Effect of pH on Methane Oxidation and Community Composition in Landfill Cover Soil

Krishna R. Reddy, F. ASCE

Professor, University of Illinois at Chicago, Department of Civil & Materials Engineering, 842 West Taylor Street, Chicago, IL 60607, USA; e-mail: kreddy@uic.edu (Corresponding author)

Raksha K. Rai, S.M. ASCE

Graduate Research Assistant, University of Illinois at Chicago, Department of Civil & Materials Engineering, 842 West Taylor Street, Chicago, IL 60607, USA; e-mail: rrai5@uic.edu

Stefan J. Green

Sequencing Core, Research Resources Center & Department of Biological Sciences, University of Illinois at Chicago, 835 S. Wolcott, Chicago, IL 60612, USA; e-mail: greendna@uic.edu

Jyoti K. Chetri, S.M. ASCE

Graduate Research Assistant, University of Illinois at Chicago, Department of Civil & Materials Engineering, 842 West Taylor Street, Chicago, IL 60607, USA; e-mail: jkc4@uic.edu

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Abstract: Municipal solid waste (MSW) landfills are regarded as one of the major sources of greenhouse gas (GHG) emissions across the world. An innovative and sustainable biogeochemical cover system that consists of soil, biochar and basic oxygen furnace (BOF) slag is being developed to mitigate fugitive landfill emissions such as methane (CH_4), carbon dioxide (CO_2) and hydrogen sulfide (H_2S). Biochar amended soil can mitigate CH_4 emissions by oxidizing CH_4 with the help of methanotrophs (CH_4 consuming microorganisms) whereas BOF slag can mitigate CO_2 and H_2S emissions by adsorption and various mineralogical reactions. However, BOF slag is highly alkaline in nature with pH values usually above 12 and the effect of such high pH on the overall performance of biogeochemical cover system is not known. This study aims at investigating the effect of pH on CH_4 oxidation and methanotrophic community structure in landfill cover soil and cultivated consortia through laboratory incubation experiments. In this regard, soil-suspension and enrichment cultures were incubated at pH values ranging from 2-12, and CH_4 oxidation rates were measured, and microbial community structure was analyzed using 16S rRNA gene amplicon sequencing**.** The optimal pH for CH_4 consumption was found to be 7 in enrichment culture and 7.6 in soil suspensions. Very low or no CH_4 consumption was observed at extreme pH values of 2 (enrichment culture) and 12 (enrichment culture and soil-suspension). A shift in microbial community structure was observed in enrichment cultures initiated at different pH values. Type II methanotrophs were enriched under acidic pH conditions and Type I methanotrophs, were enriched in incubations from pH 4-10. Soil suspensions were more stable, but also showed slight shifts in microbial community dominated by Type I methanotrophs and methylotrophs at pH 7.6-10.0. These results demonstrate that at an extreme alkaline pH (~12), CH_4 oxidation is inhibited as growth of methane oxidizing bacteria (MOB) is arrested in the landfill cover soil.

Keywords: Municipal solid waste (MSW) landfills; Biocover; Landfill cover soil; Landfill gas; Methanotrophs; Methane oxidation; pH; *Methylobacter*

Introduction

Landfills are the third largest anthropogenic source of CH_4 emissions in the USA, making up for 14.1% of the total CH_4 emissions in 2016 (USEPA 2019). Although significant amount of CH_4 is emitted from landfills, it is estimated that between 10% and 90% of CH_4 produced is being consumed by methane oxidizing bacteria (MOB) before it enters the atmosphere (Semrau et al. 2010; De Visscher et al. 2007). Eq. 1 shows reaction of CH_4 with O_2 to produce CO_2 and H_2O.

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \quad (1)$$

The enzyme CH_4 monoxygenase (MMO) catalyzes the above reaction (Reddy et al. 2014). In methane oxidizing bacteria (MOB), also called methanotrophic bacteria, the production of this enzyme is regulated by genes such as *pmoA* and *mmoX* (Reddy et al. 2014). Methanotrophs or MOB, are a subset of a larger microbial community called methylotrophs (Reddy et al. 2019). Methanotrophs utilize CH_4 as a sole source of carbon and energy, whereas methylotrophs use a broader range of C1-compounds as their source of carbon and energy (Hanson and Hanson 1996). Methanotrophs within the phylum Proteobacteria have been classified into three phylogenetically distinct groups: Type I, Type II and Type X methanotrophs, where Type I and Type X belong to the class Gamma proteobacteria and Type II to the class Alpha proteobacteria (Hanson and Hanson 1996; Semrau et al. 2010). Type I, Type II and Type X methanotrophs are ubiquitous in nature and are usually active where CH_4 and O_2 are present.

Landfill cover soils are typically dominated by Type I or Type II methanotrophs depending upon environmental factors such as moisture content, pH, temperature, substrate concentration, soil texture and nutrients (Su et al. 2014; Borjesson et al. 2004; Urmann et al. 2009; Scheutz et al. 2009). Studies have shown the greatest abundance of Type I methanotrophs in landfill cover soils

with pH values ranging from 6.7-8.2 (Chi et al. 2015; Yargicoglu and Reddy 2017a) and Type II methanotrophs at pH range of 5.0-6.5 (Wise et al. 1999; Cebron et al. 2007; Su et al.2014). Methanotrophs are known to adapt to *in-situ* pH levels in the landfill cover soil (Scheutz and Kjeldsen 2004), though environmental selection can lead to a shift in microbial community structure (Su et al. 2014). But, in recent years, many studies have shown the resiliency of methanotrophs to extreme acidic and alkaline conditions in the forest soils, peat soils, sediments, mine impoundments and soda lakes (Dunfield 2010; Baesman et al. 2015; Kalyuzhnaya et al. 2008). These studies have identified methanotroph clades in acidic environments, including members of the families Methylocystaceae, Beijerinckiaceae and Methylococcaceae; these organisms have been typically identified as acidophilic or acid-tolerant methanotrophic bacteria (Nguyen et al. 2018). Further, studies have shown a broad diversity of methanotrophs in alkaline environments. For example, an alkaliphilic methanotroph was isolated from saline Tuva soda lakes which showed fastest growth at pH 9.0-9.5 (Kalyuzhnaya et al. 2008; Khmelenina et al.1997).

Several studies have been conducted to investigate the effects of pH on CH_4 oxidation and microbial community composition using molecular techniques in different ecosystems and are summarized in **Table 1**. For example, Amaral et al. (1998) studied the effects of pH (4.0 to 8.0) on atmospheric CH_4 consumption by forest soil microbial communities using soil slurries (slurry of soil that showed high CH_4 consumption during enrichment) and culture consortia (solution of the bacteria extracted from soil slurries and nutrient media). They observed differences in optimal pH of the soil methylotrophs using slurry (4.0-6.5) or culture consortia (5.8) even though the bacteria were extracted from the same soil, and that cultivation conditions lead to selection for different taxa. Their results showed that the ambient CH_4 consumers were physiologically different from known methanotrophs. Dunfield et al. (1993) tested CH_4 consumption by peat soil obtained

99 from different locations to study the effect of pH ranging from 3.5-8.0. They observed that
100 maximum CH_4 consumption was observed at 0-1 units above the native acidic pH of the peat
101 samples (Saari et al. 2004) and concluded that the microflora involved in CH_4 metabolism was not
102 well adapted to low pH conditions. Baesman et al. (2015) studied the CH_4 oxidation potential and
103 characterized microbial community in a mercury mine impoundment using slurry enrichments.
104 Their studies showed maximum oxidation activity at pH range of 4.5-7.0, with minimal or no
105 activity at pH 3, 9 and 11. Soils contained Type I and Type II Methanotrophs as well as
106 Methylotrophs based on *pmoA* and 16S rRNA gene amplicon analysis. As it is difficult to cultivate
107 these microbes in laboratory because the environmental conditions and nutrient requirements by
108 microbes vary from genera to genera and species to species, cultivation-independent molecular
109 tools have been adopted to detect methanotrophs by employing targeted amplification and
110 sequencing of functional or structural genes, or through non-targeted shotgun sequencing
111 approaches. In shotgun sequencing, all genomic DNA from the microbiome is prepared for
112 sequencing, and portions of the sequence data can be annotated to the taxonomic level of species.
113 Species-level taxonomic resolution is more variable in targeted sequencing approaches (e.g., 16S
114 rRNA gene amplicon sequencing), though targeted approaches are beneficial when the microbial
115 group of interest is not abundant. In such cases, PCR amplification is used, most frequently
116 targeting structural genes such as microbial small subunit ribosomal RNA genes (16S rRNA gene),
117 as well as methane monooxygenase (MMO) genes and genes involved in C1 compound oxidation.
118 Relatively few studies have focused on analyzing the effect of pH on CH_4 oxidation and
119 microbial communities in landfill cover soils (Scheutz and Kjeldsen, 2004; Han et al. 2016; Su et
120 al. 2014). Scheutz and Kjeldsen (2004) studied the effect of environmental factors on CH_4
121 oxidation in the landfill cover soil. Their results showed optimum oxidation at pH 6.5-7.5,

concluding that pH was within the range of pH values found at the site (6.9) and not one of the important factors controlling CH_4 oxidation. Furthermore, due to the natural buffering capacity of soil, landfill cover soils frequently have circumneutral pH and that facilitates CH_4 oxidation. Su et al. (2014) studied various factors affecting the diversity of methanotrophs in the landfills with and without landfill gas (LFG) recovery systems. Their studies concluded that pH was the most dominant factor influencing the methanotrophic diversity in the landfill cover soil, followed by water content and organic content.

Our study focuses in understanding the broader aspect of effect of pH on both CH_4 oxidation and microbial community structure in a newly proposed biogeochemical cover for landfill, where a layer of highly alkaline BOF slag (pH > 12.0) will be overlain or mixed with biochar-amended soil. Previously, the behavior and activity of CH_4-oxidizing bacteria was characterized in a series of batch experiments with highly alkaline biogeochemical cover wherein steel slag was amended with soil and biochar-amended soil (Rai and Reddy, 2019). It was noted that the amendment of soil or biochar-amended soil with steel slag inhibited the CH_4 oxidation process. It is hypothesized that the high pH of these mixtures (>11) could have affected the enzymes of MOB, thereby inhibiting CH_4 oxidation. The present study focuses on affirming this hypothesis and hence, the specific objectives of this study are to systematically investigate the effect of pH on (1) CH_4 oxidation, and (2) the microbial community structure in landfill cover soil, by employing an experimental framework of characterizing enrichment cultures and soil suspension derived from the landfill cover soil. The results from this study will be useful in designing the cover profiles for field applications.

Materials and Methods

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146 ***Soil Enrichment***

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148 Soil samples were collected from the Zion landfill, Zion, Illinois, USA from different locations of
149 an interim cover (collection depth ~1 to 2 feet). The samples were stored in 5 gallon buckets at
150 room temperature (23 ± 2°C). Soil samples were air dried (moisture content < 0.5%), pulverized
151 and screened through a 2 mm sieve (Humbolt Mfg. Co., Elgin, IL, USA) prior to conducting
152 experiments. Physical and chemical properties of the soil are reported by Rai and Reddy (2019).
153 To obtain methanotrophic-enriched consortia, approximately 5 g of sieved soil was mixed with
154 100 mL of modified Nitrate Mineral Salts (NMS) media (Whittenbury et al. 1970) in a 500 mL-
155 serum vial (WHEATON®, Millville, NJ, USA) and sealed using long sleeved rubber septa (Fisher
156 Scientific, Lenexa, KS, USA). Approximately 80 mL of air from the headspace was replaced with
157 equal volume of synthetic LFG (50% CH_4 (*v/v)* and 50% CO_2 (*v/v)*) to achieve a headspace
158 concentration of 7% CH_4 (*v/v*) and 7% CO_2 (*v/v*) balanced in air (86%) and was incubated for 15
159 days at room temperature (23 ± 2°C). Gas samples from the headspace were analyzed at regular
160 intervals using Gas Chromatography (GC) until the headspace CH_4 concentration dropped below
161 1%. The vials containing consortia were replenished twice with the synthetic LFG for the
162 enrichment of the methanotrophic culture.

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164 ***Enrichment Culture Batch Tests***

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166 Serum vials (WHEATON®, Millville, NJ, USA), rubber septa (McMASTER-CARR®, Elmhurst,
167 IL, USA) and pipettes (Fisher Scientific, Lenexa, KS, USA) were sterilized using a Napco Model

8000-DSE autoclave (Thermo Fisher, Waltham, MA, USA) operated at >120°C for a minimum of 60 minutes to ensure sterilization prior to experiments with enrichment culture (Reddy et al. 2019). The supernatant from soil enrichments (above) was collected for use in batch tests. Briefly, 5 mL of enrichment culture supernatant was mixed with 5 mL of sterile modified NMS medium (without phosphate buffer) and placed in a 125 mL-serum vial and sealed hermetically using butyl rubber septa secured by aluminum crimp cap (DWK Life Sciences Wheaton™, Fisher Scientific, Lenexa, KS, USA). About 20 mL of air from the headspace of the vial was replaced with an equal volume of synthetic LFG to achieve a headspace concentration of ~5 to 6% CH_4 (*v/v*) and 5 to 6% CO_2 (*v/v*) balanced in air (89-90%). The pH of enrichment cultures was adjusted from 2 to 12 using either 1M H_2SO_4 or 1M NaOH. Headspace samples were analyzed using gas chromatography (described below). Headspace CH_4 concentrations were monitored until CH_4 concentrations dropped below 1%. In addition, the pH of the liquid medium in each sample was tested periodically with a pH meter (Orion 720A model, Orion Research, Inc. Beverly, MA, USA). Rates of CH_4 oxidation were determined from linear regression analysis of CH_4 concentration with respect to time based on zero-order kinetics. At the end of the incubation, microbial cells in the enrichment media were pelleted by centrifugation and frozen for later DNA extraction and microbial community analysis. Experiments were conducted in duplicates, along with the controls containing only sterile media.

Soil Suspension Batch Tests

For each sample, 10 g of sieved soil was mixed with 10 mL of sterile distilled water (1:1 ratio), placed in a 125 mL serum vial and hermetically sealed with butyl rubber septa secured by

aluminum crimp cap. The vials were spiked with 1M H_2SO_4 to achieve an acidic pH of 4 and 1M NaOH to achieve an alkaline pH of 9.0, 10.0 and 12.0. Bottles with an initial pH of 2.0 were difficult to maintain at that pH due to high buffering capacity of the soil, and hence, these samples were not included in downstream analyses in maintaining the targeted pH. As described above, synthetic landfill gas was added to each bottle to achieve a starting headspace concentration of ~5 to 6% CH_4 (*v/v*) and ~5 to 6% CO_2 (*v/v*) balanced in air (89-90%). The initial pH value was measured after 60 minutes of addition of acid or alkali, and final pH values measured at the end of the experiment (22 days). At the end of the incubation, the soil suspension samples were centrifuged at 12,500 RPM using mySPIN™ 12 Mini Centrifuge (ThermoFisher Scientific, Skokie, IL, USA) for 15 min, and the supernatant decanted. The residual soil material was frozen at -20°C for later DNA extraction and microbial community analysis. All the samples were prepared in replicate along with the controls (vials containing only LFG without soil slurry or culture).

Gas Analysis

Gas samples from headspace were collected at regular time intervals and analyzed for CH_4 and CO_2 concentrations using an SRI 9300 GC (SRI Instruments, Torrance, CA, USA) equipped with thermal conductivity detector (TCD) as previously described (Yargicoglu and Reddy, 2017). Gas samples were extracted from the vials using 1 mL syringe, and 0.5 mL of gas sample was released from the syringe, injecting remaining 0.5 mL into the GC to eliminate the gas pressure effects in the gas analysis by the GC. A calibration curve with a minimum of three points was established

using high purity standard gas mixtures ranging from 1% to 50% CH_4 (v:v) and 5% to 50% CO_2 (v:v) (Rai et al. 2018).

Analysis of Microbial Community Structure

Genomic DNA (gDNA) was extracted from soil samples and cell pellets using a DNeasy PowerSoil Kit (Qiagen) following manufacturer's instructions with a slight modification. Detailed procedure is explained in Reddy et al. (2019). DNA extraction, library preparation and sequencing were performed at the University of Illinois at Chicago (UIC) Sequencing Core (UICSQC). Raw sequence data were initially processed by merging forward and reverse reads using the software package PEAR (Zhang et al. 2013). Ambiguous nucleotides, and primer sequences were removed by trimming merged reads based on quality scores (Reddy et al. 2019). USEARCH algorithm with a comparison to Silva 132 reference sequence database was used to identify and remove chimeric sequences (Edgar 2010; Glöckner et al. 2017; Quast et al.2012). Taxonomic summaries and biological observation matrices (BIOMs) were generated following similar procedure as explained in Reddy et al. (2019). Generation of BIOMs was performed by the Research Informatics Core (RIC) at UIC.

Data Archive

Raw sequence data files were submitted in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) and are available under the BioProject identifier PRJN545136.

Statistical Analysis

One-way (ANOVA) and t-tests (equivalency of sample means) were used to perform statistical analysis of batch test results. Statistical significance was established at a significance level of alpha = 0.05 for all the tests performed in the study. Alpha-diversity indices were calculated and ordination plots (i.e., multidimensional scaling (MDS) plots) were generated by analyzing the microbial community abundance data within the software package Primer7 (Clarke and Gorley, 2015; Reddy et al. 2019). Significant differences in community structure between experimental conditions were assessed using analysis of similarity (ANOSIM).

Results

Effect of pH on Methane Oxidation in Soil-Suspension and Enrichment Culture

The enrichment culture and soil-suspension samples were exposed to a wide range of pH conditions to investigate the effect of pH on CH_4 oxidation potential and community composition. **Fig 1.** shows the CH_4 consumption with time in enrichment culture (starting pH 2-12) and soil-suspension samples (starting pH 4 -12), along with the corresponding pH measurement. An initial lag phase of 3 days was observed in enrichment cultures at starting pH of 2.0 as shown in **Fig 1a.** after which a gradual decrease in CH_4 concentration was observed from day 3 to 13. Thereafter, no change in CH_4 concentration was observed until day 29. The pH values varied from 1.9 to 2.3 throughout the course of the incubation. **Fig 1b.** shows CH_4 headspace concentration with time at

a starting pH of 4.0 in enrichment culture and soil-suspension. An initial lag phase of 2 days was observed in enrichment culture after which a gradual decrease in CH_4 headspace concentration was observed from day 3 to day 13. No change in CH_4 headspace was measured by the end of the experiment (day 22). In contrast, soil-suspension samples showed no change in CH_4 concentration throughout the course of the incubation. pH values in the enrichment culture fluctuated from 4.2 at the beginning of the experiment to 5.1 at the end of the experiment and increased from 4.2 to 6.0 in soil-suspensions. **Fig 1c.** shows CH_4 headspace concentration and corresponding pH measurements as a function of time in reactors with a starting pH of 7.0. An initial lag phase of 1 and 3 days were observed in enrichment culture and soil-suspension, respectively, after which a rapid decrease in CH_4 headspace concentration was observed in enrichment cultures and a more gradual decrease in soil-suspension reactors. pH values remained nearly constant throughout the incubation with a decrease of 0.3 - 0.4 units in enrichment cultures and 0.2 units in soil-suspension reactors by the end of the experiment. **Figs 1d** and **1e** show CH_4 headspace concentration and the corresponding pH measurement as a function of time for reactors with starting pH values of 9.0 and 10.0, respectively. An initial lag phase of 3 days was noted in enrichment culture reactors with starting pH values of 9.0 and, after which a rapid decrease in CH_4 headspace concentration was observed. By the end of the experiment, reactor pH values had dropped to approximately 7.6-7.8, despite intermittent pH adjustment through NaOH addition. In soil-suspension reactors, an initial lag phase of 2 days was observed together with a gradual decrease in CH_4 headspace concentration. Consistent with the enrichment culture reactors, elevated pH levels were not maintained, and average pH values at the end of the experiment were 7.6 and 7.4 for pH 9.0 and pH 10.0 reactors, respectively. No major change in CH_4 headspace concentration of reactors was observed throughout the course of the incubation in enrichment culture and soil-suspension reactors with

starting pH values of 12.0. In enrichment culture reactors, pH values dropped from 12.3 to 10.4 by day 4 and were adjusted by adding NaOH. Thereafter, average pH values fluctuated between 11.8 and 12.3. In soil-suspension reactors, pH remained nearly constant throughout the incubation (12.1-12.2). A comparison of all reactors is shown in **Fig 2** which demonstrates similar rates of methane oxidation in enrichment culture reactors with starting pH values of 7.0, 9.0 and 10.0, and minimal or no methane oxidation in reactors with starting pH values of 2.0, 4.0 and 12.0. Similarly, soil suspension reactors with starting pH values of 7.6 and 10.0 had similar rates of methane oxidation, and no methane oxidation was observed in reactors with starting pH values of 4.0 and 12.0.

Fig 3. shows average CH_4 oxidation rates in soil-suspension and enrichment culture reactors incubated at starting pH values ranging from 2.0 to 12.0. Maximum CH_4 oxidation rates were observed in reactors with starting pH values of 7.0-10.0 (42.9 - 47.5 μg CH_4 $mL^{-1}d^{-1}$) in enrichment culture and at starting pH values of 10.0 (20 μg CH_4 $g^{-1}d^{-1}$) in soil-suspension reactors. CH_4 oxidation rates in reactors with starting pH values of 7.0, 9.0 and 10.0 were not significantly different from each other in enrichment culture (ANOVA, $p = 0.3325$), whereas rates of CH_4 oxidation were significantly different in reactors with starting pH values of 7.6, 9.0 and 10.0 (ANOVA, $p=0.0147$) in soil-suspension reactors. Low rates of CH_4 oxidation were observed in enrichment culture reactors with starting pH values of 2.0 and 4.0 (2.4 and 7.3 μg CH_4 $mL^{-1}d^{-1}$, respectively). No methane oxidation was observed in enrichment culture reactors with a starting pH of 12.0 and in soil suspension reactors with starting pH values of 4.0 and 12.0.

Methylotroph Microbial Community Structure in Enrichment Cultures Incubated Across a pH Gradient

Microbial community structure in the enrichment cultures reactors was analyzed using deep sequencing of microbial 16S rRNA gene amplicons (**Fig 4**). The average relative abundance of 16S rRNA gene amplicon sequences from methylotrophs relative to the total microbial community ranged from 26% (starting pH 2) to 74% (starting pH 12). Microbial community structure varied by starting pH (**Fig 4c**), and the methanotrophic communities were dominated by bacteria from the genera *Methylobacter, Methylovorus, Methylocystis, Methylomicrobium, Methylosinus* and *Mehyloversatilis*. *Methylobacter,* a Type I methanotroph, was found in abundance across all pH conditions examined. More specifically, *Methylobacter marinus* A45 was the most abundant methanotrophic species detected in the enrichment reactors, and sequences from this taxon constituted 78, 22, 36, 56 and 78% of the total methylotrophic 16S rRNA sequences identified at starting pH values of 2.0, 4.0, 9.0, 10.0, and 12.0, respectively. Type II methanotrophs were also detected, including bacteria from the genera *Methylocystis*. *Methylocystis* were abundant in reactors with starting pH values in the range of 2.0-7.0, but negligible in reactors with higher pH values. In reactors with low and high starting pH values (*i.e.*, pH of 2.0 and 12.0), microbial communities were dominated (>90% relative abundance) by methanotrophs from the genus *Methylobacter*. This similarity likely represents low or no microbial activity in these reactors, leading to an observed microbial community derived from the same source enrichment. At other starting pH values, microbial growth led to shifts in observed microbial community structure.

Methylotroph Microbial Community Structure in Soil-Suspensions Incubated Across a pH Gradient

Microbial community structure in soil-suspensions reactors with starting pH values ranging from 7.6 to 12.0 were analyzed using 16S rRNA gene amplicon sequencing (**Fig 4b)**. The average relative abundance of 16S rRNA gene amplicon sequences from methanotrophs relative to sequences from the total microbial community ranged from 3% (starting pH 12.0) to 32% (starting pH 9.0 and 10.0). Four taxa dominated the active methanotrophic microbial communities in reactors with starting pH values of 7.6, 9.0 and 10.0, including bacteria from the genera *Methylobacter* and *Methylomicrobium* and bacteria from the family Methylophilaceae and Methylomonaceae. The bacteria from members of the family Methylomonaceae was found in abundance with a relative abundance of 22.5%, 32% and 29%.at starting pH 7.6, 9.0 and 10.0, respectively, followed by Methylophilaceae with a relative abundance of 28.7%, 11.6% and 22.7%. However, at starting pH 12.0, only 3% of the total sequences that belong to the methylotrophic community were identified, indicating high alkaline conditions (starting pH 12.0) did not favor the growth of methylotrophic community. Further, due to sequencing difficulties at starting pH 4.0, microbial community was not detected from these sample sets.

Discussion

This study analyzed the effect of pH on CH_4 oxidation potential and microbial community structure in landfill cover soil and methanotrophic enrichment cultures. The pH values fluctuated during the experiment, hence pH ranges are reported denoting the initial (start of experiment) and final (end of experiment) pH values. Both culture and soil suspension showed lag phases that varied from 1 to 3 days at different pH values tested which could be due to the time for microorganisms to adapt to the new pH environment. The highest CH_4 oxidation rates were

observed at circumneutral starting pH values of 6.7-7.1 (enrichment culture reactors) and 7.4-7.6 (soil-suspension reactors), consistent with prior studies on landfill covers (Scheutz and Kjeldsen, 2004, Han et al. 2016, Wang et al. 2011). This finding is also consistent with the *in situ* pH of the landfill cover soil (pH 7.6), and the long-term stability of the soil pH could limit the available microbial diversity. The highest CH_4 oxidation rates by pure cultures of methanotrophs have been shown at pH values of 6.6-6.8 (Whittenbury et al. 1970, Hanson and Hanson 1996). For environmental methanotrophic communities, the highest rates of CH_4 oxidation have been shown at pH values of 5.5-8.5 in soils and sediments from a variety of different ecosystems (Dunfield 1993, Hutsch 1994; Scheutz and Kjeldsen 2004; Sherry et al. 2016; Han et al. 2016). Our results show a slight, but not significant, decrease in CH_4 oxidation rates with increasing pH in enrichment cultures with starting pH values between 7.0 and 10.0. At starting pH values of 9.0 and 10.0, the small decrease in the oxidation rates could be a result of the initial alkaline pH. By day 6, despite addition of NaOH, the pH was measured to be 9.3 and 8.3, and corresponding decreases in CH_4 headspace concentrations were also observed. Thereafter, the pH decreased to 7.7-7.8, with continuing oxidation of CH_4. This drop in the pH could be a result of bacterial growth releasing metabolites, acids and/or production of CO_2 during CH_4 oxidation. A similar trend was observed in soil-suspension experiments, where the pH dropped to neutral pH by the end of the experiment, likely due to the metabolic activity of methanotrophic and heterotrophic bacteria in the reactors. With decreasing pH values, rates of methane oxidation increased. At an extreme alkaline starting pH 12, methanotrophic and heterotrophic activity were restricted, and pH values did not decrease substantially, with the exception of a drop in pH after 4 days in the enrichment culture reactors. After pH adjustment, no further significant change in pH levels were observed. This may be in part due to buffering by the carbonate system (pKa of 6.4 and 10.3). Since the targeted pH of 12

was maintained throughout the incubation in both enrichments and soil-suspension, no CH_4 oxidation was observed, confirming inhibition of activity of MOBs at such pH values. Currently, no studies have shown the oxidation of CH_4 at an alkaline pH >12, although alkaliphilic methanotrophs have been isolated from extreme alkaline lakes and marine environments, and these organisms can grow at pH of 9-11 in the presence of NaCl (Kalyuzhnaya et al. 2001, Sorokin et al. 2000, Khmelenina et al. 1997). These halophilic or alkaliphilic methanotrophs are genotypically and phenotypically different from taxa in freshwater and have been identified as new species within the genera *Methylobacter* and *Methylomicrobium* (Khmelenina et al. 2009). Their specific biochemical properties such as synthesis of osmoprotectants, formation of glycoprotein S-layers on the outer surface of the cell walls and the ability to modify chemical composition of cell membranes help them to adapt to highly alkaline habitats (Trotsenko and Khmelenina, 2002).

At acidic starting pH of 2 and 4, pH varied between 1.9-2.3 and 4.2-5.1 in enrichment cultures. CH_4 oxidation was significantly low or negligible at acidic pH range (pH 2.0-4.0) which shows high sensitivity of methanotrophs to acidification of the environment (Le Mer and Roger 2001). Marginal oxidation of CH_4 occurred in enrichment culture reactors with starting pH 4 in contrast to soil-suspension reactors that showed no oxidation at starting pH 4.0. This could be due to differing microbial communities in the soil suspension reactors and enrichment culture reactors. Methanotrophs capable of oxidizing CH_4 in acidic soils (pH 4.0-6.0) have been discovered previously (Wise et al.1999; Cebron et al. 2007; Kong et al. 2014; Benstead and King, 2001; Amaral et al. 1998). However, this study suggests that the landfill cover soil methanotrophic microbial community was not adapted to lower pH conditions even though the enrichments did respond to acidic pH with marginal CH_4 consumption.

It was observed that methanotrophic microbial community structure varied by pH in soil and enrichment reactors. Bacteria most closely related to *Methylobacter marinus* A45*,* a Type I methanotroph, were identified in abundance across all pH conditions tested in enrichment cultures, with lower relative abundance at circumneutral pH (2.1%). *Methylobacter marinus* A45 are aerobic methanotrophs, typically found in coastal and hydrothermal vent marine ecosystems (Flynn et al. 2016) and are halophilic or alkali-tolerant methanotrophic bacteria that require NaCl or Na ions for growth (Bowman et al. 1993; Kalyuzhnaya et al. 2008). Not all species of *Methylobacter* require NaCl for growth, however (Bowman et al. 1993), and most species are considered to be non-halophilic (Osudar et. al 2017). Our studies detected this species at both acidic and alkaline pH, but this may represent microorganisms (active or inactive) present in the source soil material.

In reactors with starting pH values of 7, 9, and 10, bacteria from the family Methylophilaceae (soil suspension), Methylomonaceae (soil suspension) and genera *Methylobacter* (enrichment culture) and *Methylomicrobium* (soil suspension) were abundant. Prior studies have identified the presence of these taxa in landfill cover soil (Gebert et al. 2009; Su et al. 2014; Wise et al. 1999; Chi et al. 2015; Xing et al. 2017). Bacteria from the genus *Methylobacter* have been shown to grow at pH ranging from 5.0-9.0 with an optimal growth at 6.5-7.0 (Bowman 1993), consistent with this study. In general, however, bacteria from the genus *Methylobacter* appear to be ubiquitous in landfill soils. Prior studies of landfill soil have demonstrated the widespread presence of *Methylobacter* species. In enrichment cultures seeded with landfill soil and with high methane headspace concentrations, Wise et al. (1999) observed that nutrient levels were more important than pH in selecting for MOB, and elevated nutrient levels selected for bacteria from the genus *Methylobacter*. Similarly, Kallistova et al. (2013) identified *Methylobacter*

419 species from landfill cover soil in mesophilic enrichments, and a recovered *Methylobacter* isolate
420 from the landfill was most similar to a psychrotolerant strain. Jugnia et al. (2009) also enriched
421 primarily *Methylobacter* from experimental landfill covers and opined that these type I
422 methanotrophs represent pioneer organisms with a high growth rate. In this study, *Methylobacter*
423 are clearly abundant in the source material, but further enriched under the cultivation conditions
424 employed.

425 Methanotrophs from the genus *Methylocystis,* Type II methanotrophs, were identified in
426 samples from enrichment culture reactors with starting pH values 2, 4 and 7. These microbes were
427 also identified in multiple studies of acidic (pH 4.8 and 6.2) and neutral (pH 7.6) landfill cover
428 soils (Wise et al. (1999), Cebron et al. (2007), Su et al. (2014), and Kong et al. (2014)). The relative
429 abundance of bacteria from the genus *Methylocystis* in soil-suspensions was low at pH values of
430 7.6 and above, possibly due to competition with Type I methanotrophs. Type I methanotrophs have
431 been shown to outcompete Type II methanotrophs at higher O_2 and lower CH_4 concentrations
432 (Amaral and Knowles 1995; Henckel et al. 2000). Our incubation conditions have likely limited
433 the growth of Type II methanotrophs in this study. Bacteria from the genus *Methylocystis* have
434 been shown to grow within a pH range from 4.5-9.0 (Bowman et al.1993). They possess diverse
435 systems of membrane transporters that ensures pH homeostasis (Nguyen et al. 2018).

436 Bacteria from the genus *Methylovorus* were also identified in enrichment cultures at
437 starting pH values of 4, 9 and 10. *Methylovorus glucosetrophus SIP3-4,* of the family
438 Methylophilaceae, are obligate methylotrophs that utilize C1 compounds as a source of carbon and
439 energy for growth (Lapidus et al. 2011). This organism was first isolated from sediments of Lake
440 Washington, growing at a pH 4.2-8.0 (optimum at 6.5) and temperature 9-37°C (Kalyuzhnaya et
441 al. 2012). These organisms may have obtained their carbon from methanol produced during

442 oxidation of CH_4 by the enzyme MMO (Cebron et al. 2007). Kallistova et al. (2005) and Han et al.
443 (2016) detected *Methylovorus glucosetrophus* in the landfill cover soil (pH 6.0-8.0) and were
444 successful in cultivating them in laboratory, suggesting that their occurrence in landfill cover soils
445 is not unusual and is consistent with the community composition found in the current study.
446

447 ## Conclusions

448

449 In the present study, enrichment cultures and soil suspensions derived from landfill cover were
450 studied to evaluate the effect of pH on CH_4 oxidation and microbial community structure. The
451 highest CH_4 oxidation was observed in reactors with pH 7.0-7.6 (pH 7.0 in culture and 7.6 in soil
452 suspension), with negligible oxidation at pH 2.0 (enrichment culture) and completely inhibited at
453 pH 12.0 (enrichment culture and soil-suspension). Analysis of microbial community structure in
454 the enrichment culture reactors demonstrated shifts in the microbial communities with Type I,
455 Type II methanotrophs and methylotrophs identified in reactors with starting pH values of 4, 7, 9
456 and 10. However, in soil-suspension no strong shift in methylotrophic community was observed
457 at starting pH values of 7.6, 9.0, and 10.0 and communities were generally dominated by Type I
458 methanotrophs, particularly *Methylobacter*. Overall, this study shows CH_4 oxidation at pH range
459 of 4.0-10.0 in enrichment culture, 7.6–10.0 in soil-suspension and no oxidation at an extreme
460 alkaline pH 12.0 (enrichment culture and soil-suspension) in the landfill cover soil. This study
461 indicates that the high pH of slag (pH >12.0) could inhibit methanotrophic activities in soil if the
462 slag comes in direct contact of the soil. However, the study also shows that the microbial
463 communities can adapt to a wide range of pH conditions (pH 4.0-10.0) suggesting that if BOF slag
464 is used as a layer above in biogeochemical cover, it may not have a significant effect on the

methanotrophic activities below the slag layer. Further studies are underway to analyze the microbial activity in various profiles of biogeochemical cover system under dynamic environmental conditions.

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Data Availability

All data generated during the study appear in this article.

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Table 1: Effect of pH on CH_4 oxidation and community composition in different ecosystem

pH Range	Optimal/ Maximum pH	CH_4 concentration	Ecosystem	Molecular Biomarker	Genus/ Species/ Type of Methanotrophs	References
4.0 – 9.0	4.0 - 6.5 (Soil)	5%	Forest soil	-	-	Amaral et al. (1998)
	5.0 – 8.0 (Culture)	20%		-	-	
3.5 – 8.0	-	0.1%	Peat soil	-	-	Dunfield et al. (1993)
4.3 – 8.0	-	<1%	Upland soils	*pmoA* PLFA	Type I Methanotrophs (*Methylocaldum*) Type II Methanotrophs (*Methylocystis, Methylosinus*)	Knief et al. (2003)
3.0 – 10.0	6.5 – 7.5	15%	Landfill cover	-	-	Scheutz and Kjeldsen (2004)
9.4	-	-	Coal mine	16S rRNA *pmoA* microarray RFLP DGGE	Type I Methanotrophs (*Methylobacter, Methylosoma, Methylococcus*) Type II Methanotrophs (*Methylocystis, Methylosinus*)	Han et al. (2009)
4.7 - 8.1	-	5%	Landfill cover	*pmoA* TRFLP	Type I Methanotrophs (pH 6.8 – 8.1) Type II Methanotrophs (pH 4.7 – 5.35)	Su et al. (2014)
4.0 – 9.0	6.0 – 8.0	5%	River estuary	*pmoA*	*Methylomonas, Methylosoma* (pH 4 - 5) *Methylomicrobium* (pH 9) *Methylobacter* (pH 6 – 9)	Sherry et al. (2016)
3.0 – 11.0	4.5	1%	Mercury mine impoundment	*pmoA* 16S rRNA	Type I Methanotrophs and Type II Methanotrophs (pH 4.5)	Baesman et al. (2015)
5.0 – 10.0	7.0	20%	Landfill cover	-	-	Han et al. (2016)

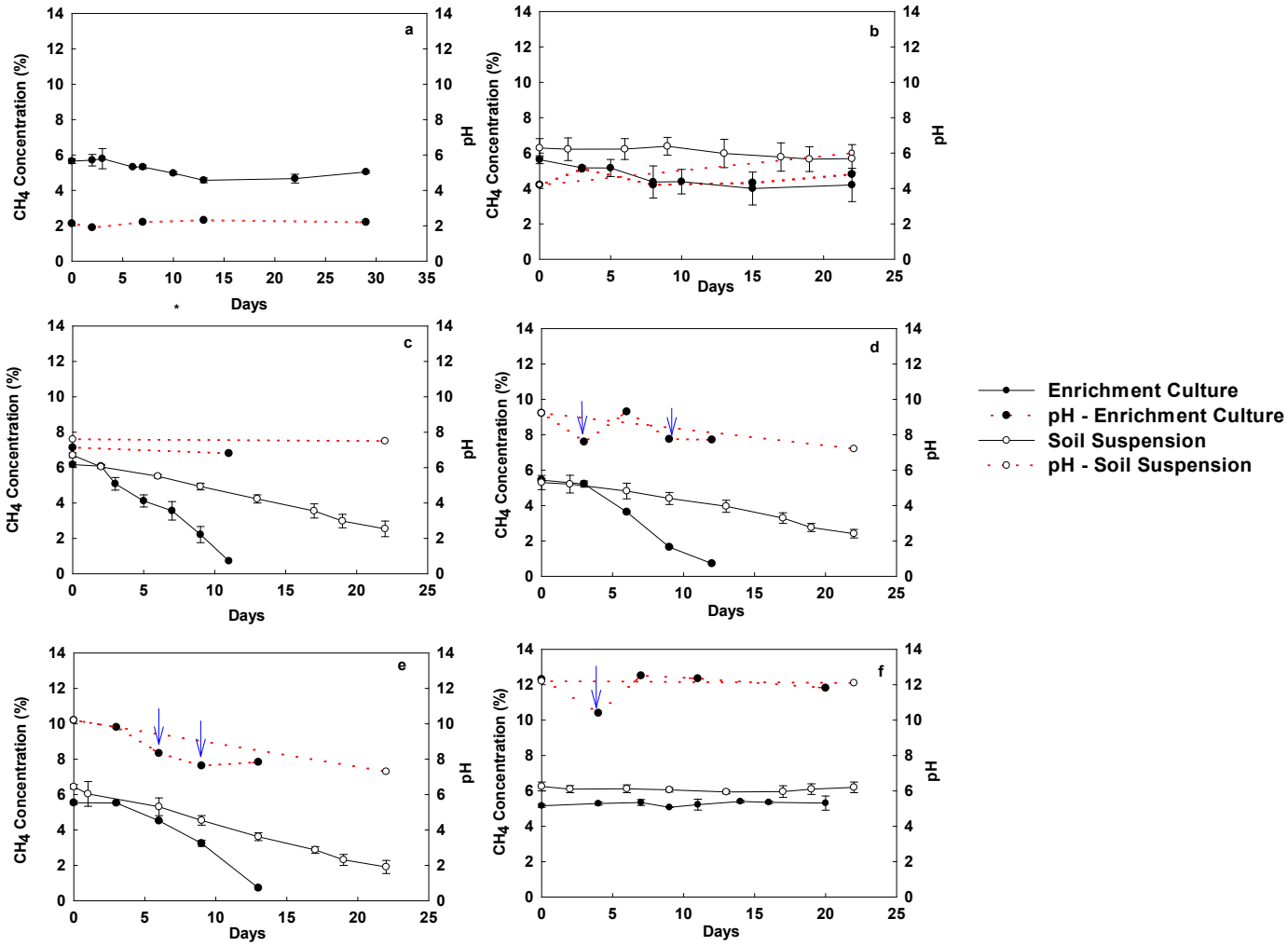
Figure Captions

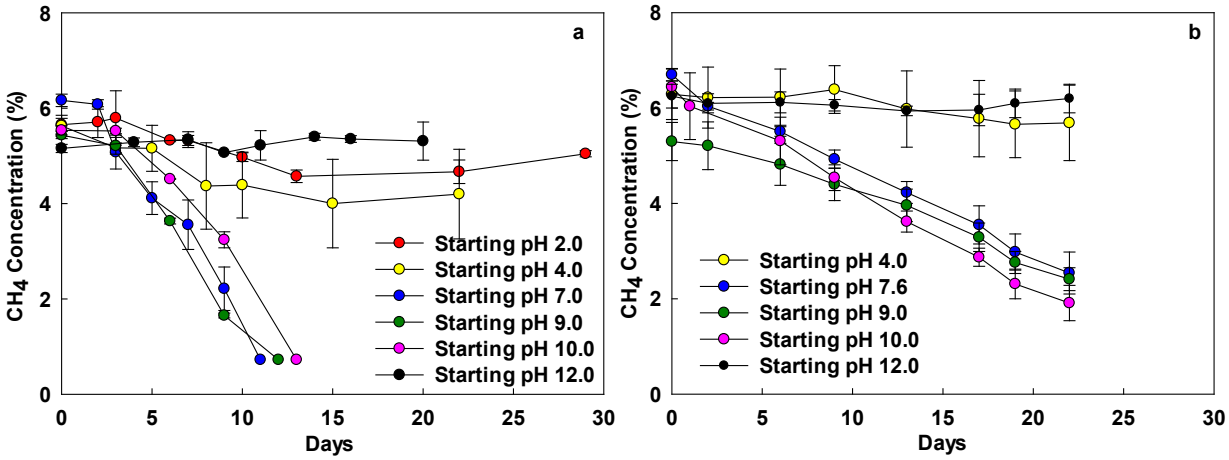
Figure 1: Methane concentration over time as a function of pH in enrichment culture and soil-suspension reactors with starting values of (a) pH 2.0, (b) pH 4.0, (c) pH 7.0/7.6, (d) pH 9.0, (e) pH 10.0 (f), and pH 12.0. (All pH values are ± 0.1 - 0.3 units). The arrows indicate addition of NaOH in enrichment cultures

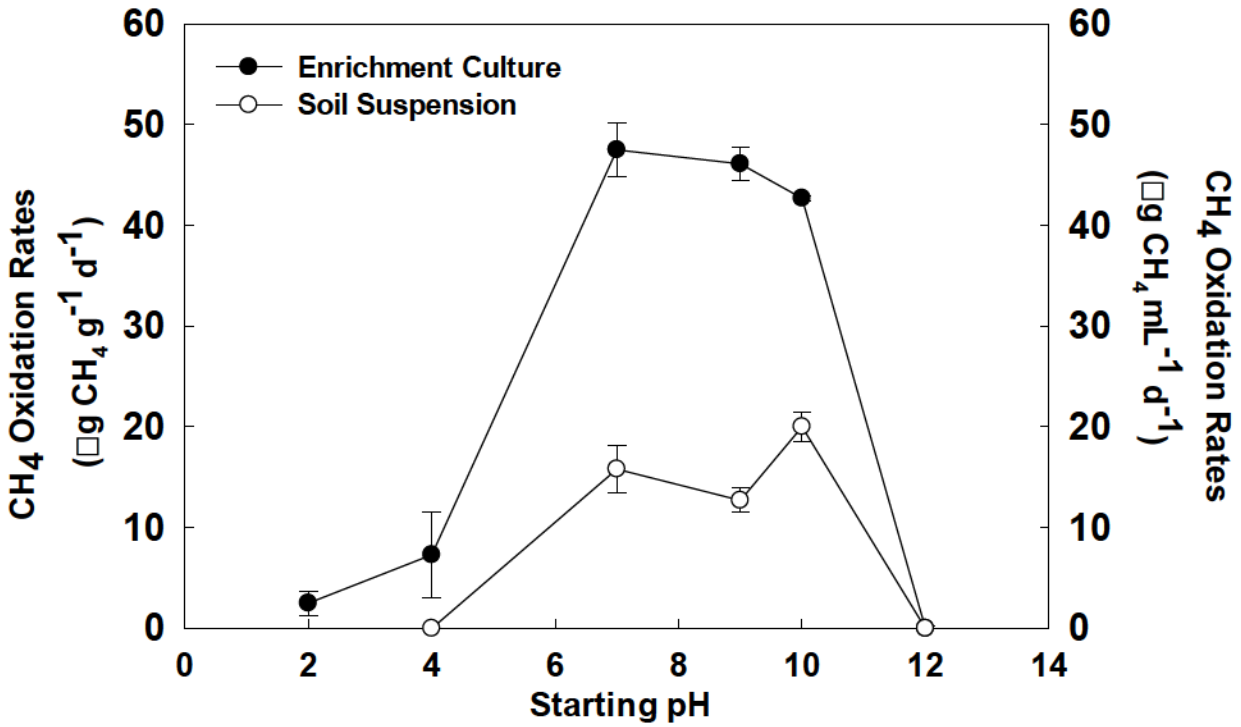
Figure 2: Methane consumption in (a) enrichment culture reactors and (b) soil suspension reactors with starting pH values ranging from 2.0 to 12.0

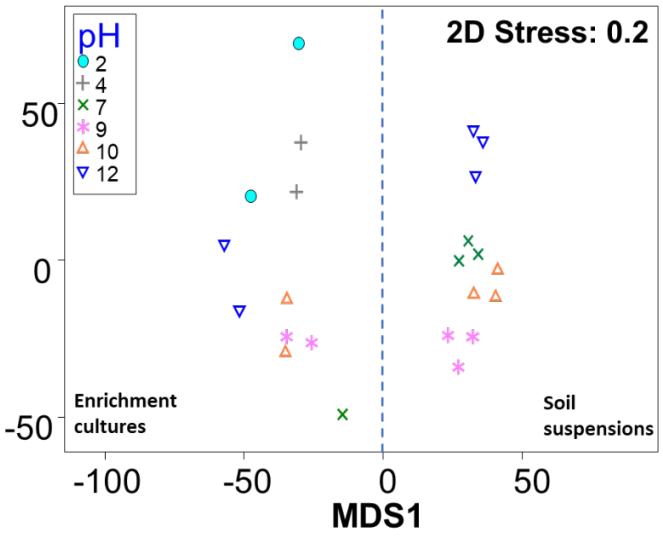
Figure 3: Methane oxidation rates in soil-suspension and enrichment culture reactors with starting pH values ranging from 2.0 to 12.0

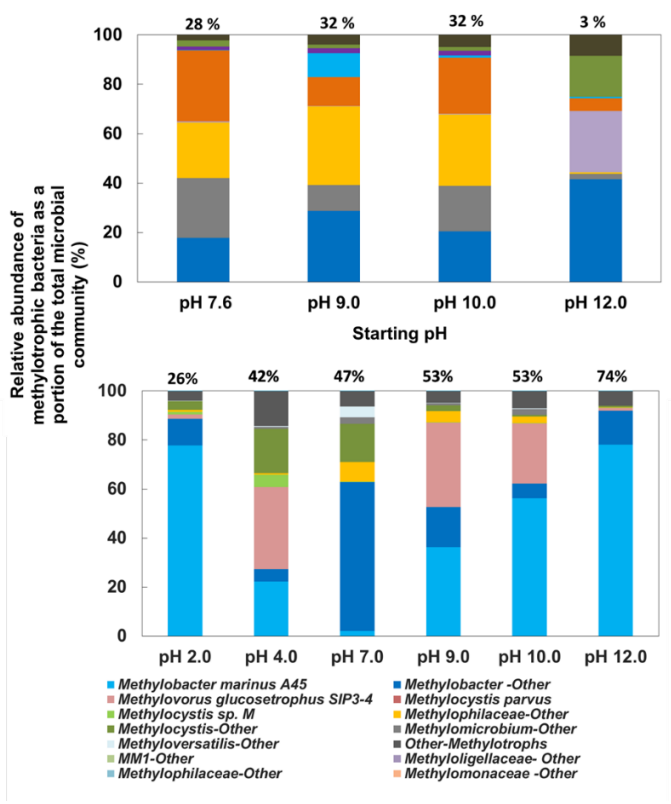
Figure 4: Microbial community structure in soil and enrichment culture microcosms as assessed by DNA-based 16S rRNA gene amplicon sequence analysis. (a) Metric multi-dimensional scaling plot of total microbial community structure by pH. (b) Bar chart of the average methylotrophic bacteria communities in soil suspension microcosms across a range of incubation pH values. The relative abundance of methylotrophic bacteria as a portion of the total microbial community is shown above each condition. (c) Bar chart of the average methylotrophic bacteria in enrichment culture microcosms across a range of incubation pH values. The relative abundance of methylotrophic bacteria as a portion of the total microbial community is shown above each condition


a
b
c
d
e
f
CH4 Concentration (%)
pH
Days
*
Enrichment Culture
pH - Enrichment Culture
Soil Suspension
pH - Soil Suspension


a
b
CH_4 Concentration (%)
Days
Starting pH 2.0
Starting pH 4.0
Starting pH 7.0
Starting pH 9.0
Starting pH 10.0
Starting pH 12.0
Starting pH 4.0
Starting pH 7.6
Starting pH 9.0
Starting pH 10.0
Starting pH 12.0


Enrichment Culture
Soil Suspension
CH4 Oxidation Rates
(□g CH4 g-1 d-1)
CH4 Oxidation Rates
(□g CH4 mL-1 d-1)
Starting pH
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14


pH
2
4
7
9
10
12
2D Stress: 0.2
Enrichment cultures
Soil suspensions
50
0
-50
-100
-50
0
50
MDS1


Relative abundance of methylotrophic bacteria as a portion of the total microbial community (%)
28 %
32 %
32 %
3 %
pH 7.6
pH 9.0
pH 10.0
pH 12.0
Starting pH
26%
42%
47%
53%
53%
74%
pH 2.0
pH 4.0
pH 7.0
pH 9.0
pH 10.0
pH 12.0
Methylobacter marinus A45
Methylobacter -Other
Methylovorus glucosetrophus SIP3-4
Methylocystis parvus
Methylocystis sp. M
Methylophilaceae-Other
Methylocystis-Other
Methylomicrobium-Other
Methyloversatilis-Other
Other-Methylotrophs
MM1-Other
Methyloligellaceae- Other
Methylophilaceae-Other
Methylomonaceae -Other