance of methane oxidizing bacteria.

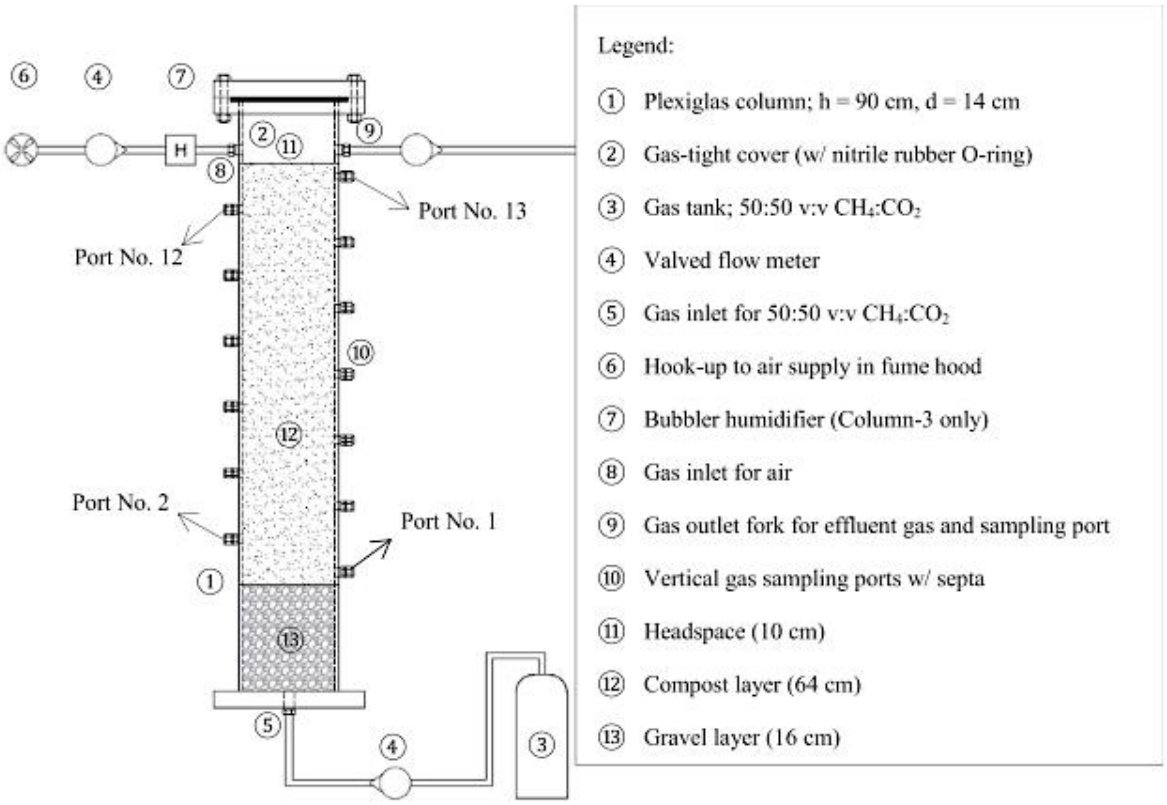


Figure 3. Laboratory column experiment set-up. Extracted from Niemczyk et al. 2022.

1.2.3 Molecular analysis of landfill microbiomes

While a number of physical variables influence methane oxidation in landfills, the widespread community of microorganisms present in this environment plays an indispensable role and can differ widely under fluctuating conditions. Although most landfill soil conditions and therefore their respective microbial communities are mesophilic, several studies have reported moderately thermophilic to thermophilic temperatures, often within biocover systems (Berenjkar et al. 2021; Yang et al. 2021). Metagenomic studies of landfills in various climates have identified a variety of mesophilic and some thermophilic methanotrophs, most commonly

Methylobacter, *Methylococcus*, *Methylocystis*, and *Methylocaldum* (Gebert et al. 2009; Lin et al. 2009; Reddy et al. 2019). Through molecular analysis it was discovered that an additional characteristic of interest in some methanotrophs is their ability to perform sulfide reduction, thereby aiding in odor mitigation of landfills (Lee et al. 2018; Schmitz et al. 2022). Recently, a number of methanotrophic organisms were identified as possessing the sulfide:quinone oxidoreductase (SQR) gene, providing them with the ability to oxidize CH_4 and H_2S simultaneously (Schmitz et al. 2022). The discovery of this unique capability is another example of the versatility of aerobic methanotrophs, and the need to explore this group of organisms further is important since H_2S is a major source of unpleasant odour associated with landfill fugitive emissions (Catena et al. 2022).

Temperature conditions within landfills have been shown to fluctuate significantly throughout seasonal changes, particularly in continental climates. The extensive impacts these oscillations may have on methanotroph communities has received minimal exploration, particularly in ecosystems that experience conditions ranging from extreme frost cover to thermophilic temperatures. This study seeks to bridge that gap in the literature by examining the effects of seasonal changes in a continental climate on the microbial community of engineered landfill biotic systems.

1.3 Research objectives

The goal of this research is to increase understanding of the microbial community present in a continental MSW landfill biowindow through seasonal changes, based on the hypothesis 1) that populations would change with seasons and that 2) there is a resident population of

thermophilic methane oxidizing bacteria. Furthermore, knowledge of the microbiome will add to the limited existing research on methanotrophy in such landfills. The specific objectives of this thesis are:

1) To analyze the diversity and abundance of methanotrophs as well as whole bacteria community in engineered landfill soil columns.
2) To collect additional field data for the BRRMF biowindow site to supplement previous studies. Field data archive currently includes 2016 – 2020.
3) To identify the entire community of bacteria present in the BRRMF biowindow across varying seasonal conditions, and compare to conventional clay cover soil and a recently degraded industrial compost windrow. While the whole microbial community is of interest, an emphasis will be placed on methanotrophic bacteria as the responsible entities for landfill methane oxidation.
4) To investigate the presence of culturable thermophilic methanotrophs in landfill soil samples through enrichment, isolation, and characterization. The prevalence of thermophilic conditions in this site has been of great interest, as thermophilic methanotrophs have not yet been isolated from a landfill environment. Given the high levels of methane oxidizing activity previously detected at thermophilic temperatures, it is hypothesized that these organisms are present and can be enriched for and isolated in pure culture.
5) To determine the abundance of methanotrophs present in biowindow soil, as compared to conventional clay cover soil and fresh compost from the Brady landfill composting facility. This will also be done across seasonal comparison.

Chapter 2: Materials and Methods

2.1 Column soil extraction

Column experiments were performed to determine optimal aeration of the biowindow based on the size of limestone gravel mixed with compost. The methods of this experiment are outlined in Berenjkar et al. (2021). Columns were constructed out of 90 cm tall PVC tubing with 15 cm diameter, and gas sampling ports every 5 cm (Figure 3). Both columns were packed with an initial 12 cm GDL comprised of ½" limestone gravel, followed by 45 cm of a unique compost and gravel mixture. For this, Column 1 used compost and ¼" limestone gravel, while Column 2 included compost plus ½" limestone gravel. The compost was retrieved from the BRRMF biowindow in November 2019. A synthetic LFG mixture of CH_4/CO_2 at a ratio of 50:50 flowed into the system through a port below the GDL, while another inlet allowed for air to pass over top of the column to simulate wind and promote the diffusion of oxygen into the system. For microbiological analysis of the methanotroph community present in these microcosms, columns at the end of their final stabilization period were unpacked individually to remove soil. This involved removing ~30 g of soil from each sampling port spaced 5 cm apart, resulting in a composite soil mixture made up of all soil samples in equal parts. Each column was unpacked in this manner individually. These two composite soil samples were later used for gDNA extraction, 16S rRNA amplicon sequencing, and qPCR (see section 2.4).

2.2 Landfill soil sampling

Soil was collected from BRRMF. Collection was performed on May 3, August 16 & 25, and October 25 of 2021 in order to examine any differences in community structure throughout

seasonal changes. Sampling sites included an engineered biowindow constructed in October 2016, conventional clay cover adjacent to the biowindow, and compost windrows (Table 2). The biowindow was comprised of a 0.55 m limestone gravel gas distribution layer beneath a 0.75 m oxidation layer, which was a 1:4 mixture of yard waste and leaf compost (YWLC) and biosolids compost (BSC) (Berenjkar et al. 2021). Both the biowindow and the clay soil samples originated from landfill cells filled and capped in 1993. Compost samples were taken from a 2020 YWLC windrow (May & August samples) and a 2020 source-separated organics (SSO) compost windrow (October). Two sampling events occurring during the month of August (Appendix Table 6). Soil samples were taken every 20 cm up to a depth of 60 cm and were collected by drilling a soil core using an auger and depositing samples into Ziploc bags (Lee et al. 2018; Reddy et al. 2019). Upon return to the lab all soil samples were sifted through a 2 mm metal sieve, which was cleaned with ethanol in between sieving each sample (Lee et al. 2018). All soil property analyses and enrichment cultures were performed within 24 hours. Soil that was not immediately used for analysis was stored at -80°C.

Table 2. Description of each soil sampling site.

Site classification	Description	Date of origin
Biowindow (BW)	Pilot biowindow filled with YWLC and BSC, covering 3.5 x 2.5 m area of a 1993 waste cell	2016
Clay cover soil (CS)	Conventional clay covering filled waste cell	1993
Compost windrow (CW)	YWLC or SSO from municipal collection program	2020

2.2.1 Soil property analysis

Soil samples were analyzed for different physicochemical parameters in order to study any correlation to methanotroph communities across various seasons. All analyses were performed using soil samples from specific depths, unless otherwise noted. Moisture content was determined gravimetrically by weighing 5 g of sieved soil into weigh boats and placing in 55°C incubator for 72 hours (Freitag et al. 2010). Final dry weight was measured and used to calculate total moisture content. Soil pH was measured by adding 1 g of sieved soil into test tubes with 10 mL of distilled H_2O. The tubes were vortexed for 5 seconds, and incubated at room temperature for 30 minutes before measuring pH using Orion 420A pH meter (Carere et al. 2017).

Initial methane oxidation potential (MOP) protocols were based off of Berenjkar et al. (2021) and Niemczyk et al. (2021). MOP was determined by weighing 1 g of soil into 120 ml serum bottles sealed with rubber stoppers and aluminum crimp caps. 25 mL of CH_4 was injected into bottles using a gas-tight sample-lock syringe, to achieve a headspace of 20% CH_4. 1 mL samples were removed from the headspace every 24 hours to monitor the levels of O_2, CH_4, and CO_2 by gas chromatography (GC) using Agilent 490 Micro GC, with nitrogen as the carrier gas. Bottles were incubated at 22°C, 45°C, and 55°C to represent mesophilic, moderately thermophilic, and thermophilic conditions. As a negative control, soil samples were added to bottles using the same method but without the addition of CH_4 to the headspace, to determine baseline heterotrophic respiration of soil microorganisms. All soil analyses were performed in triplicate.

2.3 Growth and isolation of methanotrophs

Nitrate Mineral Salts (NMS) medium was used for all growth experiments. NMS is a defined medium with no soluble organic carbon source, and was composed of 0.5 g KNO_3, 0.5 g $MgSO_4$, 0.1 g $CaCl_2$, 0.26 g KH_2PO_4, 0.33 g Na_2HPO_4, 0.26 mg Na_2MoO_4, 0.2 mg sodium ferric EDTA, and 1 mL Trace Elements solution, per 1 litre of Milli-Q® water (Whittenbury et al. 1970). The composition of Trace Elements solution was as follows: 0.25 $FeSO_4$ g 0.2 g $ZnSO_4$, 0.01 g $MnCl_2$, 0.025 g $CoCl_2$, 5 mg $NiCl_2$, 7 mg H_3BO_3, 0.125 g EDTA disodium salt, 1 L Milli-Q® water (Whittenbury et al. 1970).

For regulation of the copper switch, media was supplemented with 8 µM $CuCl_2$ to promote expression of pMMO (Semrau et al. 2018). Cerium ($CeCl_3$) was added to selected

media for the attempted isolation of Verrucomicrobia, at a concentration of 250 nM (Sharp et al. 2014). NMS plates were solidified with 1.5% Bacto agar. Wheaton-type bottles (120 mL) or Balch tubes (27 mL) were used for all liquid cultures and were sealed with rubber stoppers and aluminum crimp caps. CH_4 was added to all cultures at a concentration of 20% CH_4-in-air using a 0.2 µm filter fastened onto a syringe.

2.3.1 Enrichment Cultures

Enrichment cultures were made by adding 2 g of soil to 10 ml of NMS in a 250 mL Erlenmeyer flask. Soil slurry was then vortexed for 1 minute and placed in a shaking incubator at 200 rpm for 10 minutes to release microbes and sediment soil (Wartiainen et al. 2006). 1 ml of the supernatant was added to 9 ml of NMS in 120 serum bottles supplemented with Ce, Cu, or neither. Cultures were incubated at either 22°C, 45°C, or 55°C. Methane oxidation was monitored via gas chromatography by removing 1 mL samples from the headspace to measure O_2, CH_4, and CO_2 gas levels. Gases were replenished approximately every 7 days or when oxygen levels began to deplete, by removing the rubber stopper and allowing for oxygenation in a sterile biosafety cabinet for 30 minutes before injecting additional CH_4 (Reddy et al. 2019). After 14 days of incubation, 1 mL of each culture was passaged into 9 mL of fresh, sterile NMS under the same conditions (Wartiainen et al. 2006). Cycle was repeated for a total of 3 passages (6 weeks) to enrich for methanotrophic bacteria.

2.3.2 Serial dilution plating

Following growth of enrichment cultures, a dilution series was performed on each sample, to a factor of 10^{-7} (Bodrossy et al. 1997). Samples of 100 µL of 10^{-5}, 10^{-6}, and 10^{-7}

dilutions were spread plated onto NMS plates, with corresponding media additives and incubation temperatures from the enrichment cultures. Cycloheximide was added to select plates at a concentration of 50 µg/mL (McGenity et al. 2017). Plates for all experiments were incubated for two weeks in BD GasPak^{TM} EZ containers with the addition of CH_4 through rubber tubing, at an estimated concentration of 20% CH_4-in-air (Wartiainen et al. 2006). Gas was replenished every 2-3 days. After the two-week period, plate counts were done to determine colony forming units (CFU) per mL. Select colonies were picked and streak plated for purification until the isolate was determined to be morphologically pure. Pure strains were tested for growth in the absence of CH_4. If negligible growth was shown on control plates after two weeks, isolates were inoculated into 4 mL of NMS broth in Balch tubes with 20% CH_4, and gas levels were monitored to confirm methanotroph activity. Similarly, growth was also tested on NMS agar in 120 mL serum bottles with CH_4 added. For testing of additional carbon sources, the isolates were plated onto NMS + 0.1% Glucose, NMS + 0.1% Fructose, NMS + 0.1% Yeast Extract, and 1/10th Tryptic Soy plates (Hoefman et al. 2012). Isolates that were morphologically pure, showed a reduction in CH_4 and O_2 with an increase in CO_2 in sealed tubes or bottles, had positive growth on NMS + CH_4 plates, negative growth on NMS – CH_4 plates, and negative growth on all other carbon sources, would be considered a pure methanotroph isolate (Hoefman et al. 2012).

2.3.3 Extinction culturing

As an alternative isolation method to resolve issues with fungal contamination, extinction culturing was performed in Balch tubes as well as sterile 96-well microtiter plates (Hoefman et al. 2012; Meruvu et al. 2020). 6-week enrichment cultures were used to inoculate dilution series, and serial dilutions were performed in culture tubes and microtiter plates up to 10^{-11}. Each tube

contained 4 mL of media with a 10% inoculum of culture in NMS media, and 20% CH_4-in-air. Microtiter plates contained a total volume of 300 µL with a 10% inoculum in NMS media. Plates were placed in in BD GasPak™ EZ containers with the addition of CH_4, and all cultures were incubated at 45°C for 14 days (Hoefman et al. 2012). Initial and final optical density was measured using BioTek Epoch 2 microplate spectrophotometer or Biochrom Novapec II spectrophotometer. Gas chromatography was used to measure gas levels in culture tube dilution series at 0, 7, and 14 days, and gases were replenished as needed. The highest dilution showing growth for each series was plated on NMS agar plates to check for purity. Pure isolates underwent carbon source testing as described in section 2.3.2.

2.3.4 Whole genome sequencing using Nanopore MinION Mk1B

DNA was extracted from pure colonies using Wizard® Genomic DNA Purification Kit, according to the manufacturer's protocol. All genomes were sequenced as previously described using the Nanopore MinION Mk1B (Hawkins et al. 2022). Sequencing was carried out using a Nanopore MinION Mk1B with kits SQK-LSK-112 and EXP-NBD-112.24, R10.4 flowcells, and base-calling was handled by Guppy-GPU (Wick et al. 2019). Default parameters were used for all software in the analysis. Sequencing was stopped when on average there was enough data present to provide ~100x coverage on all genomes. Reads were trimmed using BBduk (Bushnell et al. 2017), and then De Novo genome assembly was carried out using Flye followed by 3 rounds of polishing using Minimap2 (Li 2018; Kolmogorov et al. 2019). Genome completion was estimated using checkM (Parks et al. 2015) and found to be above 99% with an estimated 1% contamination in all assemblies. Completed genomes were then annotated with the

Prokaryotic Genome Annotation Pipeline using default parameters and automatic taxa determination (Tatusova et al. 2016).

2.4 Amplicon sequencing

To examine the methanotroph community in the various landfill soils at the genomic level, genomic DNA (gDNA) was extracted from 0.25 g of sieved compost, clay cover, biowindow soil, and column soil using the DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer's instructions. DNA was also extracted from 6-week enrichment culture pelleted cells. Extracted DNA was stored at -20°C prior to sequencing. Where DNA concentrations were sufficient, samples were submitted with three biological replicates. Illumina MiSeq Paired-End 300 bp sequencing was performed at Centre d'expertise et de services Genome Québec (Montréal, Canada) (Oswald et al. 2017). Due to limitations placed on minimum DNA concentrations by Genome Québec, 16S rRNA controls were sequenced by Integrated Microbiome Research (Halifax, Canada). *Methylomonas methanica* MC09 and *Methylocella silvestris* BL2 were used for positive controls of Type I and Type II methanotrophs, respectively, while a DNA extraction of nuclease-free water was used as the negative control.

2.4.1 16S rRNA gene

DNA samples were analyzed via 16S rRNA sequencing for total bacterial community analysis. The V4 variable region of the 16S rRNA gene was amplified from the total community DNA using the universal primers 515F and 806R (Bergmann et al. 2011; Houghton and Stewart 2019; Reddy et al. 2019) (Table 3). Sequence reads were processed using the Geneious Prime®

program (Version 2022.1.1) (Valenzuela et al. 2021). All reads with average quality scores <30, lengths <150 bp or >320bp were trimmed, as were the Illumina adaptors (Lee et al. 2018; Reddy et al. 2019). Chimeric sequences were removed using the USEARCH algorithm and Silva gold alignment database (Houghton and Stewart 2019). Reads were then aligned and clustered into Operational Taxonomic Units (OTUs) at 98% sequence similarity, which were checked against the NCBI database using a Megablast search (Nguyen et al. 2016; Mysara et al. 2017).

2.4.2 *pmoA* gene

The primers 189F and mb661R were used to target the *pmoA* gene as a marker for methanotrophs, as it encodes the particulate methane monooxygenase enzyme (Ho et al. 2011; Paszczynski et al. 2011; Kizilova et al. 2014; Houghton and Stewart 2019; Wen et al. 2021) (Table 3). Sequence reads were processed using the Geneious Prime® program (Version 2022.1.1). Illumina adapters and reads with average quality scores <30 were trimmed, and minimum sequence lengths of 200 bp were kept for analysis (Dumont et al. 2014). Reads were aligned and clustered into Operational Taxonomic Units (OTUs) at 97% similarity, and sequences were searched against the known families of methanotrophs (*Methylococcaceae, Methylocystaceae, Beijerinckiaceae, Methylomirabilis, Methylacidiphilaceae,* and *Methylothermaceae*) using the NCBI BLASTn online tool (Kizilova et al. 2014).

Table 3. Primers used for quantitative PCR analysis and amplicon sequencing.

Primer	Sequence (5' → 3')	Target (locus tag)	Amplicon length (bp)
189f	GGN GAC TGG GAC TTC TGG (18 bp)	*pmoA* [1]	454
mb661r	CCG GMG CAA CGT CYT TAC C (19 bp)		
536f	CGC TGT GGA AGG GCA TGA AGC G (22bp)	*mmoX* [2]	340
898r	GCT CGA CCT TGA ACT TGG AGC C (22 bp)		
515f	GTG CCA GCM GCC GCG GTA A (19 bp)	16S rRNA V4	272
806r	GGA CTA CVS GGG TAT CTA AT (20 bp)	variable region [3]	

[1] Ho et al. 2011; Paszczynski et al. 2011; Kizilova et al. 2014; Houghton and Stewart 2019; Wen et al. 2021

[2] Fuse et al. 1998; Paszczynski et al. 2011

[3]Bergmann et al. 2011; Houghton and Stewart 2019; Reddy et al. 2019

2.4.3 Statistical analysis

Taxa that contained less than 0.01 percent relative abundance across all samples were excluded from data analysis. To determine alpha diversity of the samples, the Shannon diversity index, Pielou's evenness index, and Simpson diversity index were used (Yun et al. 2013; Lee et al. 2018).The principle coordinates analysis (PCoA) was used to visualize beta diversity, based on the Bray-Curtis dissimilarity index. The software PAST 4.11 was used for all statistical analyses (Hammer 2001).

2.4.4 Phylogenetic trees

Geneious Prime® software (Version 2022.1.1) was used to construct phylogenetic trees. Representative sequences of *pmoA* were obtained from GenBank (Sayers et al. 2019), and sequences were aligned using MUSCLE (3.8.425) (Edgar 2021). Neighbour-Joining method was used to build phylogenetic trees with the Jukes-Cantor genetic distance model, using 1000 bootstrap replicates. *Methylacidiphilum* sp. RTK17.1 was selected as the outgroup model.

2.5 Methanotroph enumeration using qPCR

Quantitative PCR was used to measure the abundance of methanotroph marker genes and total bacterial counts for all landfill soil samples and column soil. Bacterial primers for the *pmoA* and *mmoX* genes were used to detect gene copy levels of pMMO and sMMO, respectively (Table 3) (Paszczynski et al. 2011). To determine cycle threshold values, standard curves were generated by a dilution series of DNA from *Methylomonas methanica* MC09 (DSM 25384) for

pmoA, and *Methylocella silvestris* BL2 (DSM 15510) for *mmoX*. For all qPCR reactions, a BIO-RAD CFX Connect Real-Time System machine was used. Data analysis was performed using Bio-Rad CFX Manager 3.1 software. All PCR amplifications were performed with three technical replicates, using total volumes of 25 µL. Reactions included 12.5 µL of iQ SYBR Green Mastermix (Applied Biosystems), 300 nM of each primer, and 1 µl of DNA standard or sample (Paszczynski et al. 2011). The amplification cycle included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, 55–60°C for 1 min, and 72°C for 1 min (Paszczynski et al. 2011). Fluorescence readings were taken following the 72°C incubation. To confirm product specificity a melt curve analysis was performed following every run, in 0.5°C increments from 50°C - 95°C (Paszczynski et al. 2011). Statistical significance was calculated by ANOVA with the software PAST 4.11 (Hammer et al. 2001).

Chapter 3: Results

3.1 Methanotroph presence in columns

16S rRNA sequencing was performed using soil DNA from two columns that acted as landfill biowindow microcosms: one column packed with a mixture of compost and ¼" gravel (Column 1), and the other with compost and ½" gravel (Column 2) (Berenjkar 2021). Results from the column experiments (performed previously by Berenjkar) showed the highest level of methane removal efficiency with ¼" gravel and compost, at a ratio of 1:7. Column soil was extracted for the current study at the completion of the column experiment, and used for microbial analysis.

Community analysis revealed that at the class level, the proportion of Gammaproteobacteria and Alphaproteobacteria in Column 1 was 12% and 5.7% respectively, while in Column 2 it was 16% and 21% (Figure 4A). Interestingly, one of the dominant classes of organisms in both columns was that of Anaerolinae, a non-methanotrophic group of obligate anaerobes. This group accounted for 23% of all reads in each column. In each column 22% - 24% of all reads were identified to the genus level (Figure 4C). Furthermore, 7.2% of all Column 1 reads belonged to known methanotroph genera, while 20% of all Column 2 reads were methanotrophs (Figure 4D). The key differences in methanotroph community were seen in the presence of *Methylocystis* (3.2%) and *Methylosinus* (5.8%) in Column 2. These organisms were found in Column 1 at much smaller proportion of 0.06% and 0.14%, respectively. *Methylocaldum* was identified at a similar abundance in Columns 1 and 2, at 5.6% and 5.5%, respectively, while *Methylobacter* was present at 1.3% and 4.7% relative abundance.

The Shannon and Simpson diversity indices showed high diversity in the bacterial community, with scores being consistently high across both columns (Table 4). Pielou's evenness index also indicated that both communities have a reasonably high degree of evenness and are not dominated by any one species. The Bray-Curtis dissimilarity index between the two communities was 0.187, indicating that the columns share a vast majority of the same organisms. While there are key differences in the most abundant genera found in both columns, as shown in Figure 4C the Bray-Curtis index was calculated based on the entire library of OTUs. A Bray-Curtis index of 0.463 was also calculated using only the six different OTUs from known methanotrophic genera present in the columns, showing that the methanotroph community in particular had a slightly higher degree of dissimilarity at the time of sampling. These statistics reveal that differences in gravel size have minimal effect on diversity of the microbial community in the columns. A relatively high degree of similarity between the two communities is expected as the packing material originated from the same source, namely the BRRMF biowindow. Interestingly, the compost used to fill both columns was collected from the biowindow in November 2019, during a time in which thermophilic conditions were not expected to be present in the biowindow based on previous annual data retrieved by Berenjkar et al. (2021). However, as described below in Section 3.4, thermophilic methanotrophs were present in the biowindow through to late fall, so while we cannot conclusively say what the starting population was in the column soil, these results show that thermophilic methanotrophs persisted to the end of the experiment.

Enumeration of methanotrophic bacteria via qPCR revealed a slightly higher number of *pmoA* in Column 1 than Column 2 (Figure 5). Furthermore, a higher abundance of *mmoX* was detected in Column 2. Ultimately, statistical analysis using ANOVA revealed there was not a

significant difference between methanotroph gene abundance in either columns (p-value = 0.18. However, Column 1 was found to have significantly more *pmoA* gene copies than *mmoX* (p-value = 0.01).

Table 4. Statistical analysis of community diversity in columns based on defined Operational Taxonomic Units (OTUs).

Column	Observed OTUs	Shannon Index	Simpson Index	Evenness Index	Bray-Curtis
1	1665	5.45	0.986	0.723	0.187
2	1522	5.12	0.974	0.699	

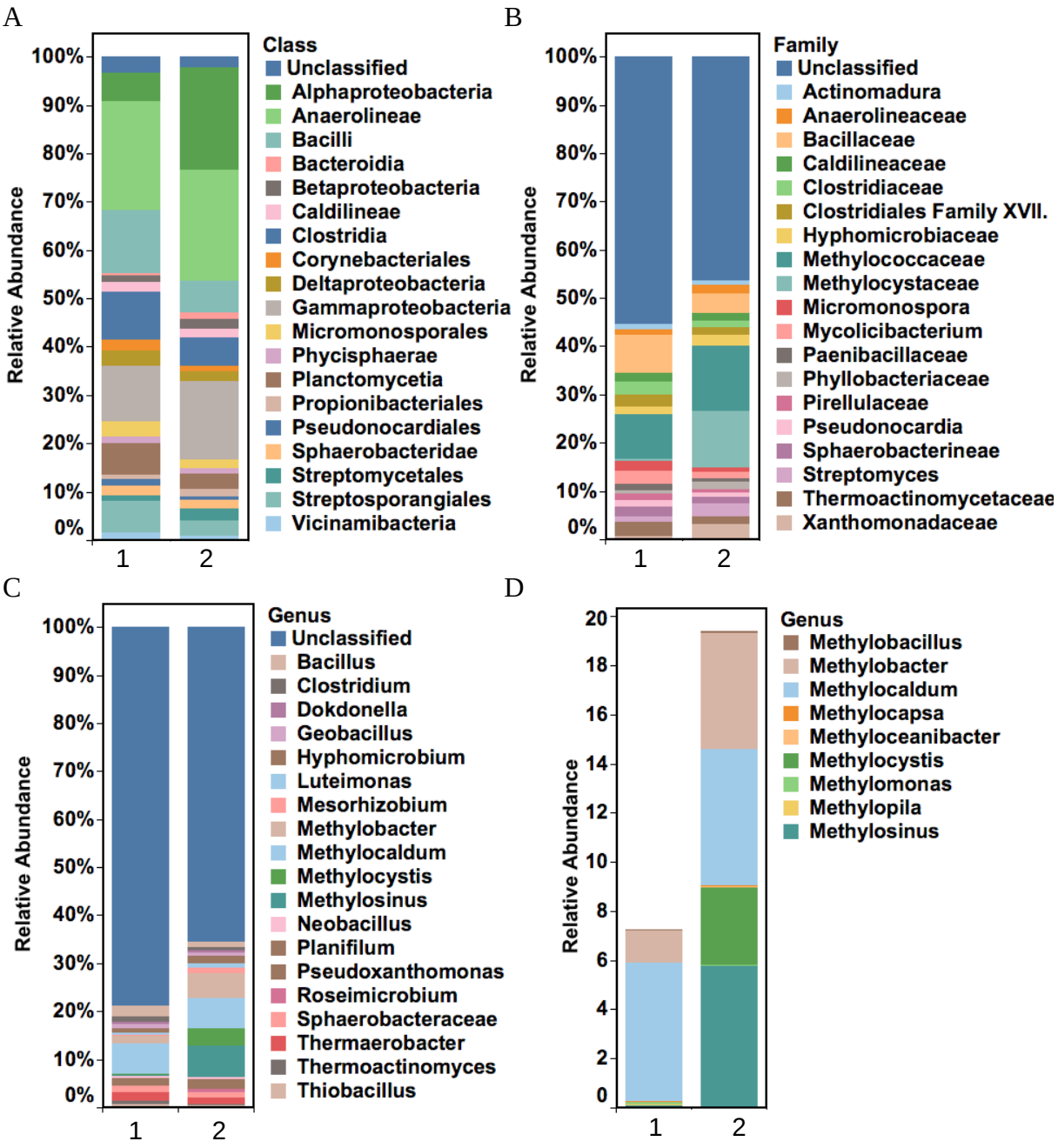


Figure 4. Organisms identified in landfill microcosm column experiments through 16S rRNA sequencing. Taxonomy is shown at the class **(A)**, family **(B)**, and genus **(C)** level. **D)** shows only known methanotroph genera identified in columns, as seen in **(C)**.

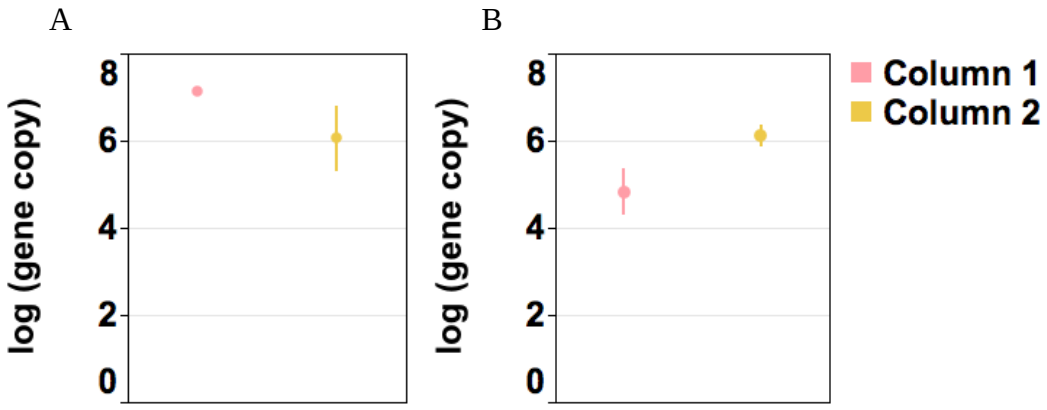


Figure 5. Methanotroph abundance of column soil DNA based on qPCR targeting of *pmoA* **(A)** and *mmoX* **(B)** genes. Values are expressed as the log of gene copy number per g of soil. Data points represent average of 3 technical replicates, and bars show standard deviation.

3.2 BRRMF site analysis

The continued presence of methanotrophs throughout Berenjkar's experiments led to a return to the biowindow site with a specific focus on the methanotrophs themselves for an additional field season. Soil samples were collected from BRRMF in May, August, and October 2021 in order to acquire a diverse array of seasonal conditions within the time frame. The samples were used as representatives of spring, summer, and fall seasons, respectively. Weather in spring 2021 was fairly representative of average conditions, with a typical amount of precipitation consistent with historical records (Figure 6A). However, during the summer of 2021 Southern Manitoba experienced extremely dry conditions, with July recording less than 40% of median precipitation (Government of Manitoba, 2021). August brought considerable amounts of precipitation later in the month, which allowed for the comparison of extremely dry, thermophilic conditions in landfill soil, to the soil following an extended period of heavy rainfall

(Figure 6B). This resulted in two back-to-back sampling excursions in the month of August, 9 days apart: August 16 and August 25. A final sampling trip on October 25 permitted analyses of sites in late fall that was reasonably representative of average annual precipitation.

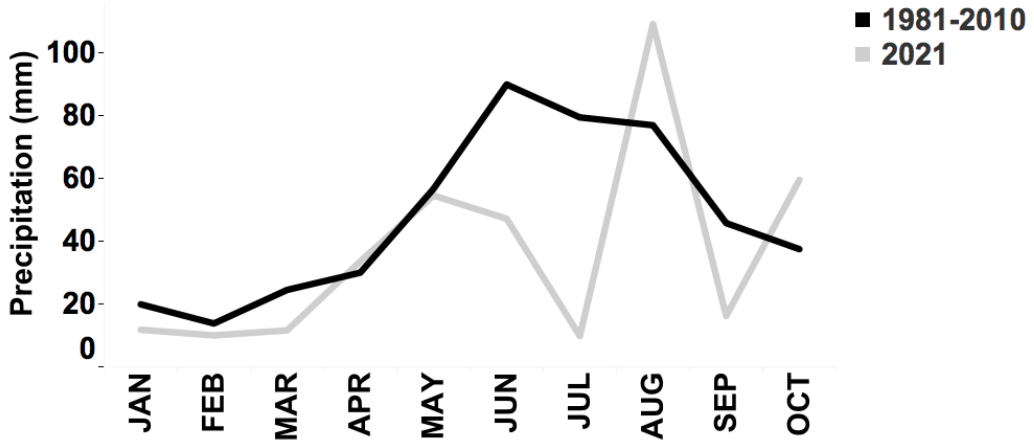
Substantial *in situ* temperature variations were found in comparing the biowindow soil (BW), conventional clay soil (CS), and compost windrow (CW). In all seasons significantly elevated temperatures were found within the biowindow, with readings 20-35°C above ambient temperature during all sampling events (Figure 7). Thermophilic temperatures up to 60°C occurred during the summer. With increased depth BW temperature increased accordingly, exhibiting temperatures 5-10°C higher at 60 cm versus 20 cm in May and August, and as much as a 22°C difference in October. *In situ* temperatures of CS and CW were much closer to the recorded ambient temperature, often either the same or a maximum of 5°C warmer. The exception to this was in the CW Summer-Wet data, a temperature 14°C warmer than the ambient temperature of 17°C was recorded. CW temperature was marginally higher with increased depth, with a maximum of 5°C increase in Summer-Wet and Fall, while in CS there was no visible temperature increase with depth throughout the entire field season.

Moisture content (% w/w) values in were measured using individual samples from each depth (Appendix Table 6) and are shown in Figure 7; averages of those depths are discussed in this paragraph. For every soil type, the highest level of moisture was recorded during Spring. BW and CW showed the same value of 46% w/w, with CS being considerably lower at 23% (Figure 7). Summer data from August 16 revealed decreased moisture content with BW, CW, and CS showing averages of 24%, 35%, and 12% respectively. Data from August 25 following a period of heavy rainfall interestingly showed that BW moisture content was unchanged from the

previous dry samples 9 days earlier. CW retained a high level of 44% following the rainfall, while CS also had a slight increase with an average value of 23%. A final sampling trip in late October had surprisingly low values again for all three sites, despite a relatively average amount of precipitation throughout the fall months. All soil types in October had an average moisture content between 20-22%.

BW soil (6-6.5) was found to have a slightly lower pH than CS and CW (6.5-8.2). These measurements were relatively consistent throughout the seasons. Spring pH data was not recorded.

A



B

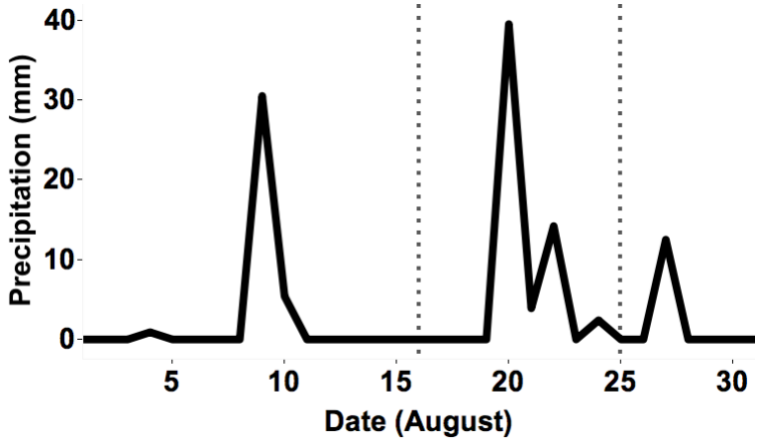


Figure 6. Precipitation data for Winnipeg, MB. **A)** Monthly historical precipitation data from January to October. **B)** Total daily precipitation in August 2021; dotted black lines represent sampling days. Data retrieved from Canadian Climate Normals Data.

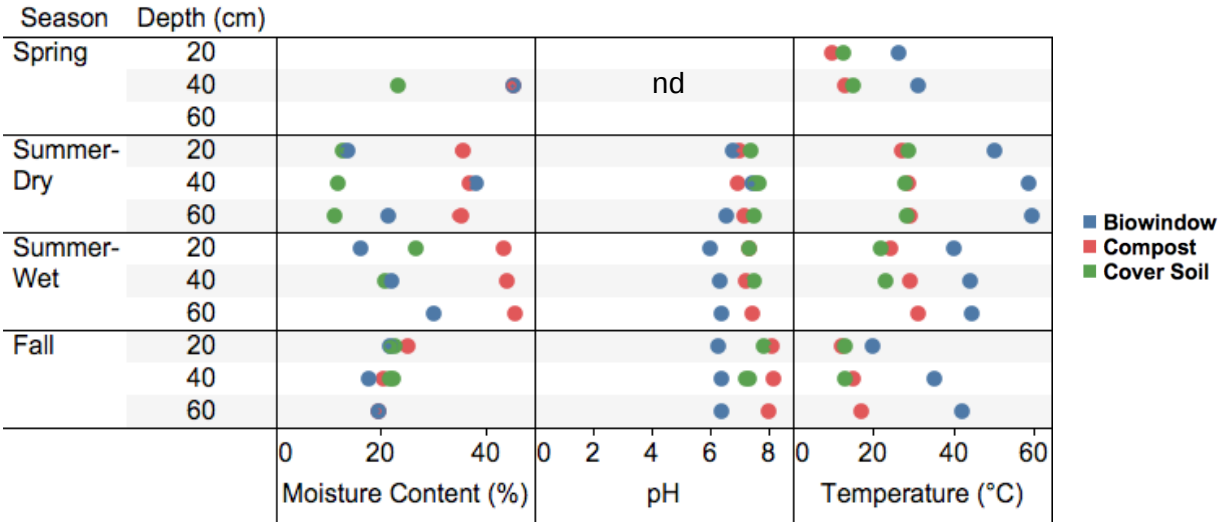


Figure 7. Soil property data of samples from BRRMF. Moisture content and pH were measured in triplicate upon return to the lab and temperature readings were recorded *in situ*. Ambient temperature on sampling days was 4°C (Spring), 29°C (Summer-Dry), 17°C (Summer-Wet), and 7°C (Fall).

3.2.1 Initial MOP of landfill soil

Methane oxidation potential tests revealed that the greatest amount of CH_4 oxidation occurred in Spring BW soil (Figure 8). The largest reduction in CH_4 occurred under mesophilic conditions with levels decreasing by half, to 10% after 72 hours. At thermophilic temperatures, both 45°C and 55°C, Spring BW also showed a noticeable decrease in CH_4. CS and CW soil showed negligible oxidation of CH_4. With all subsequent samples, there was virtually no methane oxidizing activity measured in the initial soil analysis from any of the sampling sites during Summer or Fall at the *in situ* moisture concentration.

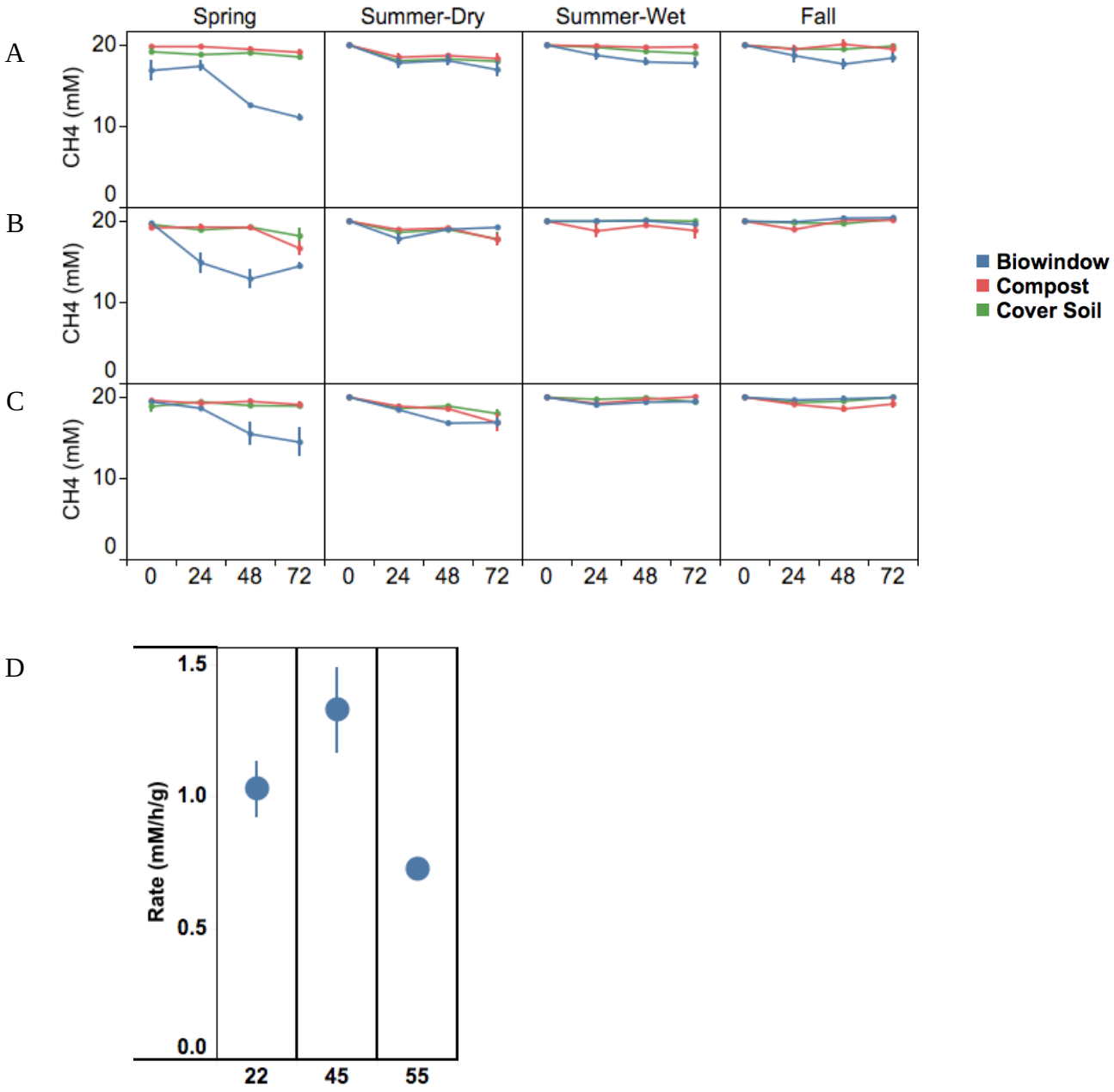


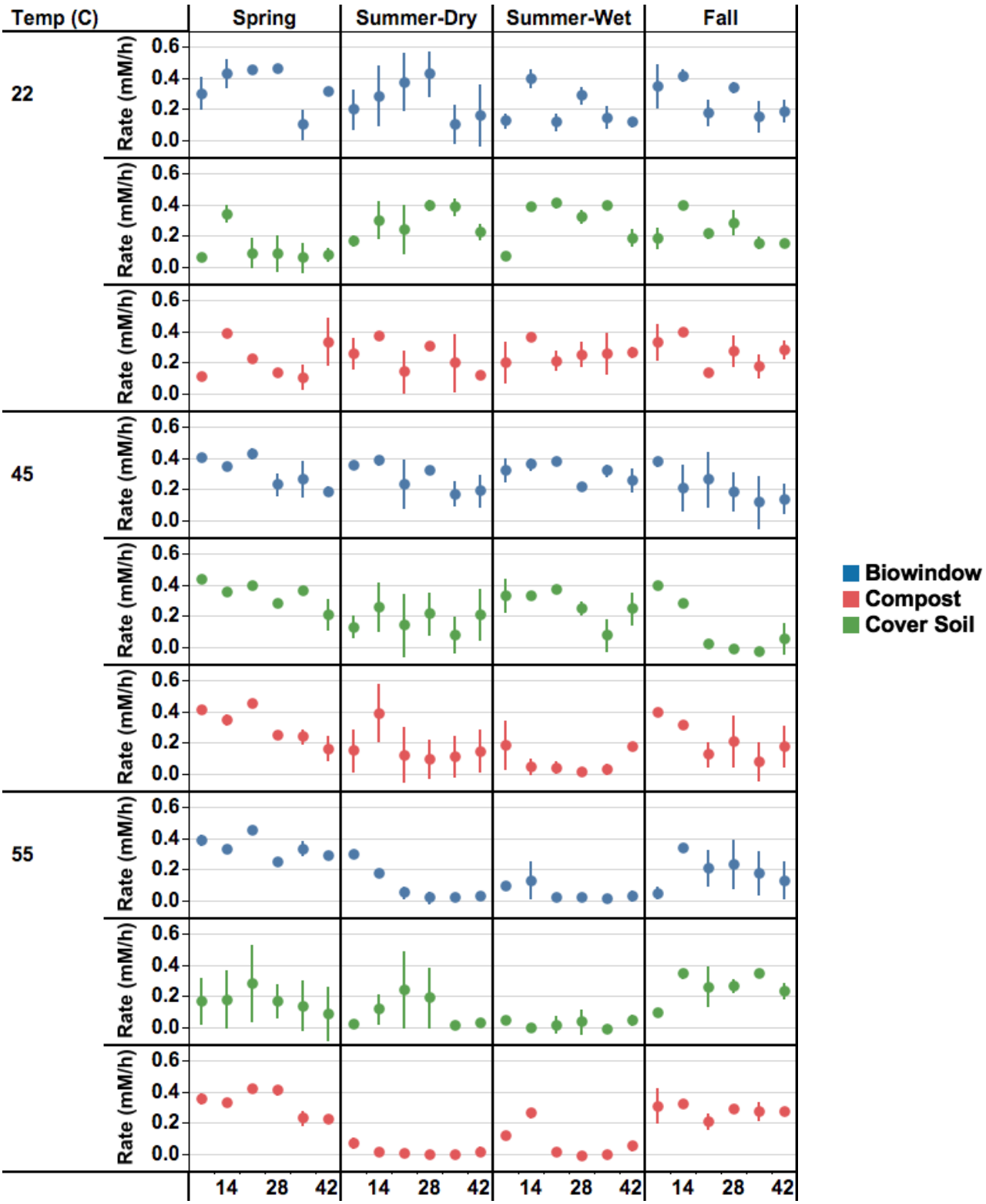
Figure 8. Initial methane oxidation potential of landfill soil. Soil was incubated at 22°C **(A)**, 45°C **(B)** and 55°C **(C)**, with time in hours is shown on x-axis. **D)** shows maximum rate of CH_4 oxidation in Spring Biowindow soil samples, with temperatures shown on x-axis. Error bars represent standard deviation from 3 technical replicates.

3.3 Enriching for thermophilic and mesophilic methanotrophs

Despite low initial MOP, enrichment cultures showed a positive indication of methanotroph presence in nearly all samples. For the purposes of this work, a positive indication of methanotroph growth was defined as a decrease in CH_4 of at least 25% along with a proportional decrease in O_2, increase in CO_2, and visible turbidity. BW samples showed the highest rates of methane oxidation overall, followed by CS, then CW (Figure 9). It was noted that there was a consistent decrease in the methane oxidation rates of some cultures over the 6-week period of passaging, i.e. the greatest reductions in methane were seen within the first 1-2 passages and were subsequently less after that. The 55°C enrichment cultures showed very little indication of growth in either the Summer-Dry or Summer-Wet samples, while the 45°C and 22°C cultures were much more consistent throughout the seasons for all soil types.

In terms of distinctions of media types, there was a marginal difference in methanotroph activity in the cultures that did not have Cu added (Figure 9B). BW samples without Cu at both mesophilic and thermophilic conditions all showed methanotrophy relatively consistent with the Cu+ samples. CS and CW samples, in particular at 22°C, both had less methane oxidation occurring in the Cu-free cultures.

A


Temp (C)
Spring
Summer-Dry
Summer-Wet
Fall
22
45
55
Rate (mM/h)
0.6
0.4
0.2
0.0
14
28
42
Biowindow
Compost
Cover Soil

B

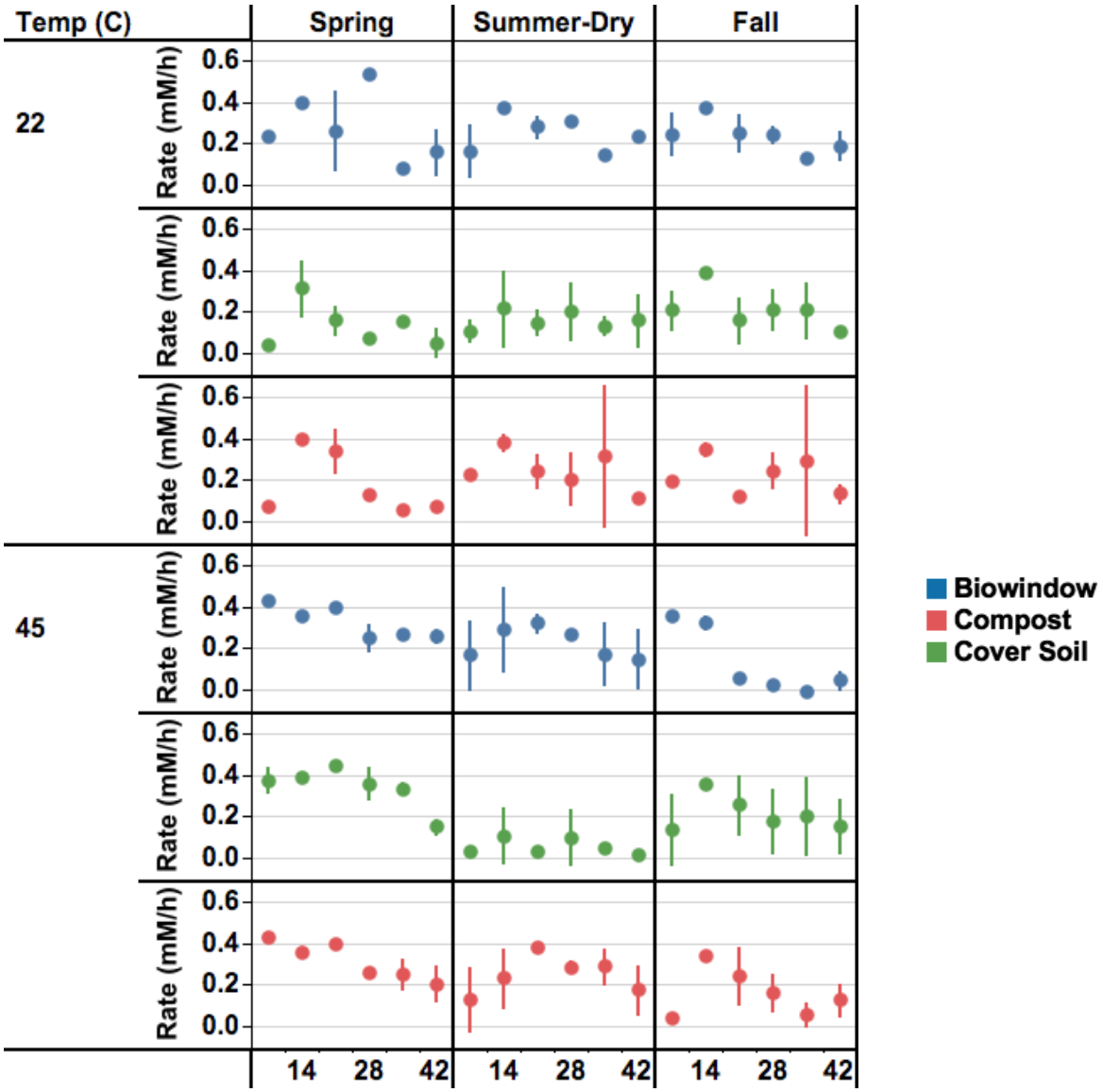


Figure 9. Rate of CH_4 oxidation in enrichment cultures measured in mM of CH_4/hour. NMS media with Cu **(A)** and without Cu **(B)**. Graphs show rates of CH_4 oxidation over 42 days, displayed as 7-day averages. Data points represent average of three replicates with standard error bars shown. Gases were replenished every 7 days and cultures passaged every 14 days.

3.3.1 Isolation and purification of methanotrophs

Serial dilution and plating of the 6-week enrichment cultures led to the isolation and visualization of a number of bacterial colonies. Unfortunately, for all plating experiments fungal contamination was found to be a continuous setback. For many of the Summer and Fall plate count experiments, excessive fungal contamination led to the inability to determine accurate bacterial counts. Where CFU per mL could be accurately determined, the data is shown in Figure 11. Prompted by the consistent fungal contamination not only for serial dilutions but also during attempted purification of isolates, cycloheximide was ultimately supplemented into NMS media. Given the high incubation temperatures and moisture production in the GasPak containers, as well as the prolonged incubation periods, this aided in reducing fungal growth but did not eliminate it entirely.

Plate counts from serial dilutions of Spring samples showed a significantly higher number of organisms in BW soil growing in the presence of CH_4, compared to CW and CS (p-value = 5.70×10^{-5}) (Figure 11). There was also a significantly higher CFU/mL under mesophilic conditions in Spring samples, compared to thermophilic (p-value = 1.32×10^{-4}). Dilution plating of Summer samples revealed no significant difference between the sources of soil, with p-values of 0.18 and 0.45 for Summer-Dry and Summer-Wet experiments, respectively. Interestingly, results from Fall dilution plating exhibited significantly higher plate counts in Compost samples (p-value = 0.01), with 22°C cultures again showing the highest counts overall (p-value = 3.18×10^{-3}). While there was notable bacterial growth under thermophilic conditions in the presence of CH_4 and CFU/mL was determined for a number of samples (Figure 11), some could not be determined because of an overabundance of fungal contamination.

For bacterial colonies that were isolated successfully on agar plates, there were difficulties in inoculating isolates into liquid culture to confirm methane oxidizing capabilities. Several isolates could be successfully grown in pure culture on agar with CH_4 but would not grow in liquid culture under the same conditions. Likewise, several liquid cultures that were successfully growing and oxidizing CH_4 were unable to grow on plates.

Extinction culture methods, both in tubes and microtiter plates, showed gradual reduction in OD and CH_4 oxidation abilities throughout the dilution series (Figure 13). While pure organisms were not able to be isolated on plates in this manner, experiments resulted in several mixed cultures. The lack of consistent growth beyond 10^{-4} in Figure 13A, and 10^{-1} in Figure 13B, despite a high initial OD, is indicative of a co-culture in which multiple organisms are required for growth.

An isolate produced from serial dilution plating that underwent whole genome sequencing was identified as *Meiothermus silvanus* (Bacteria; Deinococcus-Thermus; Deinococci; Thermales; *Thermaceae*; *Meiothermus*). This organism was isolated from thermophilic cultures inoculated with spring biowindow soil (shown as "55B2" in Figure 12). Sequencing statistics are shown in Table 5. *M. silvanus* appeared in 16S rRNA sequencing in only one enrichment culture, that of Spring 55°C compost. The family *Thermaceae* was found in two samples of compost DNA at 0.012% and 0.007% relative abundance. The class Deinococci was identified in all soil samples at the class level, ranging from 0.012% in biowindow to 1.1% in compost (Figure 10).

Table 5. Sequencing statistics for *Meiothermus silvanus* strain JP1.

Strain	Reads	Basepairs Sequenced	N50 (Kbp)	Fold coverage	Genome Size	Contigs	GC Content
JP1	130K	643Mbp	19.8Kbp	161x	3.6Mbp	2	62.4%

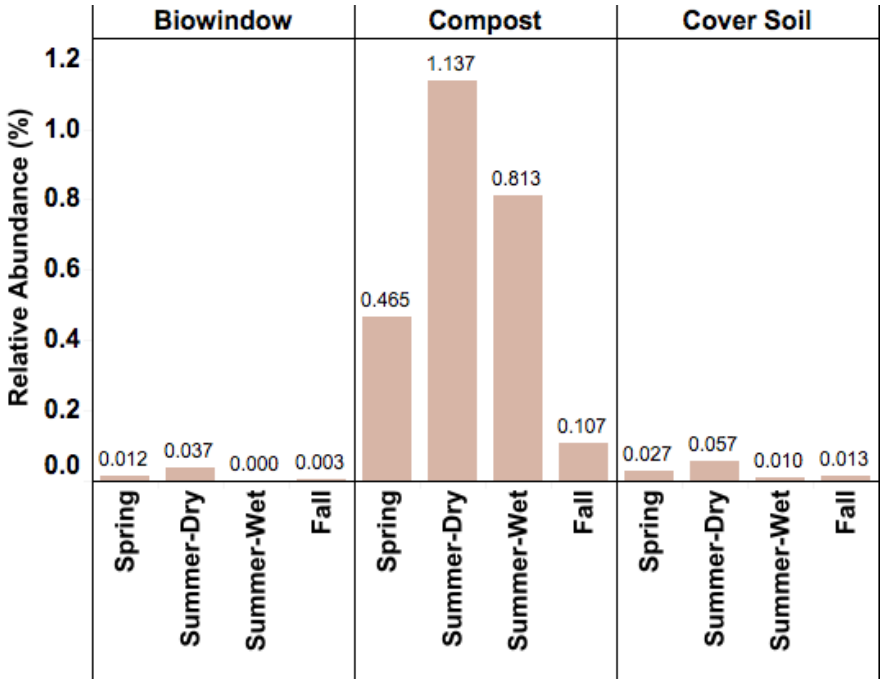
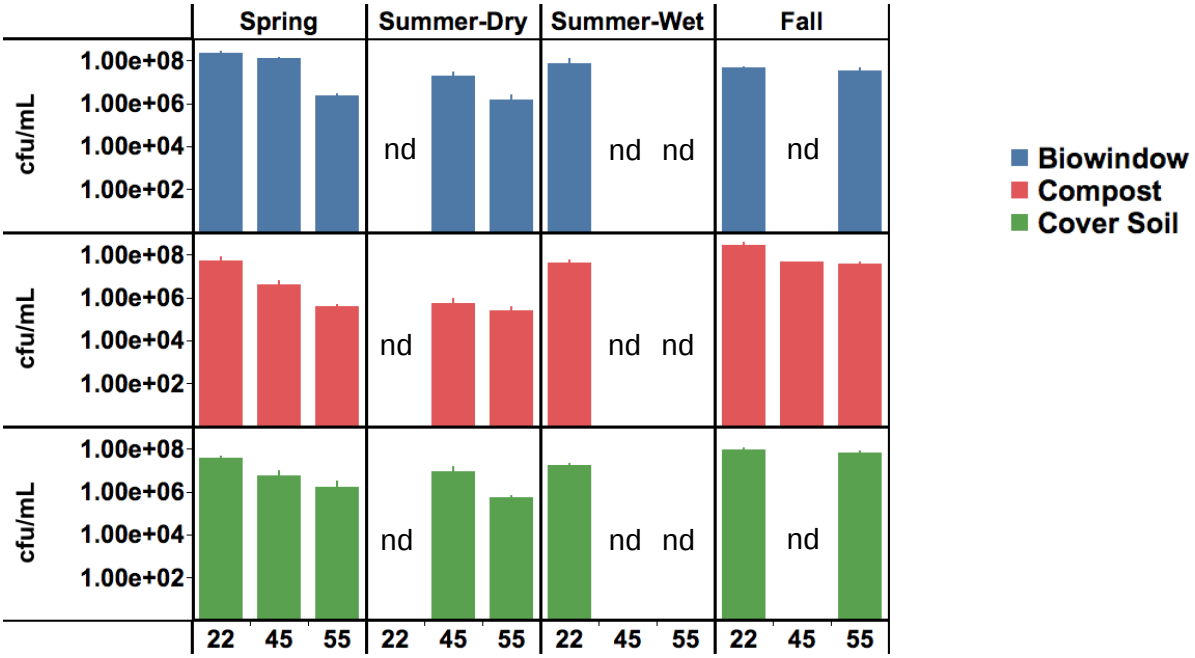


Figure 10. Relative abundance of class Deinococci in soil based on V4-region 16S rRNA sequencing.

A



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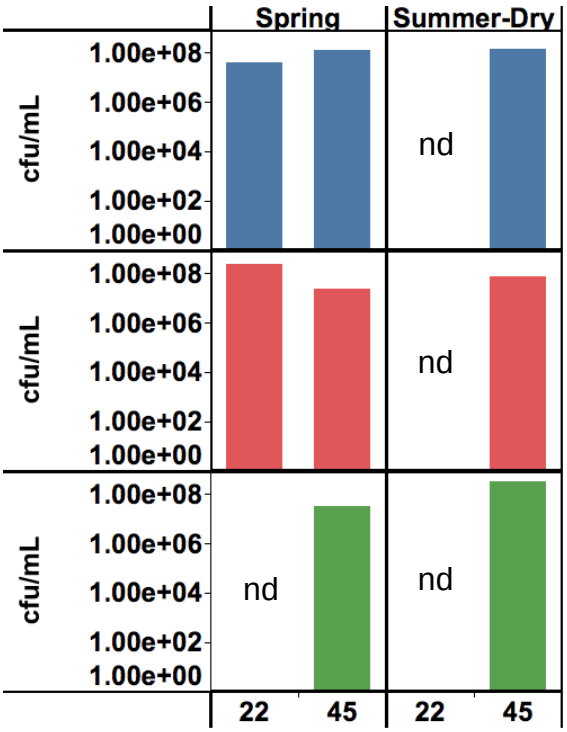


Figure 11. Colony forming units from serial plating of 6-week enrichment cultures with Cu **(A)** and without Cu **(B)** incubated in the presence of CH_4 as the sole added carbon source. Columns with "nd" indicate CFU was not determined due to fungal contamination.

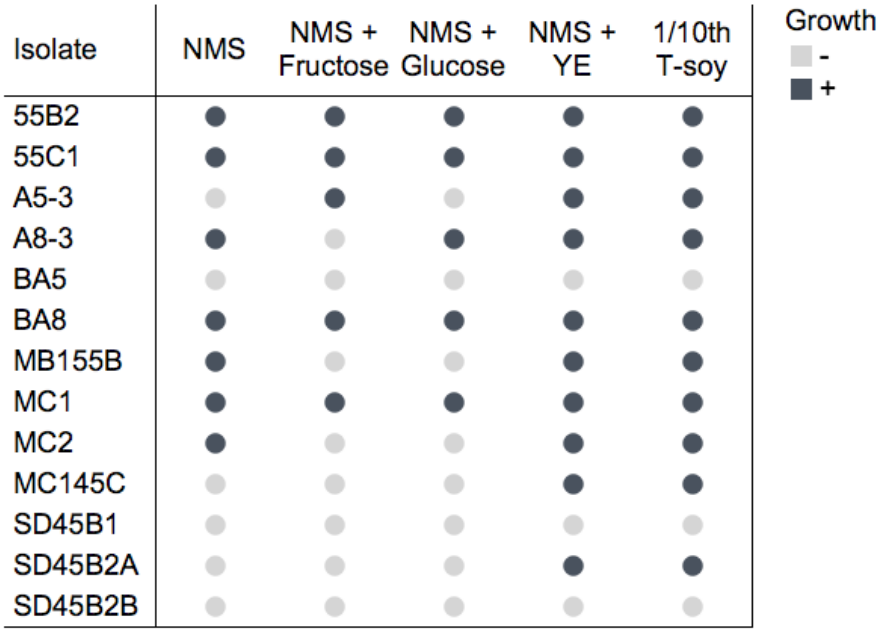
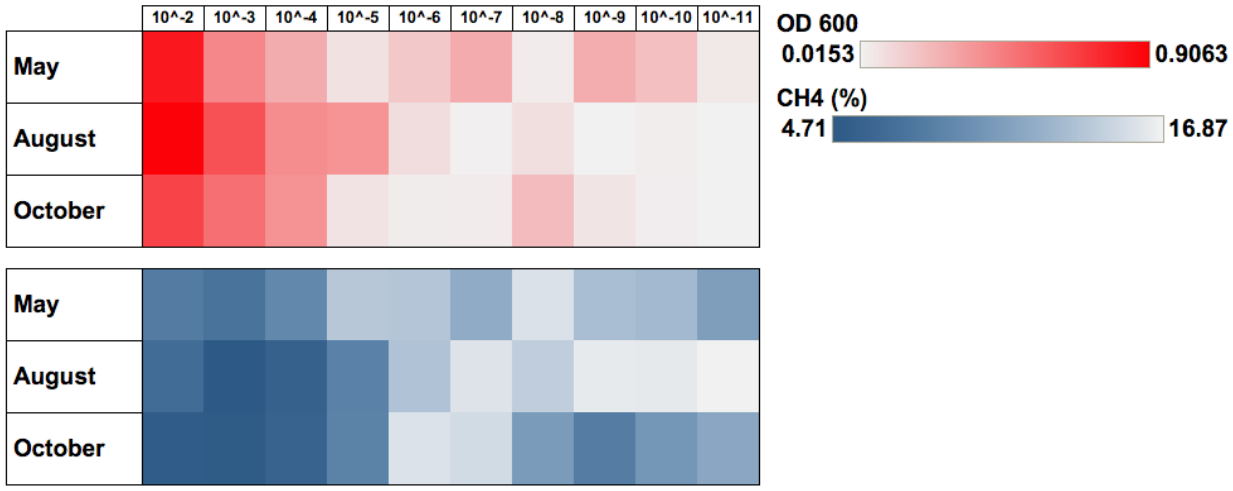


Figure 12. Carbon source testing of suspected methanotroph isolates grown in the absence CH_4.

All listed organisms were initially isolated on NMS in the presence of CH_4.

A



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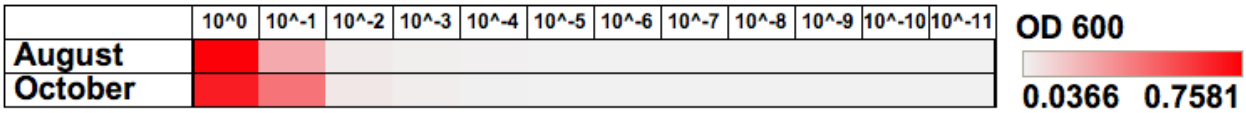


Figure 13. Heat maps depicting growth of extinction cultures in culture tubes **(A)** and microtiter plates **(B)** both incubated at 45°C. Red represents final optical density measured at 600 nm, and blue indicates final CH_4 levels based on gas chromatography, at the end of the incubation period.

3.4 Microbial community analysis

16S rRNA sequencing was performed on landfill soil DNA as well as enrichment culture DNA. Up to 35% of soil DNA reads were identified at the genus level. Community analysis of the landfill soil samples revealed a higher proportion of methanotroph genera were present in BW soil (7% - 25% of total reads), as compared to the CS (<3%) and CW (<0.2%) (Figure 14B). Throughout the four sampling events BW soil showed a substantial increase in the relative abundance of *Methylocaldum* reads, a known thermophilic methanotroph. This genus was found in CS and CW only in trace amounts of 0.2 – 0.5% relative abundance. Reads for *Methylobacter* were found consistently throughout the entire field season in both BW and CS between 0.44 – 2.8%, and in very small amounts of CW at 0.12 – 0.17%. Interestingly, *Methylocystis* reads were found primarily during the spring in the biowindow, with very small amounts found in any other samples. Other methanotroph genera that were identified through 16S sequencing included *Methylococcus, Methylophilus,* and *Methylosinus*, all of which were present in all samples. At the class level, a higher proportion of reads for Gammaproteobacteria were found in BW soil, with the exception of spring which showed Alphaproteobacteria dominating (Figure 14C). Despite the identification of known thermophilic methanotroph genera such as *Methylocaldum* and *Methylococcus,* several thermophilic methanotroph groups were absent entirely from this study. These included *Methylothermus*, *Methylomarinovum*, and the Verrucomicrobia *Methylacidiphilum* and *Methylacidimicrobium*. Likewise, *Methylomirabalis* from the Candidate Phylum NC10 was also not detected.

In addition to methanotrophs, several groups of heterotrophs were found to be particularly abundant across the soil samples. Bacteria belonging to the family *Sphaerobacteraceae* were among the most abundant organisms found during all sampling

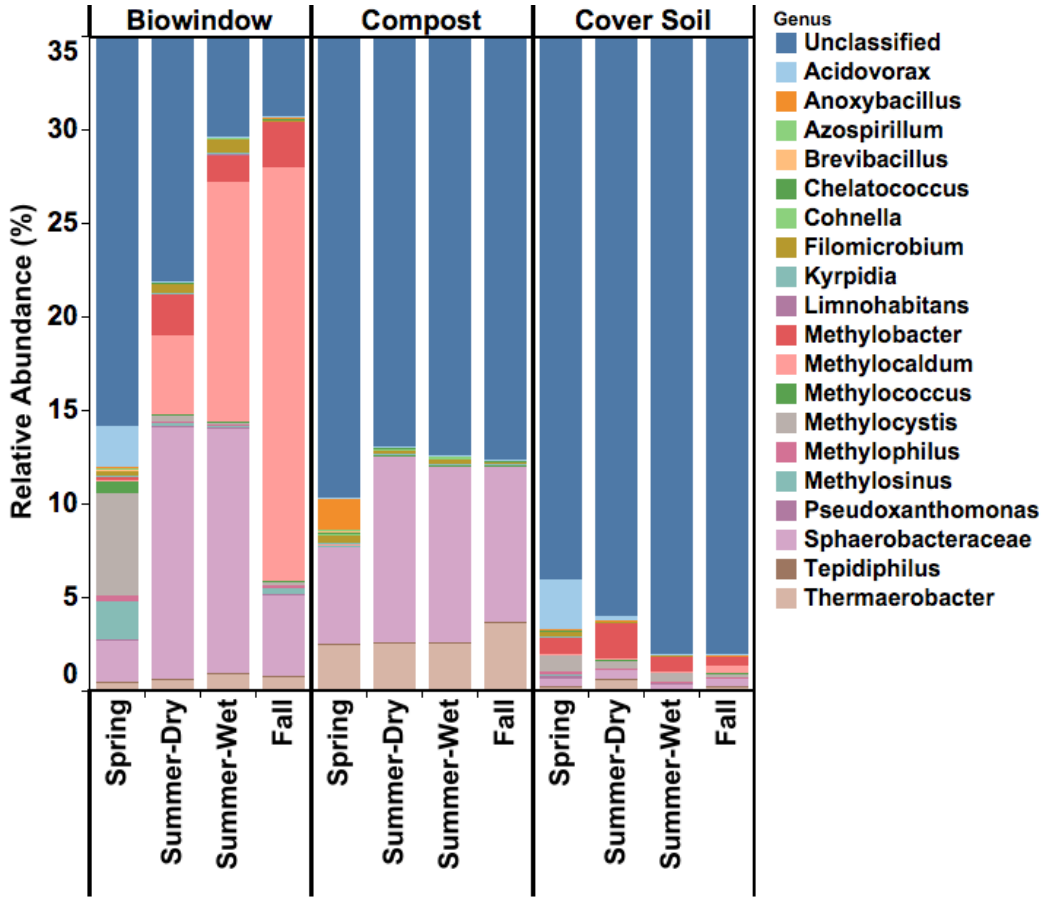
seasons in BW and CW samples (2.2% - 14%) (Figure 14A). This group was identified in CS at a frequency of 0.50%. Reads matching to *Kyrpidia*, a known thermophile, were found in all soil types. *Thermaerobacter* was another primary heterotrophic component of CW reads at 2.9 – 5.5%, small numbers of which were also found in BW and CS. Genera that have been shown to possess the SQR gene for sulfide oxidation made up 0.4 – 1.0% of all soil DNA reads on average. No key differences in relative abundance of these genera were noted between soil types and seasons.

16S rRNA analysis of 6-week enrichment culture DNA revealed largely different communities present at mesophilic versus thermophilic incubation temperatures (Figure 15A-E). All three soil types successfully enriched for methanotrophic organisms in cultures both with and without the addition of Cu. Mesophilic enrichment cultures at 22°C saw a high abundance of the methanotrophs *Methylophilus, Methylobacter, Methylocystis, Methylococcus* and *Methylosinus.* In all 45°C thermophilic cultures, the dominant methane oxidizer found was *Methylocaldum,* with all other methanotrophs being accounted for in trace amounts. The 55°C cultures also saw *Methylocaldum* as the primary methanotroph in Spring and Fall, however very few reads for this genus were present in 55°C Summer enrichments. Instead, the thermophilic heterotroph *Kyrpidia* dominated in 55°C Summer Biowindow and Compost enrichments.

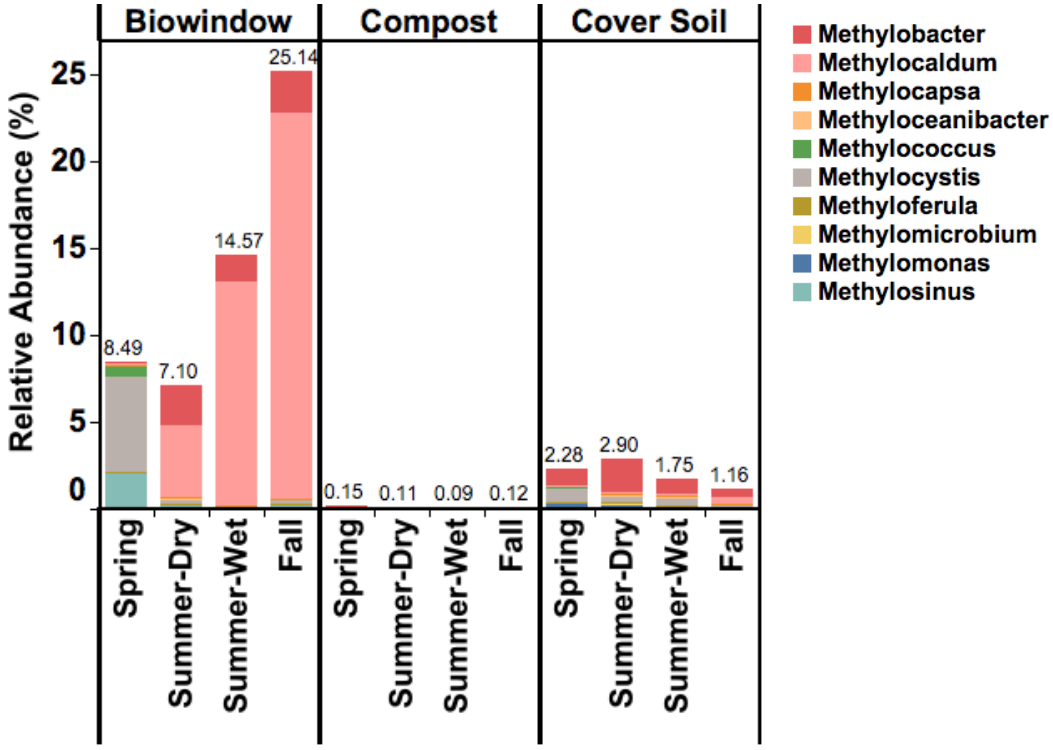
Pure cultures of *M. silvestris* and *M. methanica* used as positive controls for 16S rRNA sequencing successfully identified each organism at 99.8% and 99.9% abundance, respectively (Appendix Figure 28). Negative sequencing controls using water blanks resulted in a total of 2600 reads, relative to an average of 65 000 reads for the positive controls. Reads associated with methanotrophic genera appeared between 1 – 75 times per genus and were deemed to be

negligible amounts overall. The most abundant genera identified in the negative controls belonged to organisms that were not phylogenetically relevant to this study, e.g. *Escherichia, Pseudomonas,* and *Rouxiella.*

A


Biowindow
Compost
Cover Soil
Relative Abundance (%)
0
5
10
15
20
25
30
35
Spring
Summer-Dry
Summer-Wet
Fall
Genus
Unclassified
Acidovorax
Anoxybacillus
Azospirillum
Brevibacillus
Chelatococcus
Cohnella
Filomicrobium
Kyrpidia
Limnohabitans
Methylobacter
Methylocaldum
Methylococcus
Methylocystis
Methylophilus
Methylosinus
Pseudoxanthomonas
Sphaerobacteraceae
Tepidiphilus
Thermaerobacter

B


Biowindow
Compost
Cover Soil
Relative Abundance (%)
0
5
10
15
20
25
8.49
7.10
14.57
25.14
0.15
0.11
0.09
0.12
2.28
2.90
1.75
1.16
Spring
Summer-Dry
Summer-Wet
Fall
Methylobacter
Methylocaldum
Methylocapsa
Methyloceanibacter
Methylococcus
Methylocystis
Methyloferula
Methylomicrobium
Methylomonas
Methylosinus

C

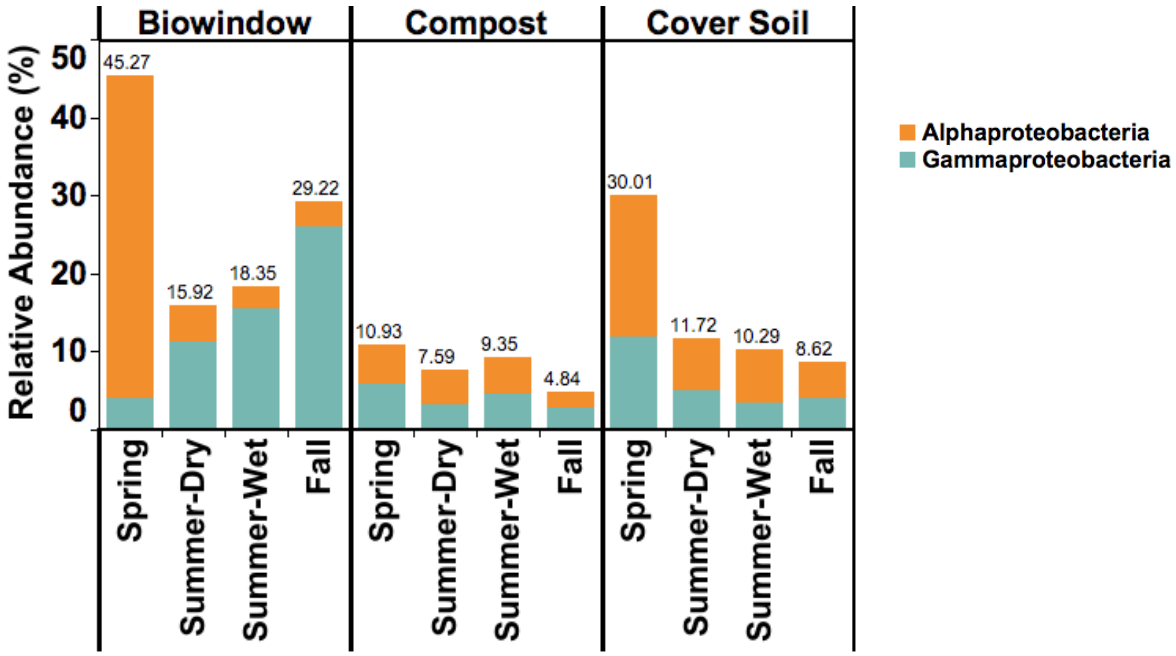
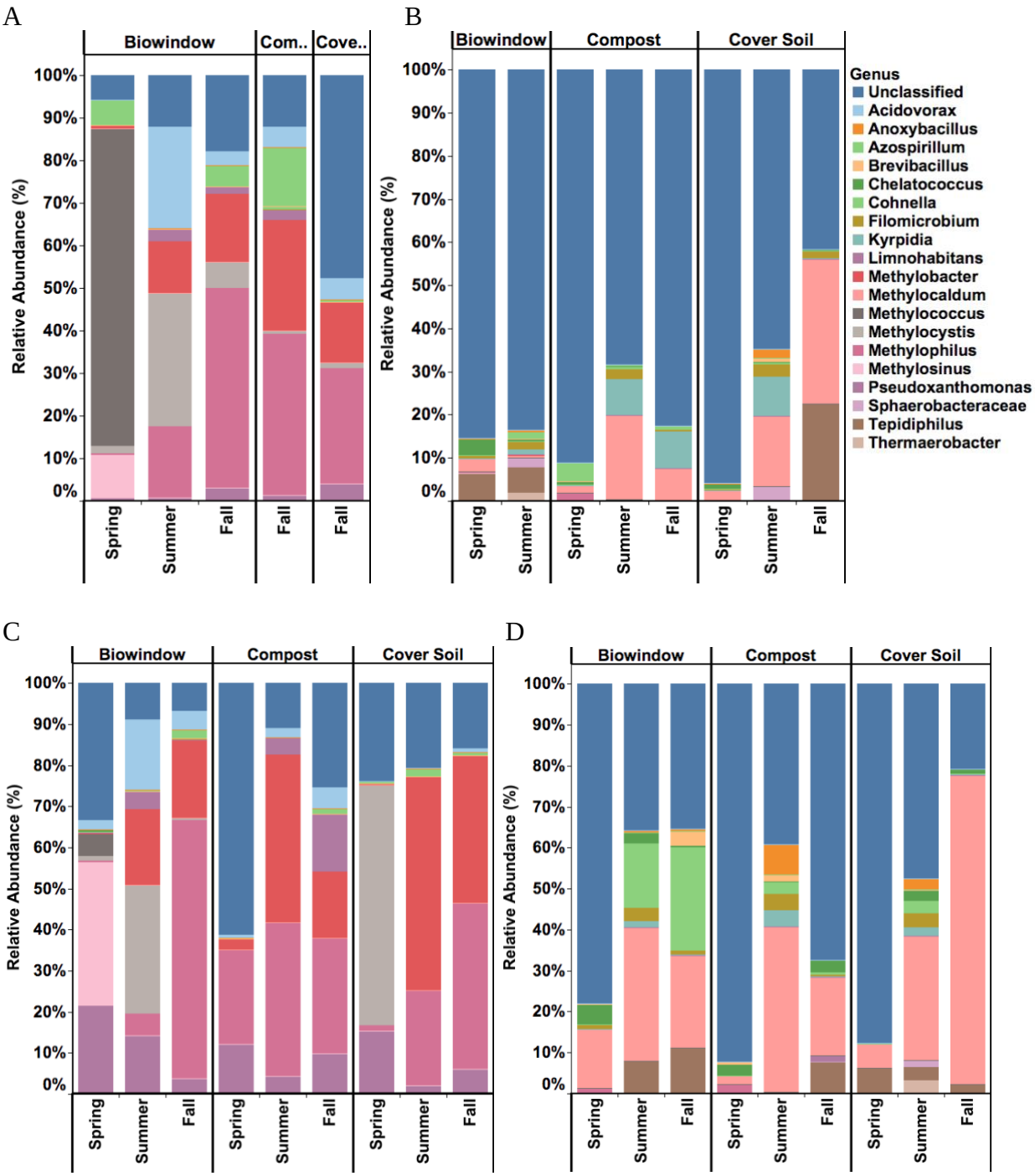


Figure 14. Taxa identified in soil DNA via V4-region 16S rRNA amplicon sequencing. **A)** Top 20 genera overall, **B)** only known methanotroph genera shown (as a proportion of all genera), **C)** only known methanotroph classes shown (as a proportion of all classes).


A
B
C
D
Biowindow
Com..
Cove..
Compost
Cover Soil
Relative Abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Spring
Summer
Fall
Genus
Unclassified
Acidovorax
Anoxybacillus
Azospirillum
Brevibacillus
Chelatococcus
Cohnella
Filomicrobium
Kyrpidia
Limnohabitans
Methylobacter
Methylocaldum
Methylococcus
Methylocystis
Methylophilus
Methylosinus
Pseudoxanthomonas
Sphaerobacteraceae
Tepidiphilus
Thermaerobacter

E

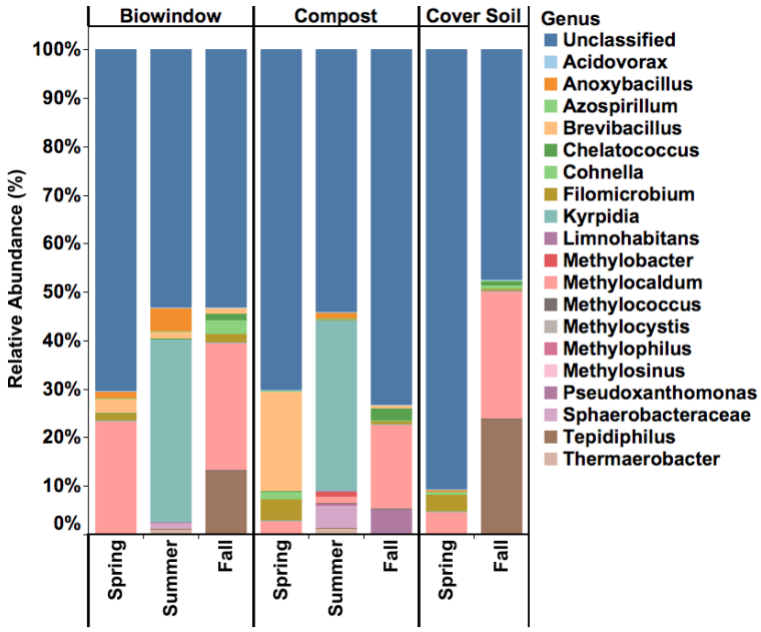


Figure 15. Genera identified in enrichment cultures via V4-region 16S rRNA sequencing. **A)** 22°C NMS – Cu, **B)** 45°C NMS – Cu, **C)** 22°C NMS + Cu, **D)** 45°C NMS + Cu, **E)** 55°C NMS + Cu + Ce.

3.4.1 Diversity indices

Beta diversity of all bacterial communities were analyzed using Principal Coordinates Analysis (PCoA) to allow for visualization of the relationships between each sample. In comparing the communities of each type of landfill soil, a distinct grouping can be seen for each soil type in Figure 16A. Within each of these groupings, there appears to be minor effects of seasonal change on the respective soil community. Biowindow soil shows the greatest amount of seasonal change, with its corresponding Summer-Wet and Fall communities reaching opposite ends of the quadrant. PCoA of all enrichment culture communities alongside landfill soil DNA revealed a very distinct grouping of mesophilic cultures, independent of sample time or location (Figure 16B). Thermophilic cultures were very distinct from the mesophilic cultures, however formed a looser knit group of populations and had little discernible differences found between the 45°C and 55°C cultures. The soil communities of compost and cover soil were well isolated from the enrichment cultures, with the major exception of Spring and Fall biowindow soil. These samples were found alongside the grouping of thermophilic enrichment cultures, indicating the microbial population of biowindow soil has a very high degree of similarity to the communities found in thermophilic laboratory enrichments.

Alpha diversity can be used to examine differences within each community. The Shannon index is an example of a statistical analysis of alpha diversity, which takes into account species richness as well as their relative abundance. In looking at the Shannon diversity of soil communities, cover soil showed the highest levels of diversity in all seasons, and biowindow soil exhibited the lowest diversity (Figure 17A). Compost had an intermediate level of diversity. The Simpson Index is an additional statistical analysis of communities that gives more weight to

more abundant species. Through this diversity index we again see cover soil with the highest diversity, with the addition of Summer-Wet biowindow soil and Fall compost showing relatively high outlying diversity (Figure 17B). The remaining biowindow samples had the lowest scores. Through analysis of the samples with an Evenness Index, data shows Biowindow soil having the least even community, and Cover Soil representing the highest level of evenness (Figure 17D). Shannon and Simpson diversity indices of enrichment cultures showed little differences seasonally or based on soil type, with the exception of several outliers (Summer 55°C Cover Soil, Fall 45°C Cover Soil) (Figure 18).

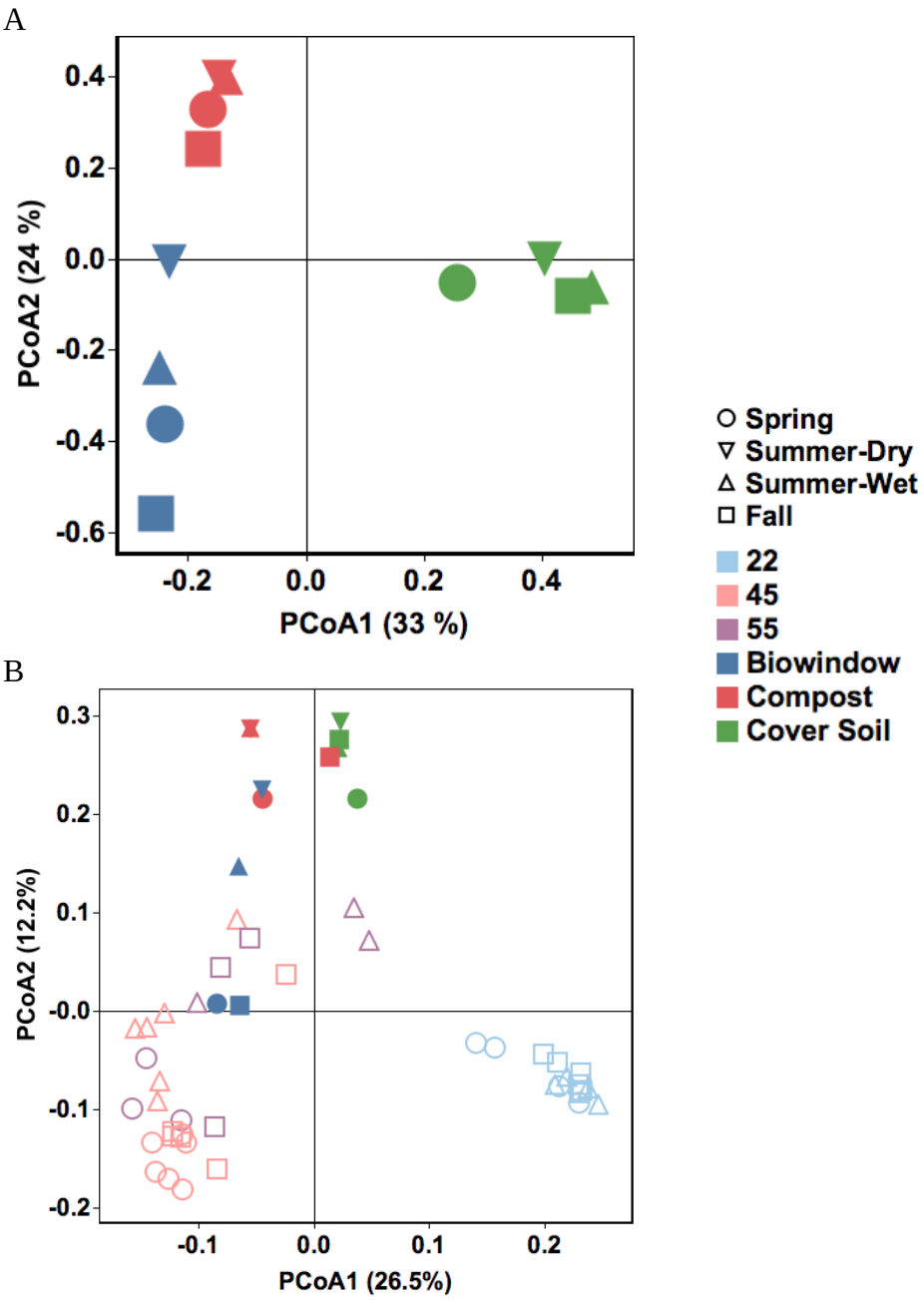


Figure 16. Principal coordinate analysis of 16S communities. Community distribution in soil only **(A)** and both enrichment cultures and soil **(B)**.

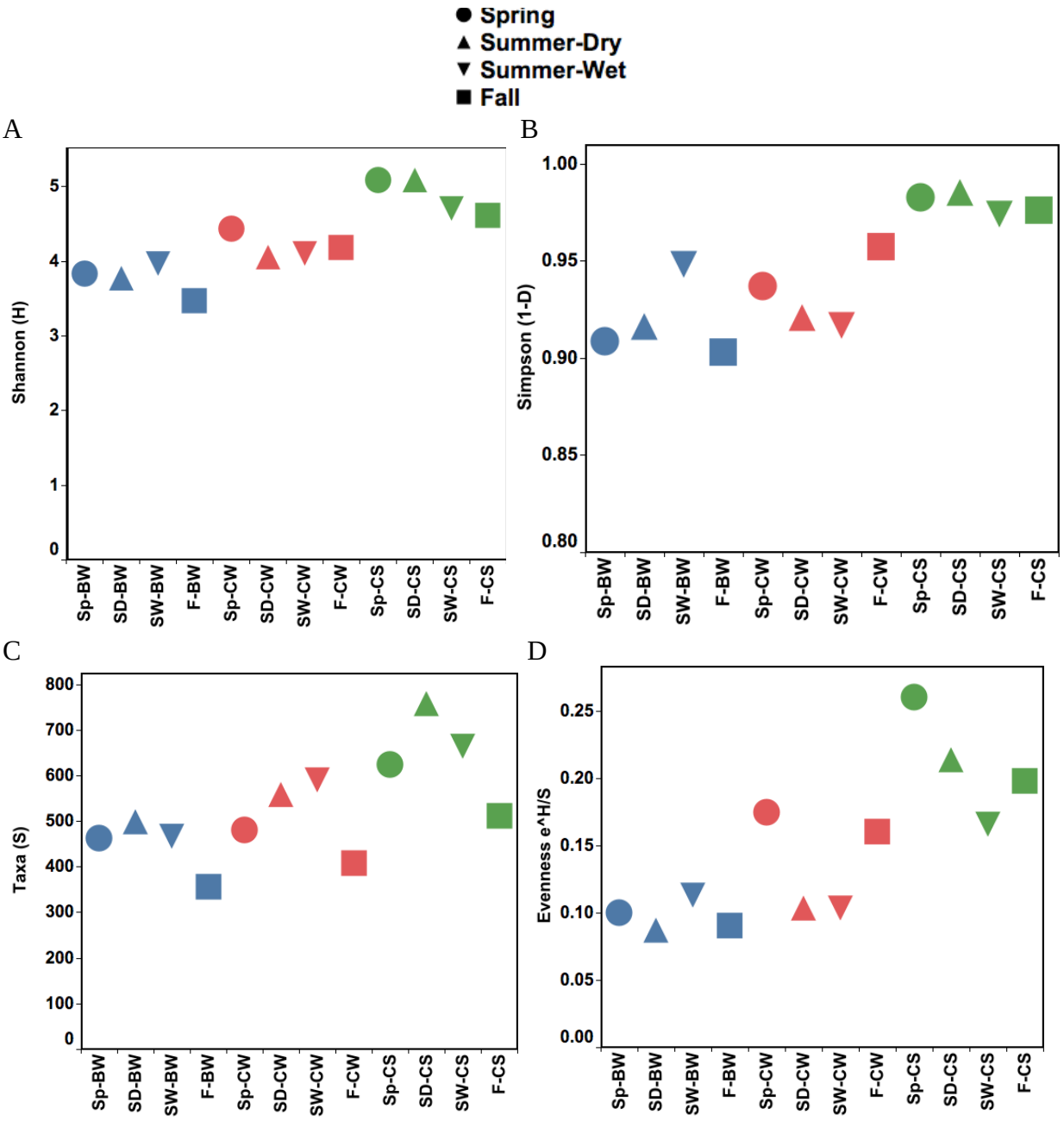


Figure 17. Diversity indices of landfill soil microbial communities. **A)** Shannon index, **B)** Simpson index, **C)** Total taxa, and **D)** Evenness.

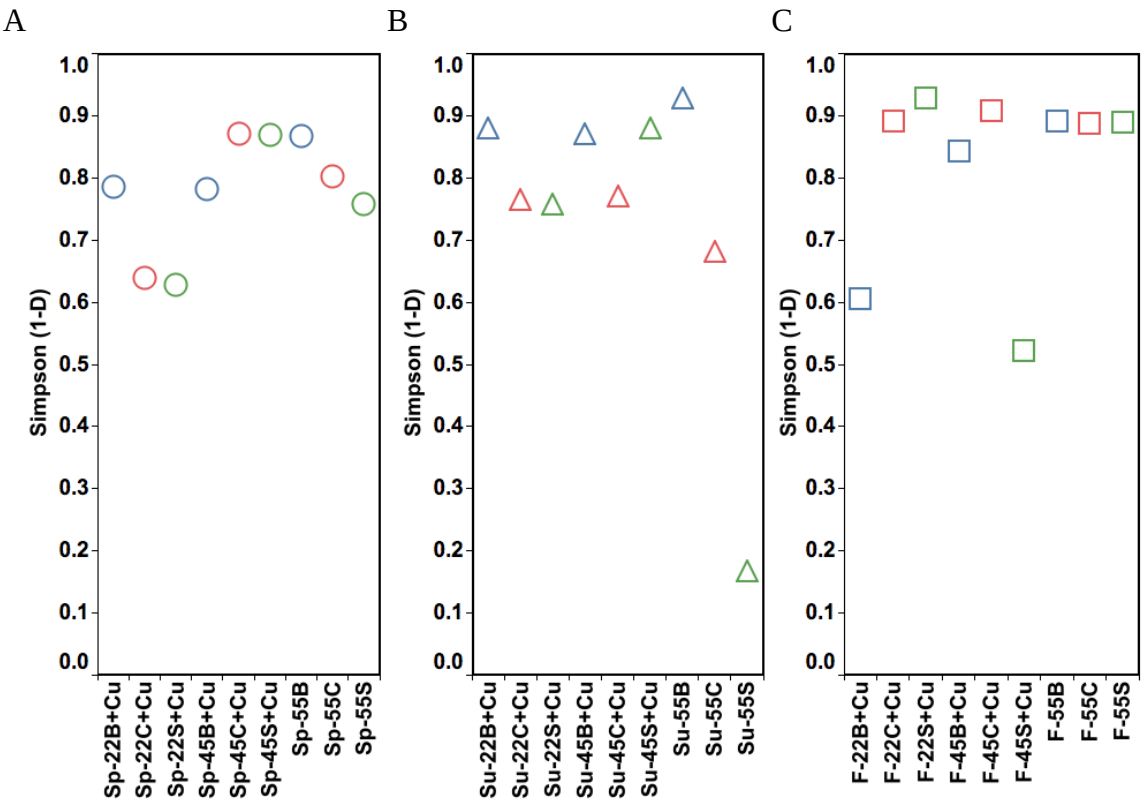


Figure 18. Simpson diversity indices of enrichment cultures. **A)** Spring, **B)** Summer, **C)** Fall.

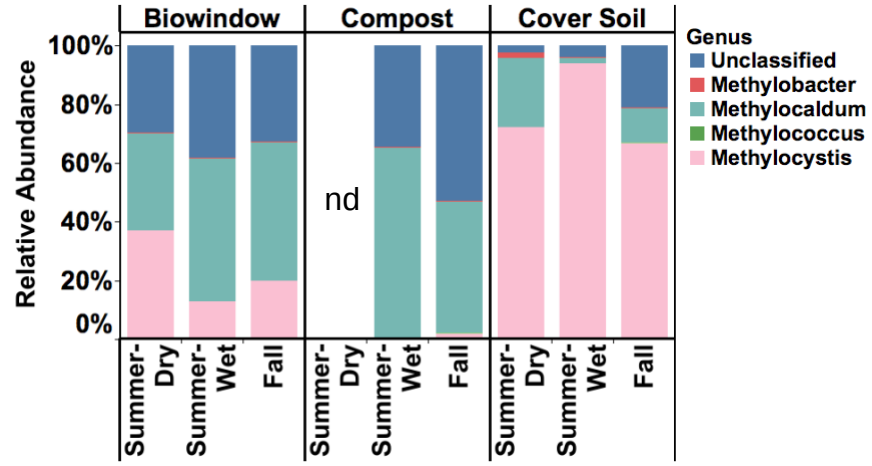
3.5 Methanotroph community analysis based on *pmoA* marker gene

In part due to the inaccuracy of sequencing a singular variable region of the 16S rRNA gene, phylogeny based on *pmoA* amplicon sequencing was established rather than make interpretations centred on methanotrophs lineage solely from 16S rRNA analysis. Deep-amplicon sequencing of the *pmoA* methanotroph marker gene allowed for further genomic characterization of the methanotroph community in soil and enrichment cultures. In the soil DNA samples, sequencing reads of Biowindow soil at the genus level were largely *Methylocaldum* (33% - 49%) or *Methylocystis* (13% - 37%) (Figure 19A). Cover Soil DNA possessed a much higher ratio of *Methylocystis* (67% - 94%) to *Methylocaldum* (2.0% – 24%). *Methylobacter* was detected in Cover Soil at a relative abundance of 0.3% - 1.9%. At the class level, Biowindow and Cover Soil showed inverse ratios of Gammaproteobacteria to Alphaproteobacteria; Gammaproteobacteria corresponded to 48% - 75% of Biowindow sequences, while Cover Soil showed a relative abundance of 68% - 96% Alphaproteobacteria (Figure 19C). Consistent with 16S rRNA sequencing, major differences in enrichment cultures were seen in the methanotroph communities under mesophilic conditions compared to thermophilic conditions (Figure 20). Cultures grown at thermophilic temperatures revealed sequencing reads belonging predominantly to *Methylocaldum*, and some *Methylocystis.* Under mesophilic conditions, cultures were found to consist of mostly *Methylocystis* or *Methylobacter.* Small numbers of reads corresponding to *Methylosinus* and *Methylomicrobium* appeared in 22°C Compost and Cover Soil cultures. For both enrichment cultures and raw soil, seasonal variation did not appear to play a significant role in the relative abundance of various *pmoA* sequences.

A large proportion of *pmoA* sequences found in Summer and Fall thermophilic cultures did not correspond to any previously classified methanotroph genera. However, when these

pmoA sequences were placed into phylogenetic trees, they were found to be close relatives of *Methylocaldum* species (Figure 21 & Figure 22). These bacteria were abundant in the enrichment cultures, but were represented by a small number of organisms that showed little novelty. A phylogenetic tree based on Summer Biowindow soil displayed a large proportion of sequences being closely related to *Methylocystis parvus* and *Methylosinus trichosporium* (Figure 23). Another branch in the tree indicates phylogenetic relations to the species' *Methylocaldum gracile* and *Methylocaldum szegediense*. Several uncultured, unknown organisms were identified that were closely related to *Methylosoma difficile*, *Methylococcus capsulatus*, *Methylocaldum szegediense*, and *Methylocystis* species.

A



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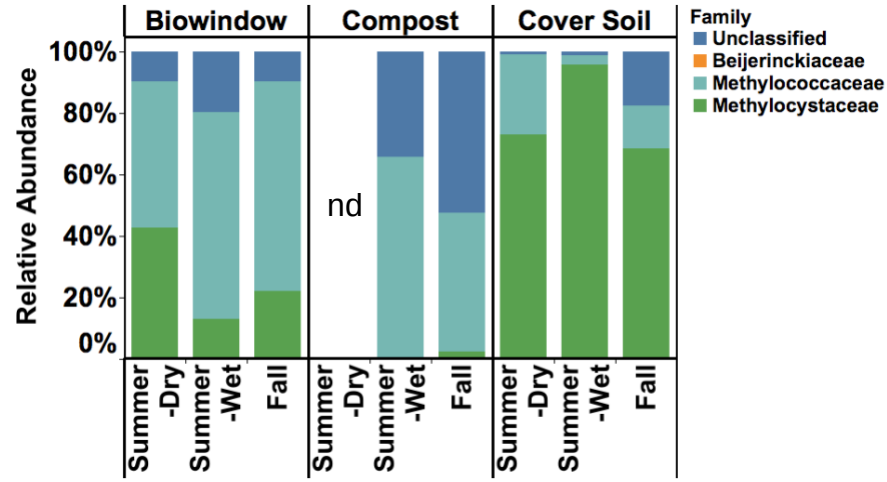

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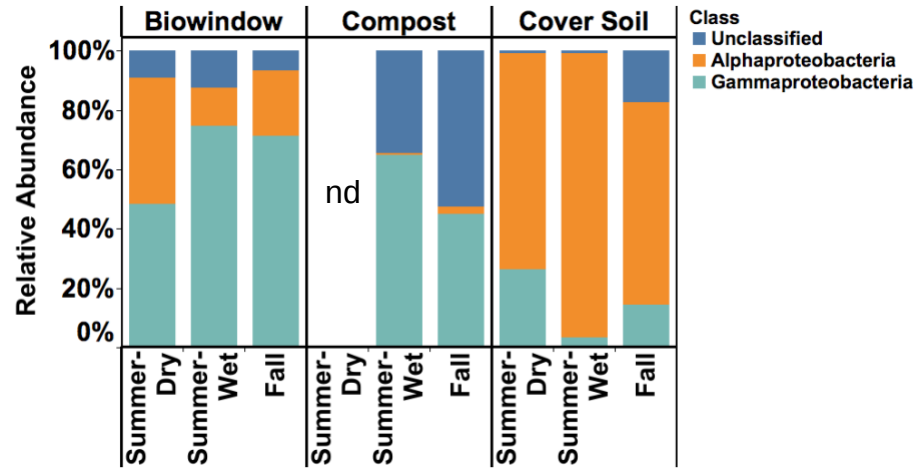

Figure 19. Methanotrophic taxa identified in landfill soil DNA via *pmoA* amplicon sequencing.

A) Genera, **B)** Family, **C)** Class. ND: not determined

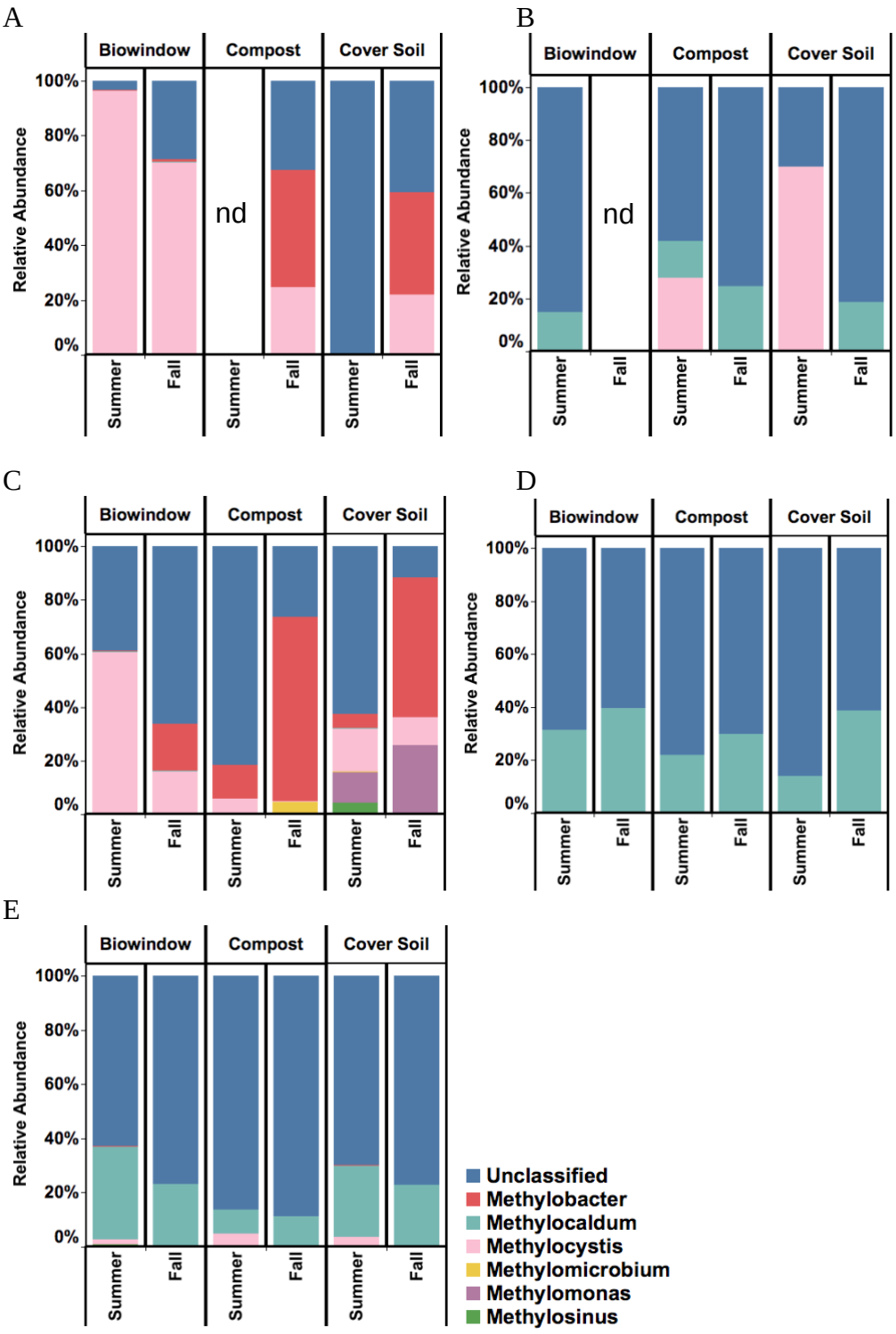

Figure 20. Genera identified in enrichment cultures via *pmoA* amplicon sequencing. **A)** 22°C NMS – Cu, **B)** 45°C NMS – Cu, **C)** 22°C NMS + Cu, **D)** 45°C NMS + Cu, **E)** 55°C NMS + Cu + Ce. ND: not determined

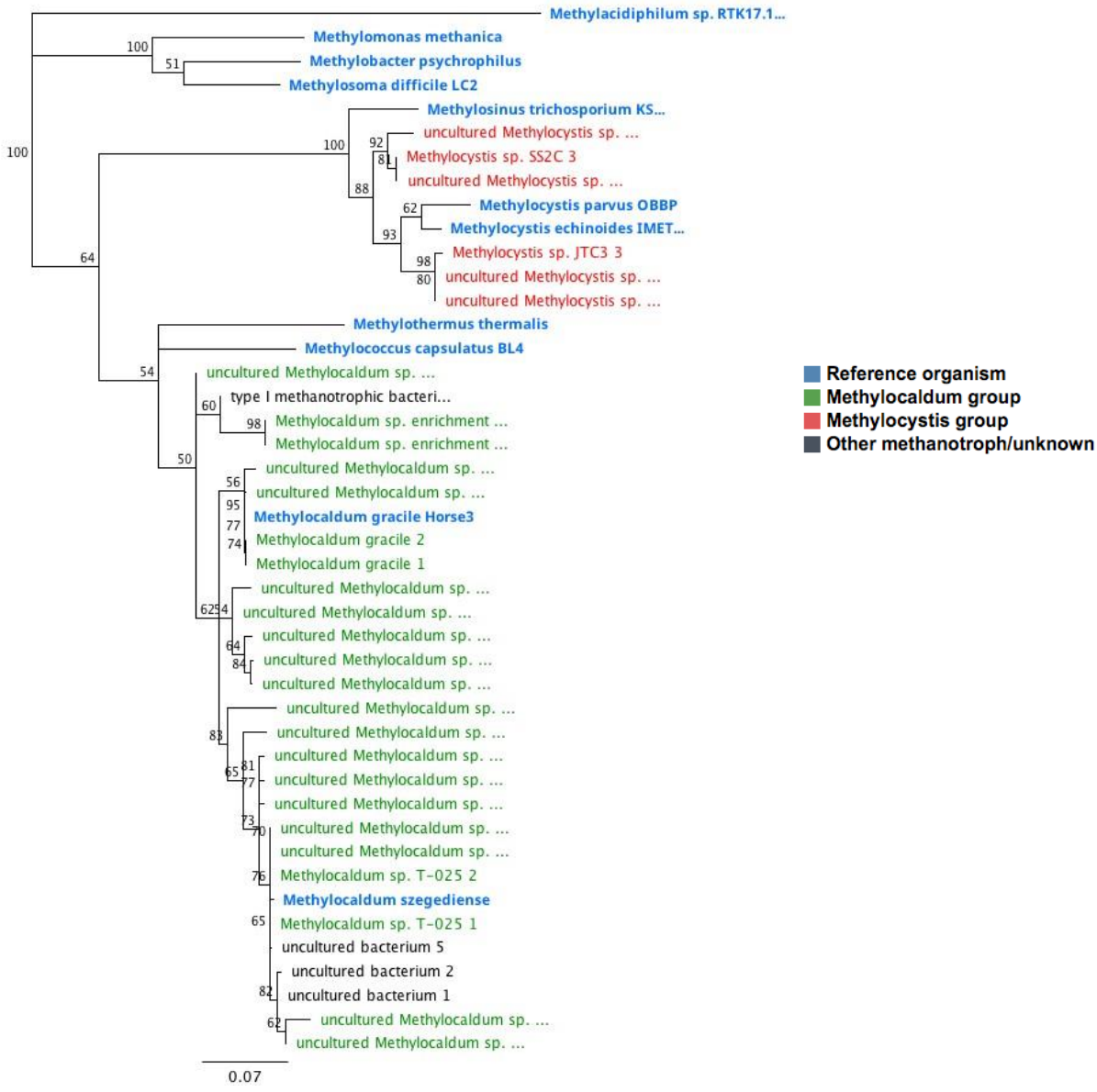

Figure 21. Phylogenetic tree of methanotrophs in Summer (Wet) thermophilic (45°C and 55°C) biowindow enrichment cultures based on *pmoA* amplicon sequencing. Bootstrap values shown on branches, while scale represents substitutions per site. *Methylacidiphilum* used as outgroup.

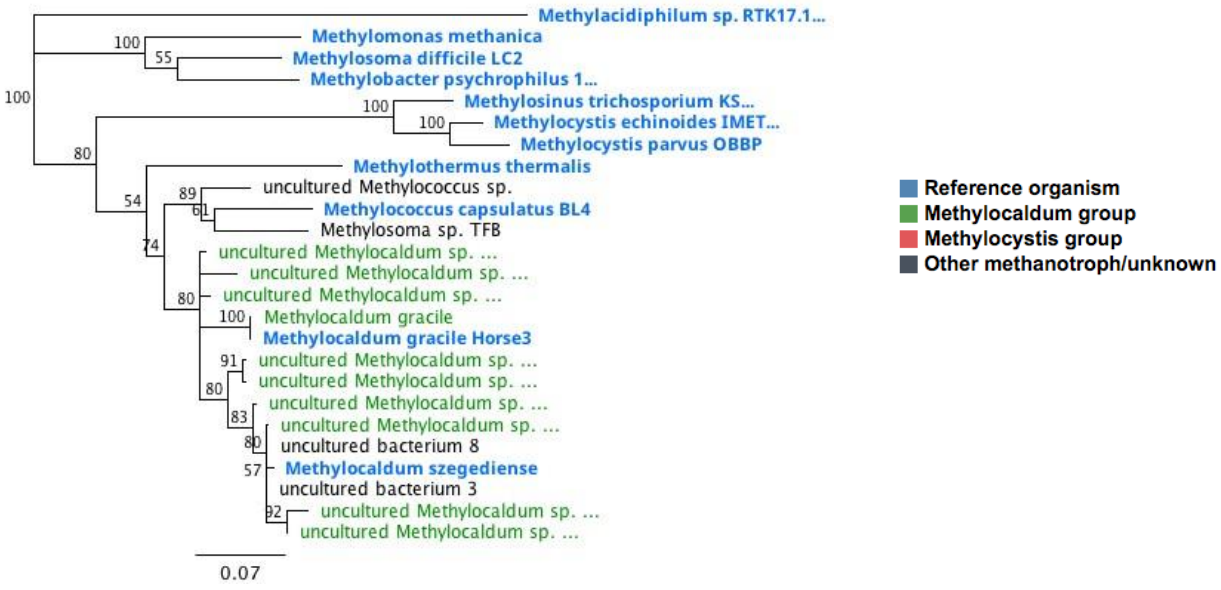

Figure 22. Phylogenetic tree of methanotrophs in all Fall thermophilic (45°C and 55°C) biowindow enrichment cultures based on *pmoA* amplicon sequencing. Bootstrap values shown on branches, while scale represents substitutions per site. *Methylacidiphilum* used as outgroup.

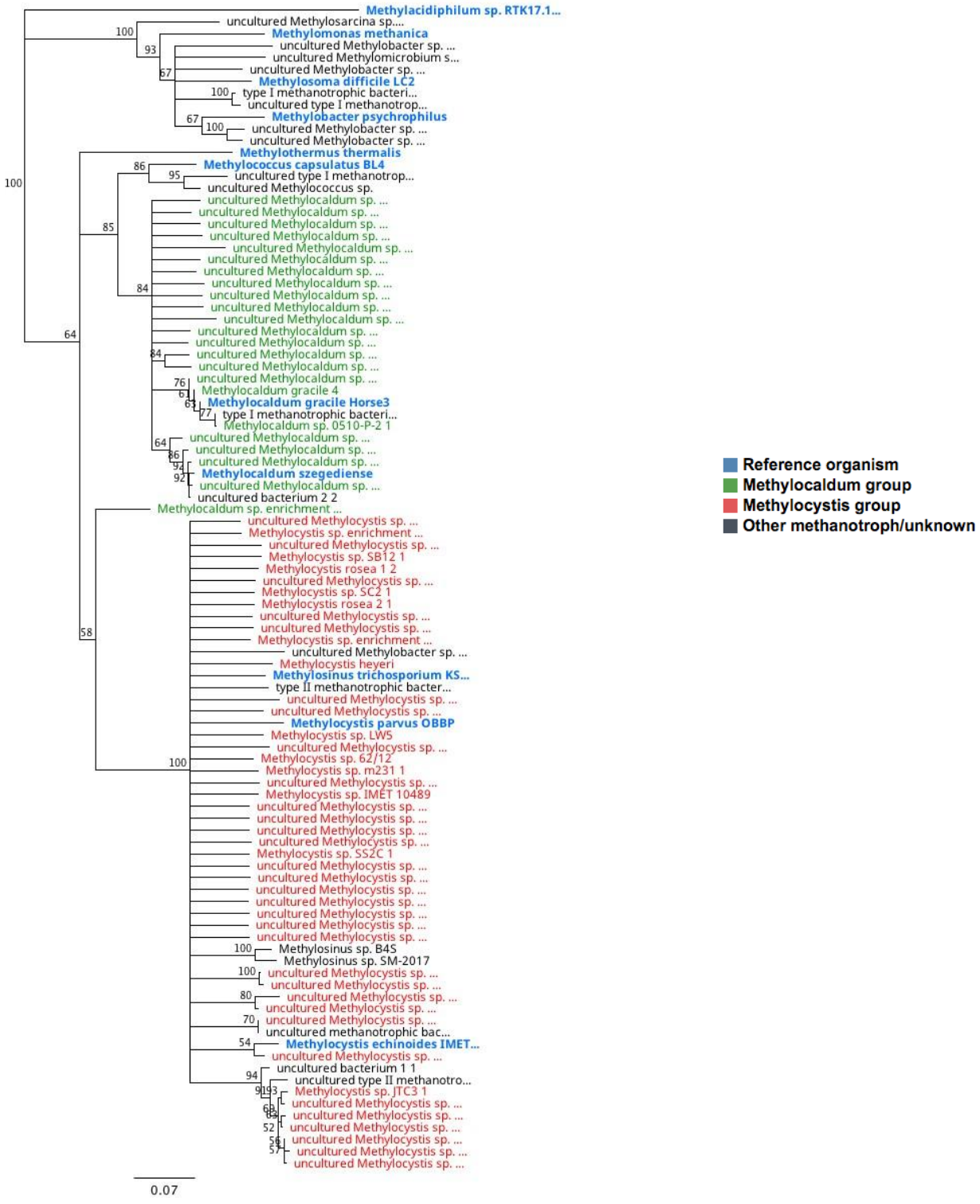

Figure 23. Phylogenetic tree of methanotrophs in Summer (Wet) biowindow soil based on *pmoA* amplicon sequencing. Bootstrap values shown on branches, while scale represents substitutions per site. *Methylacidiphilum* used as outgroup.

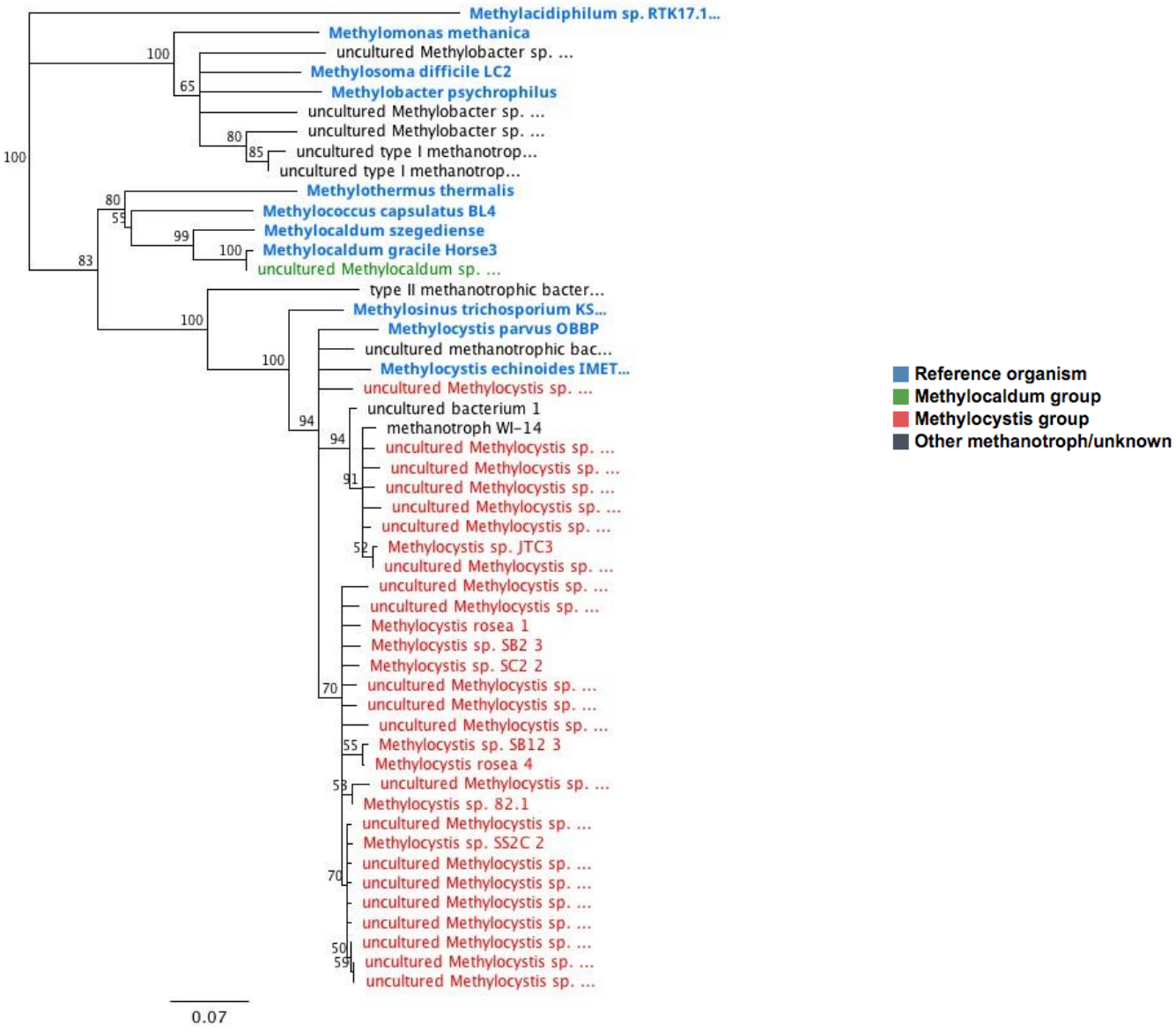

Figure 24. Phylogenetic tree of methanotrophs in Summer (Wet) mesophilic (22°C) biowindow enrichment cultures based on *pmoA* amplicon sequencing. Bootstrap values shown on branches, while scale represents substitutions per site. *Methylacidiphilum* used as outgroup.

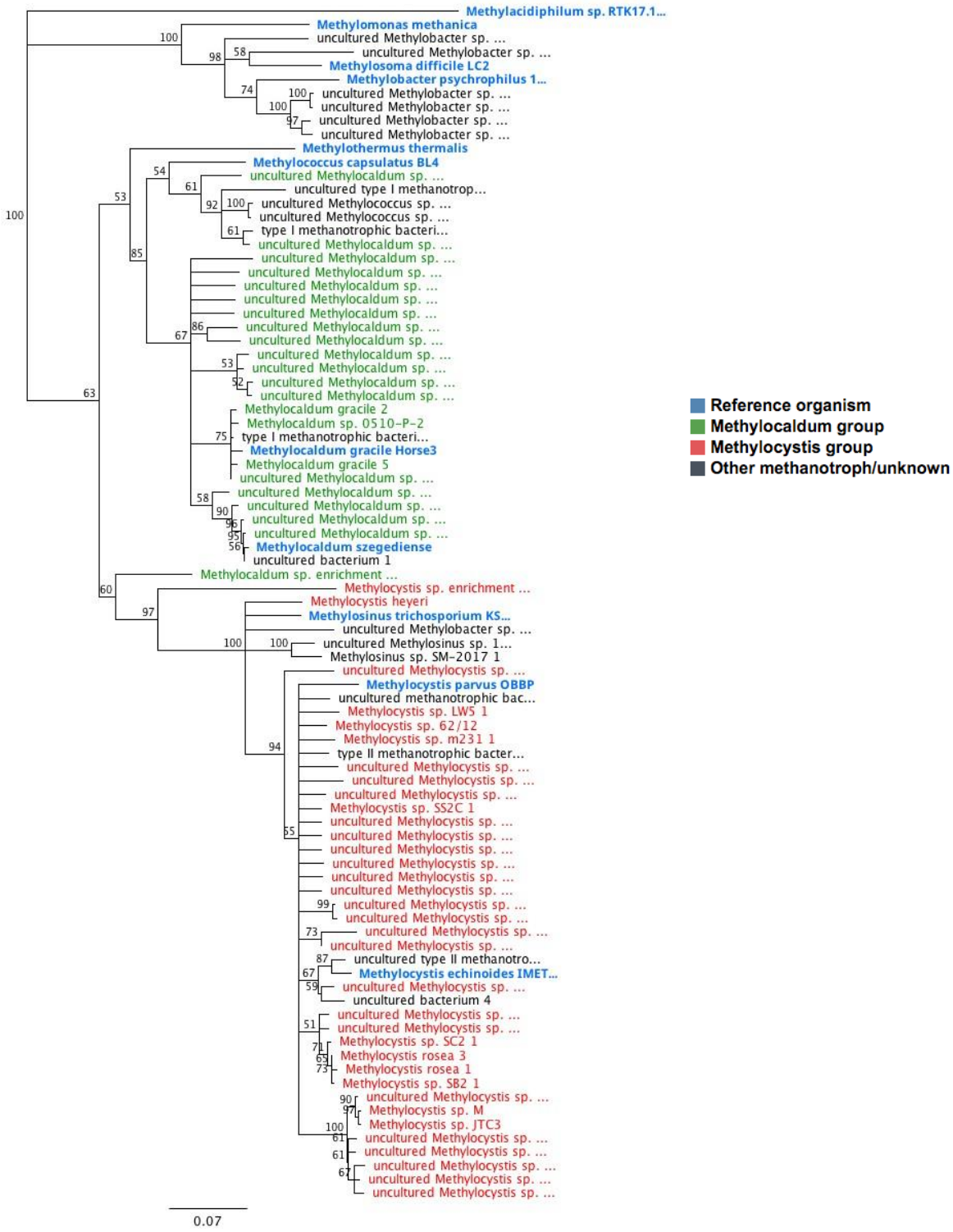

Figure 25. Phylogenetic tree of methanotrophs in Fall biowindow soil based on *pmoA* amplicon sequencing. Bootstrap values shown on branches, while scale represents substitutions per site. *Methylacidiphilum* used as outgroup.

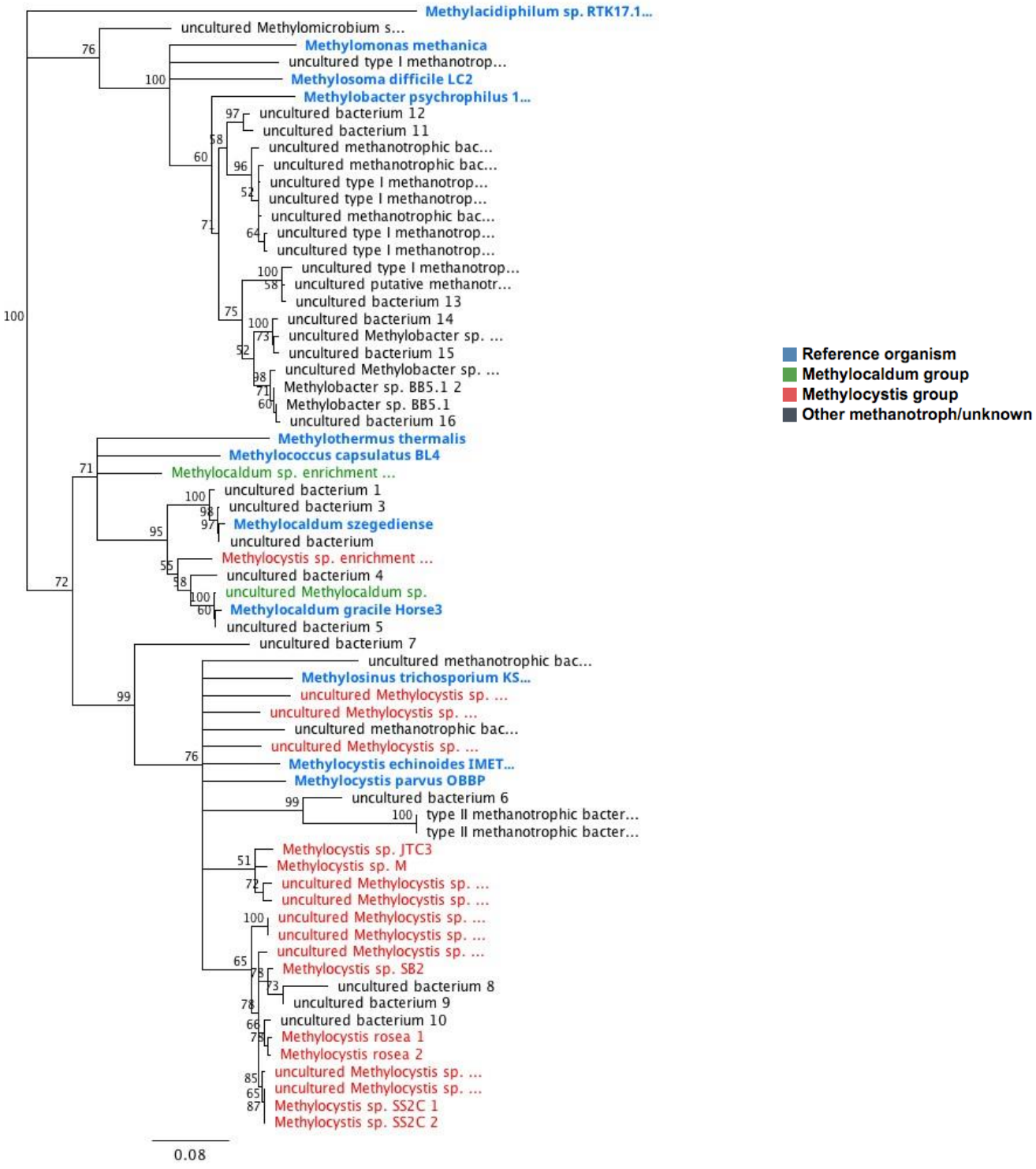

Figure 26. Phylogenetic tree of methanotrophs in all Fall mesophilic (22°C) biowindow enrichment cultures based on *pmoA* amplicon sequencing. Bootstrap values shown on branches, while scale represents substitutions per site. *Methylacidiphilum* used as outgroup.

3.6 Methanotroph abundance in landfill

Quantitative PCR was performed on landfill soil DNA samples to quantify the amount of *pmoA* and *mmoX* present in the samples, and therefore determine the abundance of methanotrophs. The results showed little variation in either gene between the Biowindow and Cover Soil, although Compost showed significantly lower numbers of *pmoA* (p-value = 0.01 (Figure 27). There was no significant difference between the different seasons, or between the quantity of *mmoX* found in the various soil types.

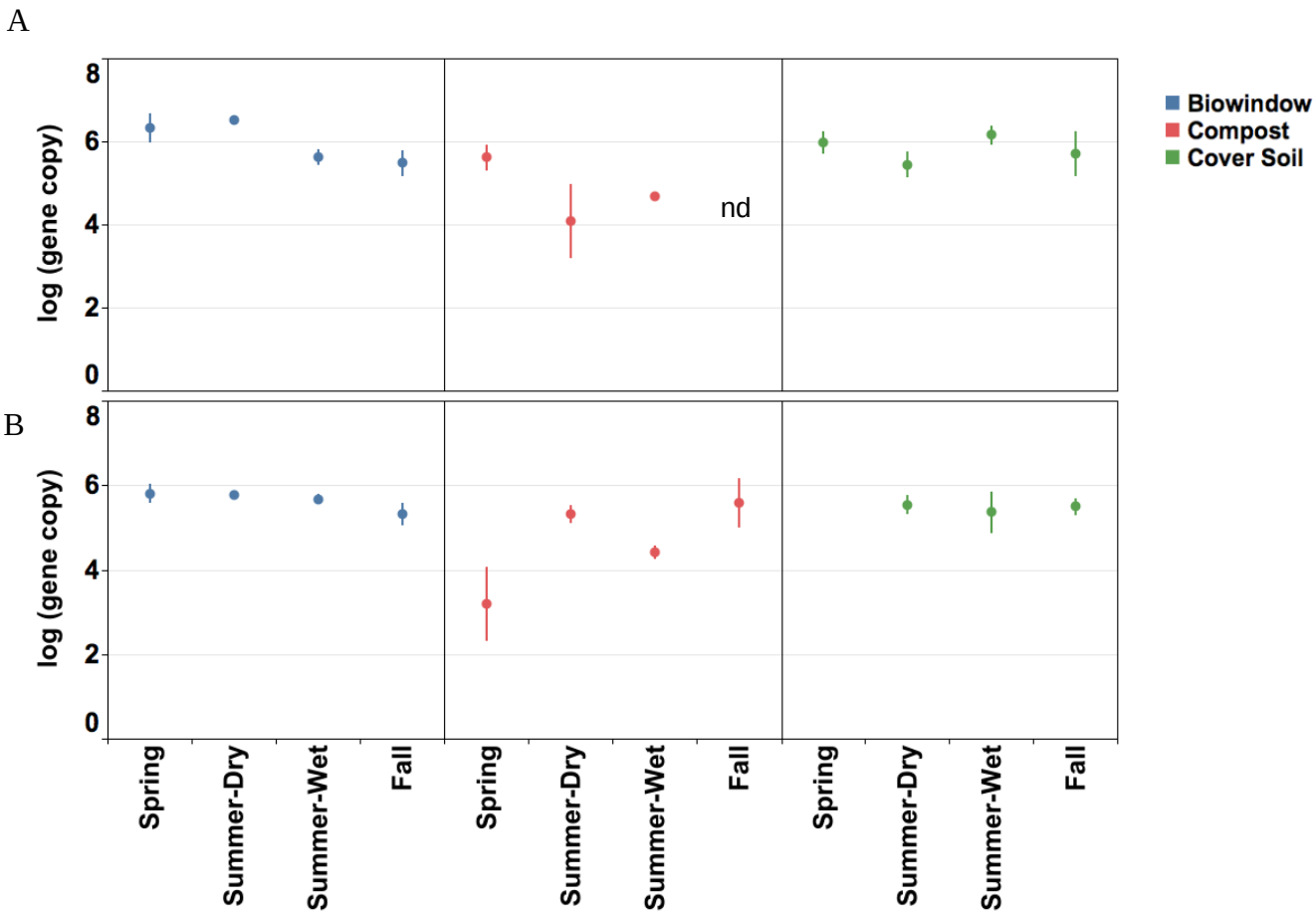

Figure 27. Methanotroph abundance based on qPCR targeting *pmoA* **(A)** and *mmoX* **(B)** genes. Values are expressed as the log of gene copy number per gram of soil. Data points represent average of 3 technical replicates, and bars show standard deviation. Nd indicates no data for that sample.

Chapter 4: Discussion

4.1 Methanotrophs in engineered landfill soil columns

Based on previous exploration of landfill soil columns, it was hypothesized that these laboratory-scale microcosms contain a substantial community of methanotrophic bacteria, which are responsible for the observed methane oxidation. The findings of our study confirmed this hypothesis, revealing that a large proportion of methanotrophs are present among a highly diverse community of organisms. While both Type I and Type II methanotrophs were present in Columns 1 and 2, the relatively high proportions of Type I methanotrophs found in both columns were consistent with previous studies of similar long-term landfill column tests (Yargicoglu and Reddy 2017; Attalage et al. 2022). Gas chromatography analysis of the columns performed by Berenjkar et al. (2021) revealed lower ratios of O_2:CH_4 in Column 1 throughout the duration of the experiment. Type II methanotrophs, belonging to the Alphaproteobacteria class, typically outcompete their counterparts in environments with low ratios of O_2:CH_4 (Börjesson et al. 2004), indicating Type II organisms would be expected in higher amounts in Column 1. However, the inverse was shown with over twice as many Type I methanotrophs, or Gammaproteobacteria found (Figure 4A). Gas profiles differed with increased depth, however the samples used for genomic analysis were composites of all depths, limiting the conclusions that can be made regarding effects of gas concentrations. Overall, differences in gravel size, used to enhance gas diffusion through the columns, proved to have minimal effect on diversity of the overall microbial communities in the columns.

The presence of *Methylocaldum* in both columns in similar amounts is consistent with the results of biowindow soil analysis from which the column compost originated (Figure 4D).

Through confirming the presence of this genus, we can assume it is responsible for a portion of active methane oxidation in the columns, although temporal changes of this group are not known as only end-point soil samples were extracted. Identification of *Methylobacter* was consistent with similar results citing *Methylobacter* and *Methylomonas* as the predominant Alphaproteobacteria in test columns (Yargicoglu and Reddy 2017). Existing literature has not cited a notable presence of organisms such as *Methylosinus* and *Methylocystis* belonging to the family *Methylocystaceae* in column analyses, although their presence here is consistent with the results of the biowindow analysis (Figure 4D, Figure 14B). The results of qPCR supported the results of the 16S rRNA sequencing, in regard to the methanotroph genera identified. The primary methanotroph found in Column 1 was *Methylocaldum*, the species of which mainly possess only *pmoA* (Semrau et al. 2018). This is consistent with the significantly higher levels of *pmoA* that were detected in this column. Column 2 displayed a wider variety of methanotrophs including those that contain both the *pmoA* and *mmoX* genes, such as *Methylosinus* and *Methylocystis*. This is consistent with qPCR results, which showed *pmoA* levels that were highly similar to that of *mmoX* in Column 2.

Alpha diversity of the column soil revealed higher Shannon index values than the biowindow samples (Table 1, Figure 17A), which could be attributed to the increased moisture content of the columns, allowing a wider variety of less resilient, heterotrophic organisms to thrive. High proportions of *Anaerolinae* detected in both columns indicate the present of an anaerobic microenvironment (Figure 4A). Interestingly, the high levels of *Anaerolineae* are comparable to an elevated proportion of this group found in a study analyzing rice field soil, an environment also highlighted by the excessive levels of methane (Cabezas et al. 2015). *Anaerolinae* are typically slow-growing, mostly anaerobic organisms, and it has been

hypothesized that they play a role in producing metabolic intermediates such as acetate or hydrogen from soil organic matter in methane-rich environments, which could then be used in electron-transfer to other microorganisms (Yamada and Sekiguchi 2009; Cabezas et al. 2015). Overall, the methanotroph community showed a high degree of similarity between the near ideal conditions for methanotrophy present in the columns compared to the observed community in the biowindow itself, therefore attributing the majority of variance to non-methanotrophic organisms.

4.2 Seasonal fluctuations of landfill soil and methanotrophs at BRRMF

Based on previous findings outlined in the literature review, it was hypothesized that Manitoba landfill biowindow soil is home to thermophilic methanotrophs, the communities of which undergo changes with the wide seasonal variation seen in this climate. To supplement the microbial analysis of this environment, physiochemical parameters of the soil were also analyzed to determine potential factors influencing the local methanotroph communities.

Thermophilic temperatures found consistently within the biowindow are a result of large amounts of heat production from within the landfill (Figure 7). Anaerobic fermentation and methanogenesis produce heat during waste degradation which rises upward from the waste layer creating a thermophilic environment in the biowindow, augmented by heat production from biodegradation of methane (Grillo 2014; Yang et al. 2021). *In situ* soil temperatures of cover soil and compost were only ever in the psychrophilic and mesophilic range as the compact, non-porous nature of the clay cover soil allows for limited heat penetration. Additionally, the compost windrows under examination were mature therefore limited active metabolic

decomposition processes were occurring. Compost soil is also exceptionally porous and light, allowing for maximum diffusion of wind currents to pass through the surface, decreasing the soil temperature in the outer layers.

Metadata collected throughout this field season was consistent with previous findings of thermophilic conditions present in the BRRMF biowindow (Berenjkar et al. 2021), however the unseasonably dry year highlighted the influential role moisture content plays in the methane oxidation potential. Total water capacity of less than 30% is defined as water deficit, while drought is classified as water capacity of 12–20% for a period of 16 days (Bogati and Walczak 2022). Considering this classification, conditions within the biowindow were at minimum under a water deficit in every season tested except for spring, while cover soil displayed a water deficit during all seasons, and compost never experienced a water deficit (Figure 7). Biowindow moisture levels fell below 20% in summer (dry) and fall, suggesting drought despite significant rain episodes. Moisture level is widely known to be a key factor in methane oxidation and is likely responsible for low initial MOP, as increased moisture content in the biowindow correlated strongly with increased MOP in the spring (Figure 8) (Dijkstra et al. 2012; Sadasivam and Reddy 2014; Ran et al. 2017). The observed low MOP of biowindow soil indicates thermophilic methanotrophs have low activity *in situ* during periods of low moisture and drought, suggesting the cells undergo a period of dormancy in response to environmental stress. Previous studies have implemented a pre-incubation period of landfill soil for 60 days with methane to induce methane oxidizing activity in presently inactive soil and determine the ultimate potential for methane oxidation, a technique that could be applied in future analysis of this topic (Spokas and Bogner 2011). The 2020 compost windrow also proved to retain its moisture content more readily than the 2016 materials in the biowindow, which suggests that

either fresh compost inoculation or irrigation of the biowindow may improve *in situ* methane oxidation conditions. A 2021 study by He *et al* tested the use of landfill leachate as irrigation to restore low methane oxidation rates in landfill biocover soil. This research took place in an arid climate in which continuous moisture loss had resulted in problems with maintaining methanotroph activity (He et al. 2021). Irrigation with leachate increased *pmoA* abundance by up to 79-fold, and resulted in a methane-removal rate 4-fold higher as compared to water irrigation (He et al. 2021). The efficacy and availability of leachate for this purpose could be a potential solution to moisture loss seen in the BRRMF biowindow that is worth exploring further.

The seasonal shift to thermophilic conditions in the biowindow correlated with a relative increase in *Methylocaldum* based on 16S rRNA findings, as the conditions enriched for known thermophilic methane oxidizers (Figure 14). This observed change in community is consistent with similar studies that have documented a shift in methanotroph community above 30°C in landfill soil microcosms (Reddy et al. 2019; Raksha et al. 2020). The same studies identified *Methylocaldum* as a dominant organism at thermophilic temperatures. *Methylocaldum szgediense* and *Methylocaldum gracile*, two of the four species in this genus, have been isolated from hot springs in Hungary (Bodrossy et al. 1997), therefore the presence of this organism in BRRMF's continental climate landfill is of interest. Under mesophilic conditions, previous studies have shown *Methylobacter* as the principal mesophile in landfill soil microcosms and biocover soil (Reddy et al. 2019; Raksha et al. 2020; Yang et al. 2021), however the results of this research found *Methylocystis* to be most abundant at mesophilic temperatures in the spring. It has been suggested that a group of thermotolerant *Methylocystis*-like organisms exists, after similar Alphaproteobacterial methanotrophs were detected in Russian hot springs at temperatures

exceeding 50°C (Kizilova et al. 2014), leading to the possibility that the *Methylocystis* detected in the biowindow may be thermotolerant or thermophilic.

Compared to 16S rRNA sequencing results, a recurrence of similar methanotrophs were found through *pmoA* amplicon sequencing (Figure 19). Few studies have been conducted using *pmoA* sequencing of landfill soil DNA, although some work focused on meadow and rice field soil, sewage sludge, or aquifer water samples have shown similar groupings in comparing 16S and *pmoA* sequencing (Kolb et al. 2003; Paszczynski et al. 2011; Ghashghavi et al. 2017). In our study, the groupings identified in landfill soil via 16S rRNA (*Methylocaldum, Methylocystis, Methylobacter, Methylococcus, Methylosinus*) were consistent with the *pmoA* identified groupings (*Methylocaldum, Methylocystis, Methylobacter, Methylococcus*). A 2003 *pmoA*-based amplicon study of soil methanotrophs by Kolb *et al.* revealed the primary genera as *Methylococcus, Methylobacter, Methylosinus,* and *Methylocapsa*, consistent with concurrent 16S analysis. Additionally, through examination of *pmoA* gene expression in landfill soil, Chen *et al* (2007) identified the presence of *Methylobacter, Methylosarcina, Methylomonas, Methylocella,* and *Methylocystis*. The prevalence of *Methylocaldum* in the landfill soil examined in our study can be attributed to the thermophilic environment of the biowindow. Moreover, in an Ontario landfill study, Gammaproteobacteria methanotrophs were documented as having more diversity than their Alphaproteobacteria counterparts (Lin et al. 2009). Through the phylogenetic tree constructed of biowindow soil reads in Figure 23, similar findings can be observed in comparing the diversity of reads related to Type I methanotrophs, e.g. *Methylocaldum, Methylobacter, Methylosoma*, to the more highly similar population of *Methylocystis* sp. This is additionally comparable in nature to the findings of Lin's group, in that most Type II methanotrophs

identified were most closely related to that of *Methylocystis,* while Type I methanotrophs were most like *Methylocaldum.*

It was hypothesized that mature YWLC compost windrows were a source of thermophilic methanotroph inoculum for the biowindows. Mature compost was examined as this would ultimately be the nature of the compost used in engineering subsequent biowindows for fugitive methane removal. Interestingly, relatively small amounts of methanotrophs were identified in these compost samples (Figure 14B). This was confirmed through qPCR in which a significantly lower abundance of *pmoA* gene was detected in compost samples compared to biowindow soil (Figure 27). It is known that methanotrophs as well as methanogens are present during composting processes while active decomposition is happening (Jäckel et al. 2005; Halet et al. 2006), however given the maturity of the windrows examined in this study it is likely that minimal active biological breakdown was occurring. The compost was still able to act as an inoculum when transferred into the nutrient and methane -rich environment of enrichment cultures, however it would be expected that the addition of CH_4 during initial MOP tests would have also restored methane oxidizing activity. Given that the biowindow was constructed with 4:1 BSC to YWLC, it could be suggested that the biosolids compost plays a more instrumental role as an initial inoculum for the biowindow's methanotroph community. As this study focused only on YWLC windrows, the influence of the BSC in methanotroph inoculum would require further examination.

Thermophiles were present in the compost in relatively high numbers but did not belong to any known methane oxidizing groups. Overall, an abundance of non-methanotroph thermophiles were identified in the biowindow and compost soil, as seen with the highly abundant *Sphaerobacter* (Figure 14), a thermophilic Actinomycete previously isolated from

thermophilically treated sewage sludge (Hugenholtz and Stackebrandt 2004). *Anoxybacillus*, *Brevibacillus*, *Chelatococcus*, *Kyrpidia*, *Tepidiphilus,* and *Thermaerobacter* all contain known thermophilic species (Manaia et al. 2003; Han et al. 2010; Xu et al. 2014; Gomri et al. 2018; Reiner et al. 2018; Jabeen et al. 2019), and were all in the top 20 most abundant genera identified.

Additionally, the widespread presence of organisms known to possess the SQR gene for sulfide reduction in all soil samples indicates the potential role this community may play in odor reduction at BRRMF (Schmitz et al. 2022). Further analysis would be required to explore the activity of this gene in the biowindow environment compared to conventional cover soil.

The microbial diversity of the landfill soils examined showed similarities to previously reported values in pilot-scale biocover soils based on Shannon diversity index, particularly in the conventional cover soil with values 4.614 – 5.088 (Figure 17), compared to literature values of 4.703 – 5.587 (Yang et al. 2021). However the biowindow soil showed a slightly lower diversity, between 3.465 in the fall to 3.961 in the summer (wet). Shannon index values of 3.872 – 4.227 were reported in a similar biocover study (Lee et al. 2018). Total OTU counts in the current study ranged from 355 (biowindow) to 757 (cover soil) and had fair overlap with numbers reported by Yang *et al* in 2021 (545 – 1060). Overall, the decreased diversity in the biowindow community suggests the environment is being enriched for bacteria that are more targeted for that specific niche. With the added selection pressures of thermophilic conditions and high methane levels permeating the system, the community appears more streamlined. This is supported by the Principal Coordinates Analysis data shown in Figure 16, in which the biowindow soil clearly falls into a grouping with thermophilic laboratory enrichment cultures, noticeably further away from all other non-biowindow soil types. Of additional note, cover soil

consisted of composite samples up to 40 cm depth, which encompassed ~10 cm of topsoil followed by clay. As the topsoil is covered with significant vegetation, the rhizosphere must be considered as a factor that may be adding to the microbial diversity of the cover soil samples.

The frequency and duration of field data collection should be taken into consideration when interpreting the fluctuations of microbial community. This study included samples that spanned a duration of seven months in one calendar year, and sample retrieval occurred an average of two months apart. A long-term analysis on this research topic would be beneficial given the current volatility of annual climate conditions, especially when dealing with a continental climate that experiences large swings in in both moisture and temperature.

4.3 Enriching for thermophilic and mesophilic methanotrophs

Based on the conditions identified in the BRRMF biowindow, it was hypothesized that through laboratory enrichments both thermophilic and mesophilic methanotrophs could be cultured. Through various culture-based techniques, this study conclusively showed that thermophilic methanotrophs are present in the biowindow. While MOP tests indicated thermophilic methanotrophs have low activity *in situ* during periods of low moisture and drought, this section demonstrated they can be re-activated and enriched for given the proper conditions.

Enrichment culture experiments in which soil with low initial MOP was inoculated into liquid media resulted in cultures regaining methane oxidizing ability (Figure 9). These results are consistent with the findings of previous studies on methanotrophs exposed to desiccation and/or heat stress, which determined the soil communities show resilience to induced stress following

rewetting of soil (West and Schmidt 1998; Ho et al. 2016b). The samples in those studies displayed no decrease in methane oxidizing abilities following rewetting of soil, however with sequential desiccation-rewetting events methanotrophs did exhibit a reduced ability to recover (Ho et al. 2016b). Ho *et al* also described a "tipping point", in which in which continuous stressors may cease methanotroph recovery over time, which can be seen here in slightly reduced methane oxidation in fall enrichment cultures, as well as reduced diversity in some cases (Ho et al. 2016a).

Of the organisms identified through amplicon sequencing of enrichment cultures, *Methylocystis* is one of particular interest. The presence of *Methylocystis* in enrichment cultures and soil samples of this study could be due to the fact that these organisms have a competitive advantage over other, non-cyst forming methanotrophs in a fluctuating environment. Certain families of methanotrophs such as *Methylocystaceae* are known to have high levels of resistance to desiccation due to their cyst-forming capabilities (Whittenbury et al. 1970; Semrau et al. 2010). The prevalence of *Methylocystis* over *Methylobacter, Methylococcus,* or *Methylocapsa* frequently seen in similar studies suggests that the continental environment being examined here selects for more resilient methanotrophic genera (Reddy et al. 2019; Raksha et al. 2020; Yang et al. 2021). Methanotrophs within the *Methylococcaceae* family have also been shown to produce cysts, primarily *Methylobacter* (Bowman 2006). Cyst formation in other *Methylococcaceae* such as *Methylocaldum, Methylmonas,* and *Methylococcus* is not as widespread (Bowman 2006). Sequencing of thermophilic methanotroph cultures also revealed that a large proportion of *pmoA* amplicon reads did not correspond at the genus level to previously classified methanotrophs (Figure 20). This may be an indication that the diversity of this group of bacteria is underrepresented in the current GenBank database, or due to the nature of amplicon sequencing

allowing for only partial coverage of the *pmoA* gene. The length of *pmoA* ranges from 744 bp to 885 bp depending on the methanotroph family (Cai et al. 2022). While high-throughput amplicon sequencing allows for 2 x 300 bp reads, this does not encompass the entirety of the gene and results in partial fragments. However, phylogenetic trees constructed based on thermophilic enrichment culture sequences revealed that the unknown, uncultured bacteria in question were very closely related to *Methylocaldum* species, and could be confidently be grouped into that genus (Figure 21 & Figure 22). These sequences were highly abundant in the enrichment cultures, yet they were represented by a very small number of organisms low in novelty.

Beta diversity of enrichment cultures were consistent with previous findings showing clear groupings based on incubation temperatures (Figure 16) (Reddy et al. 2019), with the one key difference being the close association between landfill soil and thermophilic cultures shown here. Notably, Reddy's works focused on conventional landfill soil as opposed to the unique conditions of the biowindow environment assessed in this study.

As it was originally hypothesized, methanotrophs showed positive enrichment in both copper-supplemented and copper-free media, suggesting the use of the "copper-switch" (Semrau et al. 2010, 2018). Despite a larger proportion of methanotrophs being known to possess pMMO over sMMO, very similar amounts of growth were found in both media types. Additionally, methanotrophs that are thought to possess only pMMO and would therefore not be expected to grow under copper-limited conditions were detected in copper-free media based on amplicon sequencing and dilution plating. There are two possible explanations this. It is possible given our limited knowledge of this group of organisms that species possessing sMMO exist within genera thought to only express the particulate enzyme, as the presence or absence of sMMO is known to vary between species (Semrau et al. 2010). Alternatively, given that these enrichments were

inoculated with environmental soil, sufficient trace amounts of copper may be present in the landfill soil, therefore supplementing the media with Cu. This hypothesis is consistent with the BRRMF Annual Report which acknowledges the detection of 0.00568 mg/L of copper in clay wells, and 0.21754 mg/L in leachate which is significantly above the standard criterion of 0.087 mg/L (City of Winnipeg Water and Waste 2021). The observed decrease in methane oxidation with repeated passaged also suggests there may be a soil enrichment component that is subsequently being diluted from the media, and cultures may benefit from supplementing media with a soil or compost extract (Liebeke et al. 2009). This observation also supports the copper-supplementing hypothesis.

The recurrent fungal contamination confronted in the culture-based component of this study was consistent with findings of other groups that noted difficulties in growing methanotrophs on plates (Whittenbury et al. 1970; Escoffier et al. 1997; Bowman 2006). In addition, growth of numerous amounts of non-methanotrophic bacteria and fungi has been previously noted with the use of NMS medium (Escoffier et al. 1997). Methanotroph strains have also been known to display diminished growth on agar surfaces during subculturing, as well as general intolerance to agar (Bowman 2006). As an alternative, highly purified agars like noble agar used at a lower concentration, or the use of silica gel or phytagel may prevent this problem and could be utilized in further experiments (Bowman 2006).

Co-cultures resulting from dilution-to-extinction experiments may indicate a consortium of methanotrophs and heterotrophs living together, as described in a review on this topic (Singh et al. 2019). It is expected that methanotrophs would provide carbon compounds as food sources, and non-methanotrophs would aid in detoxifying the environment through the removal of methanol, or with the production of vitamins (Singh et al. 2019). Several studies that display

noticeably beneficial relationships between methanotroph and non-methanotroph co-cultures have been reported, although the topic requires further investigation (Stock et al. 2013; Singh et al. 2019).

The isolation of *Meiothermus silvanus* from biowindow soil is the first documentation of this organism in a landfill environment, and to the best of our knowledge the first known documentation of a thermophilic organism being isolated from a continental climate landfill. Previous reports of heterotrophic thermophiles in landfills include a thermophilic *Bacillus* species isolated from a Uganda landfill (Omara et al. 2012), and a thermophilic *Streptomyces* species from a landfill in Thailand (Phithakrotchanakoon et al. 2009). *M. silvanus*, previously known as *Thermus silvanus*, has been previously isolated from hot springs in Portugal (Tenreiro et al. 1995). The phylum Deinococcus-Thermus contains both mesophiles and thermophiles, and has been detected via 16S sequencing in landfills (Huang et al. 2005) and as one of the top genera in the metagenome of tannery waste sites (Verma and Sharma 2020). *Meiothermus*, while capable oxygen respiration, is believed to have a facultative role in the reduction of Chromate (Cr) and has even been suggested to display synergy with *Methylosinus* (Lai et al. 2016). In this relationship the methanotroph would consume CH_4 and release organic metabolites that are used as electron donors for *Meiothermus*, which may then reduce Cr (VI) (Lai et al. 2016). This association is of considerable interest to this study since in the attempted isolation of a methanotroph from a methane-rich environment *Meiothermus* was instead isolated, but its methane oxidizing partner could not be, supporting the co-culture hypothesis also discussed in the above paragraph. While Chromium was not added to the enrichment cultures, the presence of chromate reducing bacteria in a landfill environment is not surprising. Chromium is used in a variety of industrial applications in Canada, and it was estimated in the early 1990s that wastes

containing over 5000 t of Cr were dumped annually in this country (Canada. Environment Canada. and Canada. Health Canada. 1994). It is worth noting that the landfill cell being examined in the current study was closed in 1993. Ground water monitoring in the BRRMF 2021 annual report documented between 0.0001 mg/L to <0.001 mg/L of dissolved Cr present in grounds well, and up to 0.43693 mg/L in leachate (City of Winnipeg Water and Waste 2021). The standard level of concern for Chromium is listed at 0.81 mg/L, therefore while there is not an environmentally alarming amount being found, it may have further implications in the context of this study.

While methanotrophs were not isolated in pure culture through this work, various methods proved that methanotrophs are present throughout the biowindow and are consistently tolerant of thermophilic temperatures. Further attempts at isolation of these organisms may take different approaches of methodology, including amended media constituents.

Chapter 5: Conclusions, Applications, and Future Directions

5.1 Conclusions

The aim of this work was to explore the presence of thermophilic methanotrophs in a continental climate biowindow in the hopes of understanding their involvement with methane oxidation within the engineered system under some of the major environments stressors expected from this climate. We demonstrated through various culture-based and culture-independent techniques that the fluctuating thermophilic conditions present in the Brady Road Resource Management Facility biowindow lead to the survival of thermophilic methanotrophs, confirmed through the widespread occurrence of the thermophile *Methylocaldum*. While their activity and presence are largely impacted by environmental factors such as drought, the results of enrichment cultures conclusively showed that whether or not the methanotrophs are active *in situ* they can be re-activated and enriched for given the proper conditions. The prevalence of the cyst-forming *Methylocystis* suggested that organisms capable of entering a resting stage or dormancy period may have an evolutionary advantage in this niche. Comparison of multiple microbial communities showed the population of the *in situ* biowindow most closely resembled that of thermophilic methanotroph enrichment cultures, demonstrating that the biowindow is successfully enriching for a community of organisms highly similar to those in ideal laboratory conditions.

Additionally, analysis of landfill soil columns incubated at room temperature revealed the persistence of the thermophilic population to end of the experiment. Furthermore, while the addition of gravel of different sizes to the compost in the columns to facilitate gas diffusion did

affect methanotrophic rate but had minimal effect on diversity of the methanotroph community and general microbial community in the columns.

5.2 Applications

As rising landfill emissions continue to the influence the current climate emergency we find ourselves in, mitigative strategies must be implemented on a wider scale. Important takeaways of this research in regards to real world applications of biowindows include the observed reduction in methane oxidizing potential in a drought year, and the persistence of such conditions through subsequent non-drought seasons. Based on the results of this study, further exploration into the efficacy of implementing irrigation in landfill biotic systems is recommended. As such, a biowindow without the additional stress of persistent drought in a climate that already faces highly fluctuating weather conditions is presumed to host a higher diversity of methanotrophs, aiding in the reduction of fugitive emissions. However, the presence of a strong thermophilic population may not be the best adapted to the shoulder months, particularly in the fall when the weather drops and the population diversity may not be optimal for that time of year. Furthermore, while the organisms may be poised to work even in thermophilic conditions, their activity may be mitigated due to lack of moisture consistent with the findings by Berenjkar et al. 2021.

The low maintenance, low-cost model of the landfill biotic systems that were explored in this study could have potential implementation in previously capped landfills, as well as small rural landfills such as those in First Nations communities in Canada. The waste management challenges faced in these communities stem from environmental injustice and discrimination and

have led to insufficient funding and resources for these groups of people in our well-developed country. A common mode of practice in First Nations communities is often open dumping and open-air burning, and while these methods were once acceptable due to the largely organic nature of the waste, impacts of colonization have led to increased toxic materials and higher volumes of waste (Oyegunle and Thompson 2018). In addition to environmental impacts, significant health and safety risks are a major concern of these open sites. A biowindow in particular requires minimal materials and relatively little active monitoring due to the nature of its size and function, and can be strategically placed in areas of high LFG flux for maximal efficiency (Huber-Humer et al. 2008). With relatively little additional funding, First Nations could implement biotic waste management systems and be an example for developing communities everywhere as they continue to embrace their roles as stewards of this sacred land. The research carried out in our study adds to our knowledge of biowindows and their role in greenhouse gas mitigation, leading us closer to bringing them forth more readily into society.

5.3 Future directions

Several ways this work could be built on for future research include the investigation of effects of irrigation or reinoculation of the biowindow with fresh YWLC compost on the methanotroph community. Given the trajectory of the climate crisis and the increasing prevalence of weather extremes, the tolerance of methanotrophs in biowindows to a changing climate should be investigated further. It would be of additional interest to attempt further isolation of thermophilic methanotrophs using modified culturing techniques, including the addition of soil extract into media, and substituting phytagel for agar plates, for example. The

further examination of a potential synergistic relationship between *Meiothermus* and methanotrophs in the thermophilic biowindow is also of interest.

Appendix

Table 6. Summary of soil samples taken from BRRMF.

Date	Location	Maximum Depth (cm)
May 3, 2021	Biowindow	45[1]
	Clay cover	30[1]
	YWLC windrow	45[1]
August 16, 2021	Biowindow	60
	Clay cover	60
	YWLC windrow	60
August 25, 2021	Biowindow	60
	Clay cover	40[1]
	YWLC windrow	60
October 25, 2021	Biowindow	60
	Clay cover	40[1]
	SSO windrow	60

[1] Maximum depth of 60 cm was not reached due to excess moisture and soil compaction or inadequate excavation tools.

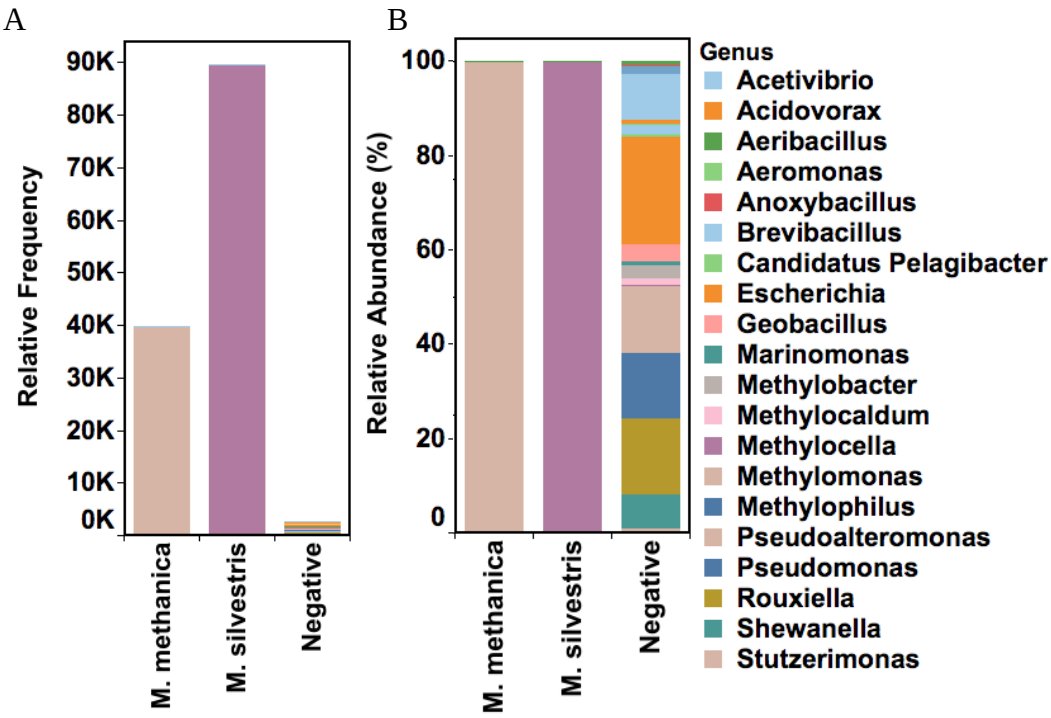

Figure 28. Positive and negative controls for 16S rRNA sequencing. **A)** shows frequency of reads, and **B)** shows percentage of reads for each genus. Genera with a relative abundance of <0.01% were excluded.

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