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EFFECT OF MEDIA COMPOSITION ON THE ENRICHMENT OF METHANOTROPHS FROM SOILS

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TABLE OF CONTENTS

ACKNOWLEDGMENTS iv
ABSTRACT vi
INTRODUCTION 1
METHODS AND MATERIALS 6
Sample collection and initial methane enrichment setup 6
Media composition and methane enrichment cultures in the different media types 6
DNA extraction, PCR amplification, and Illumina sequencing 8
Sequence and phylogenetic analysis 9
RESULTS 11
Methane oxidation by methanotrophs in the enrichment cultures 11
Relative diversity of methanotrophs in the enrichment cultures 12
Effect of enrichment media type on methanotrophs 20
Phylogenetic analysis of the methanotrophs 21
Phylogenetic diversity of methanotrophs in the enrichment cultures 26
DISCUSSION 28
Methane oxidation in the enrichment cultures 28
Methanotrophic abundance 29
Abundance of methylotrophic bacteria in methanotrophic enrichment cultures 30
Effect of media type on growth of different methanotrophs 31
REFERENCES 36
SUPPLEMENTAL DATA 43

ABSTRACT

Methanotrophic bacteria play a crucial role in regulating methane (an important greenhouse gas) release into the environment by oxidizing it. In the last 50 years, several new genera and species of methanotrophs have been documented, and many methanotrophs have been cultivated. However, several lineages of methanotrophs lack cultivated representatives and this creates a challenge in studying their metabolism, physiology, and biochemistry. To cultivate more diverse groups of methanotrophs, we wanted to determine the effect of using 11 different NMS based media types on the growth of methanotrophic enrichment cultures. Soil samples were inoculated from Norman landfill and duckpond. Methane oxidation was monitored, and cells were harvested from the highest two dilutions, which exhibited methane oxidation. 16S rRNA and *pmoA* gene amplicon analysis was done from the DNA of the harvested cells. AMS had an inhibitory effect on the initial methane oxidation rate of methanotrophs in the enrichment cultures. We found there was a relatively higher abundance of methanotrophs in landfill (12%) soil sample compared to the duckpond (< 3%). The most abundant cultivable methanotrophic OTU in enrichment cultures was *Methylocystis* in landfill cultures and *Methylomonas* in duckpond cultures. These methanotrophs were present in all the enrichment media cultures. The methanotrophic OTUs found in the enrichment cultures were from genera *Methylocystis, Methylomonas, Methylomicrobium* and *Methylobacter*. Methanotrophic sequences from *Methylococcus* and *Methyloparacoccus* were present in the soil samples but not detected in any of our enrichment cultures. Methylotrophic sequences were present (10%) in our duckpond methanotrophic enrichment cultures, but not in the landfill cultures. The co-existence of methanotrophs and methylotrophs is likely due to nutrient sharing. The media type with largest number of methanotrophic OTUs were NMS and NMS (1:5) at pH 7. $NaHCO_3$ and 1% O_2

(11/12 OTUs) were effective in duckpond enrichments whereas NMS (1:5) pH 6 (5/5 OTUs) were effective in landfill enrichments. There was a pH effect on the methanotrophic OTUs where more OTUs (11/12) were present at pH 7 compared to pH 6 and 8 (6/12) in duckpond cultures. An effect of catalase (50 µg/ml) was observed in duckpond cultures which led to lowest number of methanotrophic OTU (only *Methylomonas*) present in duckpond cultures. Our results from this study suggest that medium type affects the methane oxidation activity of methanotrophs and their relative abundance in enrichment cultures.

INTRODUCTION

Methanotrophic bacteria can use methane as their sole carbon source (Whittenbury et al., 1970). They play a crucial role in regulating methane (an important greenhouse gas) release into the environment by oxidizing it. Since the 1970s, many new genera of methanotrophs have been documented, and some have also been cultivated. However, several lineages of methanotrophs lack cultivated representatives. This poses a serious challenge in studying their metabolism, physiology, biochemistry, and other characteristics (Dedysh and Knief, 2018). Apart from reducing methane emissions into the atmosphere, methanotrophs have been studied extensively for years for their biotechnology applications like the production of polyhydroxyalkanoates (PHA), methanol, extracellular ectoine (EC), fatty acids, lipids, and organics acids (Gęsicka et al., 2021). Cultivation of methanotrophs in a lab is vital as it gives an opportunity to grow, genetically modify and test these organisms to further enhance their performance for various biotechnological purposes. Researchers can optimize their use by understanding how methanotrophs respond to various growth conditions (Gęsicka et al., 2021).

There are numerous sources of atmospheric methane emissions, wetlands, ruminants, paddy fields, lakes, landfills, and many others(Hanson and Hanson, 1996; Kong et al., 2013; Rahalkar et al., 2009). Wetlands are considered the largest natural source (~23%) of methane emissions into the atmosphere (Conrad, 2009). Methanotrophs consume most of the methane produced in these wetlands at the sub-oxic sediment surface of the water. Previous studies in alpine swamps and marshes (Sanjiang wetland) have found a very high abundance of *Methylobacter* (Mo et al., 2020; Yun et al., 2015). Some other abundant methanotrophs found in the wetlands are *Methylomonas, Methylosinus*, *Methylocystis,* and *Methylovolum* (Chen et al., 2018; Danilova and Dedysh, 2014).

A major source of anthropogenic methane emissions is landfills which amount to around 15% of the total methane emissions in the US (USEPA, 2021). Anaerobic degradation of municipal solid waste leads to the production of methane in landfills. Cover soil is applied as an interface between the waste material and the atmosphere that allows methanotrophs in the soil to oxidize the methane being generated before it escapes into the atmosphere (Wang et al., 2011). Some of the methanotrophs isolated from landfills are of genera *Methylosarcina, Methylomicrobium, Methylobacter, Methylococcus,* and *Methylocystis* (Gebert et al., 2009; Su et al., 2014). Previously it has been found that the pH of the soil affects the diversity of the methanotrophs in landfill (Su et al., 2014). However, there is very little knowledge about other factors that might be influencing the abundance and diversity of methanotrophs in landfills. More methanotrophs must be isolated from landfill to better understand their community compositions and diversity. Ultimately this would help us develop better landfill soil coverage techniques to maximize the methane capture by the methanotrophs.

Isolation of methanotrophs has been done predominantly by using two growth media, ammonium mineral salts (AMS) and nitrate mineral salts (NMS) (Conrad, 2009). So far, very few studies have been conducted to increase the cultivation efficiency of methanotrophs. It has been shown that high concentrations of magnesium (>1 mM) and nitrate (> 500 µM) significantly reduced the number of methanotrophs grown in a mineral medium (Bussmann et al., 2004). A study conducted on an organic waste landfill soil sample showed that adding NH_4^+-N at 100 – 300 $mgkg^{-1}$ had a stimulatory effect on methane oxidation, whereas a higher concentration of 600 $mgkg^{-1}$ has an inhibitory effect (Zhang et al., 2014). A previous study on subtropical paddies showed an increase in methanotrophic abundance by 3-6 times due to the addition of slag and biochar (Wang et al., 2020). Dimethyl sulfide (DMS) at the concentration of

0.1% - 0.2% has been demonstrated to have an inhibitory effect on methane oxidation activity (Wang et al., 2023). Due to the limited understanding of how the growth medium affects the methanotrophic abundance and community composition, it is crucial to conduct more studies that would allow us to cultivate novel and diverse methanotrophs.

The media used to cultivate methanotrophs depends on the target methanotrophs and the environment it is being sampled/isolated from. A wide variety of neutrophilic methanotrophs have been isolated using Nitrate mineral salts (NMS) medium with minor modifications. Some of the methanotrophs isolated with NMS media are psychrophilic *Methylobacter* sp. from boreal lake (Khanongnuch et al., 2022), Type I marine methanotroph *Methylomonas* (NaCl added) (Lidstrom, 1988), Type II methanotroph *Methylocystis hirsuta* from a groundwater aquifer (Lindner et al., 2007), and endophytic methanotrophs *Methylomonas* and *Methylosinus* from peatland plants (Stępniewska et al., 2017). Modified dilute nitrate mineral salts (dNMS, NMS 1:5) medium was used to isolate different groups of methanotrophs from Rice fields in India: Type Ia (*Methylomonas, Methylomicrobium, and Methylocucumis*), Type Ib (*Methylocaldum* and *Methylomagnum*), and Type II (*Methylocystis*, and *Methylosinus*) (Rahalkar et al., 2021). These studies indicate that an NMS medium with modifications can be used to cultivate a variety of methanotrophs in enrichment cultures. Although NMS medium has been used widely in isolating methanotrophs, other media types have also been used to isolate from environments that are not that common. Some of the methanotrophs isolated using different media types are Type Ib (*Methylococcus*) moderately thermophilic from a hot spring in Ethiopia using low-salt mineral medium (LMM) amended with KNO_3 (Islam et al., 2020). Thermoacidophilic methanotroph *Methylacidiphilum fumariolicum SolV* from hot and acidic mud pool in Naples, Italy using assorted medium of Methanethiol ($MgCl_2 \cdot H_2O$ (0.2 mM), $CaCl_2 \cdot H_2O$ (0.2 mM), Na_2SO_4 (1 mM),

K_2SO_4 (2 mM), $(NH_4)_2SO_4$ (2 mM), and $NaH_2PO_4 \cdot H_2O$ (50 μM)) (Schmitz et al., 2022, 2020). *Methylocystis Bryophila S285* isolated from acidic peat soil in Russia using liquid mineral medium M2 (KNO_3 (0.25 g), KH_2PO_4 (0.1g), $MgSO_4.7H_2O$ (0.05g), $CaCl_2.2H_2O$ (0.01g), NaCl (0.02g), distilled water (100 ml)) (pH 5) (Han et al., 2018; Meruvu et al., 2020). *Methylobius crimeensis* halophilic methanotroph from hypersaline lake in Crimea using Medium S (NH_4NO_3 (0.5 g), $Na_2HPO_4.2H_2O$ (0.5 g), KH_2PO_4 (0.1 g), $FeSO_4.7H_2O$ (0.005 g) (l^{-1})) (5-10% NaCl) (Heyer et al., 2005). The type of media used to cultivate methanotrophs plays a crucial role in growing and isolating them from different environments.

In this study, we took samples from two sites in Norman, Oklahoma, USA. A soil sample was collected from a decommissioned landfill, and air exposed sediment from a local pond. To examine the presence of methanotrophs in these sites, enrichment cultures were set up in 11 different media types (Table 1). Most of our enrichment media were NMS based. Some modifications made to our different media types were pH, NMS dilution strength, $NaHCO_3$, catalase, soil extract, and headspace oxygen concentrations. These modifications for the different media types were carefully selected based on their effect on methanotrophs. It has been found that some aerobic methanotrophs thrive under oxygen-limited conditions (Guerrero-Cruz et al., 2021), $NaHCO_3$ acts as a buffering agent (Valença et al., 2021), and soil extract was used to mimic the nutrient composition in the sampling environment. Under oxidative stress, methanotrophs have shown changes in the expression of genes encoding enzymes like catalase as a defense (Guerrero-Cruz et al., 2018; Kim et al., 2016). We expect to see the different media types affecting methane oxidation and the abundance of methanotrophs in the enrichment cultures. The abundance of methanotrophs was measured using 16S rRNA gene and functional marker gene *pmoA*. In the past, no study has looked extensively at how different media types

affect the abundance and diversity of the methanotrophic community. The goal of this study was to 1) determine the abundance of methanotrophs in these two sites. 2) what is the effect of the different media types on the methanotrophic community abundance and diversity? This study would allow us to have a better understanding of methanotrophic communities in landfill and wetland environments. It would also give us insight into the best media to use to grow abundant and diverse groups of methanotrophs from these environments.

METHODS AND MATERIALS

Sample collection and initial methane enrichment setup

Two soil samples were collected. One sample came from a decommissioned solid waste landfill in Norman, Oklahoma, USA. It has been designated as a research site by the U.S. Geological Survey (USGS) (Scott C. Christenson and Isabelle M. Cozzarelli, 2003). The soil sample was collected in a 50 ml sterile centrifuge tube from a shallow depth of 4 cm (35°10'3"N, 97°26'44"W) on sunny day (29°C). On the same day, a sample was collected from top sediment (~2 cm deep) in a 50 ml centrifuge tube from a duck pond located in Brandt Park, Norman, Oklahoma (35°12'21"N, 97°26'10"W). The samples were stored in a refrigerator at 4°C for around 24 hrs. To determine the presence of methanotrophs in the soil samples collected, an initial incubation was created in 160 ml sterile serum bottles. Soil (5 g) was added into the serum bottles with 2% (v/v) CH_4 and 5% (v/v) O_2 in the headspace, sealed with rubber stoppers and incubated at 28°C for 5 days. Methane oxidation by the methanotrophs in the soil samples was measured every day with a Shimadzu GC-14A FID gas chromatograph having a Porapak Q column (Supelco, Bellefonte, US). Helium was used as the carrier gas. Methane oxidation by the methanotrophs was confirmed after observing the complete depletion of the methane from the headspace in the serum bottles.

Media composition and methane enrichment cultures in the different media types

After confirming that methanotrophs were actively oxidizing methane from the headspace of the soil and sediment samples, samples were serially diluted into 11 different sterile modified nitrate mineral salts (NMS) and ammonium mineral salts (AMS) media (Whittenbury et al., 1970). NMS medium included $MgCl_2 . 6H_2O$ (4.4 mM), $MgSO_4 . 7H_2O$ (0.4 mM), KNO_3 (9.9 mM), $CaCl_2 . 2H_2O$ (0.9 mM), KH_2PO_4 (2 mM), and Na_2HPO_4 (2 mM), Nitrilotriacetic acid (0.08

mM), Alkaline trace element solution (0.2 ml/L), Acidic trace elements solution (0.5 ml/L), vitamin solution (10 ml/L). Acidic trace element solution contained (per liter): $FeSO_4 . 7H_2O$ (2g), $ZnSO_4 . 7H_2O$ (0.07 g), $MnCl_2 . 4H_2O$ (0.5 g), $CoCl_2 . 6H_2O$ (0.12 g), $NiCl_2 . 6H_2O$ (0.01 g), $CuSO_4$ (0.5 g), H_3BO_3 (0.01 g), $LaNO_3$ (0.06 g), $CeNO_3$ (0.06 g), and HCl (100 mM). Alkaline trace elements solution contained (per liter): Na_2SeO_4 (0.07 g), Na_2NO_4 (0.05 g), Na_2WO_4(0.2 g), and NaOH (0.04 g). 100x vitamin solution had the following components (per liter): 4-Aminobenzoic Acid (5 mg), D-Biotin (2 mg), Folic Acid (2 mg), Pyridoxine-HCl (10 mg), Riboflavin (5 mg), Thiamine-HCl x $2H_2O$ (5 mg), Nicotinic Acid (5 mg), Calcium D-Pantothenate (5 mg), Thioctic Acid (α-Lipoic acid) (5 mg), and Vitamin B12 (0.10 mg).

Eleven different media types were tested for the enrichment cultures, as shown in Table 1. Most of the media used were NMS based. For the 1/5 NMS medium (NMS 1:5), the first 6 components (above primary minerals) were used at 1/5 concentration. The AMS medium was changed by using ammonium (9.35 mM NH_4Cl) instead of KNO_3. Individual media had either $NaHCO_3$ (8.3 mM), Catalase (50 µg/ml) or soil extract (20% v/v) added (Table 1). Soil extract was made by making a sterile mixture of soil (30 g) and water (150 ml). The sterile soil mixture was centrifuged for 20 min at 5950 g (JLA, Avanti J-25, Beckam Coulter) and supernatant was collected into a sterile serum bottle. The pH of the different media types was adjusted using HCl and NaOH solutions. To create enrichment cultures in different media types, 1.5 g of soil sample from the initial incubation was put into 10 ml NMS (1:20) and a standard 1:10 serial dilution was performed (10 tubes). Each sterile enrichment media tube (15 ml) was inoculated (0.15 ml) from the same serially diluted tubes. Enrichment cultures were prepared at 5 dilutions (10^{-4} – 10^{-8}) for each media. For each dilution triplicates were made. Headspace of cultures were first flushed with N_2, then pressure was adjusted to ambient levels and the headspace was amended with

methane (2% v/v) and oxygen 5% (v/v) (except, where indicated otherwise, see Table 1). The enrichment cultures were incubated at 28 °C for around 47 days. During the incubation period, methane depletion from the enrichment tube was monitored every 2-4 days. Tubes were replenished with methane and oxygen 2-3 times after methane depletion.

Table 1. Media types used for methanotrophic enrichment cultures.

NMS Dilutions	Additions	pH	O_2 %
1:5		7	5
1:5		6	5
1:5		8	5
Undiluted		7	5
1:5	$NaHCO_3$	8	5
1:5	Catalase	7	5
1:5	Soil extract	7	5
1:20		7	5
1:5		7	1
1:5		7	20
AMS		7	5

DNA extraction, PCR amplification, and Illumina sequencing

After the incubation, cells were immediately harvested from the highest two dilutions in each medium that showed complete methane depletion. Cells were harvested by centrifugation at 4347 g for 25 minutes. The pellet collected was recentrifuged at 9700 g for 10 min in a 1.5 ml centrifuge tube and stored at – 80 °C . DNA was extracted from the frozen cell pellets using the DNeasy Blood and Tissue DNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the instruction provided in the kit.

Extracted DNA was used for the amplification of 16S rRNA gene and *pmoA* gene. 16S rRNA was amplified using M13F – Bac341F (5’ CCTACGGGNGGCWGCAG 3’) and Bac 785R (5’ GACTACHVGGGTATCTAATCC 3’) (Li et al., 2021) primers using Dream Taq^{TM} Green PCR Master Mix (2x) (K1082, Thermo Fisher Scientific, USA). PCR cycling conditions

for 16S rRNA gene amplification were as follows: initial denaturation 94 °C for 3 min; 28 cycles of 94 °C for 30 sec, 56°C for 60 sec, and 72°C for 75 sec; followed by final extension for 10 min at 72°C (Eppendorf AG, Hamburg, Germany). *pmoA* genes were amplified using methanotroph targeted primers M13F-A189F (5' GGNGACTGGGACTTCTGG 3') and Mb661R (5' CCGGMGCAACGTCYTTACC 3') (Li et al., 2021). The cycling conditions for *pmoA* were identical to 16S rRNA apart from annealing temperature being 55 °C instead of 56°C. The template DNA used for the PCR was 5 ng for each sample. PCR products were examined using gel electrophoresis. Samples with correct PCR products were purified and prepared for sequencing using bead purification (Seramag) using the 'Sera-Mag™ Select Size Selection and PCR Clean-up reagent' protocols along with the 'NEBNext Ultra II DNA library Prep Kit for Illumina'. The bead purified PCR product (5µl) was used as a template DNA for the second round of PCR using appropriate index primers for both 16S rRNA and *pmoA* gene (Li et al., 2021). The cycling conditions were identical to round 1 apart from that 8 cycles were used. This step allowed us to assign unique barcodes to each sample to be used during the demultiplexing (Li et al., 2021). Samples obtained from the same primer set were combined and gel purified. Gel purified products were combined into one tube for Illumina MiSeq Sequencing at Oklahoma Medical Research Foundation.

Sequence and phylogenetic analysis

Paired-end demultiplexed reads from Illumina MiSeq sequencing platform were analyzed using QIIME2 (qiime2-2022.2). For 16S rRNA and *pmoA* amplicon analysis, primers were trimmed from their paired end reads. Denoising of the sequences was performed using DADA2 (qiime2-2022.2) with a quality score >20. Denoising parameters used for the *pmoA* sequences were trunc-f = 260 and trunc-r = 250 base. 16S rRNA sequences were denoised using parameters trunc-f =

345 and trunc-r = 200 base. After the denoising process, a feature table was generated that was used for the downstream taxonomic and phylogenetic analysis. For taxonomic annotation, Silva 128 SEPP reference database (qiime2-2022.2) was used for 16S rRNA amplicon analysis. For *pmoA* taxonomic annotation, the qiime classifier was trained using *pmoA* gene reference database from GFZ data services (Yang et al., 2016) along with the primer set (A189F and Mb661R) . For taxonomic analysis of methanotroph OTUs in the enrichment cultures, OTUs were filtered out for the following classes Gammaproteobacteria, Alphaproteobacteria, and phylum Verrucomicrobiota as per recent classification of methanotrophs (Guerrero-Cruz et al., 2021). Methanotrophic OTUs with a relative abundance greater than 0.01% were included in the analysis for 16S rRNA and *pmoA* genes. Representative OTU sequences were compared to the NCBI BLAST database to determine their taxonomy. Phylogenetic analysis of the methanotrophic OTUs was done using representative sequences obtained during feature table formation. Reference 16S rRNA and *pmoA* sequences were obtained from NCBI database. FASTA sequences of the methanotrophic OTUs were aligned for constructing maximum likelihood phylogenetic tree for 16S rRNA(nucleotide) and *pmoA* (amino acid) using MEGA version X (Kumar et al., 2018).

RESULTS

Methane oxidation by methanotrophs in the enrichment cultures

Enrichment cultures were prepared for methanotrophs from Norman landfill soil and OU duckpond sediment samples. Methane oxidation by the methanotrophs in these samples was determined in 11 different NMS based enrichment media (Table 1). In each enrichment culture, the time taken by the methanotrophs to oxidize 2% methane from the headspace was measured using gas chromatography (supplemental data, Figure 1.1, 1.2). In landfill cultures, methanotrophs were able to oxidize the initial 2% methane in all the enrichment media tested except for the enrichment with a high O_2 concentration of 20% (supplement data Figure 1.1). Methane was oxidized in all other enrichment cultures in 9 – 28 days at dilution 10^{-7} (supplemental data Figure 1.1). In the duckpond enrichment cultures, methane oxidation occurred in all the enrichment cultures. The initial methane was oxidized by the methanotrophs in the duckpond enrichment cultures in 5 – 15 days at dilution 10^{-6} (supplement data, Figure 1.2). In landfill enrichment cultures, methanotrophs from $NaHCO_3$, NMS (1:5) pH 6 and 1% O_2 oxidized the methane significantly faster ($p < 0.05$) than soil extract and AMS cultures (supplemental data Figure 1.1). In duckpond cultures, methanotrophs from 20% O_2, catalase, NMS pH 7, soil extract, NMS (1:5) pH 8, and $NaHCO_3$ oxidized methane significantly faster than AMS pH 7 and 1% O_2 (supplemental data, Figure 1.2). In both systems, AMS pH 7 took the longest to oxidize initial methane and $NaHCO_3$ was significantly faster than other media types. We found that landfill cultures oxidized methane in lower oxygen concentrations (1-5%), and duckpond cultures oxidized methane faster at higher oxygen concentration of 20% and slower at 1% oxygen concentration (supplemental data, Figure 1.1, 1.2).

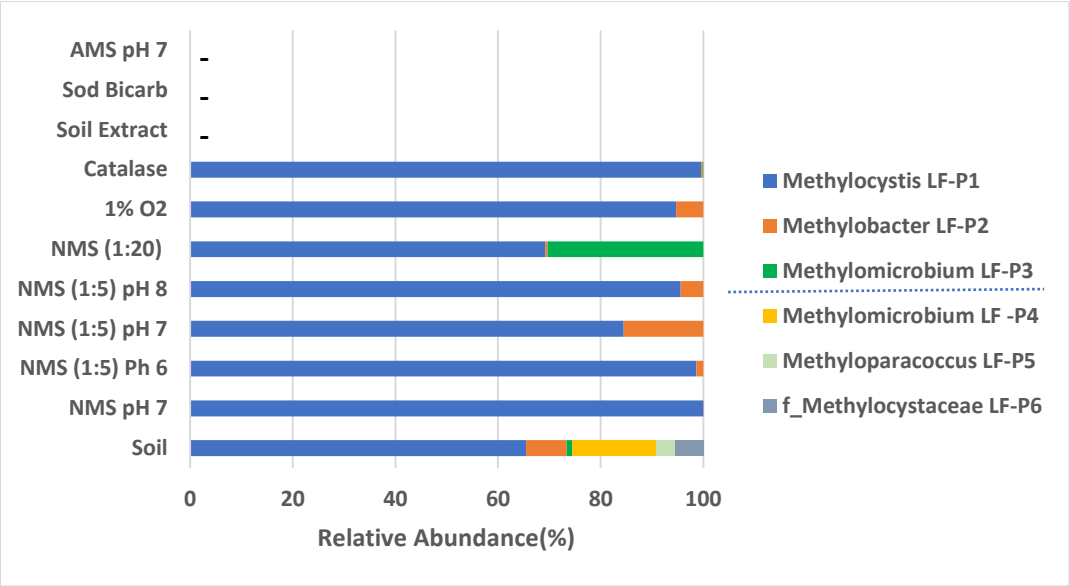
Relative diversity of methanotrophs in the enrichment cultures

Methanotrophic communities were studied in the landfill and duckpond enrichment cultures with different media using *pmoA* (Figure 1) and 16S rRNA sequence analysis (Figure 2) from all active enrichment cultures. Cells were harvested from the highest two dilutions that showed methane oxidation in each medium type. For most enrichment cultures, the highest two dilutions in which methane oxidation occurred were 10^{-6} and 10^{-7}. The three most abundant methanotrophic OTUs found during *pmoA* sequence analysis in Norman landfill soil were *Methylocystis* LF-P1, *Methylobacter* LF-P2, and *Methylomicrobium* LF-P3 (Figure 1). *Methylocystis* LF-P1 was also the most abundant cultivable methanotrophic OTU in the landfill soil during *pmoA* sequence analysis with a relative abundance of 87.9% of the cultivable community (Figure 1). Relative abundance of Methylocystis LF-P1 *pmoA* reads was very high in all the tested media types at both dilutions (Figure 1). This suggests that Methylocystis LF-P1 grows rapidly in different NMS based enrichment media and can outcompete other methanotrophs in these enrichment cultures.

To examine the abundance of methanotrophs as well as other genera relative to the whole community, 16S rRNA sequences were analyzed from all the enrichment cultures. 16s rRNA sequence analysis compares the relative abundance of methanotrophic OTUs with the abundance of the total bacterial community present in the enrichment cultures (Figure 2). The results revealed similar methanotrophic OTUs to the *pmoA* analysis. There were 5 abundant methanotrophic OTUs found in the landfill enrichment cultures (Figure 2). The most abundant methanotrophic OTU was *Methylocystis* LF-1 (4.5% at 10^{-6}, and 8.3% at 10^{-7}) (Figure 2). The relative abundance of total methanotrophic OTUs (16S rRNA sequences) in landfill soil sample was around 12.2% of the total bacterial community (Figure 2,5). *Methylobacter* LF4 and LF5

combined (1.6%) were more abundant in landfill soil than *Methylocystis* LF1 (0.7%) (Figure 2). The increase in relative abundance of *Methylocystis* in enrichments further supports the argument that *Methylocystis* methanotrophic OTUs can outcompete the growth of other methanotrophic OTUs in NMS based enrichment medium.

A



B

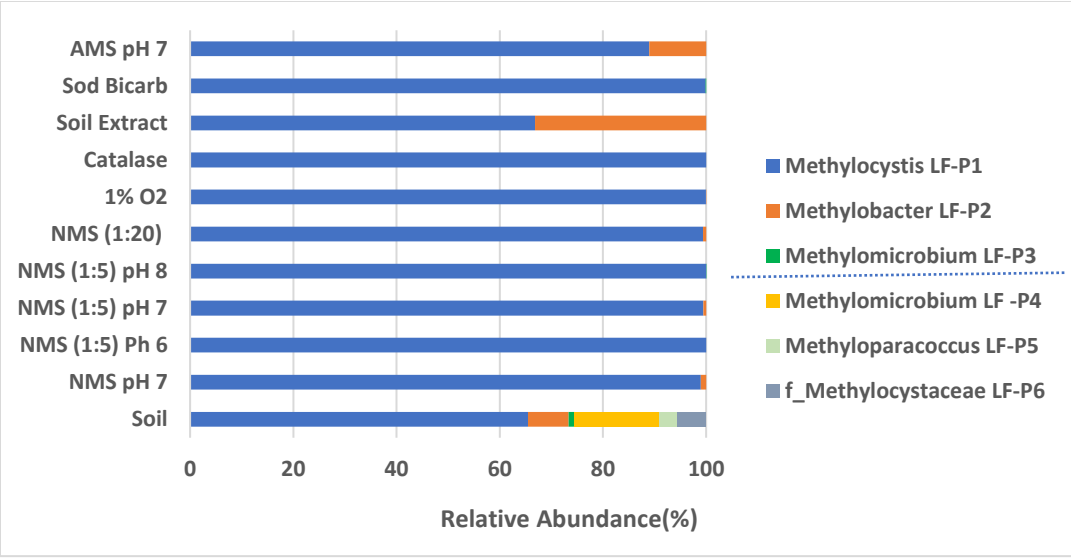
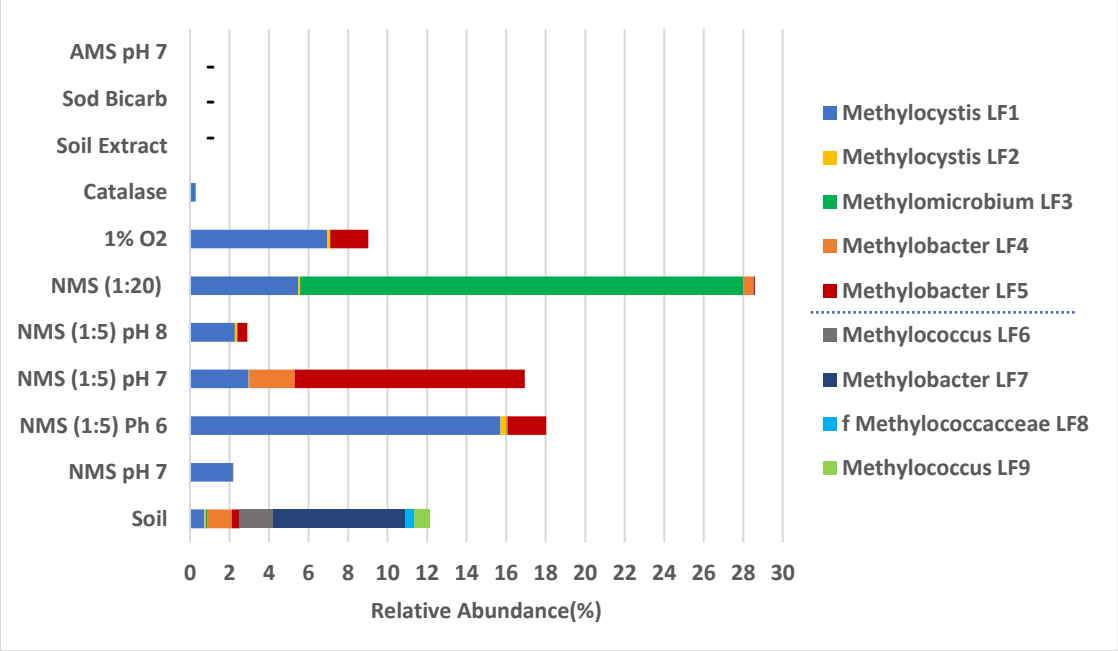


Figure 1. Relative abundance of methanotrophic *pmoA* sequences from Norman landfill in 10 different enrichment media types. Relative abundance of the methanotrophic OTUs was measured using the *pmoA* sequence analysis at dilution 10^{-6} and 10^{-7}. Note - OTUs presented above the dashed line (….) are cultivable. **A.** The relative abundance of methanotrophic OTUs at

dilution 10^{-6} in 7 different media types and the soil sample. (-) Cells not harvested from these enrichment media types as they were only harvested from the highest two dilutions. **B.** The relative abundance of methanotrophic OTUs at dilution 10^{-7} in 10 media types and the soil sample.

A



B

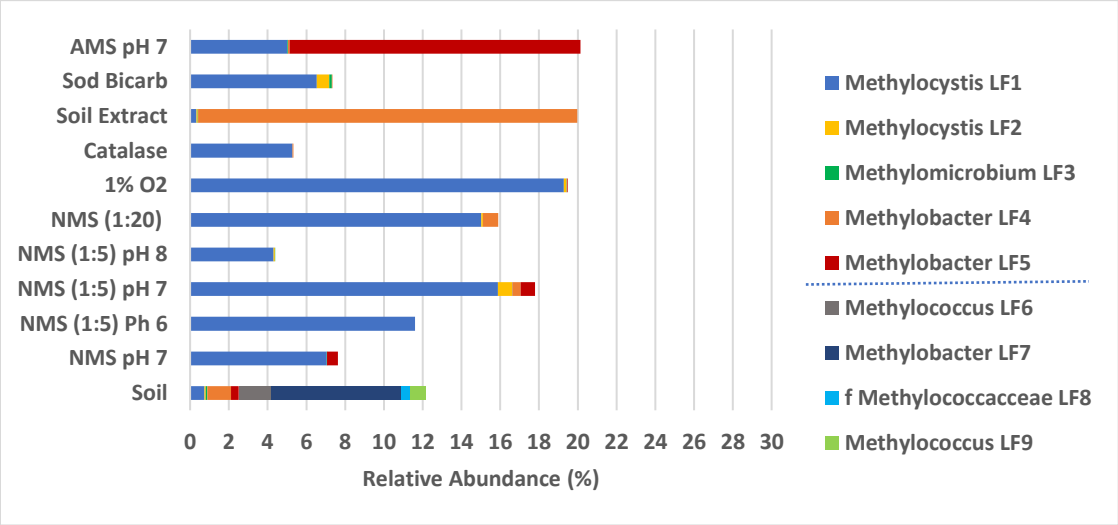


Figure 2. Relative abundance of methanotrophic 16S rRNA sequences from Norman landfill in 10 different enrichment media types. Relative abundance of the methanotrophic OTUs was measured using the 16S rRNA sequence analysis at dilution 10^{-6} and 10^{-7}. Note - OTUs presented above the dashed line (….) are cultivable. **A.** The relative abundance of methanotrophic OTUs at dilution 10^{-6} in 7 different media types and the soil sample. (-) Cells not harvested from these enrichment media types as they were only harvested from the highest two

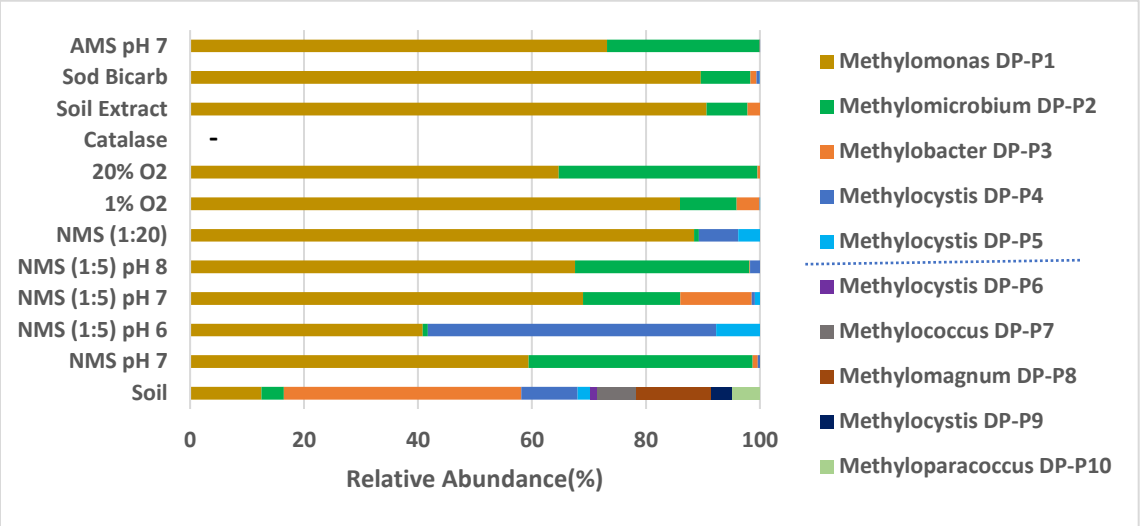
dilutions. **B.** The relative abundance of methanotrophic OTUs at dilution 10^{-7} in 10 media types and the soil sample.

pmoA sequence analysis of duckpond enrichment cultures revealed that the most abundant methanotrophic OTUs on average were *Methylomonas* DP-P1 (72.3% at 10^{-6} and 49.5% at 10^{-7}), *Methylomicrobium* DP-P2 (17.6% at 10^{-6} and 25.3% at 10^{-7}), *Methylobacter* DP-P3 (2.12% at 10^{-6} and 7.64%), *Methylocystis* DP-P4 (6.08% at 10^{-6} and 10.9% at 10^{-7}), and *Methylocystis* DP-P5 (1.25% at 10^{-6} and 6.60% at 10^{-7}) (Figure 3). *Methylomonas* DP-P1 was the most abundant cultivable methanotrophic OTUs at both dilutions. The relative abundance of *Methylomonas* DP-P1 was 17.8% in duckpond soil, which was lower than the most abundant methanotrophic OTU *Methylobacter* DP-P3 (59.27%) (Figure 3). It appears that *Methylomonas* DP-P1 grows better compared to the other methanotrophic OTUs in the 11 different NMS media used.

Our analysis of 16S rRNA sequences also included methylotrophic OTUs because of the relatively high abundance of methylotrophs in the duckpond enrichment cultures. Certain methylotrophs like *Methylotenera sp*. are known to utilize methane-derived carbon from methanotrophs (Van Grinsven et al., 2021). There were total of 9 methanotrophic and 3 methylotrophic OTUs (*Methylotenera* DP2, *Methyloversatilis* DP8, and *Methyloradius* DP12) found during 16S rRNA sequence analysis of enrichments (Figure 4). Methylotrophic OTUs were not found in landfill enrichment cultures (Figure1, 2).16S rRNA analysis produced similar results to *pmoA* sequence analysis. The most abundant cultivable methanotrophic OTU in the duckpond enrichment culture was *Methylomonas* DP1 (18% at 10^{-6} and 10.23% at 10^{-7}). *Methylomicrobium* DP3 was also present at high abundance with 5.85% at 10^{-6} and 4.70% at 10^{-7}. The rest of the methanotrophic OTUs had an average relative abundance of 0.07% - 3.61% at

10^{-6} and 0.001-4.73% % at 10^{-7} (Figure 4). The most abundant methylotrophic OTU found was *Methylotenera* DP2 (10.24% at 10^{-6} and 11.19% at 10^{-7}) (Figure 4). The relative abundance of total methanotrophic and methylotrophic OTUs in duckpond soil was much lower than landfill with 2.6% of the total bacterial community (Figure 6). *Methylomonas* DP1 was present at 0.26%, and *Methylotenera* DP2 at 0.15% in the duckpond soil sample (Figure 4). Based on the 16S rRNA analysis and *pmoA* sequence analysis, *Methylomonas* DP1 seems abundant in all the different enrichment media tested. It should also be noted that methylotroph like *Methylotenera* DP2 is in fairly high abundance in the methanotrophic enrichment cultures possibly due to their ability to utilize methane-derived carbon from the methanotrophs (Van Grinsven et al., 2021).

A.



B

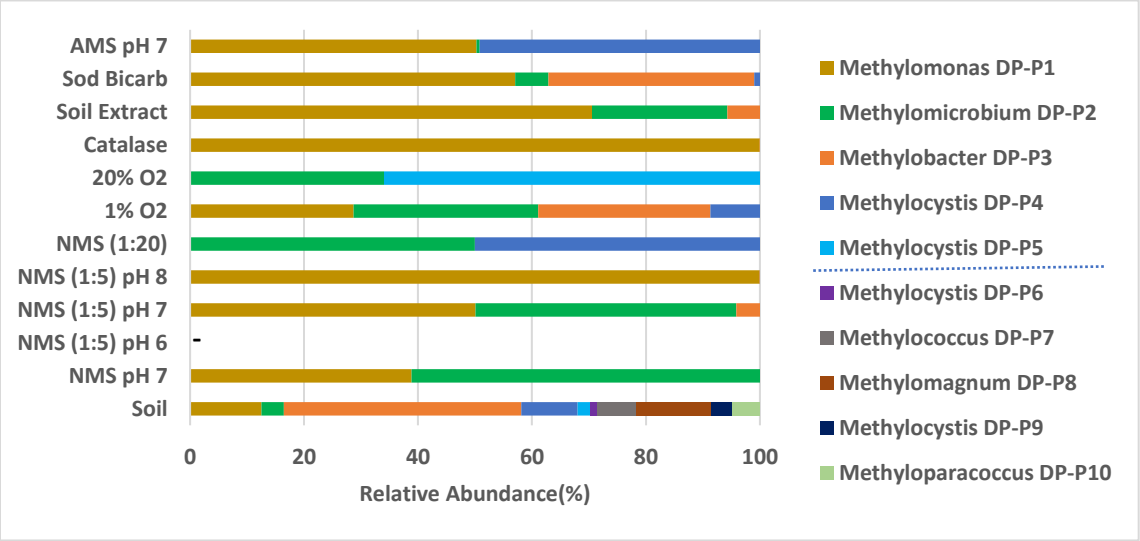
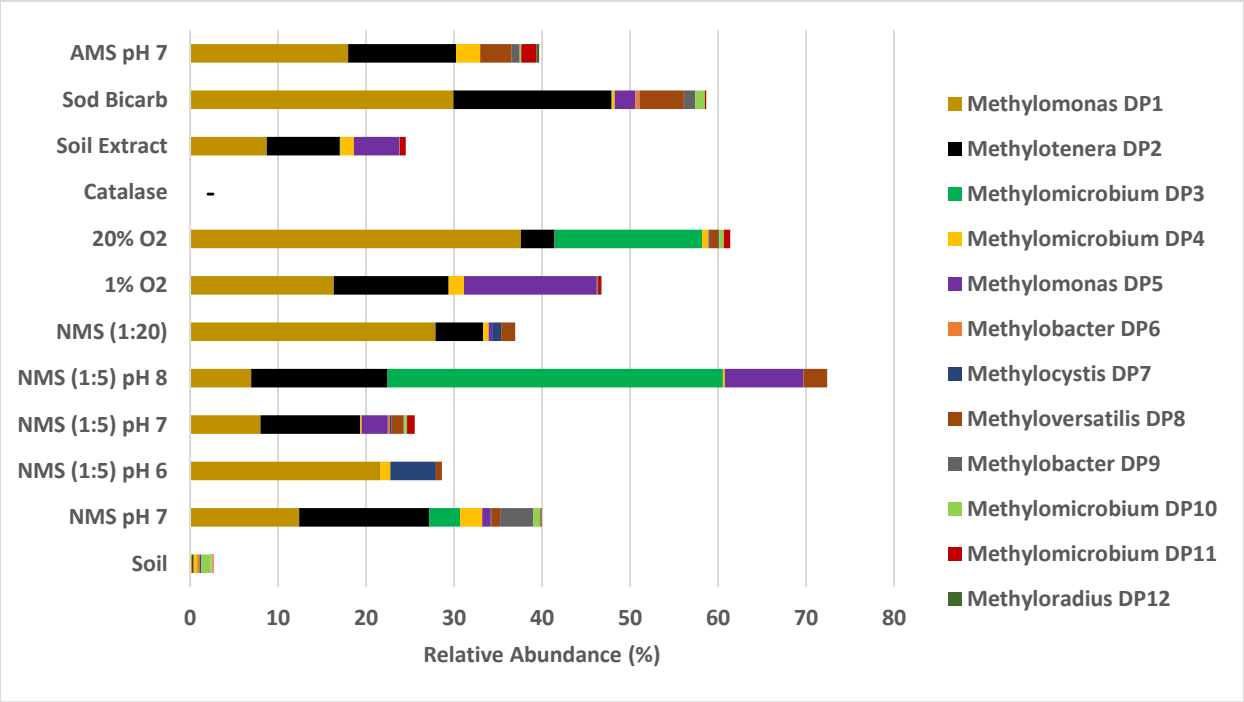


Figure 3. Relative abundance of methanotrophic *pmoA* sequences from OU duckpond in 11 different enrichment media types. Relative abundance of the methanotrophic OTUs was measured using the *pmoA* sequence analysis at dilutions 10^{-6} and 10^{-7}. Note – OTUs presented above the dashed line (….) are cultivable. **A.** The relative abundance of methanotrophic OTUs at dilution 10^{-6} in 10 different media types and the soil sample. (-) Cells were not harvested from these enrichment media types as they were only harvested from the highest two dilutions. **B.** The

relative abundance of methanotrophic OTUs at dilution 10^{-7} in 10 media types and the soil sample.

A



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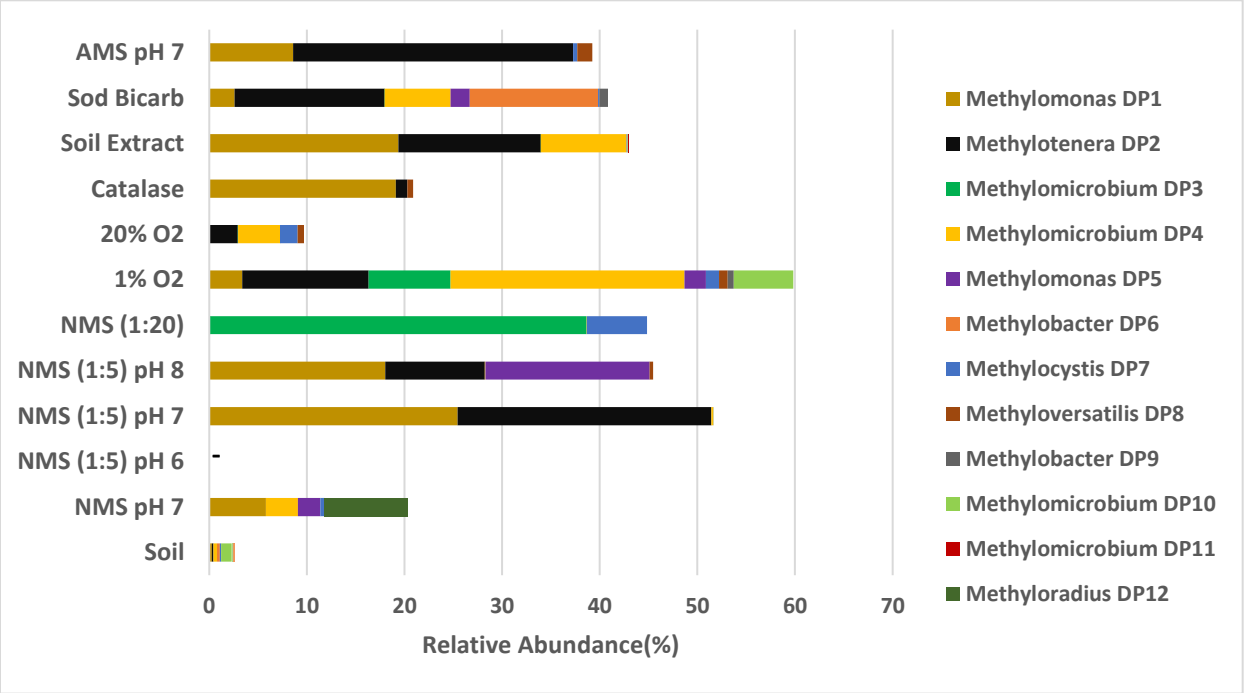

Figure 4. Relative abundance of methanotrophic and methylotrophic 16S rRNA sequences from OU duckpond in 11 different enrichment media types. Relative abundance of the methanotrophic and methylotrophic OTUs was measured using the 16S rRNA sequence analysis at dilution 10^{-6} and 10^{-7}. There were a total of 3 methylotrophic OTUs (*Methylotenera* DP2, *Methyloversatilis* DP8, and *Methyloradius* DP12). (-) Cells were not harvested from these enrichment media types as they were only harvested from the highest two dilutions. Note – Only cultivable OTUs are presented (refer to Figure 6 for uncultivable OTUs). **A.** The relative abundance of methanotrophic and methylotrophic OTUs at dilution 10^{-6} in 11 different media types and the soil sample. **B.** The relative abundance of methanotrophic and methylotrophic OTUs at dilution 10^{-7} in 11 media types and the soil sample.

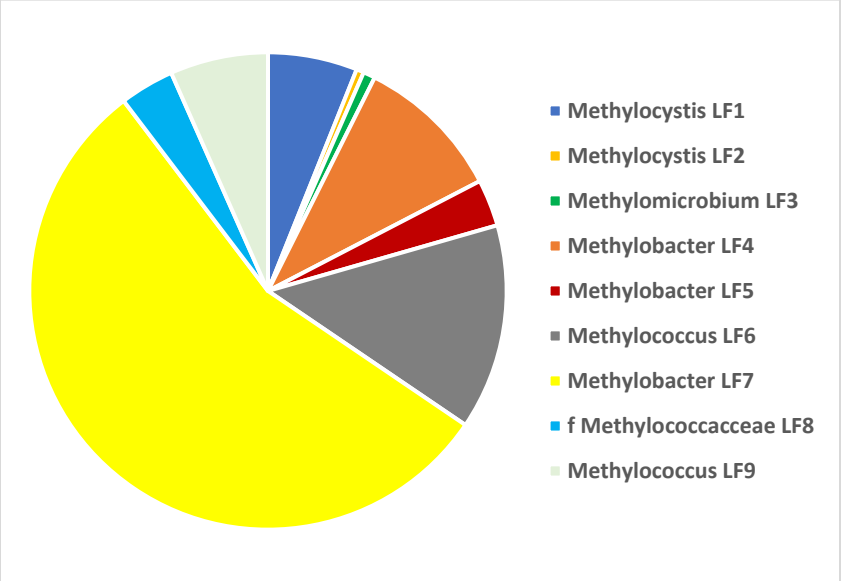

Figure 5. Abundance of methanotrophic OTUs in the Norman landfill soil sample. The relative abundance of total methanotrophic OTUs (16S rRNA sequences) was around 12.2% of the bacterial community.

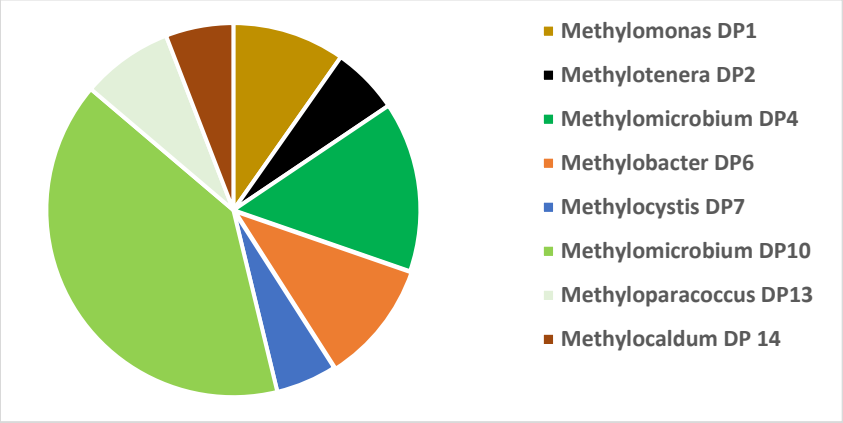

Figure 6. Abundance of methanotrophic OTUs in the OU duckpond soil sample**.** Relative abundance of methanotrophic and Methylotrophic OTUs (2.6%) in the total bacterial community

was determined using 16S rRNA sequences. The only methylotrophic OTU found in was *Methylotenera* DP2.

Effect of enrichment media type on methanotrophs

16s rRNA and *pmoA* sequence analysis at 10^{-6} and 10^{-7} dilutions were used to measure the abundance of methanotrophic OTUs in different enrichment media cultures (Figure 1,2,3,4). The most abundant cultivable methanotrophic OTUs found in landfill and duckpond enrichment cultures were *Methylocystis* and *Methylomonas* respectively (Figure 1,2,3,4). They were abundant in all the enrichment media tested (Figure 1,2,3,4). In duckpond enrichment cultures, the media types that led to growth of the greatest number of methanotrophic OTUs were $NaHCO_3$, 1% O_2, NMS pH 7 and NMS 1:5 pH 7 (Figure 3,4). These enrichment cultures had 11/12 OTUs (16S rRNA) and 4-5/5 OTUs (*pmoA*) (Figure 3,4). Most of these enrichment cultures favored the growth of all the cultivable methanotrophic OTUs except $NaHCO_3$ which had *Methylocystis* OTU as the least abundant (Figure 3,4). The media type that led to the growth of lowest number of methanotrophic OTUs in duckpond enrichment culture was catalase, 3/12 OTUs (16S rRNA), and 1/5 OTU (*pmoA*), where *Methylomonas* was the only methanotrophic OTU present (Figure 3,4). In Landfill samples, the media types that supported the growth of most methanotrophic OTUs were NMS pH 7, NMS (1:5) pH 7, NMS (1:20), NMS (1:5) pH 6. These enrichment cultures had 5/5 OTUs (16S rRNA) and 2-3/3 OTUs (pmoA) (Figure 1,2). All these enrichment cultures had a very high abundance of *Methylocystis* OTU. They had a low abundance of *Methylomicrobium* OTU (NMS (1:5) pH 6, 7) and *Methylobacter* OTU (NMS 1:20) (Figure 1,2).

The most dilute medium (NMS 1:20) did not support growth of *Methylobacter* as there was no *Methylobacter* OTU in duckpond cultures and it was at lower abundance in landfill

cultures (Figure 1,2,3,4). In duckpond cultures, there was a higher number of methanotrophic OTUs at lower oxygen concentrations(11/12 and 4/5) than at higher oxygen concentrations (7/12 and 3/5) (Figure 3,4) . Higher oxygen concentrations showed a lower abundance of *Methylobacter* OTU (Figure 3,4). In both systems, AMS and soil extract media led to growth of a higher number of methanotrophic OTUs (Figure 1,2,3,4). The effect of pH was seen slightly in NMS (1:5) pH 6 and pH 8 in duckpond cultures where fewer methanotrophic OTUs (6/12 OTUs, 16S rRNA) were present compared to NMS (1:5) Ph7 (11/12 OTUs, 16S rRNA) (Figure 3,4).

Phylogenetic analysis of the methanotrophs

Phylogenetic analysis was done for the methanotrophic OTUs found in the enrichment cultures and soil sample from Norman landfill and OU duckpond using 16S rRNA sequences (Figure 8) and *pmoA* sequences (Figure 7). The three common OTUs identified in the landfill enrichment cultures using 16S rRNA and *pmoA* were *Methylocystis, Methylomicrobium,* and *Methylobacter* (Blue squares in Figure 7,8). There were additional OTUs of methanotrophs from genera (*Methylococcus*, *Methyloparacoccus*, and *f_Methylocystaceae*) which were identified in the soil but were not detectable in the enrichment cultures (yellow circles in Figure 7,8). Phylogenetic analysis of methanotrophic OTUs in the duckpond enrichment cultures revealed similar methanotrophic communities to landfill cultures (Figure 7,8,9,10). There were 4 common methanotrophic OTUs (*Methylocystis, Methylomonas, Methylobacter* and *Methylomicrobium*) found in duckpond cultures during 16S rRNA and *pmoA* analysis (blue squares, Figure 9,10). The *Methylomonas* OTU was only present in the duckpond enrichment cultures and not in the landfill cultures (Figure 7,8,9,10). There were methanotrophic OTUs in duckpond soil that were not detected in the enrichment cultures like *Methylococcus*, *Methyloparacoccus*, *Methylomagnum* and *Methylocaldum* (Figure 9,10). *Methylocaldum* and *Methylomagnum* OTUs

were only detected in the duckpond soil and not in the landfill (Figure 7,8,9,10). The overall phylogenetic analysis of duckpond and landfill samples using 16S rRNA and *pmoA* sequences suggests that methanotrophs like *Methylocystis*, *Methylomonas*, *Methylobacter*, and *Methylomicrobium* grew well in the NMS based enrichment cultures. The media did not support the growth of members of the *Methylococcaceae* including genera *Methyloparacoccus* and *Methylococcus* observed here.

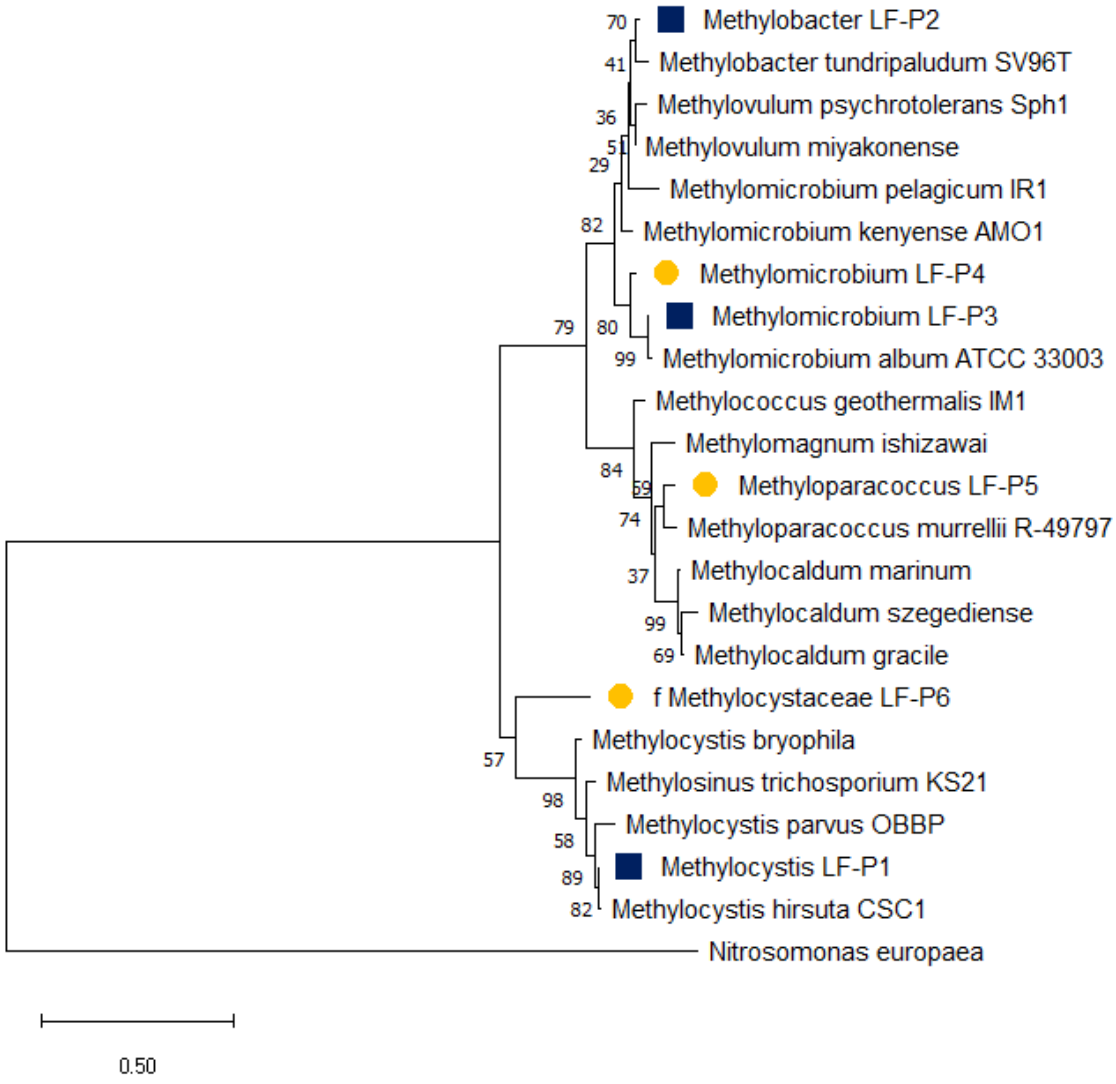

Figure 7. Maximum likelihood phylogenetic tree for methanotrophic OTUs from Norman

landfill based on partially aligned amino-acid sequences of *pmoA* gene. Evolutionary analysis was conducted using MEGA X with 100 bootstrap iterations (Kumar et al., 2018). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 23 amino acid sequences, and there were a total of 299 positions in the final dataset. Blue colored squares show the methanotrophic OTUs found in enrichment culture whereas yellow circles represent the methanotrophic OTUs found in soil but not in the enrichment culture. *Nitrosomonas europaea* was used as an outgroup.

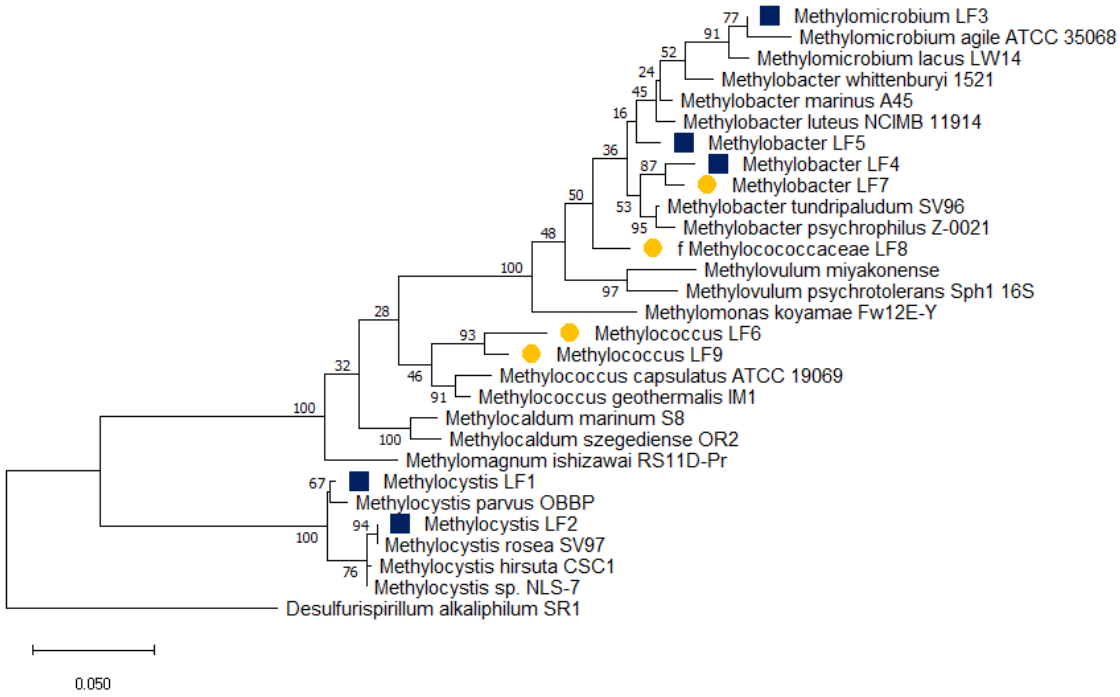

Figure 8. Maximum likelihood phylogenetic tree for methanotrophic OTUs from Norman landfill based on partially aligned 16S rRNA gene. Evolutionary analysis was conducted using MEGA X with 100 bootstrap iterations (Kumar et al., 2018). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involves 29 nucleotide sequences, and there were a total of 1555 positions in the final dataset. Blue colored squares show the methanotrophic OTUs found in the enrichment culture, whereas yellow circles represent the methanotrophic OTUs found in soil but not in the enrichment culture. *Desulfurispirillum alakaliphilum SR1* was used as an outgroup.

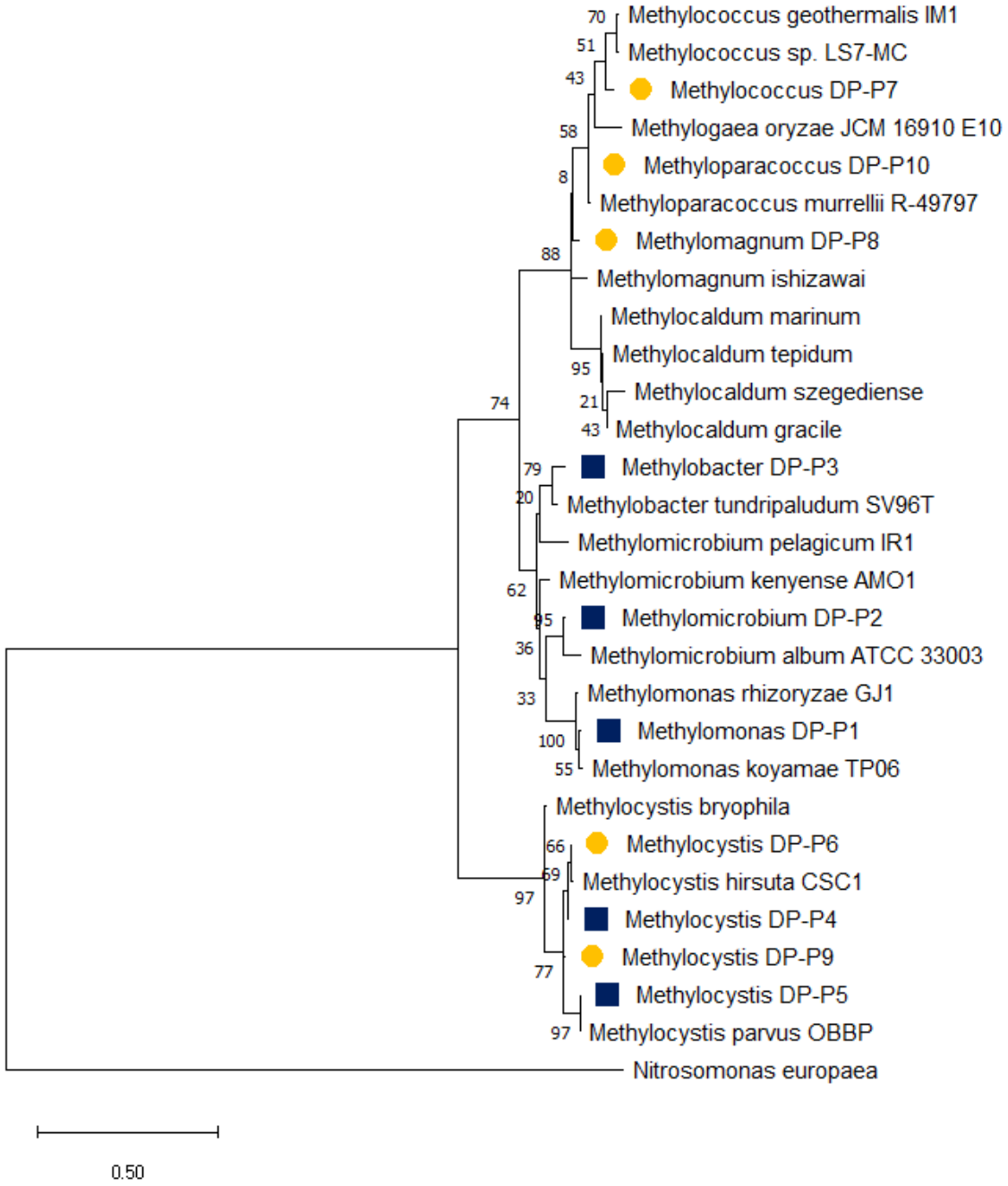

Figure 9. Maximum likelihood phylogenetic tree for methanotrophic OTUs from OU duckpond based on partially aligned amino-acid sequences of *pmoA* gene. Evolutionary analysis was conducted using MEGA X with 100 bootstrap iterations (Kumar et al., 2018). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 29 amino acid sequences and there were a total of 301 positions in the final dataset. Blue colored squares show the methanotrophic OTUs found in the enrichment culture, whereas

yellow circles represent the methanotrophic OTUs found in soil but not in the enrichment culture. *Nitrosomonas europaea* was used as an outgroup.

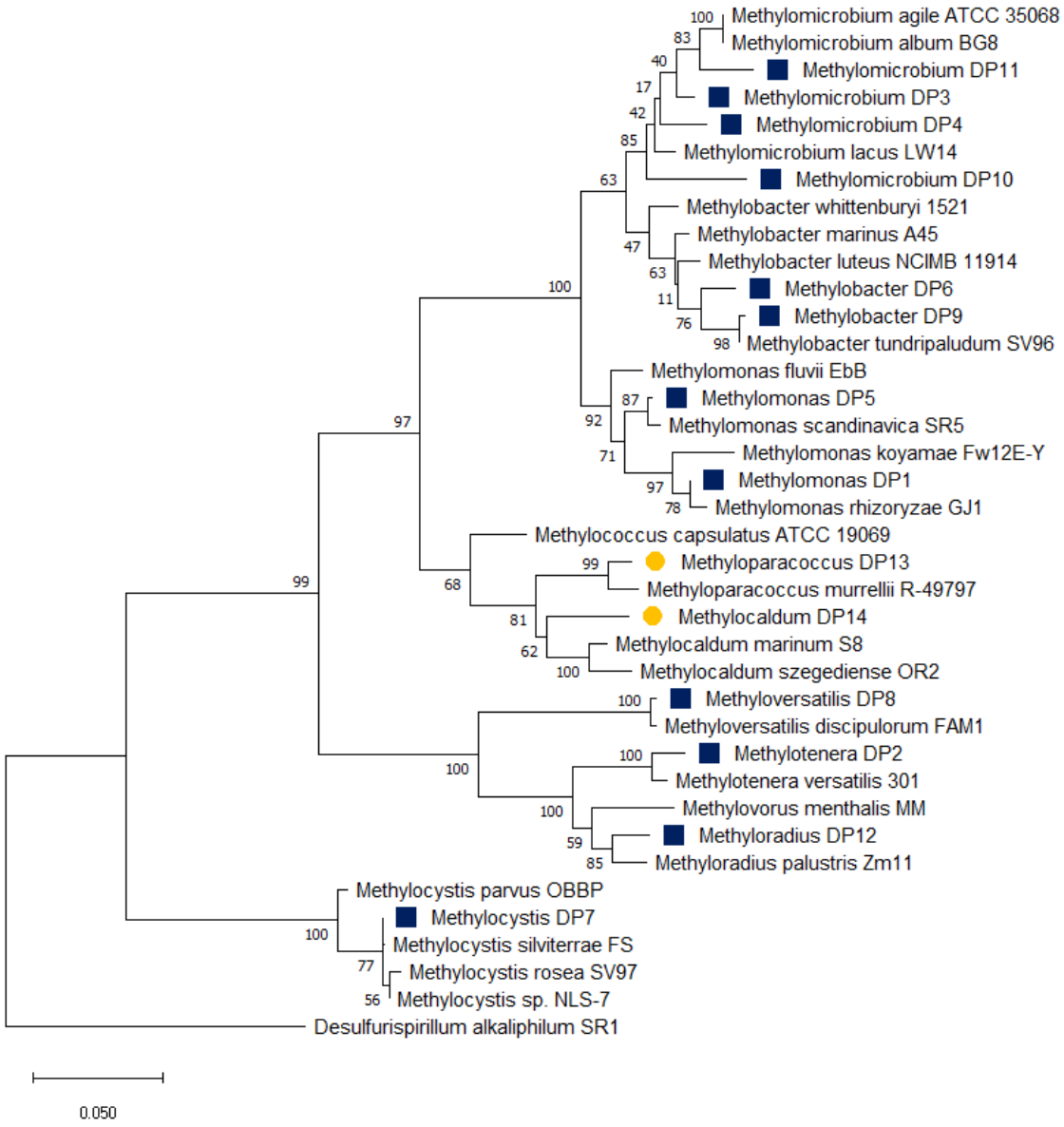

Figure 10. Maximum likelihood phylogenetic tree for methanotrophic and Methylotrophic OTUs from OU duckpond based on partially aligned 16S rRNA gene. Evolutionary analysis was conducted using MEGA X with 100 bootstrap iterations (Kumar et al., 2018). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involves 38 nucleotide sequences. There were a total of 1491 positions in the final dataset. Blue colored squares show the methanotrophic OTUs found in the enrichment culture, whereas yellow circles represent the methanotrophic OTUs found in soil but not in the enrichment culture. There were 3 Methylotrophic OTUs (*Methylotenera* DP2, *Methyloversatilis* DP8, and *Methyloradius*

DP12) present in enrichment cultures. *Desulfurispirillum alakaliphilum SR1* was used as an outgroup.

Phylogenetic diversity of methanotrophs in the enrichment cultures

Phylogenetic diversity of the methanotrophs growing in the different enrichment cultures was determined using Faith's phylogenetic diversity (PD) index. Two dilutions (10^{-6} and 10^{-7}) from Norman landfill enrichment cultures were used for each media type except for AMS pH 7, $NaHCO_3$, and catalase, for which diversity was measured only at dilution 10^{-7}(Figure 11). To measure the phylogenetic diversity of methanotrophs in each enrichment media culture, *pmoA* sequences were used as they are functional marker genes specifically for methanotrophs. The median value for Faith's PD ranged from 0.86 – 3.77 (solid blue line) (Figure 11). It appears from the box plot results that AMS pH 7 (0.86) has lower phylogenetic diversity compared to catalase (3.77). However, a Kruskal Wallis test was performed (H = 11.414, test priori = 0.8113) to determine the significance of the phylogenetic diversity difference between the enrichment cultures, and it was found that it was not significant (p = 0.2484). Phylogenetic diversity of the duckpond enrichment cultures were studied using two dilutions (10^{-6} and 10^{-7}) of each media type except for NMS 1:5 pH 6 (only 10^{-6}) and catalase (only 10^{-7}) (Figure 12). The median value of Faith's PD for duckpond enrichment cultures ranged from 0.88 (catalase) to 2.49 (NMS (1:5) pH 7). phylogenetic diversity between the methanotrophs present in different duckpond enrichment cultures (H = 14.46, test priori = 0.9629) was also found to be non-significant (p = 0.153). However, duckpond had lower p value than landfill. This suggests that the phylogenetic diversity between the methanotrophs in duckpond enrichment might be higher than the methanotrophs in landfill enrichment cultures.

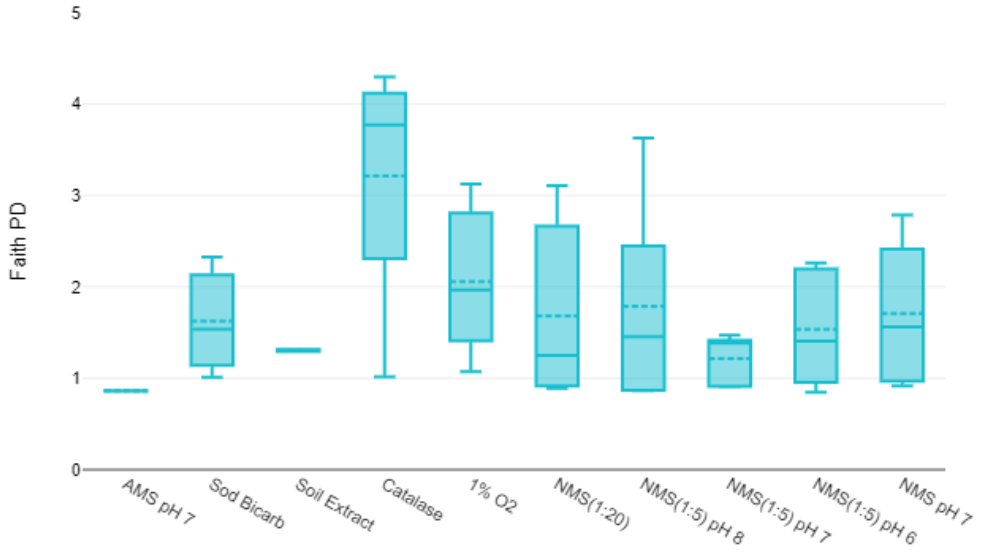

Figure 11. Faith's phylogenetic diversity (PD) for the methanotrophic OTUs from Norman landfill in 10 different enrichment media types using *pmoA* sequence analysis.

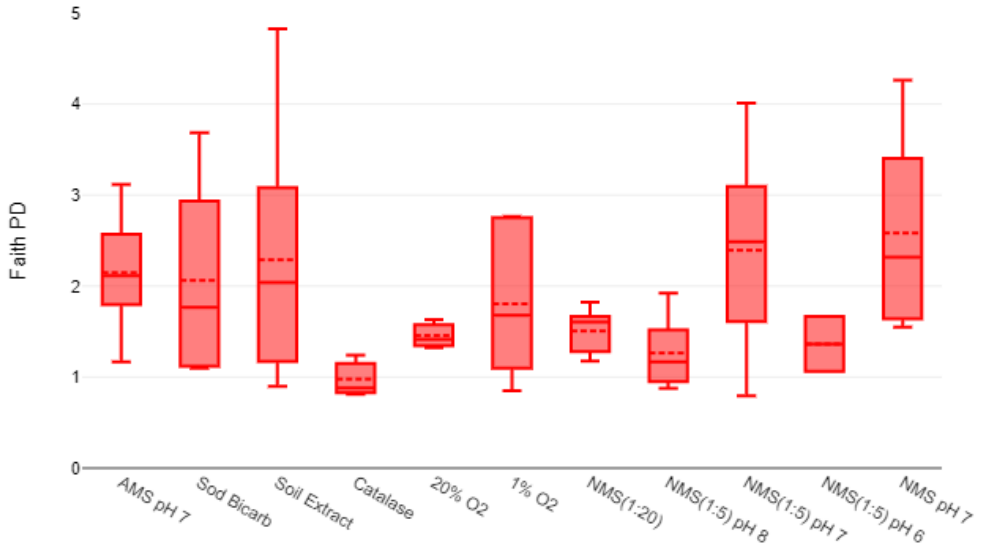

Figure 12. Faith's phylogenetic diversity (PD) for the methanotrophic OTUs from OU duckpond in 11 different enrichment media types using *pmoA* sequence analysis.

DISCUSSION

Methane oxidation in the enrichment cultures

In this study, methane enrichment cultures were prepared for duckpond sediment and landfill soil samples. Methane consumption from the headspace was observed in each enrichment culture except for the enrichment from a landfill with a high O_2 concentration of 20% in the headspace (supplemental data, Figure 1.1,1.2). These results align with another study that looked at methane oxidation at different oxygen levels in soil and they found that methane oxidation at 20% oxygen was lower than at lower oxygen concentration of 2% – 10% (Walkiewicz et al., 2018). Our study also supports the argument that methane oxidation is inhibited for some methanotrophs at higher oxygen concentrations. In both the enrichment systems, the initial time taken by the methanotrophs to consume 2% of methane from the headspace was 9-12 days and 5-15 days in landfill and duckpond enrichments, respectively (supplemental data, Figure 1.1,1.2).

It was noted that in both the enrichment systems, AMS at pH 7 took the longest to oxidize the initial 2% of the methane from the headspace (supplemental data, Figure 1.1,1.2). These results suggest that AMS might have an inhibitory effect on the methanotrophs. Many studies have looked into the effect of ammonium on methane oxidation by methanotrophs (King and Schnell, 1994; Yang et al., 2020; Zhang et al., 2014). A study on landfill cover soil found out that the addition of high level of ammonium (600 $mgkg^{-1}$) had an inhibitory effect on initial methane oxidation(Zhang et al., 2014). 500 µM of ammonium had an inhibitory effect on the *Methylobacter albus BG8* (King and Schnell, 1994). One of the main reasons for the inhibitory effect of ammonium presented in these studies is the substrate competition between methane and ammonium for the active site of enzyme methane monooxygenase (MMO), responsible for

methane oxidation (Zhang et al., 2014). Our study further supports the present body of work showing the inhibitory effect of ammonium on methane oxidation.

Methanotrophic abundance

There is a very high abundance of methanotrophs (12%) in the landfill soil sample (Figure 2) relative to the methanotrophic community in the duckpond sample (2.5%) (Figure 6). The methanotrophic OTUs found in landfill soil were predominantly from the genera *Methylocystis, Methylobacter, Methylococcus* and *Methylomicrobium* (Figure 1,2). Type I methanotrophs *Methylobacter* and *Methylomicrobium* and Type II methanotroph *Methylocystis* together, have abundance of around 9% in landfill soil (Gebert et al., 2009). We and others have also observed type I methanotrophs *Methylococcus* and *Methyloparacoccus* (Figure 3,4,5) (Su et al., 2014). Some of the abundant methanotrophic OTUs found in the duckpond sediment were similar to the landfill community and included *Methylobacter, Methylomicrobium, Methylocystis*, *Methyloparacoccus,* and *Methylococcus.* Other methanotrophic OTUs like *Methylomonas*, *Methylocaldum*, and *Methylomagnum* were only present in the duckpond samples (Figure 3, 4, 6) (Rahalkar et al., 2021). *Methylomonas* is often abundant in aquatic sediments (lakes, wetlands, wastewater) (Bowman, 2016). Some species of *Methylomonas* have polysaccharide-containing cysts that are sensitive to desiccation. These polysaccharides cysts may help them to regulate methanol transport (Sandford and Laskin, 1977).

The most abundant cultivable methanotrophic OTU found in the enrichment cultures were the Type II methanotrophs *Methylocystis* from the landfill and Type I methanotroph *Methylomonas* from the duckpond (Figure 1,2,3,4). Their relative abundance was supported by both *pmoA* and 16S rRNA analysis. These most abundant methanotrophic OTUs grew well in all the media types we tested. The cultivable methanotrophs found in both landfill and duckpond

enrichment cultures were from the genera *Methylomicrobium*, *Methylobacter*, and *Methylocystis* (Figure 7,8,9,10). These methanotrophic genera have been previously isolated using NMS media from similar environments (Rahalkar et al., 2021; Lindner et al., 2007; Lee, 2011).

There were certain methanotrophic OTUs that were relatively abundant in soil samples but were not detected in the NMS based enrichment cultures (Figure 7,8,9,10). No single methanotrophic OTU was present in any of the enrichment cultures from the genera *Methyloparacoccus*, *Methylococcus*, *Methylocaldum*, and *Methylomagnum* (Figure7,8,9,10). All these methanotrophs are Type Ib methanotrophs (Knief, 2015). 16S rRNA and *pmoA* gene analysis did not detect any of the type 1b methanotrophs in the enrichment cultures (Figures 6,8) indicating that this group was truly not present. Previously these member of type Ib have been isolated using NMS media (Hoefman et al., 2014; Lieberman et al., 2003; Rahalkar et al., 2021). The absence of Type Ib methanotrophs might be due to the competition between the different methanotrophs for resources in the enrichment cultures (Graham et al., 1993; Hršak and Begonja, 2000).

Abundance of methylotrophic bacteria in methanotrophic enrichment cultures

Three Methylotrophic OTUs (*Methylotenera*, *Methyloversatilis*, and *Methyloradius*) were found to be present on average at 0.4% to 10.7% in the duckpond methanotrophic enrichment cultures based on 16S rRNA analysis (Figure 4). Methylotrophs can obtain energy from C1 compounds like methane and methanol (Anthony, 1982). Co-occurrence of methanotroph *Methylobacter* and non-methane oxidizing methylotroph *Methylotenera* has been shown in Lake Washington sediment samples (Krause et al., 2017). In the co-culture, a cross-feeding took place between *Methylobacter* and *Methylotenera* where MxaF-type methanol dehydrogenase (MDH) gene was over expressed by methanotroph that led to methanol production. The methylotrophs used this

methanol (Krause et al., 2017). Another study on lake samples showed the incorporation of ^{13}C-labeled carbon from $^{13}CH_4$ into the methanotroph *Methylobacter* and also into the methylotroph *Methylotenera* in a co-culture study (Van Grinsven et al., 2021). These studies argue for the transfer of carbon from methanotrophs to methylotrophs. Our study where enrichment cultures have methanotrophs like *Methylobacter* along with methylotrophs *Methylotenera* and *Methyloradius* (Figure 4) provides strong support for this type of interaction (Miyadera et al., 2021) as the only known carbon source in these enrichment cultures is methane in the headspace. Therefore, the energy and carbon source for non-methane oxidizing methylotrophs is likely the methanol produced by the methanotrophs (Ho et al., 2014; Krause et al., 2017; Takeuchi et al., 2019).

Effect of media type on growth of different methanotrophs

Some of the media types that led to the growth of the largest number of methanotrophic OTUs in the enrichment cultures of landfill and duckpond were NMS and NMS (1:5) at pH 7 (Figure 1,2,3,4). NMS and diluted NMS (1:5) have been used previously to cultivate neutrophilic methanotrophs from different environments like rice fields, boreal lakes, freshwater lake sediment, and landfill soils (Auman et al., 2000; Ghashghavi et al., 2019; Khanongnuch et al., 2022; Lee, 2011; Rahalkar et al., 2021). Some of the methanotrophs cultivated included genera observed in our enrichment cultures including *Methylomonas*, *Methylobacter*, *Methylocystis*, and *Methylomicrobium* (Auman et al., 2000; Khanongnuch et al., 2022; Lee, 2011; Rahalkar et al., 2021). Our study also found that certain media types favored a greater number of methanotrophs in only one enrichment culture. NMS (1:20) and NMS (1:5) pH 6 were effective in landfill enrichments, whereas $NaHCO_3$ and 1% O_2 were effective in duckpond enrichments (Figures 1,2,3,4). Almost all the cultivable methanotrophic OTUs were present in $NaHCO_3$ media

possibly due to its pH buffering effect in the enrichment media (Valença et al., 2021). pH buffers like phosphate and HEPES buffers are used in the enrichment media while cultivating methanotrophs (Rahalkar et al., 2021). $NaHCO_3$ could also be a potential source of CO_2 which might be utilized by the aerobic methanotrophs in the enrichment cultures (Acha et al., 2002). Optimum pH is important for the growth of methanotrophs in enrichment cultures. NMS (1:5) media at a lower pH of 4.8 has been used to isolate type I and II methanotrophs from landfill soil (Wise et al., 1999). We have also observed a higher number of type I and II methanotrophic OTUs at pH 6-7 in landfill enrichments relative to pH 8 (Figure 1,2). In duckpond samples as well, we have observed the influence of pH on the number of methanotrophic OTUs where methanotrophs are more in pH 7 (11/12 OTUs) compared to pH 6 and 8 (6/12 OTUs) (Figures 2,4). Multiple studies have isolated various methanotrophs from lake water/sediment and wetland environments using NMS at pH 6.8 (Auman et al., 2000; Rahalkar et al., 2021; Stępniewska et al., 2017; Whittenbury et al., 1970).

Here, we observed that the addition of catalase had a big effect on the composition of the methanotrophic community in the enrichment cultures. Catalase media had the lowest number of methanotrophic OTUs in duckpond cultures, with *Methylomonas* being the only methanotrophic OTU present (Figure 3,4). Catalase is used as a defense mechanism by bacteria to overcome oxidative stress (Zámocký et al., 2012). Catalase has been previously observed in a range of methanotrophs and methylotrophs (Nguyen et al., 2019; Waturangi et al., 2011). Work in our lab observed an increased methane oxidation rate by *Methylomonas* in the presence of 20µg/mL catalase compared to no catalase (unpublished data). In duckpond cultures, *Methylomonas* was the only methanotroph shown to be growing with two methylotrophs (Figure 3, 4). This suggests

that *Methylomonas* in the presence of catalase outcompetes other methanotrophs in enrichment culture, perhaps with accompanied nutrient sharing.

AMS media had the lowest initial methane oxidation rate in the enrichments cultures (supplement Figure 1.1, 1.2). Although it appeared to have the slowest growth rate, it did not affect the composition of the methanotrophic community in the enrichment cultures much (all five methanotrophic genera were present) (Figure 1,2,3,4). In the past AMS media has been used to cultivate methanotrophs (Whittenbury et al., 1970). Methanotrophs like *Methylocystis, Methylosinus*, *Methylomicrobium,* and *Methylomonas* have been shown to oxidize methane while using ammonium as their nitrogen source (Tays et al., 2018).

Soil extract enrichment medium was prepared to grow methanotrophs found in the natural habitats by providing them with the necessary growth factors and micronutrients present in the soil (Atlas, 2010). Soil extract medium has been used to study the composition of methanogenic and methanotrophic communities in the rhizosphere (Rzehak et al., 2022) and the bacterial community composition in alpine soil (Lipson and Schmidt, 2004). Soil extract was used to cultivate soil bacteria that preferred oligotrophic conditions and soil-borne micronutrients which led to a relatively higher abundance of cultivable microbes compared to other media used (Rzehak et al., 2022). We were able to see the presence of all the cultivated methanotrophic OTUs from different genera (Figure 1,2,3,4).

In duckpond cultures, there was a higher number of methanotrophic OTUs in lower oxygen concentrations. Similar preferences for microaerobic environments (3-5% O_2) by methanotrophs were observed in a study done on groundwater methanotrophic-heterotrophic community (Hršak and Begonja, 2000). There was suppressed growth of heterotrophs and a significantly higher proportion of methanotrophs (90%) in microaerobic environments

suggesting that there might be competition for oxygen between heterotrophs and methanotrophs (Hršak and Begonja, 2000). Methanotrophs have been cultivated and isolated widely in microaerobic conditions from soil and aquatic environments (Kalyuzhnaya et al., 2013; Knief, 2015; Rissanen et al., 2021; Walkiewicz et al., 2018). It has been argued that some methanotrophs (*Methylobacter*) possess a high affinity cytochrome which allows them to oxidize methane at very low oxygen levels (Skennerton et al., 2015; Smith et al., 2018).

The Faith's Phylogenetic diversity (PD) of methanotrophs was determined in the enrichment cultures using *pmoA* gene analysis. There was no significant difference found in the PD between the different enrichment media (Figure 11,12). It has been observed that the phylogenetic diversity of cultivable methanotrophs measured using *pmoA* gene is limited to just less than 3% of the total *pmoA* sequences in the Genbank database. Apart from around 20 sequences, the rest of them are mostly attributed to the known genera *Methylocystis*, *Methylosinus*, *Methylomonas*, *Methylobacter*, *Methylocaldum*, or *Methylomicrobium* (Knief, 2015). Due to the limited reference sequences available, the methanotrophic OTUs in the different media types used here were assigned to just two families of methanotrophs (*Methylococcaceae* and *Methylocystaceae*) divided into 3 main groups in the phylogenetic tree (Figure 7, 9). This resulted in similar branch length of the methanotrophic OTUs, leading to a less phylogenetic difference between the groups as Faith's PD is calculated using the sum of branch length (Faith, 1992).

The methanotrophic OTUs found in both sets of cultures were phylogenetically similar except for the fact that the *Methylomonas* OTU was only present in duckpond cultures (Figure 9,10). This might have resulted in duckpond cultures being slightly more diverse (lower p value) than landfill cultures (Figure 11,12). Some of the PD data supports our methanotrophic OTU

abundance analysis in different media. We see relatively higher PD value in duckpond cultures for NMS (1:5) – NMS pH 7, 1% O_2, and $NaHCO_3$ compared to media with less abundant OTUs like catalase and 20% O_2 (Figure 12). We see a similar pattern with landfill data as there is relatively less difference in PD values, just like the methanotrophic OTU abundance analysis (Figure 11).

In conclusion, our study found that the use of different media types has an effect on the methane oxidate activity of the methanotrophs and their relative abundance in the enrichment cultures. This study encourages further investigation into the influence of media type on the enrichment of diverse methanotrophs. Further study should be done to understand better the relationship between the cultivable methanotrophs (Type Ia and II) and non-cultivable (Type Ib). More enrichment studies should be done to examine the relationship between methanotrophs and heterotrophs.

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SUPPLEMENTAL DATA

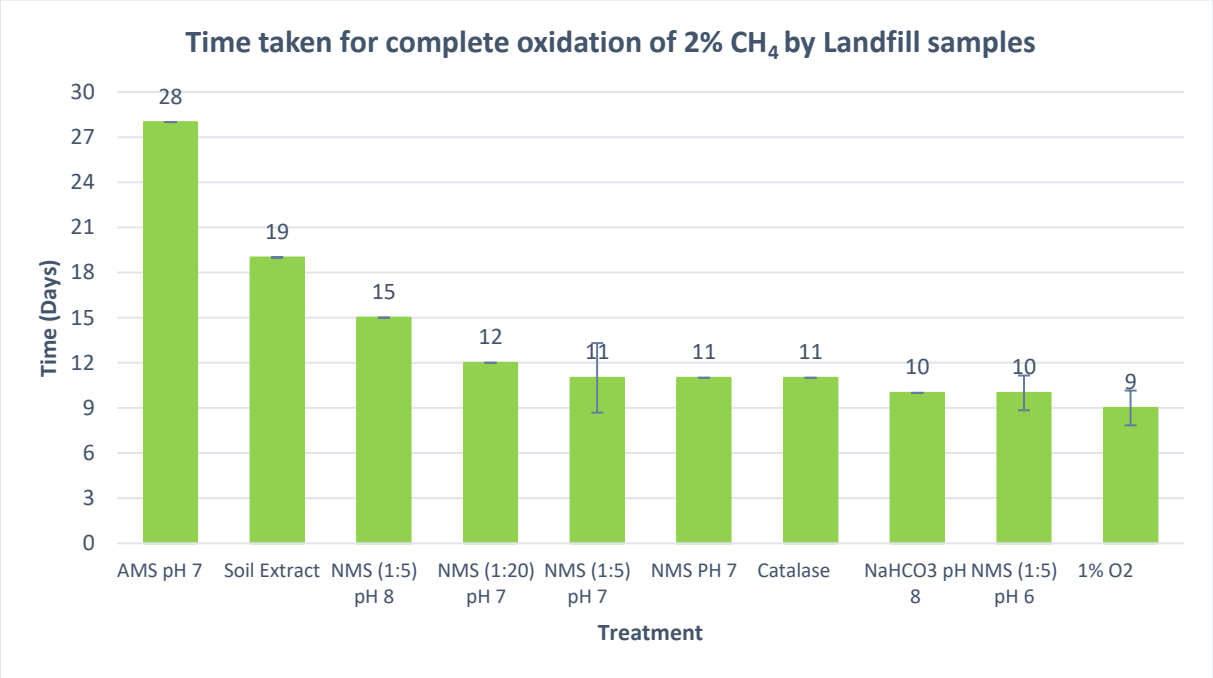

Figure 1.1 The initial time taken by the different Norman landfill enrichment cultures to deplete 2% methane from the headspace. This methane oxidation data was taken from 10^{-7} dilution. 2 % methane was depleted from every enrichment culture apart from the 20% O_2 (not shown) concentration in the headspace. Kruskal-Wallis test (multiple comparison) p = 0.002694, H=25.26, priori power = 0.7781(medium).

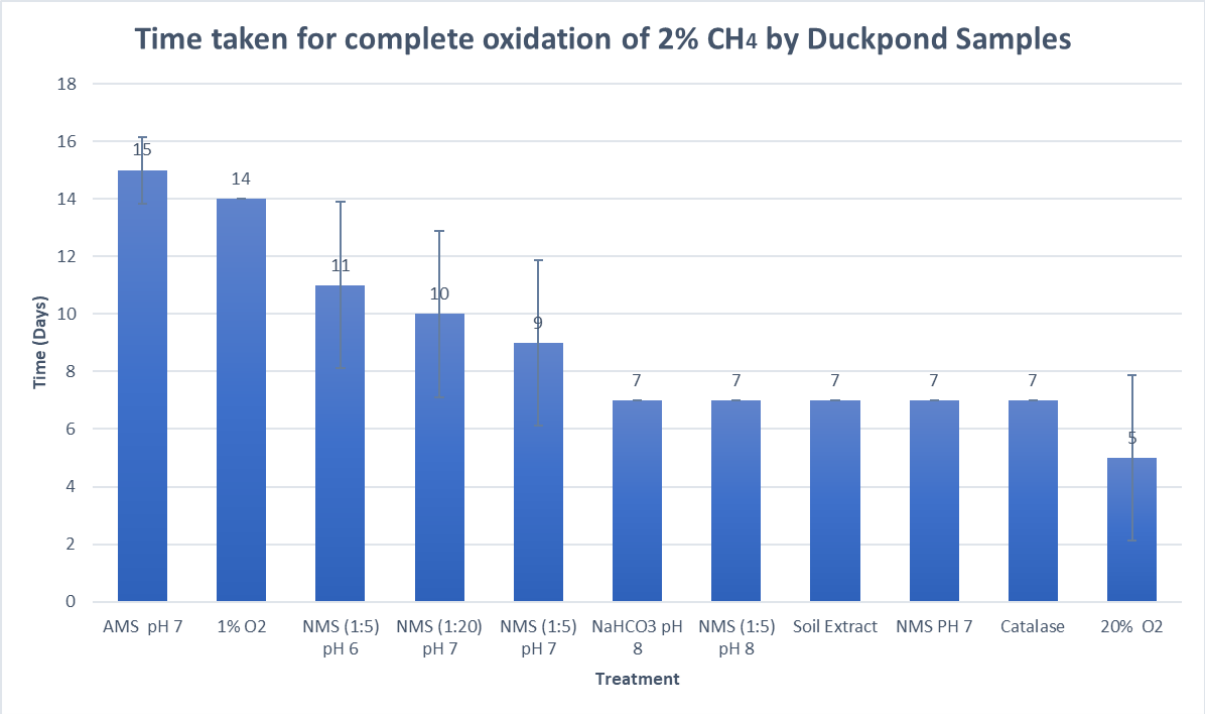

Figure 1.2 The initial time taken by the different OU duckpond enrichment cultures to deplete 2% methane from the headspace. This methane oxidation data was taken from 10^{-6} dilution. 2 % methane was depleted from every enrichment culture. Kruskal-Wallis test (multiple comparison) p = 0.002492, H=27.12, priori power = 0.7781(medium).