Identifying Active Methanotrophs and Mitigation of CH_4 Emissions in Landfill Cover Soil

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Abstract. In the USA, municipal solid waste (MSW) landfills constitute one of the major anthropogenic sources of methane emissions. In the landfill cover soils employed at MSW landfills, aerobic methane-oxidizing bacteria (MOB) convert CH_4 to CO_2, thereby partially mitigating the CH_4 emissions to the atmosphere. In this study, culture-dependent and culture-independent techniques were employed to evaluate methane oxidation capacity and to characterize the microbial community in landfill cover soil. Microcosms with synthetic landfill gas headspace were used to measure potential methane oxidation rates in landfill cover soil and in methanotrophs-enriched microbial consortia. The results demonstrate that the enriched landfill cover soil supported the growth of a diverse group of methanotrophic and methylotrophic microorganisms, and were dominated by Type I methanotrophs showing positive correlation with CH_4 oxidation rates.

Keywords: Methanotrophs · CH_4 oxidation activity · Landfill cover soil Microbial community

1 Introduction

In the USA, landfills are estimated to be the third largest anthropogenic source of CH_4 emissions making up 16.4% of the total CH_4 emissions in 2016 (USEPA 2018). Despite significant amount of methane emitted from landfills, it is estimated that between 10 and 90% is actively been consumed by the methane oxidizing bacteria (MOB) in landfill cover soils as reviewed by Semrau et al. (2010). MOB, also known as methanotrophs, are a subset of a larger microbial community called Methylotrophs. The methanotrophs utilize methane as a sole source of carbon and energy, whereas the methylotrophs use C1-compounds as their source of carbon and energy (Hanson and Hanson 1996). Methanotrophs within the phylum Proteobacteria are classified into three phylogenetically distinct groups: Type I, Type II and Type X methanotrophs, where Type I and

L. Zhan et al. (Eds.): ICEG 2018, ESE, pp. 308–316, 2019.
https://doi.org/10.1007/978-981-13-2224-2_38

Type X are grouped in Gamma-proteobacteria and Type II within Alpha-proteobacteria. Type I, Type II and Type X methanotrophs are ubiquitous in nature and are usually found in abundance where low to high concentration of methane prevails.

Landfill cover soils are typically dominated with either by Type I or Type II methanotrophs as reported in many studies (Yargicoglu and Reddy 2017; Cébron et al. 2007). Molecular ecology studies have utilized two distinct approaches for identification of methanotrophs from the environmental samples: cultivation-based enrichment and isolation approaches, and cultivation-independent molecular tools (Murrell et al. 1998) employing targeted amplification and sequencing of functional or structural genes or shotgun sequencing approaches. In some cases, both approaches are used concurrently, with molecular tools used to monitor and characterize enrichments and isolates. Targeted amplification protocols typically target structural genes such as the microbial small subunit ribosomal RNA genes (*i.e.*, 16S rRNA gene), as well as methane monooxygenase (MMO) genes and genes involved in C1 compound oxidation.

This study focuses on adopting PCR-based high-throughput amplicon sequencing technique to analyze microbial structure in landfill cover soil (LFCS) and in microcosms studies inoculated with LFCS. The specific objectives of this research were to: (1) characterize methanotrophic communities in enriched landfill cover soil using 16S rRNA gene analysis, (2) Conduct batch enrichments methanotrophic bacteria from LFCS, and follow enrichment patterns using 16S rRNA gene analysis, and (3) assess the relationship between methane oxidation rates and the relative abundance of methanotrophic community in LFCS. This study is a part of broader on-going study funded by the U.S. National Science Foundation with ultimate goal to evaluate system parameters that control microbial diversity and activity and design optimal and efficient biocover systems to mitigate CH_4 emissions at landfills.

2 Materials and Methods

2.1 Soil Enrichment

Soil was collected from Zion landfill site, located in Greater Chicago area, Illinois, USA. Soil samples were collected from an interim cover layer at a depth of ~1 to 2 feet and were shipped to the Geotechnical and Geoenvironmental Engineering Laboratory at the University of Illinois at Chicago (UIC) where it was stored at room temperature (23 °C). Soil samples were air dried (moisture content < 0.5%), pulverized and screened through 2 mm sieve prior to the inoculation of batch reactors. To obtain methanotroph-enriched consortia, approximately 5 g of sieved soil was mixed with 100 ml of modified NMS medium (Whittenbury et al. 1970) in a 500 ml serum vial and stoppered using long sleeved rubber septa. Approximately 80 ml of air from the headspace was replaced with equal volume of mix gas CH_4/CO_2 to achieve a headspace concentration of 7% CH_4 (v/v) and 7% CO_2 (v/v) balanced in air (86%) and were incubated for 20 days at 23 °C. To determine the activity of Methanotrophs and methane oxidation rates, gas samples were analyzed at regular intervals using Gas Chromatography (GC) and were monitored until the methane concentration dropped to

less than 1%. To enrich the methanotrophic culture the mix gas (CH_4/CO_2) was replenished twice throughout the enrichment. The enriched soil was later stored in micro-centrifuge tubes and frozen at −20 °C for DNA extraction and molecular analysis. Similarly, the supernatant enriched with methanotrophic cells consortium were also pelletized in 2 ml micro-centrifuge tubes by centrifuging at 12,500 RPM for 15 min, decanting the supernatant and freezing at −20°C for DNA extraction and molecular analysis.

2.2 Culture Experiments

Prior to culture experiments, serum vials, rubber septa and pipettes were sterilized using a Napco Model 8000-DSE autoclave operated at >120 °C for a minimum of 60 min to ensure complete sterilization. The supernatant obtained from the enrichment mentioned above was used in this experiment. 1 ml of enriched culture was inoculated in 9 ml modified NMS medium (total of 10 ml), placed in 125 ml serum vials and sealed air tight using butyl rubber septa followed by crimp cap. Approximately 20 ml of air from the headspace was replaced with equal volume of synthetic landfill gas comprising of 50% (v/v) CH_4 and 50% (v/v) CO_2 to achieve a headspace concentration of 5% CH_4 (v/v) and 5% CO_2 (v/v) balanced in air (90%). To determine changes in the headspace concentration, gas samples were analyzed every alternate day using Gas Chromatography (GC) until the headspace concentration dropped to less than 1%. All the experiments were conducted in triplicates along with the controls (media-NMS). The rates of CH_4 oxidation were determined from linear regression analysis of methane concentration with respect to time based on zero-order kinetics observed during testing. pH of the culture along with controls (NMS only) were also measured at the beginning and end of the experiment to examine any changes in the pH due to microbial activity.

2.3 DNA Extraction, PCR Amplification and Next Generation Sequencing

To measure microbial diversity in enriched soils and in culture consortia, genomic DNA was extracted from the samples using DNeasy Power Soil Kit (Qiagen). Extractions were performed according to the manufacturer's instructions, with slight modifications. Briefly, samples were heated at 65 °C for 10 min before homogenization with FastPrep-24 5G bead-beating device (MP Biomedicals) at 6 m/s for 40 s. Genomic DNA was used as template for PCR amplification with primers 515F-modified and 926R (Walters et al. 2016), targeting the V4–V5 variable region of the microbial small subunit ribosomal RNA gene using a two-stage "targeted amplicon sequencing (TAS)" protocol (Green et al. 2015; Bybee et al. 2011). The primers contained 5′ common sequence tags (known as common sequence 1 and 2, CS1 and CS2) as described previously (Moonsamy et al. 2013). The CS1_515F and CS2_926R primer sequences were ACACTGACGACATGGTTCTACAGTGYCAGCMGCCG CGGTAA and TACGGTAGCAGAGACTTGGTCTCCGYCAATTYMTTTRAGTTT, respectively, with the underlined regions indicating the common sequence tags.

First stage PCR amplifications were performed in 10 µl reactions in 96-well plates, using the MyTaq HS 2X mastermix (Bioline, Taunton, MA). PCR conditions were 95 °C for 5 min, followed by 28 cycles of 95 °C for 30″, 50 °C for 60″ and 72 °C for 90″. Subsequently, a second PCR amplification was performed in 10 µl reactions in 96-well plates. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876), as well as 1 µl of 1st stage PCR product. Cycling conditions were as follows: 95 °C for 5 min, followed by 8 cycles of 95 °C for 30″, 60 °C for 30″ and 72 °C for 30″. Libraries were loaded onto a MiSeq v3 flow cell and sequenced using an Illumina MiSeq sequencer. Raw sequence data were processed and merged using software package PEAR (Zhang et al. 2013), followed by quality checking (Q20), length trimming (>300 bp) and chimera checking using the UCHIME algorithm as compared with the Silva 119 16S.97 database (Edgar 2010). After chimera removal, the software package QIIME (Caporaso et al. 2010) was used to annotate sequences and generate annotation tables using a sub-OTU protocol. Briefly, all sequences were pooled, and unique sequences were de-replicated from the combined sequenced. Those sequences with counts greater than 10 were used as seed or master sequences for clustering. Low abundance sequences (fewer than 10) were queried against the master sequences using USEARCH to find the master sequence with a minimum percent identity of 98%; for matching sequences, the counts of the low abundance sequences were incorporated into the counts for the cluster. Taxonomic annotations were assigned to each seed and independent low abundance sequence using USEARCH and the Silva 119 reference database. Taxonomic and abundance data were merged into a single sequence table (seq table. biome) and summaries of absolute abundances of taxa were generated for all phyla, classes, orders, families, genera, and species present in the dataset. Library preparation, pooling and Illumina sequencing were performed at the University of Illinois at Chicago Sequencing Core (UICSQC), and basic bioinformatics processing of the data were performed at the UIC Research Informatics Core (RIC).

2.4 Gas Analysis

Gas samples were collected at regular time intervals and analyzed for CH_4, CO_2 and O_2 concentrations using an SRI 9300 Gas Chromatography (GC) equipped with thermal conductivity detector (TCD) and CTR-1 column that separates N_2 and O_2 for simultaneous analysis of CO_2, CH_4, O_2 and N_2. Gas samples were withdrawn using 1-ml syringe where 0.5 ml of gas sample was injected into the GC equipped with TCD. A calibration curve for a minimum of three points was established using high purity standard gas mixtures ranging from 1 to 50% CH_4 and CO_2.

3 Results and Discussion

3.1 Methane Oxidation

Figures 1(a) and (b) show a decrease in the headspace methane concentration with time in both enrichment and culture batch tests, respectively. An initial lag phase of four days was observed in enrichment batch tests, after which there was a rapid decrease in the methane headspace concentration, consistent with microbial methane oxidation. This is in agreement with prior studies showing similar lag phases (10–12 days) in microcosms inoculated with field samples without pre-incubation (Spokas and Bogner 2011). Culture batch tests also showed a lag phase of 24 h. Lag phases are generally due to the inoculation of microorganisms into the fresh media (NMS) and the time taken for responding or adjusting to the new environment. Methane oxidation rates were calculated using linear regression that followed zero-order kinetics. Maximum oxidation rates in the soil suspension and culture batch tests were calculated to be 123.3 μg CH_4/g dry-soil/day and 73.1 μg CH_4/ml/day, respectively. The pH of the culture sets was also measured at the beginning and end of the experiment to examine if changes in the pH had any impacts on methane oxidation rates. Results showed that the pH remained stable and was in the range of 6.7–7.1 throughout the experiment.

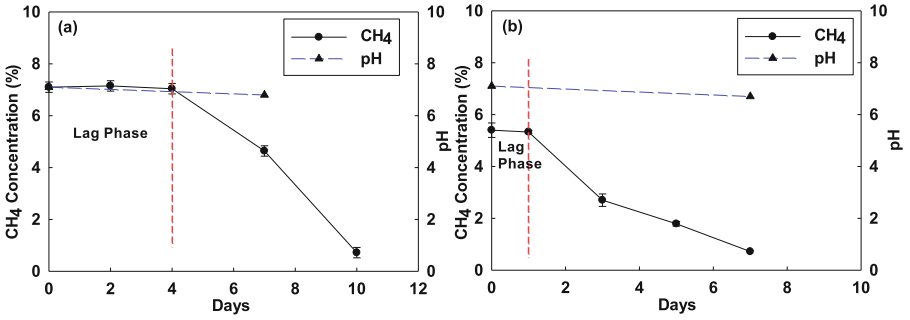

Fig. 1. Methane oxidation in: (a) Enriched soil, and (b) Culture consortium

3.2 Microbial Community Composition

The microbial community present in the enriched soil and culture consortium were analyzed using PCR amplification and high-throughput next generation sequencing of 16SrRNA genes. The results of the taxonomical classification for both samples are summarized below, with methanotrophic and methylotrophic taxa indicated.

Figure 2 shows the phylum-level composition of enriched soil and culture consortium microbial communities. The enriched soil sample was dominated by the phyla Proteobacteria (73%) and Bacteriodetes (9.1%), with lower levels of Acidobacteria (4.1%), Actinobacteria (2.8%), Verricomicrobia (2.31%), Firmicutes (1.1%), Planctomycetes (0.8%) and others. In the culture consortium, 79% of all sequences were annotated to the phylum Proteobacteria followed by Verrucomicrobia (5.7%), Bacteriodetes (2.9%) and others (<1%).

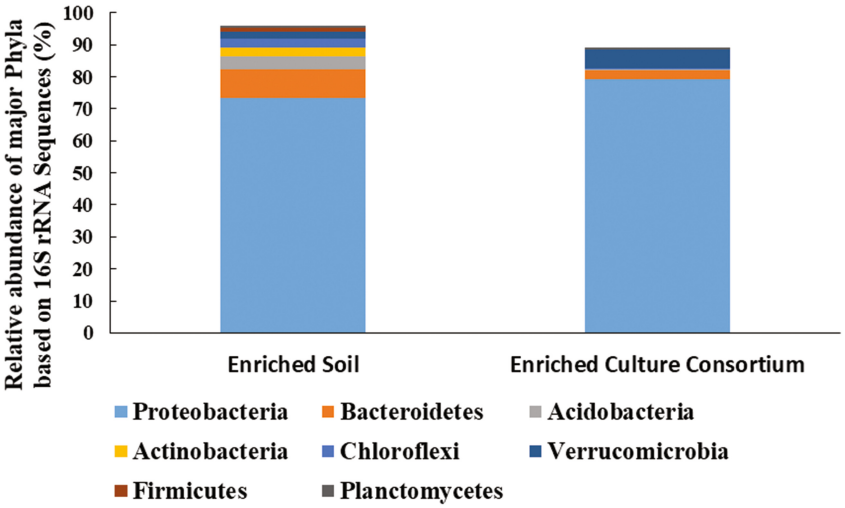

Fig. 2. Relative abundance (%) of sequences related to dominant phyla

Figure 3 shows the genus-level composition of enriched soil and culture consortium microbial communities. Sequences from different clades of methane-oxidizing bacteria, along with non-methane oxidizing methylotrophs, were identified in these samples. The most abundant methanotrophic taxa detected in both soil and cultures were *Methylobacter* accounting for 31% and 39% of the total 16SrRNA sequences identified, followed by *Methylovorus* (6.6% in soil and 4.7% in culture), and *Methylocystis* (2.6% in soil and 1.6% in culture). Some sequences recovered from these samples were not annotated at the taxonomic level of genus but could still be identified as being derived from putative methylotrophs. These sequences were derived from members of the families Methylophilaceae, Methylocystaceae, Methylococcacea, Crenotrichaceae, and Methylobacteriaceae. The majority (7.4%) of these sequences were derived from bacteria belonging to the family Methylophilaceae.

Kallistova et al. (2013) and Han et al. (2016) detected *Methylovorus* in landfill cover soil and were successful in cultivating them in laboratory, suggesting that their occurrence in landfill cover soils is not unusual and is consistent with the community composition found in the current study.

Methylobacter has been identified as one of the major genera of methanotrophic community present in abundance in most of the landfill cover soil (Yargicoglu and Reddy 2017; Cébron et al. 2007). *Methylobacter* are Type I methanotrophs that were found in abundance in the cover soil in the present study and could be responsible for oxidizing methane at faster rates, but this cannot be affirmed with our results alone as the 16SrRNA gene analysis does not determine the active members or function of the microbes/microbial community (Cébron et al. 2007). However, DNA profiling of microbial community structure can identify active microorganisms when sampling is performed longitudinally, and strong shifts are observed, or if analysis of RNA is performed.

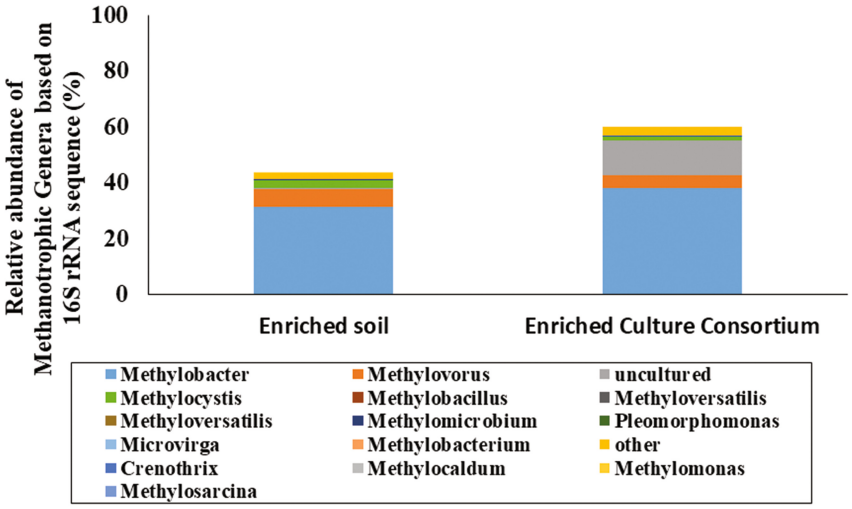

Fig. 3. Relative abundance (%) of sequences related to Methanotrophic genera

Overall, a number of methanotrophic genera (Type I and Type II) were detected in both soil and culture samples including *Methylobacter, Methylocystis, Methylomicrobium, Pleamorphomonas, Methylomonas, Methylobacterium, Methylosarcina, Methylocaldum, and Crenothrix*. More broadly, methylotrophic bacteria from the genera *Methylovorus, Methyloversatilis, Methylobacillus, Microvirga*, and from *OM43 clade* were detected. Sequences derived from methanotrophs were most frequently annotated as belonging to the genus *Methylobacter*, and second most frequently annotated as *Methylovorus glucosetrophus SIP3-4*. Due to the limited taxonomic resolution of the V4-V5 region of the microbial 16S rRNA gene, species-level annotation could not be obtained. Future studies in which shotgun metagenome sequencing (i.e., non-targeted, PCR-independent deep sequencing of microbial genomic DNA) will be performed, that will be used to identify taxa to the level of species. Shotgun metagenomic sequence data will also be used to identify taxon-specific DNA sequences of functional genes that will be used to develop quantitative assays for MMO messenger RNA molecules.

Of the total microbial community analyzed, the landfill cover soil that was enriched in laboratory with modified NMS showed a relative abundance of 39.5% of Type I methanotrophs, 1.5% of Type II methanotrophs, and 18.9% of methylotrophs in the culture consortium. Similarly, 33% of Type I methanotrophs, 2.8% of Type II methanotrophs, and 7.6% of methylotrophs were identified in enriched soil. From Fig. 4, it can be concluded that enrichment favored for cultivating majority of Type I methanotrophic bacteria and methylotrophic bacteria. The reason for not cultivating Type II methanotrophs in our cultures could possibly be the substrate (methane) limitation, as Type II methanotrophs usually dominate at high CH_4 concentration and low O_2 concentration or the nutrient rich NMS that usually promotes the growth of Type I methanotrophs inhibiting the growth of Type II methanotrophs (Wise et al. 1999). In addition, high methane oxidation potential from both enrichment and culture batch tests could possibly confirm the presence of abundant methanotrophic/methylotrophic community.

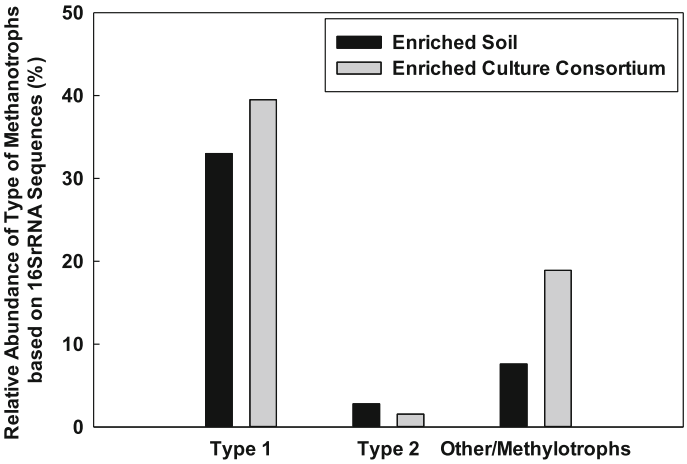

Fig. 4. Relative abundance (%) of sequences based on the types of methanotrophs

4 Conclusions

The following conclusions can be drawn from this study:

- Enrichment technique favored the maximum growth of Type I methanotrophs of the genera *Methylobacter* that were present in abundance constituting about 31% (enriched soil) and 39% (enriched culture) of the total 16SrRNA gene amplicon sequences identified. The growth of Type II methanotrophs were possibly inhibited due to lack of substrates needed for growth (1.5–2.6%). In future, shotgun metagenome sequencing shall be performed to identify taxa at the species level.
- High methane oxidation rates of 123.3 μg CH_4/g dry-soil/day (enriched soil) and 73.1 μg CH_4/ml/day (microbial culture) were observed in this study that correlate to the relative abundance of methanotrophic and methylotrophic community in both the enriched LFCS and the culture consortium.
- Overall, the enriched landfill cover soil was dominated by Type I methanotrophs (33%) followed by Methylotrophs (7.6%) and Type II methanotrophs (2.6%).

The present study provided initial insight into microbial diversity in the landfill cover soil under typical field condition. Additional DNA profiling and/or RNA analyses are being performed to identify active microorganisms. Series of microcosms are also being tested under variable moisture, pH, and temperature conditions to assess the resiliency of methanotrophs for methane oxidation. In addition, enhanced methane oxidation with an organic amendment such as biochar in the cover soil is also being investigated.

Acknowledgements. This project is funded by the U.S. National Science Foundation (Grant CMMI #1724773), which is gratefully acknowledged.

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