MICROBIAL MITIGATION OF GREENHOUSE GAS EMISSIONS FROM LANDFILL COVER SOILS

by

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ABSTRACT

Landfills are one of the major sources of methane (CH_4), a potent greenhouse gas with a global warming potential (GWP) ~23 times higher than that of carbon dioxide (CO_2). Although some effective strategies have been formulated to prevent methane emissions from large landfills, many landfills allow methane to be freely emitted to the atmosphere. In such situations, it is often proposed to stimulate methanotrophs, a group of bacteria that consume methane, in the cover soil to prevent fugitive methane emissions. Several factors, however, must be addressed to make such a biogenic removal mechanism effective. First, methanotrophic activity can be inhibited by nonmethane organic compounds (NMOCs) that are commonly found in landfill soil gas. Second, although methanotrophs can be easily stimulated with the addition of nitrogenous fertilizers, biogenic production of nitrous oxide with a GWP ~296 times higher than that of carbon dioxide, is also stimulated. To consider these issues, two general areas of research were performed. First, a dimensionless number was developed based on Michaelis-Menten kinetics that describes the effects of the presence of multiple NMOCs on methanotrophic growth and survival. This model was validated via experimental measurements of methanotrophic growth in the presence of varying amounts of NMOCs. Second, the effects of nutrient amendments on methane oxidation and nitrous oxide production were examined by constructing soil microcosms using landfill cover soils. Here, it was shown that the addition of ammonium in the presence of phenylacetylene stimulated methane

oxidation but inhibited nitrous oxide production. Furthermore, to understand the methanotrophic community structure and activity in response to these amendments, DNA microarray and transcript analyses were performed. The results indicated the predominance of Type II methanotrophs but that Type I methanotrophs responded more significantly to these amendments. Also, substantial activity of pMMO-expressing methanotrophs was observed, suggesting that these methanotrophs were responsible for nitrous oxide production. Collectively, these data demonstrate that methanotrophic activity and community structure can be differentially affected by both landfill gas composition and amendments, thus providing insights as how best to manipulate methanotrophic processes to better mitigate greenhouse gas emissions.

CHAPTER 1. Introduction

1.1. Production of methane in landfills

Methane, a major greenhouse gases that contributes to global warming, has a global warming potential (GWP) ~23 times higher than that of CO_2 (93). Specifically, GWP is an index that provides the relative impact a specific gas could have on the global climate over a defined time scale. However, because CH_4 has a short life (~8 years) relative to other greenhouse gases such as CO_2 (50-200 years) and N_2O (120 years) (93, 116), controlling the emission of CH_4 seems likely to be favorable in terms of short-term control of global warming.

Methane is produced through the decomposition of organic wastes in landfills along with CO_2. Typically, CH_4 and CO_2 comprise the majority of landfill gas (143), as shown in Table 1-1.

Table 1-1. Typical constituents in municipal solid waste landfill gas (143)

Component	%
CH_4	45-58
CO_2	35-45
N_2	< 1-20
O_2	< 1-5
H_2	< 1-5
H_2O	1-5
Trace constituents (e.g., nonmethane organic compounds, H_2S)	< 1-3

In 2005, it was estimated that US emitted 132 Tg CO_2 equivalent of CH_4 from landfills (municipal solid waste and industrial landfills combined) (62). This amount of CH_4 accounts for 25 % of the anthropogenic CH_4 emission in the US, putting landfills as one of the major anthropogenic sources of CH_4 along with natural gas systems and enteric fermentation (62). Global estimates of CH_4 emissions show that CH_4 emissions from landfills are 35-40 Tg $CH_4 \cdot yr^{-1}$, which is 6-8 % of the total emission of CH_4 including both natural and anthropogenic sources (68, 77, 115). A detailed list of sources of anthropogenic CH_4 is shown in Table 1-2 (62).

Table 1-2. Recent trends of CH_4 emissions in the US (62). Numbers in parentheses represent the proportion to total (%)

	1990	**1995**	**2000**	**2005**
	Tg CO_2 Eq.	Tg CO_2 Eq.	Tg CO_2 Eq.	Tg CO_2 Eq.
Landfills	161.0 (26.4)	157.1 (26.2)	131.9 (23.4)	132.0 (24.5)
Natural Gas Systems	124.5 (20.4)	128.1 (21.4)	126.6 (22.5)	111.1 (20.6)
Enteric Fermentation	115.7 (19.0)	120.6 (20.1)	113.5 (20.1)	112.1 (20.8)
Coal Mining	81.9 (13.5)	66.5 (11.1)	55.9 (9.9)	52.4 (9.7)
Manure Management	30.9 (5.1)	35.1 (5.9)	38.7 (6.9)	41.3 (7.7)
Petroleum Systems	34.4 (5.7)	31.1 (5.2)	27.8 (4.9)	28.5 (5.3)
Wastewater Treatment	24.8 (4.1)	25.1 (4.2)	26.4 (4.7)	25.4 (4.7)
Stationary Sources	8.0 (1.3)	7.8 (1.3)	7.4 (1.3)	6.9 (1.3)
Rice Cultivation	7.1 (1.2)	7.6 (1.3)	7.5 (1.3)	6.9 (1.3)
Abandoned Coal Mines	6.0 (1.0)	8.2 (1.4)	7.3 (1.3)	5.5 (1.0)
Others	14.8 (2.4)	11.5 (1.9)	20.7 (3.7)	17.2 (3.2)
Total (Tg CO_2 Eq.)	**609.1**	**598.7**	**563.7**	**539.3**

Methane emission from landfills, however, has decreased from 161.0 Tg CO_2 equivalent in 1990 to 131.9 Tg CO_2 equivalent in 2000. This decrease has been attributed to the installation of gas collection systems in landfills (62). Specifically, as shown in Table 1-3, although the amount of CH_4 generated from landfills has increased between

1990 and 2005 from 188.7 to 249.6 Tg CO_2 equivalent, the amount of CH_4 captured for energy generation or flaring also increased such that the overall emission of CH_4 decreased between 1990 and 2005.

Table 1-3. Historical emission of CH_4 from landfills (Tg CO_2 equivalent) (62)

	1990	1995	2000	2005
Landfills	201.6	217.6	232.7	265.7
Recovered				
Gas-to-energy	17.6	22.3	49.0	58.6
Flared	5.0	21.8	37.1	60.4
Oxidized	17.9	17.5	14.7	14.7
Total Emitted	**161.0**	**157.1**	**131.9**	**132.0**

1.2. Methane capture strategies in landfills

Capturing CH_4 for energy generation was first put into full-scale use at the Palos Verdes sanitary landfill in California in 1975 (174). However, at that time, installing landfill gas collection systems were not required. In 1991, Subtitle D of Resource Conservation and Recovery Act (RCRA) (40 CFR Part 258) went into effect. Subtitle D of RCRA requires that: i) landfill gas be controlled such that CH_4 concentrations do not exceed 25 % of the Lower Explosive Limit (LEL) in the facilities' structures (for CH_4 LEL is 5% by volume), and ii) the concentration of CH_4 should not exceed the LEL at the boundary of the facilities.

There are two general types of systems to collect gas from landfills in order to meet these regulations: i) passive, and; ii) active gas collection systems (143). The general concept of a passive gas collection system is to provide avenues for soil gases to be emitted into the atmosphere without the use of mechanical equipment as shown in Figure 1-1. Therefore, passive gas collection systems are relatively inexpensive but as it vents soil gas directly into the atmosphere, it can pose some environmental risk.

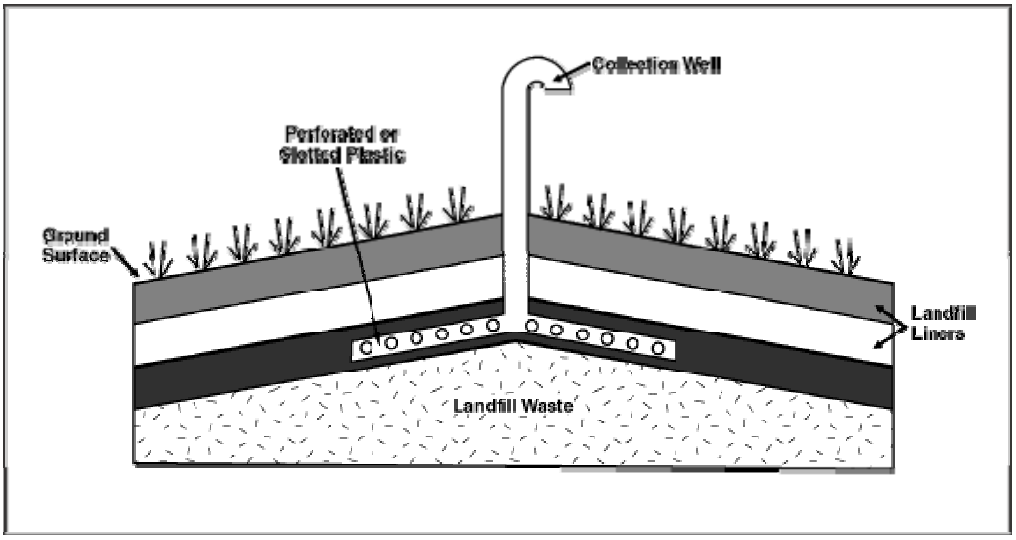

Figure 1-1. Passive gas collection system for release of landfill gas into the atmosphere (5)

Alternatively, active gas collection systems utilize mechanical equipment such as blowers and pumps to enhance the gas collection rate. Such gas collection systems are now enforced by the Landfill Rule (New Source Performance Standards and Emissions Guidelines) promulgated under the Clean Air Act in March 1996 and amended in June 1998 (63, 64). These rules require landfill gases to be collected and either flared or utilized at landfills that: i) have a design capacity larger than 2.5 million metric tons and 2.5 million cubic meters, and; ii) emit more than 50 metric tons of nonmethane organic compounds. Because of the Landfill Rule, landfills that must meet its criteria have either flaring or CH_4 recovery systems following the active gas collection system. A schematic diagram of a typical active gas collection system is provided in Figure 1-2.

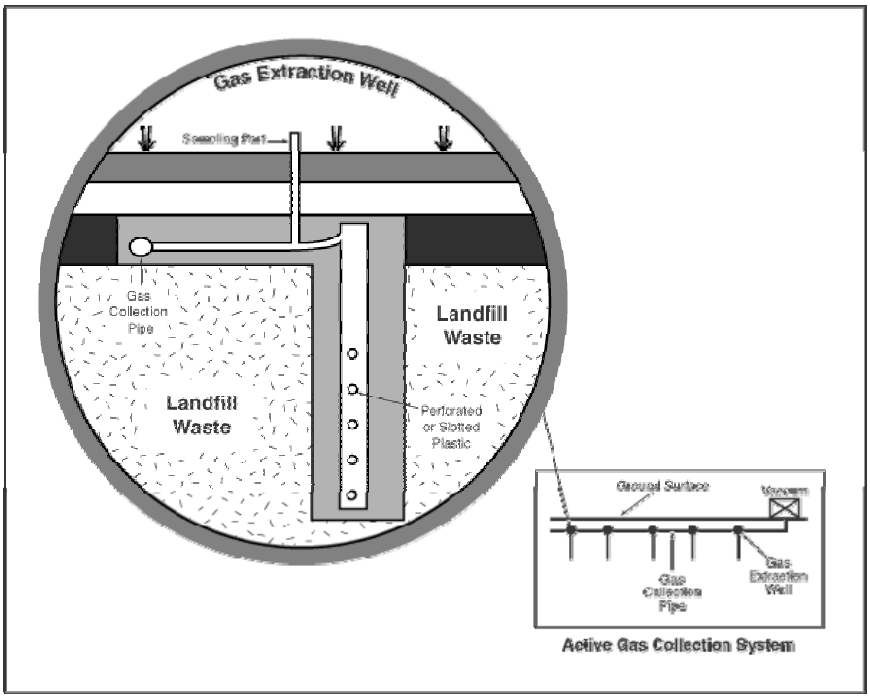

Figure 1-2. Active gas collection system for treatment of gas, e.g. energy generation, incineration (5)

Landfills that are large enough to be in compliance with the Landfill Rule can thus pose less environmental risks, compared to landfills with smaller capacity, via active gas collection systems whether the collected CH_4 is either flared or used for energy. The US EPA, however, recognizes that even with the Landfill Rule, in 2020, the projected CH_4 emissions from landfills will still be greater than 40 million metric tons of carbon equivalent (65).

It is estimated that as of 2004, more than 100,000 closed landfills exist in the US (163), as well as 1654 active landfills (156). However, not all landfills are required to either have gas collection systems installed and or possess gas generation properties suitable for energy production/flaring due to their age and/or the materials landfilled (69, 162). Therefore, it is important to develop methodologies that will reduce the emission of

CH_4 from landfills where installation of active gas collection systems is either not cost-effective or where such systems do not prevent all fugitive emissions of CH_4.

1.3. Sinks of CH_4

Natural sinks of CH_4 consist of reaction with OH radicals in the troposphere, OH, Cl, and $O(^1D)$ radicals in the stratosphere, and soil microbes (116). The major sink of atmospheric CH_4 is the reaction of CH_4 with $OH\bullet$ in the troposphere. In the troposphere CH_4 initially reacts with $OH\bullet$ to produce $CH_3\bullet$ (145). $CH_3\bullet$ then further undergoes chemical reaction and produces CO, CO_2, and H_2O among other compounds (145). The $OH\bullet$ sink in the troposphere is reported to be responsible for ~510 $Tg(CH_4)\cdot yr^{-1}$ (93, 116). In the stratosphere, CH_4 reacts with compounds such as OH, Cl, and $O(^1D)$ radicals, but this process plays a minor role in removing CH_4, being responsible for ~40 $Tg(CH_4)\cdot yr^{-1}$ (116).

Yet another sink of CH_4 is via soil microbial activity. In soils, CH_4 can be oxidized into other forms of carbon via microorganisms, i.e., methanotrophs. Methanotrophs are a group of bacteria that utilize CH_4 as its sole carbon and energy source in the presence of O_2. It has been estimated that anywhere from 10 to 100 % of the CH_4 generated in landfills is oxidized by these bacteria (24, 37, 38, 44, 118, 178). Interestingly, there have been reports where landfills have acted as sinks of CH_4 rather than as sources (21, 22). Therefore, stimulating the activities of such bacteria in landfill cover soils could possibly reduce emission of CH_4 from landfills, especially in landfills where active gas collection is not required. In attempts to stimulate methanotrophic activities, the addition of nitrogen-based fertilizers have been shown to be promising in terms of stimulating CH_4 oxidation in soils (14, 48, 129) as the nitrogenous fertilizers are

used as nitrogen sources by the soil microorganisms. However, addition of nitrogen-based fertilizers to soils generally results in stimulation of production of yet another greenhouse gas, N_2O, which has a GWP ~300 times greater than that of CO_2 (93). Thus, a strategy to mitigate one greenhouse gas, CH_4, could result in the production of a relatively more potent greenhouse gas, N_2O.

1.4. Biogenic N_2O production

Nitrous oxide, can be produced through both biological and abiotic processes. Many groups of microorganisms have the ability to produce N_2O but bacterial-mediated nitrification and denitrification appear to be the predominant sources of N_2O production (67). Microbial production of nitrous oxide can be achieved through three different processes; (i) a by-product of nitrification, (ii) nitrifier denitrification, and/or (iii) denitrification. The first process is via autotrophic ammonia oxidizing bacteria, or nitrifiers, which are a group of bacteria that oxidize NH_3/NH_4^+ to generate energy and CO_2 as the main carbon source (106). Oxidation of NH_3/NH_4^+ to NO_2^- via hydroxylamine is shown in Figure 1-3 (4). It has been shown that ammonia oxidizing bacteria can produce N_2O as a by-product of oxidation of hydroxylamine to NO_2^- (87). A study using highly purified hydroxylamine oxidoreductase from *Nitrosomonas europaea* showed that NO_2^- and NO_3^- composed 87% of the product of hydroxylamine while the remaining 13% were N_2O and NO with N_2O comprising the majority (87).

Recently, the archaeal counterpart of the autotrophic ammonia-oxidizing bacteria has been gaining interest as it appears that their abundance could exceed that of ammonia-oxidizing bacteria in soils (114). However, the ability/inability of ammonia-oxidizing archaea to produce N_2O is not known, nor is their general activity *in situ*.

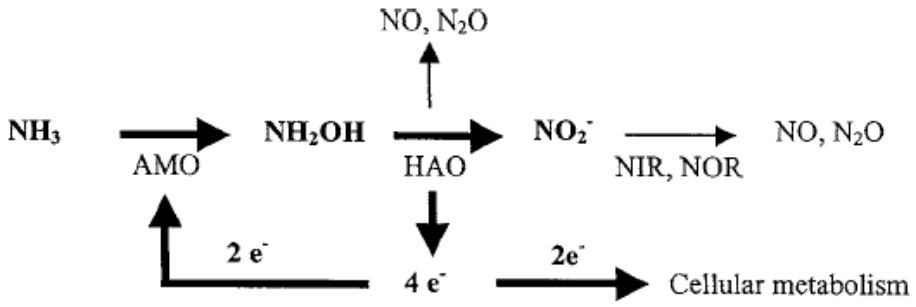

Figure 1-3. Ammonia oxidizing pathway (4)
AMO: ammonia monooxygenase, HAO: hydroxylamine oxidoreductase, NIR: nitrite reductase, NOR: nitric oxide reductase

The second process, nitrifier denitrification is another process that produces N_2O which can be attributed to autotrophic ammonia oxidizing bacteria. Specifically, ammonia oxidizing bacteria have been shown to be able to reduce NO_2^-, the product of NH_3/NH_4^+ oxidation, to N_2O (95, 142, 155). Studies using *Nitrosomonas* and *Nitrosospira* species have shown that ammonia-oxidizing bacteria are capable of reducing NO_2^- and produce N_2O while doing so. Initially, it was thought that nitrifier denitrification occurs in O_2-limiting environments (71, 142) but recently it has been shown that it can occur at atmospheric levels of O_2 (i.e., ~ 20% (v/v)) (155). However, the mechanism for production of N_2O via nitrifier denitrification is still unclear. At first, it was speculated that nitrite reductase reduced NO_2^- to produce N_2O in ammonia-oxidizing bacteria. Homologues of genes encoding copper-containing nitrite reductase (*nirK*) were found in the genome of *Nitrosomonas europaea* (34). However, disruption of the gene *nirK* in *Nitrosomonas europaea* actually resulted in larger production of N_2O suggested that nitrite reductase had a minimal role in producing N_2O from NO_2^- (11). It was further suggested that HAO could have a role in producing N_2O from NO_2^- (11, 86).

A third process that can produce N_2O is through anaerobic denitrification i.e., use of nitrate (NO_3^-) as terminal electron acceptor as shown in Figure 1-4. Denitrifying abilities are wide spread among a number of taxonomic and physiological groups of bacteria (67).

$$NO_3^- \xrightarrow[Nar]{} NO_2^- \xrightarrow[Nir]{} NO \xrightarrow[Nor]{} N_2O \xrightarrow[Nos]{} N_2$$

Figure 1-4. Reduction of nitrate to nitrogen gas in denitrifiers (140)
NAR: Nitrate reductase, NIR: Nitrite reductase, NOR: Nitric oxide reductase, NOS: Nitrous oxide reductase

For denitrification to occur there are several conditions that are necessary; i) presence of bacteria capable of denitrification, ii) availability of a suitable reductant such as organic carbon, iii) restriction of O_2 availability, and iv) availability of nitrogen oxides, e.g., NO_3^-, NO_2^-, and/or NO (67). As an intermediate of reduction of NO_3^- to N_2, N_2O is formed. Thus, when nitrogen-based fertilizer (e.g., NH_4^+ or NO_3^-) is added to soils, it is not surprising to see increased production of N_2O. Indeed, nitrogen-based fertilizers are commonly applied to agricultural soils, making these systems one of the major sources of N_2O (62).

Interestingly, methanotrophs have been reported to be capable of producing N_2O (125, 165, 182). In one study, *Methlyosinus trichosporim* OB3b was shown to be able to produce N_2O but it was concluded that methanotrophs do not play a significant role in N_2O production in the environment because the production of N_2O by this particular strain was only 1.6% of that of *Nitrosomonas europaea* (182). In another study using landfill cover soils, however, methanotrophs were suggested to be directly linked to N_2O production via nitrification (125). Methanotrophs can also be involved in N_2O production

via cross-feeding of denitrifying through metabolite excretion (2). In these studies methanotrophs in high CH_4 and low O_2 conditions were associated with a *Hyphomicrobium*-like bacterium which can denitrify using methanol (2). Therefore, understanding the processes involved in CH_4 oxidation and N_2O production individually and holistically is important in the mitigation of greenhouse gases from landfill cover soils.

1.5. Nonmethane organic compounds

As shown in Table 1-1, nonmethane organic compounds (NMOC) are also emitted from landfill cover soils. These NMOC include compounds such as alkanes, alkenes, halogenated hydrocarbons, aromatic hydrocarbons, and also sulphur compounds. NMOCs are reported to pose risk on human health as some of these compounds are considered carcinogenic/mutagenic (29). Another important issue is that some of the compounds categorized as NMOC can inhibit CH_4 oxidation since methanotrophs are capable of co-metabolizing compounds such as vinyl chloride, dichloroethylene, and trichloroethylene (74, 76, 122). Thus, understanding the effects of NMOC on methanotrophs will be crucial in mitigation of both CH_4 and NMOC.

1.6. Physiology and phylogeny of methanotrophs

Methanotrophs are a group of bacteria that utilize CH_4 as their sole carbon and energy source. These microorganisms oxidize CH_4 to CO_2 via methanol, formaldehyde, and formate. While doing so, a portion of the carbon from CH_4 is utilized to produce cell materials as can be seen in Figure 1-5. It has been shown that 30-50 % of the carbon in

CH_4 can be incorporated into biomass by methanotrophs depending on the source of nitrogen, i.e., NH_4^+ or NO_3^- (112).

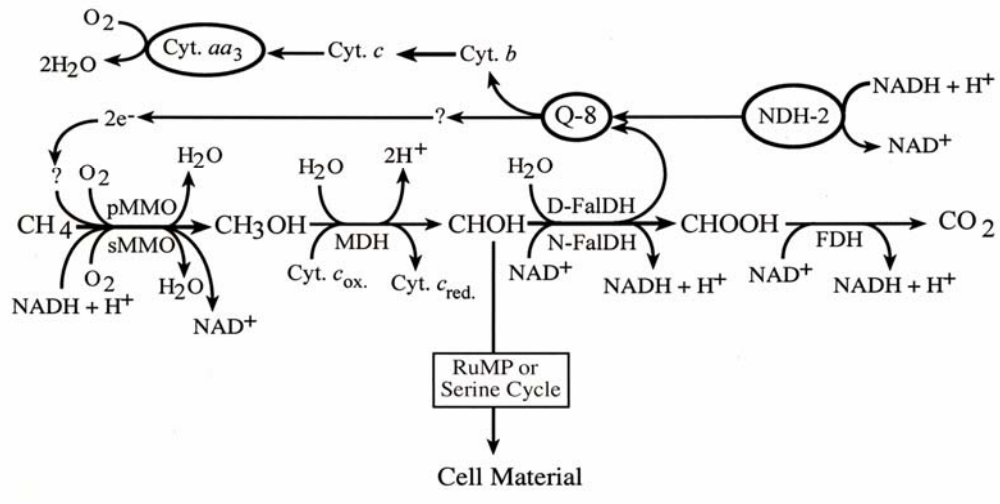

Figure 1-5. Metabolic pathway of methanotrophs (55)
pMMO: particulate methane monooxygenase, sMMO: soluble methane monooxygenase, MDH: methanol dehydrogenase, FalDH: formaldehyde dehydrogenase, FDH: formate dehydrogenase

Traditionally, methanotrophs were classified into two general groups (Type I and II) based on several characteristics such as cell morphology, membrane arrangement, carbon assimilation pathway, and predominant phospholipid fatty acids (PLFA) (76). Recently, however, acidophilic bacteria belonging to the phylum Verrucomicrobia exhibiting CH_4 oxidation capabilities have been isolated (60, 94, 141) while the traditional Type I and Type II methanotrophs belong to Proteobacteria. Some of the characteristics of Type I and II methanotrophs and methanotrophic Verrucomicrobia are summarized in Table 1-4.

Table 1-4. Characteristics of Type I and II methanotrophs and methanotrophic Verrucomicrobia (18, 28, 49-52, 59, 60, 76, 82, 94, 141, 170, 181)

Characteristic	Type I	Type II	Verrucomicrobia
Cell morphology	Short rods, usually occur singly; some cocci, ellipsoids, or pleomorphic	Crescent-shaped rods, rods, pear-shaped cells, ovoids, sometimes occur in rosettes or cocci	Rods
G+C content of DNA (mol%)	43-65	60-67	Not determined
Membrane arrangement			
Bundles of vesicular disks	Yes	No	No
Paired membranes aligned to periphery of cells	No	Most except *Methylocella* and *Methylocapsa*	No
Vesicular membranes or polyhedral organelles	No	Only in *Methylocella*	Yes
Membranes aligned to one side of cells	No	Only in *Methylocapsa*	No
Nitrogen fixation	Some strains	Yes	No
Resting stages formed			
Exospores	No	Some strains	Unknown
Cysts	Some strains	Some strains	Unknown
RuMP pathway	Yes	No	No
Serine pathway	Sometimes in *Methylococcus* and *Methylocaldum*	Yes	Yes
Calvin-Benson pathway	Partially in *Methylococcus* and *Methylocaldum*	No	Yes
Major PLFAs	14:0, 16:0, 16:1ω8c, 16:1ω7c, 16:1ω6c 16:1ω5t, 18:1ω9c, 18:1ω7	16:1ω8c,18:1ω8c, 18:1ω7	18:0, 16:0, aC15:0, 14:0
Bacterial affiliation	γ-Proteobacteria	α-Proteobacteria	Verrucomicrobia

Type I methanotrophs, which belong to γ-Proteobacteria, are comprised of *Methlyomonas, Methylococcus, Methylomicrobium, Methylosarcina, Methylosphaera, Methylothermus, Methylosoma, Methylohalobius, Methylocaldum*, and *Methylobacter (56, 76, 144)*. Type I methanotrophs can further be divided into two different groups, Type Ia and Ib, where Type Ib methanotrophs are comprised of *Methylococcus*, *Methylocaldum* and *Methylothermus* whereas the remaining genera are classified as Type Ia methanotrophs. Genera that are members of Type II methanotrophs, which belong to α-Proteobacteria, include *Methylosinus, Methylocella, Methylocapsa*, and *Methylocystis* (51, 52, 76). Additional to the traditionally accepted Type I methanotrophs that belong to γ-Proteobacteria, filamentous microorganisms *Crenothrix polyspora* and *Clonothrix fusca* have been reported to be CH_4 oxidizing γ-Proteobacteria (160, 175). Phylogenetic analysis based on 16s rRNA sequences of selected methanotrophs are shown in Figure 1-6.

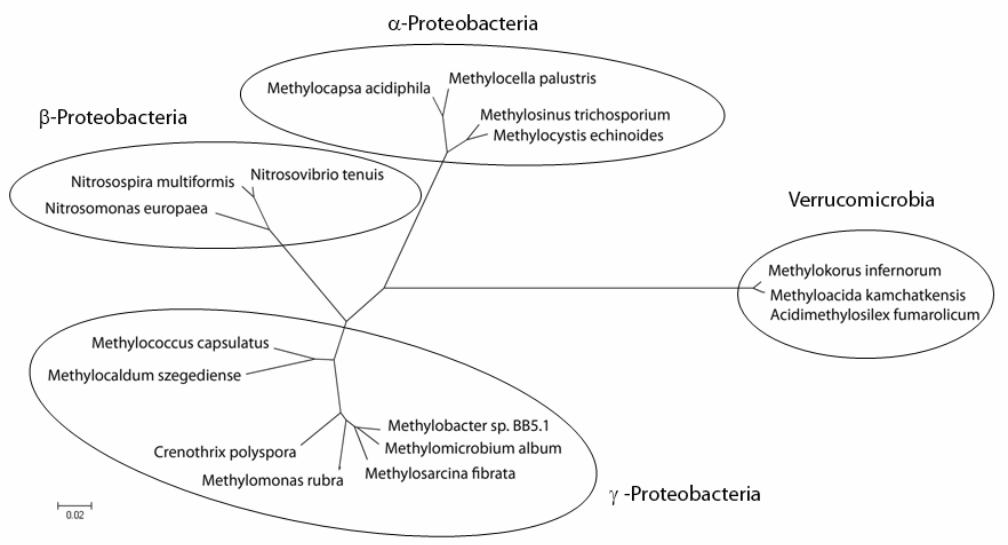

Figure 1-6. Phylogenetic tree constructed from 16s rRNA gene sequences using MEGA4 (166). The tree was constructed using the Neighbor-Joining method (148) with 1304 positions of 16s rRNA. The bootstrap consensus tree was inferred from 500 replicates (66). Evolutionary distances were computed using the Maximum Composite Likelihood method (167) with the scales indicating 0.02 base substitutions per site (153).

The most significant difference found among Type I and II methanotrophs and methanotrophic Verrucomicrobia was the structure of intracytoplasmic membranes (ICM). All the Type I methanotrophs that have been characterized possess type I ICM, stacks of vesicular disks. Meanwhile, most of the Type II methanotrophs possess type II ICM, paired membranes aligned to the periphery of the cells. However, recently characterized methanotrophs that belong to the genera *Methylocella* and *Methylocapsa* was identified to have different ICM systems compared to both type I and II ICM. *Methylocella* did not possess the type II ICM found in other Type II methanotrophs. Rather, *Methylocella* appeared to have vesicular membrane system connected to the cytoplasmic membrane, found on the periphery of the cytoplasm (50, 52, 59). Also, *Methylocapsa* contained packed vesicular membranes aligned to only one side of the cell (51). The methanotrophs belonging to the phylum Verrucomicrobia had yet another different membrane system, i.e., vesicular/tubular membranes or filled with polyhedral organelles (60, 94, 141). The identity of the unique membrane system of methanotrophic Verrucomicrobia has yet to be elucidated. However, it was speculated to be either similar to the vesicles found in *Methylocella* (141) or carboxysomes which are normally found in cyanobacteria and chemoautotrophs (94).

Another significant difference was found in what type of carbon assimilation pathway was present among these cells. In Type I methanotrophs, the ribulose monophosphate (RuMP) pathway is used while in *Methylococcus* and *Methylocaldum*, the serine pathway is found in some strains (18, 76). All known Type II methanotrophs possess only the serine pathway. Interestingly, in methanotrophic Verrucomicrobia, possibly a variation of the serine pathway was present supported by identifying some

genes that encode enzymes part of the serine pathway (60, 141). Additionally, in these studies, all the genes to form a complete Calvin-Benson cycle were identified (60) or genes for Ribulose-1,5-bisphosphate carboxylase/oxylase (RuBisCO) was detected (141). Unlike the RuMP and serine pathway where formaldehyde, a product of the oxidation of methanol shown in Figure 1-5, is utilized to produce cell materials, the Calvin-Benson cycle utilizes CO_2. It has been shown that certain methanotrophs, specifically cells belonging to the genera *Methylococcus* and *Methylocaldum,* also possess genes encoding RuBisCO, which is part of the Calvin-Benson cycle (10). The study reported that *Methylococcus capsulatus* (Bath) was only able to grow autotrophically, i.e., utilize CO_2, only on solid medium when H_2 was present and not in liquid medium (10). It will be interesting to see what the role of the complete Calvin-Benson cycle or RuBisCO genes would be in the methanotrophic Verrucomicrobia especially considering the speculation on the possible function of the polyhedral organelles found in a methanotrophic Verrucomicrobia species. In the study, it was suggested that the identity of the polyhedral organelles could be a carboxysome, which are known to contain RuBisCO in autotrophic microorganisms (94).

Abundant PLFA are also different among methanotrophs. Type I methanotrophs are abundant in 14:0, 16:0, 16:1ω8c, 16:1ω7c, 16:1ω6c 16:1ω5t, 18:1ω9c, 18:1ω7 while 16:1ω8c, 18:1ω8c, 18:1ω7 are generally found in large portions among Type II methanotrophs. Fatty acid 18:1ω7 was considered a marker for Type I methanotrophs but recently a Type II methanotroph, *Methylocystis heyeri* was found to have 18:1ω7 (49). Methanotrophic Verrucomicrobia were identified to have 18:0, 16:0, aC15:0, 14:0 (60). However, PLFA information of methanotrophic Verrucomicrobia is limited to just one

strain at this time since PLFA analysis was not performed on the other two strains that have been studied.

The first step of CH_4 oxidation is carried out by methane monooxygenase (MMO). The MMO can be found in two different forms. The particulate methane monooxygenase (pMMO) is found in the membrane whereas the soluble methane monooxygenase (sMMO) resides in the cytoplasm. Most methanotrophs are known to express the pMMO whereas only a few methanotrophs have the ability to also express sMMO. For methanotrophs that are capable of expressing both forms of MMO, the copper to biomass ratio strongly regulates which form of MMO is expressed. High copper to biomass ratios trigger the expression of pMMO whereas low copper to biomass induces the expression of sMMO. It appears that no other metal ions regulate the expression of MMOs in methanotrophs (133).

1.7. Molecular biology of MMO

Genes that encode for pMMO are clustered on the chromosome and are normally found in the order of *pmoCAB* as shown in Figure 1-7. Similar to ammonia monooxygenase genes in *Nitrosomonas europaea* (34), some methanotrophs have been found to have multiple copies of pMMO genes (176).

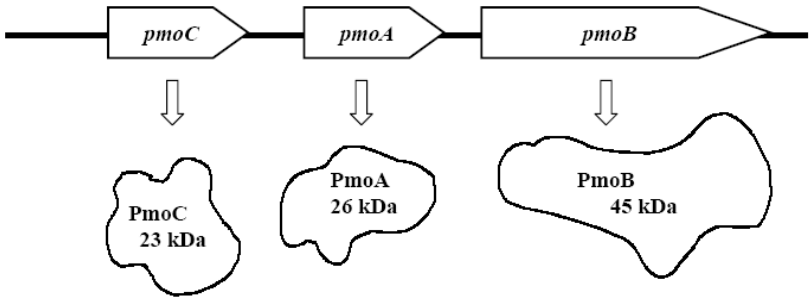

Figure 1-7. Gene cluster of pMMO (132)

Genes that encode for sMMO are also clustered in the chromosome of methanotrophs (132) as shown in Figure 1-8. Genes *mmoX, mmoY*, and *mmoZ* encode the α, β, and γ subunit of the hydroxylase subunit while *mmoB* and *mmoC* encode a small regulatory protein protein B and the reductase component, respectively. *orfY* which encodes protein MMOD, may possibly be involved in the assembly of hydroxylase diiron center (128), but its function is still unclear.

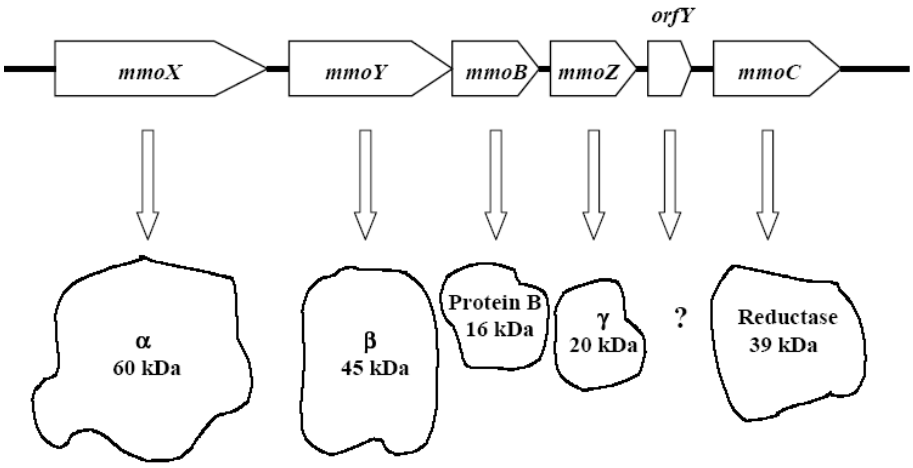

Figure 1-8. Gene cluster of sMMO (132)

1.8. Kinetics of MMO

The kinetics of CH_4 oxidation by both MMOs have been reported as shown in Table 1-5. Cells expressing pMMO had higher affinities for CH_4 than cells expressing sMMO (i.e., lower K_s values). Conversely, in *Methylosinus trichoporium* OB3b, the maximal uptake rate of CH_4 was higher when cells were expressing sMMO. Therefore, it is important to know which MMO the methanotrophs are expressing *in situ* to effectively utilize these cells for mitigation of CH_4 from landfills.

Table 1-5. Whole cell kinetics of CH_4 by a Type I (*Methylomicrobium album* BG8) and a Type II (*Methylosinus trichosporium* OB3b) expressing either soluble MMO or particulate MMO

Strain	Enzyme	Cu^{2+} (μM)	V_{max} (nmol·(min·mg protein)$^{-1}$)	K_s (μM)	V_{max}/K_s (ml·(min·mg protein)$^{-1}$)
Methylomicrobium album BG8	pMMO[a]	10	453	19	25
Methylosinus trichosporium OB3b	sMMO[b]	0	726*	92	7.9
	pMMO[c]	10	110	14	7.9

*Assumed 50% of cells in mass were protein; [a] (74); [b] (138); [c] (122)

The form of MMO methanotrophic communities are expressing *in situ* could be especially important if chlorinated solvents such as trichloroethylene (TCE), *trans*-dichloroethylene (*t*-DCE), or vinyl chloride (VC) are also present in the system. Although, both forms of MMO are capable of co-metabolizing a broad range of compounds including these chlorinated solvents (74, 122), sMMO-expressing cells have been reported to have a faster initial degradation rates for chlorinated solvents compared to pMMO-expressing cells (119, 138). Pseudo-first-order rate constants of MMO mediated degradation of different chlorinated solvents are shown in Table 1-6.

Table 1-6. Pseudo-first-order rate constants of methanotrophic cells, *Methylosinus trichosporium* OB3b and *Methylomicrobium album* BG8, expressing either pMMO or sMMO

	Pseudo-first-order rate constant (ml·min^{-1}·mg protein^{-1})		
Compound	sMMO-expressing *M. trichosporium* OB3b* (172)	pMMO-expressing *M. trichosporium* OB3b* (172)	pMMO-expressing *M. album* BG8 (74)
Dichloromethane	16	1.4	0.45
Vinyl chloride	15.2	3.8	0.23
trans-dichloroethylene	6.6	1.8	0.72
cis-dichloroethylene	9.8	0.12	0.15
1,1-dichloroethylene	6.4	<0.06	0.092
Trichloroethylene	6.2	<0.06	0.072

* Assumed 50% of cell mass is protein

Methane and co-substrates such as chlorinated solvents may be co-mingled *in situ*, leading to difficulties in modeling methanotrophic growth and substrate turnover due to competitive binding by MMOs, possible substrates and product toxicity, as well as long-term reductant limitation. Indeed, chlorinated ethylenes have been reported to be emitted from landfills along with CH_4 (8, 61). Therefore, it is important to understand what the effects of co-metabolites on the methanotrophs, i.e., growth, expressing either form of MMO.

Although chlorinated solvents are hazardous chemicals posing risks to groundwater, in terms of global warming, CH_4 could also be considered a hazardous compound. If there exists any differential effects due to the presence of NMOC, namely chlorinated solvents which can be co-metabolized by methanotrophs expressing either form of MMO, it is important to know what the effects could be on both CH_4 consumption and NMOC removal when expressing either form of MMO. If indeed there are such differential effects, what form of MMO methanotrophs are expressing *in situ* would provide great value in determining what strategy to apply in order to stimulate CH_4 consumption and NMOC removal.

1.9. Ecology of methanotrophs

Methanotrophs are ubiquitous in the environment. These cells have been found in environments that are rich in CH_4 such as landfills (25, 40) and rice paddies (78, 123), as well as those poor in CH_4 such as upland forest soils (103, 111) . They have also been found in alkaline environments (117) and even at extremely acidic environments (53, 60, 94, 141). The traditional Type I and II methanotrophs are ubiquitous and can be found frequently in the previously mentioned environments such as landfills, rice paddies, and

upland soils, while the newly discovered methanotrophs that belong to phylum Verrucomicrobia and the filamentous methanotrophs have yet to been found only in specified environments. Although the traditional Type I and II methanotrophs are often found in the same environment, it appears that some environmental conditions favor one type over the other. Specifically, in CH_4-rich and O_2-limiting environments, Type II methanotrophs seemed to outcompete Type I methanotrophs while the inverse has been observed in CH_4-limiting and O_2-rich environments (3, 31). In nitrogen- and/or nutrient-limiting environments, it appears that Type II methanotrophs have an advantage over Type I methanotrophs while in nitrogen-rich environments Type I methanotrophs appear to predominate (17, 72, 129, 180). Therefore, biogeochemical parameters can regulate methanotrophic distribution and possibly activity.

Beside the traditional Type I and II methanotrophs that belong to the phylum Proteobacteria, newly isolated three methanotrophic strains that belong to the phylum Verrucomicrobia have been discovered in extremely acidic locations (pH=1-2.5) and at high temperatures above 50 ˚C (60, 94, 141). Filamentous sheathed methanotrophs, *Crenothrix polyspora* and *Clonothrix fusca* have been found in filters of pumping wells and sand filtration systems in a groundwater treatment plant (160, 175).

Studies have investigated the vertical distribution of methanotrophs in various locations such as landfill cover soils (96, 97, 161), rice paddies (79, 123), wetlands (16, 54), deep-sea hydrothermal plumes (110) and forest soils (81, 103, 114). In these findings, methanotrophs were found in relatively large numbers in the vicinity where the counter gradients of CH_4 and O_2 met.

1.10. Objectives

The objective of this work was to understand what affects CH_4 consumption by methanotrophs. First, the effects of nonmethane organic compounds (NMOCs) that are commonly found in landfill gas on methanotrophic growth were examined. As methanotrophs co-metabolize such NMOCs via MMO activity without any benefit, it was hypothesized that the presence of NMOCs would inhibit the methanotrophic growth. Another important factor in understanding the effects of NMOCs on methanotrophic growth is the form of MMO the methanotrophs express as MMOs have different characteristics. In order to examine the effects of nonmethane organic compounds on methanotrophic growth, growth studies of methanotrophs with various amounts of NMOCs were performed using custom-made vials. Additionally, a dimensionless number was developed to investigate its use as a predictive tool in assessing the effects of NMOCs on methanotrophic growth.

Secondly, the effects of amendments on CH_4 oxidation and N_2O production in landfill cover soils were examined by constructing soil microcosm studies using landfill cover soils. As amendments that affect CH_4 oxidation can also affect N_2O production, soil microcosm studies were carried out to assess the effects of single amendments, that included moisture content, different forms of nitrogen, copper, organic carbon, and selective inhibitors chlorate and phenylacetylene, on CH_4 oxidation and N_2O production. Based on the results obtained from single amendment tests, the amendments were combined to investigate if there were any synergistic effects on CH_4 oxidation and N_2O production. Additionally, DNA microarray and transcript analyses were performed to

understand the composition of methanotrophic community and activity in response to the amendments that showed stimulation of CH_4 oxidation and inhibition of N_2O production.

CHAPTER 2. Methods and Materials

2.1. Effect of nonmethane organic compounds on methanotrophs

Culture conditions *Methylosinus trichosporium* OB3b was grown on nitrate mineral salt (NMS) medium (179) at 30 ºC in Erlenmeyer side arm flasks shaken at 260 rpm in a CH_4 to air ratio of 1:2 at 1 atm of pressure. The culture medium was 30 % of the total flask volume. For pMMO-expressing conditions, 20 μM copper was added aseptically in the form of $CuCl_2$ after autoclaving and was equilibrated for at least 1 day before the media were inoculated. This concentration was used to prevent any limitations of copper during the entire growth period and also as it has been shown that pMMO-expressing cells degraded TCE at this concentration of copper (122). For sMMO expressing conditions, no copper was added.

Chemicals Highest purity CH_4 (>99.99 %) and acetylene (99.6 %) were obtained from Matheson Gas Company, Newark, NJ. Trichloroethylene, TCE, (>99.9 % GC grade) and methanol (Biotech grade) was purchased from Fisher Scientific Company, Fair Lawn, NJ. *Trans*-dichloroethylene, *t*-DCE (99 % GC grade) was purchased from Aldrich, Milwaukee, WI. Vinyl chloride, VC, (>99.5 % GC grade) was purchased from Fluka, Ronkonkoma, NY. Distilled deionized water from a Corning Millipore D2 system was used for all experiments. All glassware was washed with detergent and then acid washed

in 2 N HNO_3 for 24 hours to remove trace metals, including copper. Nitric acid was removed by repetitive rinses with distilled-deionized water.

For chlorinated solvents liquid at room temperature, i.e. TCE and *t*-DCE, saturated stock solutions were prepared by the method of Chang and Alvarez-Cohen (35). Water saturated with either TCE or *t*-DCE was added to sample vials by using Hamilton 1700 series gas-tight syringes (Hamilton Company, Reno, NV), with care to exclude any non-aqueous-phase liquids. For compounds gaseous at room temperature, i.e., CH_4, acetylene and VC, samples were added to vials using Precision Lok® gas-tight syringes (Precision Sampling Corp. Baton Rouge, LA). Formate was added in the form of sodium formate to an initial concentration of 20 mM from a stock solution of 500 mM. The appropriate amount of chlorinated solvents to add was calculated using following dimensionless Henry's constants: VC, 1.262 (130), *t*-DCE, 0.474, and; TCE, 0.458 (169). For CH_4 and acetylene, a dimensionless Henry's constant of 27.02 and 0.87, respectively, was used (89, 130).

Measurement of Michaelis-Menten Parameters of vinyl chloride and *trans*-dichloroethylene degradation The procedure described previously (21) was used to measure V_{max} and K_s for the degradation of vinyl chloride and *t*-DCE by *M. trichosporium* OB3b expressing pMMO and vinyl chloride by *M. trichosporium* OB3b expressing sMMO. Specifically, *M. trichosporium* OB3b was grown to mid-exponential phase (OD_{600}=0.75-0.8) and then diluted to OD_{600} ~0.25 with pre-warmed NMS medium with the same amount of copper as the initial growth medium.The cells were then allowed to grow to an OD_{600}=0.3. To normalize rates to biomass, the protein

concentrations were measured using the Bio-Rad protein assay kit with bovine serum albumin as a standard. After CH_4 was removed from the growth flasks by evacuating and re-equilibrating the flasks with air at least 10 times, 3 ml aliquots were then transferred to 20 ml serum vials and 20 mM formate added as sodium formate. The vials were then capped with teflon-coated rubber butyl stoppers (National Scientific Co., Duluth, GA) and crimp sealed with aluminum caps. Triplicate samples were prepared for all degradation assays. For analysis of *t*-DCE degradation, aqueous concentrations from 28 to 222 μM were used for both pMMO- and sMMO-expressing cells. For the analysis of VC degradation aqueous concentrations from 8 -153 μM and 8- 383 μM were used for pMMO- and sMMO-expressing cells, respectively. After adding either VC or *t*-DCE, the vials were incubated at 30 °C with shaking at 270 rpm. The initial rates of degradation were determined from using a time interval from $t = 0$ to $t = 3$ minutes for VC degradation by *M. trichosporium* OB3b expressing sMMO, and from $t = 0$ to $t = 40$ minutes for VC degradation by *M. trichosporium* OB3b expressing pMMO. Furthermore, a 30 minute time frame from $t = 0$ to $t = 30$ minutes was used to measure the initial rates of *t*-DCE degradation by *M. trichosporium* OB3b expressing pMMO. An automated headspace sampler (Tekmar 7000, Tekmak Co., Cincinnati, OH) was used to inject samples onto an HP 5890 Series II gas chromatograph with a flame ionization detector (FID) and a 75 m DB-624 0.53 mm I.D. (J&W Scientific Co.). The temperatures of the injector, oven, and detector were 160, 210 and 250 ºC, respectively with a N_2 carrier gas rate of 39 ml·min^{-1}. Nonlinear regression analysis of whole cell kinetics was performed using KaleidaGraph® v.4.0 (Synergy Software)

Effect of chlorinated solvents on cell growth *M. trichosporium* OB3b was grown to the late exponential growth phase (OD_{600} ~ 0.8 as measured using a Spec20 (Milton Roy, Co., Ivyland, PA) and then diluted to an OD_{600} of 0.04-0.05 with the appropriate pre-warmed fresh medium (i.e., either not amended with copper or with 20 μM copper as $CuCl_2$). The cells were then allowed to grow to an OD_{600} of 0.06 to ensure active growth before adding chlorinated ethenes. Methane was then removed from the flasks by evacuating the flasks ten times and allowing air to re-equilibrate after each evacuation. 5 mL aliquots were then aseptically transferred to specially constructed 32.5 mL serum vials created by attaching a 12 mL Pyrex test tube to a 20 mL GC headspace analysis serum vials. The design enabled continuous non-invasive measurement of growth using a Spec20 as well as ensured gas-tight systems for the measurement of CH_4 and chlorinated solvents when capped with teflon-coated butyl-rubber stoppers (National Scientific Co., Duluth, GA). Formate as sodium formate was then added to achieve an initial concentration of 20 mM, and the vials capped with Teflon-coated butyl-rubber stoppers and crimp sealed. Using a gas-tight Dynatech A-2 syringe, 5 mL of the headspace was then removed and replaced with 5 mL of CH_4 to achieve an initial concentration of 270 μM in solution. Varying amounts of VC, *t*-DCE, and TCE were then added to achieve aqueous concentrations ranging from 10 to 100 μM.

Experimental Measurement of CH_4 and Chlorinated Ethene Concentrations

Immediately after addition of chlorinated ethenes, 100 μL headspace samples were taken using Precision Lok® gas-tight syringes for confirmation of initial concentration of all substrates using the following GC configuration: HP 5890 series II gas chromatograph

with a flame ionization detector (FID) with a 75 m DB-624 0.53 mm I.D. column and injector, oven, and detector temperatures set at 160, 80, and 250 °C, respectively and a N_2 carrier gas rate of 39 ml·min^{-1}. The vials were then incubated at 30 ºC with shaking (260 rpm) until the stationary growth phase was reached as measured using a Spec20. Methane and chlorinated ethene concentrations in the sample vials were then determined by again taking 100 μL headspace samples using Precision Lok® gas-tight syringes and analyzed as described above using a HP 5890 gas chromatograph. Before and after each experiment, the naphthalene assay specific for sMMO activity (30) was performed on all samples to verify what form of MMO was active.

Analysis of substrate and product toxicity associated with VC, *t*-DCE, and TCE To determine if substrate and/or product toxicity occurred during the incubation with mixtures of VC, *t*-DCE, and TCE, 5 mM methanol was used as a growth substrate to avoid competition for binding to pMMO and sMMO. 5 mL aliquots of cells prepared in the same methods as in growth experiments and 20 mM formate was added. The vials were then capped with teflon-coated butyl-rubber stoppers and crimp sealed and 100 μM each of the chlorinated solvents added. In some vials, 100 μM acetylene was also added as a general inactivator of MMO to monitor the possibility of substrate toxicity associated with these concentrations of chlorinated ethenes. 100 μM acetylene was chosen as complete inactivation of both sMMO and pMMO expressed by *M. trichosporium* OB3b was observed at this level in previous studies (119).Growth was monitored via OD_{600} measurements as described earlier for 30 hours.

2.2. Landfill soil microcosm experiments

Soil Collection, preparation, and analyses Landfill cover soil at a depth between 40-60 cm below land surface was collected from King Highway Landfill (Kalamazoo, MI) in 28 February 2006 on the southwestern corner of the landfill as indicated on Figure 2-1.

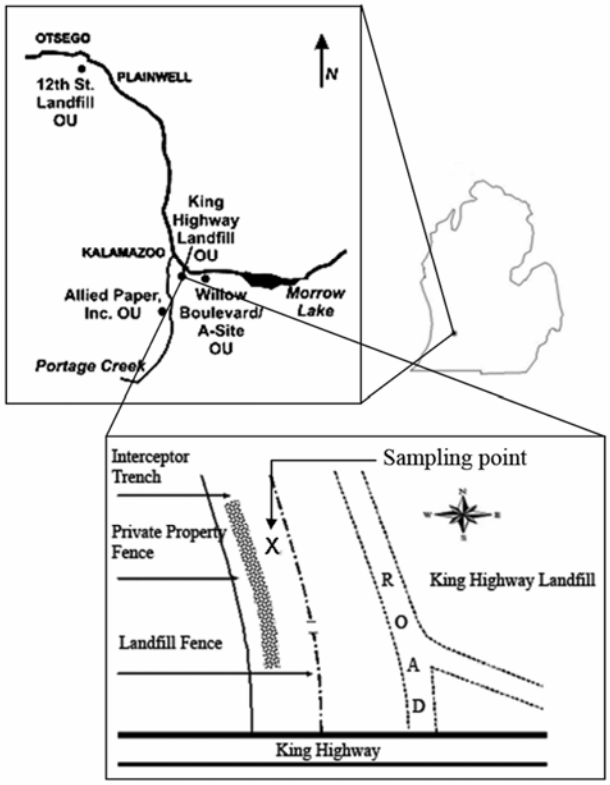

Figure 2-1. Location of King Highway Landfill and sampling site (90)

The soil was air-dried, sieved to exclude soil particles less than <2mm, homogenized, and stored at 4 ˚C in the dark until further use for up to 2 years. The pH of the soil was measured after mixing 5 g of air-dried soil with 10 ml 0.01 M $CaCl_2$ and shaking at 220 rpm for 30 min. Moisture content of the soil was measured gravimetrically by measuring

the weight before and after placing the soil in 120 ˚C oven overnight. Inorganic N, i.e., NH_4^+-N and (NO_3^-+NO_2^-)-N was extracted using 30 g of air dried soil mixed with 60 mL of 2 M KCl. The solution was shaken on an orbital shaker (220 rpm) for 20 min and then passed through Whatman #42 filter paper. The filtrate was collected for measurement of inorganic N. NH_4^+ and NO_3^-+NO_2^- were measured colorimetrically from the filtrate using a rapid flow analyzer (OI Analytical, College Station, TX). Bioavailable copper was measured by using a "hot extract" method developed elsewhere (126). Briefly, 5 g of air dried soil was mixed with 12.5 mL of 0.01 M $CaCl_2$. The solution was then heated at 90 ˚C for 30 min. The resulting solutions were filtered through #42 Whatman filter paper and 10 µl nitric acid (Fisher Scientific Co., Fair Lawn, NJ, Trace metal grade) added. To measure the total copper associated with the soils, 0.5 g air dried soils were digested in 12 ml *Aqua regia* (1:3 ratio of 70 % nitric acid (trace metal grade) and 35 % hydrochloric acid (trace metal grade)) at 110 ˚C for 3 hours. The resulting solution was heated at 60 ˚C for ~3 hours. Nitric acid (2% vol·vol^{-1}) was then added to adjust the total volume to 20 ml and filtered using #42 Whatman filter paper (39). Copper was then measured using inductively coupled plasma mass spectrometry (ELAN DRC-e, PerkinElmer Sciex). ^{63}Cu was used for measurement of copper. ^{71}Ga was added as an internal standard.

Soil Microcosms For microcosm studies, 160 ml serum bottles were soaked in 2N nitric acid bath for at least 2 days, rinsed with MilliQ water at least 5 times and autoclaved prior to use. Soils were stored at 25 ˚C for 24 hours immediately prior to soil microcosm study, and then 5 g of air dried soil added to individual serum bottles along with various amendments. Amendments tested to investigate the effects on CH_4 oxidation and N_2O

production were: (1) moisture content (added as MilliQ water with resistivity above 18 mΩ) to provide values between 5-30 %; (2) copper (added as $CuSO_4 \cdot 5H_2O$ (JT Baker Chemcial Co., Phillipsburg, NJ, Baker Analyzed)) to increase copper content to 5-500 mg Cu·(kg soil)$^{-1}$ above background levels; (3) NH_4^+ (added as NH_4Cl (Sigma-Aldrich, St. Louis, MO, cell culture tested)) to increase NH_4^+ associated nitrogen levels 25-100 mg-N $NH_4^+ \cdot$(kg soil)$^{-1}$ above background levels, and; (4) NO_3^- (added as KNO_3 (Fisher Scientific Co., Fair Lawn, NJ, ACS grade)) to increase NO_3^- associated nitrogen levels 25-100 mg-N $NO_3^- \cdot$(kg soil)$^{-1}$ above background levels.

To examine the possibility of selectively inhibiting N_2O production by either nitrifers or denitrifiers, phenylacetylene and chlorate were added respectively to some microcosms. Briefly, phenylacetylene (Sigma-Aldrich, St.Louis, MO, 98%) was dissolved in dimethylsulfoxide (Fisher Scientific Co., Fair Lawn, NJ, 99.7%) and then added to give final concentrations of 0.01-0.5 mg phenylacetylene·(kg soil)$^{-1}$. Chlorate was added as $KClO_3$ (Sigma-Aldrich, St.Louis, MO, ACS reagent) to give final concentrations of 1-10 mg chlorate·(kg soil)$^{-1}$. After amendments were added, the vials were then capped with Teflon coated butyl rubber septum (National Scientific, Rockwood, TN) and crimp sealed with aluminum caps.

To ensure consistent initial amounts of CH_4 and O_2 in all microcosms, predetermined amounts of CH_4 and O_2 was added via a custom made apparatus to flush the sealed bottles in order to achieve the desired concentrations of CH_4 and O_2. Briefly, pre-determined mixing ratios of air (Metro Welding Supply Corp., Detroit, MI, Dry grade), CH_4 (Airgas, Inc., Radnor, PA, >99.999%), and N_2 (Metro Welding Supply Corp., Detroit, MI, Pre-Purified) were generated by mixing using a series of three way valves to

control the flow of the air, CH_4, and N_2. A schematic diagram of the gassing system is shown in Figure 2-2. The entire vial headspace was flushed for 3 min at a flow rate of approximately 300 ml·min^{-1} to achieve the desired headspace composition.

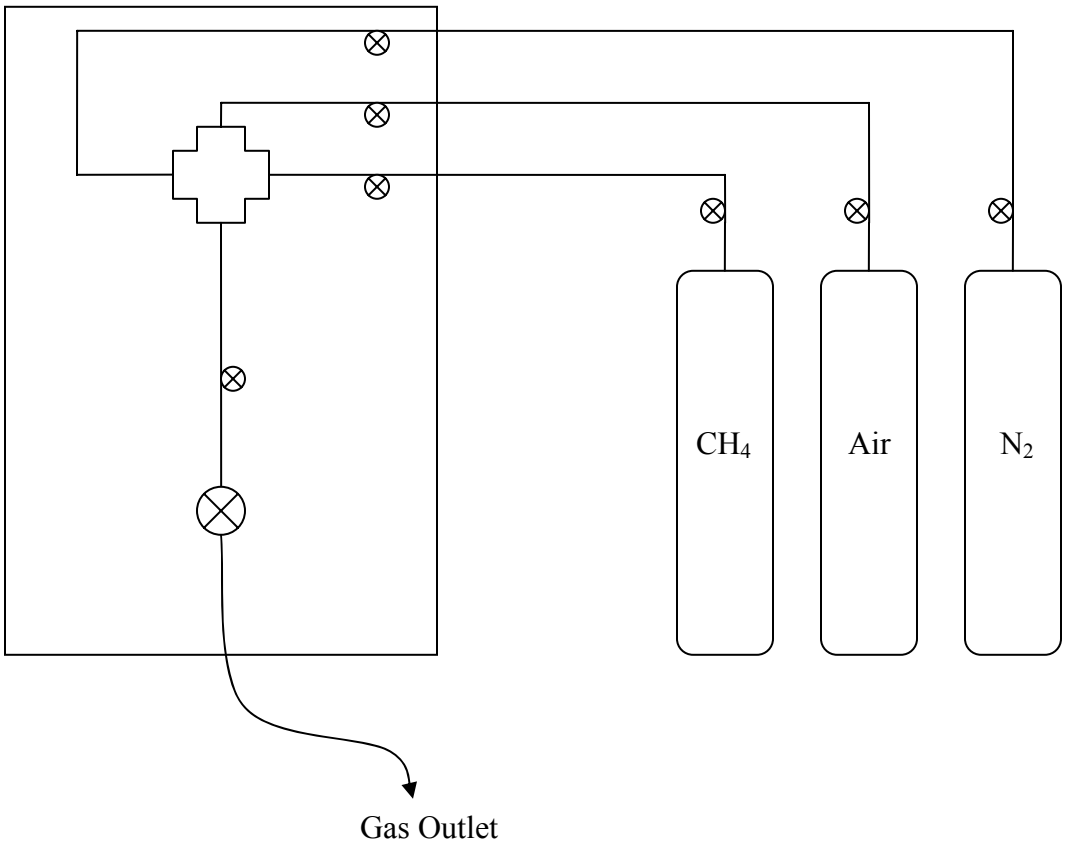

Figure 2-2. Schematic diagram of the gassing system to introduce desired concentration of CH_4 and O_2 using CH_4, air, and N_2

For initial soil microcosm experiments, the impact of individual geochemical parameters on CH_4 consumption and N_2O production was examined. For these soil microcosms, 15 % moisture content and 20 % CH_4, and 10 % O_2 were used as baseline conditions. For subsequent soil microcosm experiments, possible synergistic or antagonistic effects of multiple geochemical parameters were considered, using 5 %

moisture content and 20 % CH_4, and 10 % O_2 as baseline conditions. The vials were then stored at 25 °C in the dark during the course of each microcosm experiment, which lasted approximately 120-150 hours depending on the amendments applied. All conditions were prepared in triplicates.

Analytical Methods Methane was measured using an HP 6890 series equipped with a GS-Molesieve column (0.53 mm I.D. x 30 m) and a flame ionization detector. 100 µl of vial headspace were manually injected using a PressureLok® gas-tight syringe (Baton Rouge, LA). Temperature settings were: oven 75 °C; inlet temperature 185 °C, and detector temperature 250 °C with gas flow rate of 25 $ml \cdot min^{-1}$. H_2 was used as carrier gas while air and H_2 was introduced into the detector. Nitrous oxide was measured using an HP 5890 series II equipped with a Poraplot-Q column (0.53 mm I.D. x 25 m) and an electron capture detector. 400 µl of headspace were manually injected using a PressureLok® gas-tight syringe (Baton Rouge, LA). Temperature settings were: oven -10 °C, inlet temperature 125 °C; and detector temperature 275 °C with gas flow rate of 56 $ml \cdot min^{-1}$. Nitrogen gas was used as both carrier and makeup gas. The oven temperature was maintained below room temperature by injecting liquid nitrogen into the oven chamber using an automated cryogenic valve.

2.3. Nucleic acid extraction

DNA extraction For molecular studies, DNA was extracted from i) soil microcosm studies, and; ii) soils collected as core samples from King Highway Landfill on May 2007. DNA extraction from these samples was performed using UltraClean Soil DNA kit (MoBio Inc., Solana, CA) following the manufacturer's instructions.

mRNA extraction RNA from the soils were extracted following previously developed methods with minor modifications (75). Briefly, 0.5 g of soil (wet weight) was added to 1.0 ml extraction buffer containing 0.2% cetyl trimethyl ammonium bromide (CTAB), 1 mM 1,4-dithio-DL-threitol (DTT), 0.2 M sodium phosphate buffer (pH 8.0), 0.1 M NaCl, 50 mM EDTA (40), with 1 g of 0.1 mm silica glass beads and 1 % β-mercaptoethanol into the 2 ml screw cap microcentrifuge tubes. Six 30s bead beating procedure was performed to lyse the cells using Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK) while put on ice for 1 min in between. The bottom of the microcentrifuge tubes was then pierced using a sterile 22 gauge needle and a sterile collection tube was placed on the bottom of the microcentrifuge tube. Tubes were then centrifuged at 2500 rpm for 5 min using a swinging bucket centrifuge IEC Centra CL2 (International Equipment Co., Needham Heights, MA). The flow-through was then mixed with 1 volume of 70 % ethanol. The resulting mixture was then passed through an RNeasy column (Qiagen, Valencia, CA) via centrifugation at 4000 rpm for 1 min using a Eppendorf benchtop centrifuge (Brinkman Instruments, Westbury, NY). Afterwards, 700 µl "RW1" and then 500 µl "RPE" solutions, where both RW1 and RPE solutions were part of the RNeasy Mini Kit (Qiagen, Valencia, CA), were added to the RNeasy column and was centrifuged at 4000 rpm for 1 min each. RNA was eluted using 100 µl DEPC treated water and was treated with RNase-free DNase I (Promega, Madison, WI) to remove any DNA contamination. DNase treated RNA was then purified using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen, Vanlencia, CA). To check for any DNA contamination, PCR was performed with the extracted RNA as a template. After confirming the complete removal of DNA from RNA samples, RNA was

then reverse-transcribed to obtain cDNA by using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and stored at -20 ˚C until further PCR amplification.

Expression of functional genes To target *pmoA*, *mmoX*, and *amoA*, primer sets pmoA189-mb661 (43), mmoX206f-mmoX886r (92), and amoA1F-amoA2R (147) were used. PCR conditions were as follows. PCR was carried out with 1x PCR buffer, 1.5 mM $MgCl_2$, 20 μg of bovine serum album (107), 15 pmole of each primer, 200 μM dNTPs, 2.5 U *Taq* DNA polymerase, and 20 ng of cDNA. PCR conditions were 95 ˚C for 5 min for initial denaturation, 34 cycles consisting of 95 ˚C for 1 min, annealing temperature at 58 ˚C for 1 min, 72 ˚C for 1 min, with final elongation at 72 ˚C for 10 min. Products were ran by electrophoresis on 1 % (w/v) agarose gel stained with ethidium bromide.

2.4. Microarray analysis

Microarray sample preparation The concentration and purity levels of the collected nucleic acids were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). Samples for microarray analysis were prepared following previously described methods (161). Briefly, DNA collected from soils was amplified using either primer sets targeting *pmoA* using pmoA189-mb661 with the T7 promoter site attached to the 5' end of primers mb661. The T7 promoter site allowed the *in vitro* transcription of the PCR products via T7 RNA polymerase. Detailed information on primers pmoA189, mb661, and T7 promoter site is shown in Table 2-1. Each PCR reaction was carried out in triplicates with 25 μl of 2X MasterAmp PCR Premixture F

(EpiCentre Technologies, Madison, WI), 15 pmol of each primers either pmoA189-mb661, 1 ng environmental DNA, and 1 U Taq polymerase (Invitrogen, Carlsbad, CA). PCR conditions were 95 ˚C for 5 min before the addition of template, 32 cycles consisting of 95 ˚C for 1 min, annealing temperature at 58 ˚C for 1 min, 72 ˚C for 1 min, with final elongation at 72 ˚C for 10 min. Triplicates of PCR reactions were then pooled and purified using Qiagen PCR Purification Kit (Qiagen, Valencia, CA). Methods used for *in vitro* transcription and hybridization as described previously were used (19, 161). Briefly, *in vitro* transcription was carried out under RNase-free conditions, with either Cy3 or Cy5-labelled UTP. The Cy3 or Cy5-labelled product of *in vitro* transcription was then purified and chemically fragmented. The fragments were then used for hybridization, which was carried out on a commercial aluminum block overnight.

Table 2-1. Primers used for amplification of *pmoA*

	Name	(5' → 3')	Reference
Forward	pmoA189	GGBGACTGGGACTTCTGG	(84)
Reverse	mb661	CCGGMGCAACGTCYTTACC	(43)
5' end of reverse primer	T7 promoter site	TAATACGACTCACTATAG	(19)

B=C,G,T; M=A,C; Y=C,T; S=C,G; N=A,C,G,T

Description of probes A brief summary of the probes used for microarray analysis is shown in Table 2-2. A detailed list of the sequences each probes are designed to target can be found in Table A-1 (in the appendix) along with its characteristics. Briefly, the probes can be categorized into 12 groups, "Type Ia", "Type Ib", "Type Ic", "Type II", "Second copy of *pmoA* in Type II", "RA14", "Watershed 1 & 2", "*Methylocapsa* related", "Universal methanotrophs", "Ammonia oxidizers", "Possible novel methanotrophs", and "Unknown identity" which contain possible homologues of *pmoA*. Probes of "Type Ia" are intended to cover *pmoA* sequences that belong to Type I methanotrophs other than

Methylococcus and *Methylocaldum*, i.e., *Methlyomonas, Methylomicrobium, Methylosarcina, Methylobacter,* and probably *Methylosoma.* Group "Type Ib" also target Type I methanotrophs but is limited to the genera *Methylothermus*, *Methylococcus, Methylocaldum,* and probably *Methylohalobius*. Group "Type Ic" targets sequences retrieved from the environment such as deep sea and upland soil (88, 102, 135). Group "Type II" and "Second copy of *pmoA* in Type II" target the first and second copy of *pmoA* in Type II methanotrophs. "RA14" and "Watershed 1 & 2" target environmental sequences that are distantly related to Type II methanotrophs that are presumably utilizing atmospheric methane and sequences retrieved from a specific watershed and flooded upland soils, respectively (85, 137). "*Methylocapsa* related" and "Universal methanotrophs" target specifically *Methylocapsa* related sequences and all known sequences that can be amplified, respectively. "Ammonia oxidizers" target sequences that are related to ammonia oxidizers that can be amplified. Groups "Possible novel methanotrophs" and "Unknown identity" hybridize sequences of no further knowledge.

Table 2-2. Summary of probes used for microarray analysis

Probes	Group	Target
1-35	Type Ia	*Methylomicrobium*, *Methylobacter*, *Methylosarcina*, *Methylomonas*, and probably *Methylosoma*
36-60	Type Ib	*Methylococcus*, *Methylocaldum*, *Methylothermus*, and probably *Methylohalobius*
61-68	Type Ic	Environmental sequences from deep sea and upland soil
69-91	Type II	*Methylosinus* and *Methylocystis*
92-98	Type II 2nd	2nd copy
99-100	RA14	Environmental sequences from upland soil
101-103	Watershed 1 & 2	Environmental sequences from watersheds
104-107	*Methylocapsa* related	*Methylocapsa*
112-116	Universal	All methanotrophs
117-122	Ammonia oxidizers	Ammonia oxidizing bacteria
123-128	Possible novel methanotrophs	Environmental sequences
129-137	Unknown identity	Environmental sequences

Analysis of methanotrophic diversity Visualization of microarray results was performed using GeneSpring GX 7.3.1 (Agilent Technologies, Palo Alto, CA). Analysis of methanotrophic diversity using indices of diversity, richness, evenness, and dominance was performed following previously described methods (70). Briefly, the number of species was replaced with the number of probes with positive signals (signals retrieved were equal to or greater than 5 % of the maximum signals achievable), and the number of abundance of each species was replaced with the relative signals of each probes. Information from generalist probes were discarded for analysis of diversity as generalist probes overlap with other probes in terms of coverage. The indices used are as follows;

Simpson index of diversity (1/D)

$$D = \sum_{i=1}^{S} p_i \times p_i$$

where p_i: the proportion of individuals in the *i*th species.

For p_i, the proportion of relative signals retrieved in each signals compared to the sum of all relative signals was used. Simpson index of diversity, used as the reciprocal of the calculated value D, increases as diversity increases.

Menhinick index of richness (D_{Mn})

$$D_{Mn} = \frac{S}{\sqrt{N}}$$

where S: total number of objects,
N: total number of individuals.

For S, total number of probes that resulted in positive signals was used and for N, the sum of all relative signals from probes that resulted in positive signals was used. Increasing values of D_{Mn} indicates increasing richness.

Simpson index of evenness (E)

$$E = \frac{1/D}{S}$$

where 1/D: Simpson index of diversity
S: total number of objects

Berger-Parker index of dominance (d)

$$d = \frac{N_{max}}{N}$$

where N_{max}: the number of individuals in the most abundance species

N: total number of individuals

For N_{max}, the highest relative signal retrieved among the probes was used. The increase in d indicates the decrease in diversity and increase of dominance (124).

CHAPTER 3. Effect of presence of nonmethane organic compounds on methanotrophs

3.1. Introduction

Nonmethane organic compounds (NMOCs), which include trace organic compounds such as chlorinated ethylenes, have been reported to be emitted from landfills (1, 61, 152). Nonmethane organic compounds are formed through either the anaerobic decomposition of wastes originating from human activities or volatilization of the NMOC containing wastes (29). Nonmethane organic compounds have been reported be of a threat to human health as well as have corrosive effects on gas collection systems when it is installed (29). Therefore, it is important to prevent NMOCs from exiting landfill cover soils along with CH_4, a greenhouse gas.

Compounds such as vinyl chloride, dichloroethylene, and trichloroethylene, which are known NMOCs that can be found in landfill gas, can be co-oxidized by methanotrophs via both MMO enzymes, i.e., pMMO and sMMO (74, 122). However, when the primary substrate for the MMO, i.e., CH_4, coexists with the previously mentioned chlorinated ethylenes, competition for binding sites in the MMO could cause problems in both CH_4 oxidation and removal of the chlorinated ethylenes (74, 121). As both MMOs play an important role in mitigation of CH_4 in landfill cover soils, understanding how the methanotrophs react when chlorinated ethylenes coexist with CH_4

could assist in making predictions on how the landfill cover soils will perform in similar situations where CH_4 and various NMOC are found together in landfill gas.

Another problem associated is that MMO comes in two forms that have shown two different co-oxidizing patterns. One form of MMO, sMMO, has been reported to have a broad range of substrates compared to the other form of MMO, pMMO (76). Also, sMMO-expressing cells have been shown to have faster initial rates of chlorinated ethylene degradation compared to pMMO-expressing cells (138). However, because most methanotrophs are capable of expressing pMMO while only a selected few are known to express sMMO, the form of MMO the cells are expressing is also of importance in understanding CH_4 oxidation and degradation of chlorinated ethylenes.

In this chapter, the effect of coexistence of CH_4 and varying concentrations of chlorinated ethylenes, i.e., vinyl chloride, *trans*-dichloroethylene, and trichloroethylene, on methanotrophic growth and degradation of chlorinated ethylenes was investigated using *Methylosinus trichosporium* OB3b expressing either pMMO or sMMO in order to examine if methanotrophic growth will be affected with increasing concentrations of NMOCs and consequently the overall degradation of the pollutants. As the growth-substrate CH_4 will be competing for binding sites on MMO against the non-growth-substrates NMOCs, it was hypothesized that when concentrations of NMOCs increase with fixed amount of CH_4, methanotrophic growth will be inhibited. However, as pMMO and sMMO have different specificity towards NMOCs, i.e., sMMO can more effectively bind NMOCs compared to pMMO, sMMO-expressing cells were hypothesized to be more affected by the presence of NMOCs relative to pMMO-expressing cells. The

understanding on the effects of NMOCs on methanotrophic growth will be important in what to expect in landfills where high NMOC emissions are observed.

Additionally, a dimensionless number based on substrate specificity was developed that could possibly be used to predict which form of the enzyme could be more effective in degradation of mixtures of chlorinated ethylenes. This dimensionless number, Ω, was initially developed based on specificity of CO_2 and O_2 on ribulose bisphophate carboxylase, or known as RuBisCO (109). From the dimensionless number Ω, similar dimensionless numbers were developed that could effectively describe the methanotrophic growth in the presence of mixtures of chlorinated ethylenes using kinetic information of chlorinated ethylenes for pMMO and sMMO (122, 138). Such dimensionless number could be useful in predicting the outcome of enzymatic processes that can bind substrates that are either beneficiary or inhibitory to cellular activities. The data presented in this chapter have been previously published elsewhere (113).

3.2. Results

3.2.1. Development of dimensionless numbers.

In the study of RuBisCO it is common to characterize the ratio of CO_2 and O_2 turnover by different forms of RuBisCO with the substrate specificity factor, or Ω, specifically:

$$\frac{v_c}{v_o} = \Omega \frac{[CO_2]}{[O_2]} \qquad (1)$$

Where

$$\Omega = \frac{V_{\max}^{CO_2}}{K_S^{CO_2}} * \frac{K_S^{O_2}}{V_{\max}^{O_2}} \qquad (2)$$

and $V_{\max}^{CO_2}$ and $V_{\max}^{O_2}$ represent the maximum velocity of CO_2 and O_2 turnover, respectively, and $K_S^{CO_2}$ and $K_S^{O_2}$ being the half-saturation constants for binding of CO_2 and O_2 by RuBisCO respectively. Equation 1 is useful when one can consider pseudo-first order rates to be applicable (i.e., both CO_2 and O_2 concentrations are much less than the $K_S^{CO_2}$ and $K_S^{O_2}$, respectively).

This can be extended to situations where pollutants compete for binding with an obligate growth substrate during bioremediation. In the case of a single pollutant or non-growth substrate, equation 1 can be simply re-written as:

$$\Omega = \frac{V_{\max}^{G}}{K_S^{G}} * \frac{K_S^{P}}{V_{\max}^{P}} \qquad (3)$$

Where $V_{\max}^{G}$ and $V_{\max}^{P}$ are the maximum velocities of growth substrate and pollutant transformation, respectively, and K_S^G and K_S^P are the half saturation constants for binding of growth and pollutant. This equation can also be easily broadened to consider mixtures of *n* pollutants all bound by the same enzyme in the presence of the growth substrate as follows:

$$\Omega' = \frac{V_{\max}^{G}}{K_S^G} * \sum_{i=1}^{n} \frac{K_S^{P_i}}{V_{\max}^{P_i}} \qquad (4)$$

This concept of specificity for pollutant degradation can be very useful as it is a simple methodology that can provide some useful preliminary information as to how specific different enzymatic systems might behave/respond in any given polluted situation.

The concept of Ω and Ω', although useful, do not explicitly consider the growth of cells during long time periods that is imperative for continued pollutant degradation. Such growth will depend on the relative rate of growth substrate vs. pollutant turnover, which will be a function of the relative concentration of growth substrates and pollutants. One method to do this would be to consider the relative ratio of growth vs. pollutant transformation, as is implied in equation 1 for RuBisCO. This equation, however, is limited to low substrate concentrations. A broader model would be based on simple Michaelis-Menten kinetics, i.e.,

$$\Phi = \frac{v_G}{\sum_{i=1}^{n} v_{P_i}} = \frac{\frac{V_{\max}^{G} * S^G}{K_S^G + S^G}}{\sum_{i=1}^{n} \frac{V_{\max}^{i} * P_i}{K_s^{P_i} + P_i}} \qquad (5)$$

Here the dimensionless values of Φ can range from near zero (in the near absence of growth substrate) to near infinity (in the near absence of pollutant). Finally, a third model giving a dimensionless number potentially useful as a predictive tool can be constructed that considers the net rate of growth substrate turnover in the presence of pollutants competing for binding as follows:

$$\Delta = \frac{v_G - \sum_{i=1}^{n} v_{P_i}}{v_G} = \frac{\frac{V_{\max}^{G} * S^{G}}{K_S^{G} + S^{G}} - \sum_{i=1}^{n} \frac{V_{\max}^{P_i} * P_i}{K_s^{P_i} + P_i}}{\frac{V_{\max}^{G} * S^{G}}{K_S^{G} + S^{G}}} \qquad (6)$$

The values of Δ can vary from 1 (i.e., no pollutants present) to values less than zero (i.e., the rates of pollutant turnover are greater than that of growth substrate turnover).

3.2.2. Calculation of Ω', Φ, and Δ for mixtures of chlorinated solvents for *Methylosinus trichosporium* OB3b expressing either sMMO or pMMO.

To determine the effects of mixtures of chlorinated ethylene on methanotrophic growth, the kinetic parameters shown in Table 3-1 were used for *M. trichosporium* OB3b expressing either pMMO or sMMO. These values were determined in the presence of formate, as it is well-known that reductant limitation can affect chlorinated ethylene degradation by methanotrophs expressing either form of MMO. Using these values, if one assumes that *M. trichosporium* OB3b is exposed to a mixture of CH_4, vinyl chloride (VC), *trans*-dichloroethylene (*t*-DCE), and trichloroethylene (TCE), an Ω' value of 32 is calculated for these cells expressing pMMO and 5.3 when expressing sMMO. Such a large difference suggests that *M. trichosporium* OB3b expressing pMMO would be able to withstand and grow better in the presence of these solvents than when expressing sMMO due to a higher affinity for CH_4.

Table 3-1. Michaelis-Menten kinetics of chlorinated ethylene degradation by *M. trichosporium* OB3b expressing either pMMO or sMMO.

Enzyme	Substrate	V_{max} (nmol•min^{-1}•mg protein^{-1})	K_s (μM)	Reference
pMMO	CH_4	82	8.3	(122)
	VC	42	26	This study
	t-DCE	61	42	This study
	TCE	4.1	7.9	(122)
sMMO	CH_4	726*	92	(138)
	VC	2100	160	This study
	t-DCE	662*	148	(138)
	TCE	580*	145	(138)

*Converted from reported units of nmol•min^{-1}•mg cell^{-1} assuming cell dry weight is 50% protein.

To consider the effect of chlorinated ethylene concentration on the specificity of *M. trichosporium* OB3b to bind CH_4, one can calculate values of either Φ and/or Δ. If one assumes the maximal rate of CH_4 oxidation (i.e., very high concentrations of CH_4)

with varying equimolar amounts of VC, t-DCE, and TCE ranging from 0 to 100 μM, the trends in Figure 3-1A and B can be generated for Φ and Δ, respectively. As can be seen in Figure 3-1, a greater difference is seen between sMMO and pMMO-expressing cells when Δ is calculated. For this reason, further discussion will focus on this model to compare the ability of *M. trichosporium* OB3b to grow in the presence of varying amounts of VC, *t*-DCE, and TCE, as well as how much of these compounds are degraded during active growth.

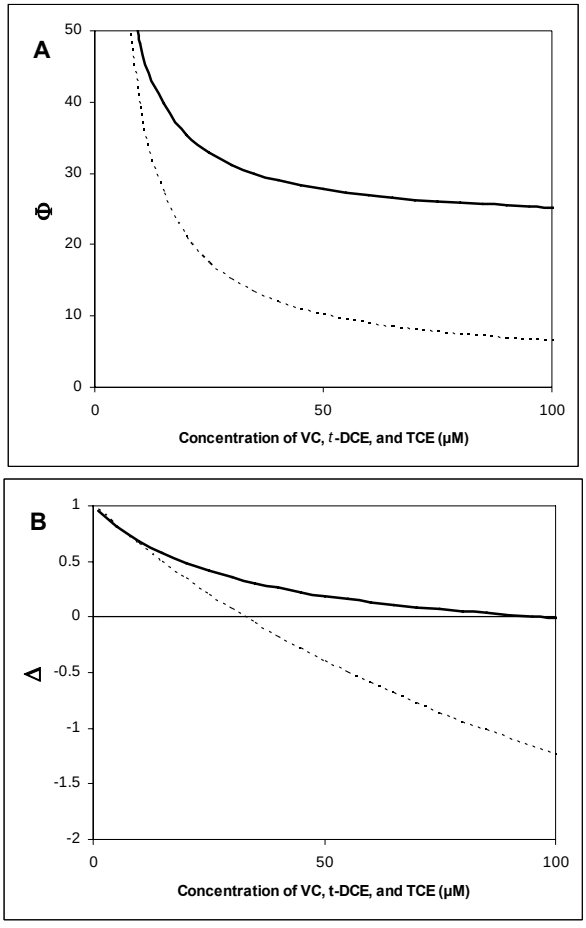

Figure 3-1. Selectivity of *M.trichosporium* OB3b cells expressing either pMMO and sMMO for CH_4 in the presence of various equimolar amounts of vinyl chloride, *trans*-dichloroethylene, and trichloroethylene. (A) Predicted specificity for cells expressing either pMMO (solid lines) or sMMO (dashed lines) using the Φ model (i.e., the raio of the predicted CH_4 oxidation rate and the sum of chlorinated ethane oxidation rates). (B) Predicted specificity for cells expressing either pMMO (solid lines) or sMMO (dashed lines) using the Δ model (i.e., the rate of predicted CH_4 oxidation less the sum of chlorinated ethane oxidation rates normalized to the rate of CH_4 oxidation).

3.2.3. Growth of *M. trichosporium* OB3b in the presence of chlorinated solvents.

Growth of *M. trichosporium* OB3b when expressing either MMO in the presence of various concentrations of chlorinated solvents, VC, *t*-DCE, and TCE are shown in Figure 3-2A and B and summarized in Table 3-2. For initial development and validation of the Δ model proposed here, equimolar amounts of VC, *t*-DCE, and TCE were used although the Δ model allows one to use any combination of growth substrate and co-metabolic pollutant concentrations. In these experiments, formate was also added at an initial level of 20 mM to prevent any limitation of reducing equivalents from affecting the turnover of VC, *t*-DCE, and TCE. From these experiments, it is apparent, and expected that the growth of both pMMO- and sMMO-expressing cells decreased with increased concentrations of each chlorinated solvent. It should be stressed that the relative growth rates, defined as μ/μ_0 where μ is the specific growth rate measured during active growth observed in Figure 3-2, and μ_0 is the specific growth rate in the absence of solvents, were always higher for *M. trichosporium* OB3b expressing pMMO regardless of the amount of solvents added. It is also interesting to note that in the presence of either 10 or 30 μM VC, *t*-DCE and TCE, the maximal cell density was equivalent for both sMMO and pMMO-expressing cells, although at higher concentrations, cells expressing sMMO grew to significantly lower densities.

Table 3-2. Growth and extent of NMOC degradation by *M. trichosporium* OB3b cells expressing either pMMO or sMMO in the presence of various amounts of VC, *t*-DCE, and TCE.

Enzyme	Substrate(s)	μ^a (h^{-1})	$\mu/\mu_0{}^a$	Max OD_{600}
pMMO	CH_4	0.052 (0.005)	1.0	0.51
	CH_4 + 10 µM each VC, *t*-DCE, and TCE	0.030 (0.002)	0.58 (0.07)	0.43
	CH_4 + 30 µM each VC, *t*-DCE, and TCE	0.036 (0.001)	0.69 (0.07)	0.50
	CH_4 + 50 µM each VC, *t*-DCE, and TCE	0.018 (0.0005)	0.35 (0.04)	0.42
	CH_4 + 100 µM each VC, *t*-DCE, and TCE	0.014 (0.0005)	0.27 (0.03)	0.27
sMMO	CH_4	0.064 (0.004)	1.0	0.49
	CH_4 + 10 µM each VC, *t*-DCE, and TCE	0.025 (0.001)	0.39 (0.03)	0.40
	CH_4 + 30 µM each VC, *t*-DCE, and TCE	0.031 (0.001)	0.48 (0.03)	0.42
	CH_4 + 50 µM each VC, *t*-DCE, and TCE	0.016 (0.001)	0.25 (0.02)	0.20
	CH_4 + 100 µM each VC, *t*-DCE, and TCE	0.007 (0.001)	0.11 (0.02)	0.14

[a]Numbers in parentheses represent the standard deviation of collected samples.

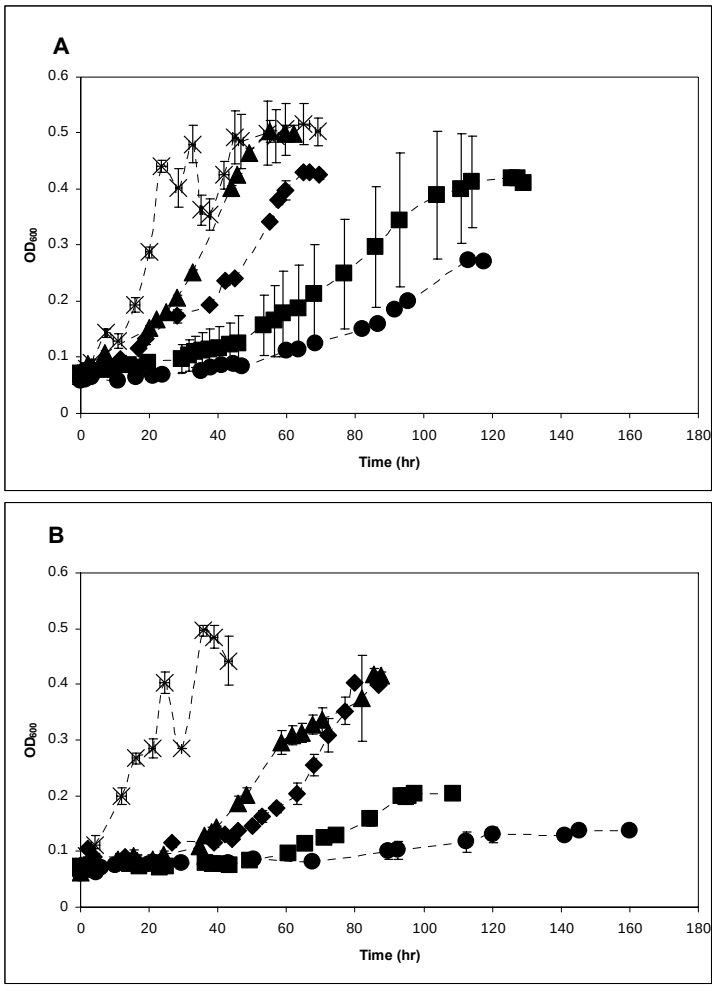

Figure 3-2. Growth of *M.trichosporium* OB3b cells expressing either (A) pMMO or (B) sMMO in the presence of various equimolar amounts of VC, *t*-DCE, and TCE. Symbols: X – CH_4 only (positive control); ▲ – 10 µM each of VC, *t*-DCE, and TCE; ♦ – 30 µM each of VC, *t*-DCE, and TCE; ■ – 50 µM each of VC, *t*-DCE, and TCE; ● – 100 µM each of VC, *t*-DCE, and TCE. Error bars represent the range of duplicate samples. Where error bars are not visible, the symbol size is greater than the measured range.

3.2.4. Degradation of VC, *t*-DCE, and TCE during growth of *M. trichosporium* OB3b.

The slower growth rates exhibited by sMMO-expressing cells are likely due to the faster oxidation rates for VC, *t*-DCE, and VC by these cells during growth. As shown in 3-3, each of these compounds was completely degraded by sMMO-expressing cells when initial concentrations were either 10 or 30 μM. At these concentrations, pMMO-expressing cells degraded only VC and *t*-DCE completely, with a significant amount of TCE remaining (60-70%). The broader substrate range and reduced competition with CH_4 apparently enabled sMMO expressing cells to degrade a larger fraction of the added solvents than pMMO-expressing cells at these initial values.

Table 3-3. Extent of NMOC degradation by *M. trichosporium* OB3b cells expressing either pMMO or sMMO in the presence of various amounts of VC, *t*-DCE, and TCE during active growth

Enzyme	Substrate(s)	% NMOC degraded (range)			Degradation time (hr)
		VC	*t*-DCE	TCE	
pMMO	CH_4				
	CH_4 + 10 μM each VC, *t*-DCE, and TCE	100 (0)	100 (0)	31 (3)	65
	CH_4 + 30 μM each VC, *t*-DCE, and TCE	100 (0)	100 (0)	39 (1)	60
	CH_4 + 50 μM each VC, *t*-DCE, and TCE	97 (1)	98 (1)	35 (9)	110
	CH_4 + 100 μM each VC, *t*-DCE, and TCE	79 (1)	74 (1)	33 (4)	100
sMMO	CH_4				
	CH_4 + 10 μM each VC, *t*-DCE, and TCE	100 (0)	100 (0)	100 (0)	80
	CH_4 + 30 μM each VC, *t*-DCE, and TCE	100 (0)	100 (0)	100 (0)	80
	CH_4 + 50 μM each VC, *t*-DCE, and TCE	97 (0)	97 (0)	74 (1)	100
	CH_4 + 100 μM each VC, *t*-DCE, and TCE	60 (4)	63 (4)	39 (6)	150

[a]Numbers in parentheses represent the range from duplicate samples

At initial concentrations of either 50 or 100 μM of these chlorinated ethylenes, neither pMMO nor sMMO-expressing cells completely degraded these compounds during active growth. In the presence of 100 μM of VC, *t*-DCE, and TCE, pMMO-expressing actually degraded more of the compounds than sMMO-expressing cells, and did so over a shorter time frame. It appears that the greater specificity of pMMO-

expressing cells for CH_4 allowed a faster growth rate which over time resulted in more degradation of chlorinated ethylenes.

3.2.5. Substrate and product toxicity.

To determine if either substrate or product toxicity associated with chlorinated ethylenes may have been affecting the growth of pMMO- and sMMO-expressing cells of *M. trichosporium* OB3b, a series of experiments were performed to monitor the growth of such cells in the presence and absence of acetylene, a known potent inhibitor of MMO activity. In these experiments, methanol was added instead of CH_4 as the growth substrate to prevent any competitive binding to either pMMO or sMMO that could obfuscate the findings. Furthermore, formate was added to prevent any limitation of reducing equivalents occurring during the oxidation of the chlorinated ethylenes.

As can be seen in Figure 3-3, in the presence of 100 μM each of VC, *t*-DCE, and TCE, no growth over 30 hours was observed in cells with active pMMO (i.e., not acetylene-treated). If these cells were treated with acetylene, however, they did grow in the presence of these chlorinated ethylenes, but to lesser degree than if the cells were grown in the presence of methanol and acetylene only (significant at a 95% level using a Students t-test). Collectively these results indicate that the growth of *M. trichosporium* OB3b expressing pMMO in the presence of these chlorinated ethylenes was significantly reduced from toxic products generated from the oxidation of these compounds although that the compounds were also toxic at these concentrations.

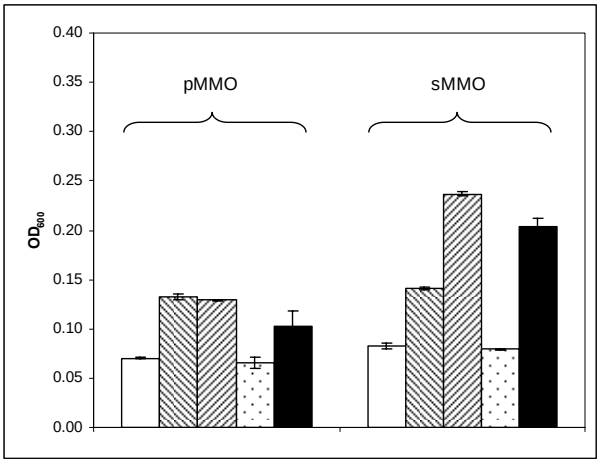

Figure 3-3. Analysis of product vs. substrate toxicity effects on growth of *M. trichosporium* OB3b expressing either pMMO or sMMO while exposed to 100 µM each of vinyl chloride (VC), *trans*-dichloroethylene (*t*-DCE), and trichloroethylene (TCE). 5 mM methanol was added as the growth substrate to avoid competition for binding to either MMO as well as 20 mM formate to prevent reductant limitation affecting turnover rates of chlorinated ethenes. 100 µM acetylene was added to some cases to inactivate the MMOs. Growth was measured after 30 hours. □ = initial OD_{600}; ▨ = methanol only; ▨ = methanol + acetylene, ▩ = methanol + VC, *t*-DCE, and TCE, ■ = methanol + VC, *t*-DCE, TCE, and acetylene. Error bars represent the range of duplicate samples.

For sMMO-expressing cells of *M. trichosporium* OB3b, again it is clear that product toxicity was a major factor in limiting growth in the presence of 100 µM each of VC, *t*-DCE, and TCE. Specifically, as shown in Figure 3-3, no growth was seen after 30 hours in the presence of these compounds in the absence of acetylene. Growth was observed, however, following the addition of acetylene to inactivate sMMO. Also, as found for pMMO-expressing cells, substrate toxicity was observed as growth in the presence of these solvents and acetylene was less than in the presence of methanol and acetylene (significant at a 95% level using a Students t-test). It is also interesting to note that the addition of acetylene to sMMO-expressing cells without chlorinated solvents enhanced growth on methanol. This indicates that the sMMO can effectively bind methanol and oxidize it as previously observed (42), possibly to formaldehyde that inhibits cell growth.

3.3. Discussion

From the broad range of substrates oxidized by methanotrophs expressing sMMO and the relatively fast kinetics of chlorinated ethylene degradation (Table 3-1), it may appear that methanotrophic-mediated removal of NMOC, specifically chlorinated ethylenes and/or other compounds that could be co-oxidized via MMO, make sMMO an ideal enzyme to utilize when the primary objective is to remove NMOC. Although a great deal of research has examined how cells, when grown under sMMO-expressing conditions (i.e., no added copper) degrade a wide range of halogenated hydrocarbons, both separately and in mixtures (6, 35, 36, 138, 139), there have been no empirical data showing how methanotrophs, when expressing either sMMO or pMMO, grow in the presence of mixtures of chlorinated solvents, and if degradation of such compounds is limited over time due to poor growth. From the growth and degradation studies presented here, it is clear that pMMO-expressing cells can actually grow faster, rapid consumption of CH_4, in the presence of high concentrations of chlorinated ethylenes, and degrade more of these compounds than cells expressing sMMO, most likely due to such greater growth. Such inhibition of growth is due to the greater ability of sMMO-expressing cells to bind and transform co-metabolic substrates that not only is of no benefit to the cells, but also results in the consumption of reducing equivalents and formation of toxic products.

Collectively these data suggest that in landfill cover soils where primary substrate CH_4 and co-metabolites in the form of NMOC are present in mixtures, those cells expressing pMMO may actually be predominant as they are better able to tolerate the presence of co-metabolites, and may play a significant role in consuming CH_4 and

preventing NMOCs from exiting the system, particularly if the initial concentrations of co-metabolic substrates are high. At high concentrations of chlorinated ethylenes, it appears that pMMO-expressing cells could utilize CH_4 for growth while degrading chlorinated ethylenes relatively effectively compared to sMMO-expressing cells. Thus, in landfill cover soils where naturally occurring CH_4 and chlorinated ethylenes coexist, although CH_4 consumption will be inhibited by the presence of chlorinated ethylenes, pMMO-expressing methanotrophic community could be relatively effective in CH_4 consumption compared to sMMO-expressing methanotrophic community while reducing the concentrations of chlorinated ethylenes. This behavior where the presence of single chlorinated ethylene inhibited CH_4 consumption in landfill cover soils has been reported in other studies although what type of MMO was being expressed was not investigated using molecular techniques (149, 150). Thus, in order to degrade chlorinated ethylenes while actively consuming CH_4 when landfill gas comprises of CH_4 and mixtures of NMOC, understanding the type of MMO expressed will be important. This hypothesis, if true, would also imply that strategies for sites with high NMOC concentrations should purposefully stimulate pMMO expression until the NMOC concentrations are reduced to the point whereby sMMO-expressing cells can effectively bind CH_4 to support their growth.

The question then arises, how can one determine which form of MMO should the methanotrophs be expressing in order to efficiently prevent both CH_4 and chlorinated ethylene from being emitted from landfill cover soils? A methodology based on Δ analysis can provide at least a preliminary assessment. As discussed earlier and described in Figure 3-1B, when one considers the net rate of growth substrate turnover in the

presence of chlorinated ethylenes, different enzymatic systems will have different profiles, and at some point, negative values are predicted depending on the amount of co-metabolic substances present. For the analysis shown in Figure 3-1B, this occurs when *M. trichosporium* OB3b expressing sMMO or pMMO is exposed to either 30 or 97 μM each of VC, *t*-DCE, and TCE, respectively. As shown in Figure 3-2 and Table 3-2 and 3-3, although growth of *M. trichosporium* OB3b expressing sMMO was significantly reduced in the presence of as little as 10 μM each of VC, *t*-DCE, and TCE, these compounds were completely degraded when as much as 30 μM of each were provided. If the initial concentration of each was increased to 50 μM, however, not only was growth affected, but so was the degradation of the co-metabolites. Growth of *M. trichosporium* OB3b expressing pMMO was also significantly impacted by the presence of as little as 10 μM each of VC, *t*-DCE, and TCE, but the degradation of these compounds during growth was not significantly affected until at least 100 μM of each was added. Thus, it appears a simple analysis based on Michaelis-Menten kinetics can provide useful information as to what microbial process can be expected to be more effective for CH_4 consumption and NMOC removal in specific situations.

Since soils comprise of diverse microorganisms, it is always possible to have non-methanotrophic microorganisms capable of degrading NMOCs present. In order to uncouple CH_4 consumption and degradation of NMOCs, stimulating microorganisms that can rapidly degrade NMOCs that cause the inhibition of CH_4 consumption could be another strategy. As lowering the concentrations of chlorinated ethylenes resulted in less inhibition of methanotrophic growth, if another microbial process degrades such NMOCs, it can be expected to be beneficial in terms of CH_4 consumption. On the other hand, one

should consider the consequence of the method applying to the system to stimulate such microorganisms as it can result in other unintended results e.g., stimulation of N_2O production.

3.4. Conclusions

Because of the ability of MMO to bind non-growth-substrates NMOCs as well as the growth-substrate CH_4, it was first hypothesized that methanotrophic growth will be inhibited by the increasing amounts of NMOCs in the presence of CH_4. Also, as sMMO can more effectively bind NMOCs compared to pMMO, growth of sMMO-expressing cells were expected to be more affected than that of pMMO-expressing cells. This chapter showed what the possible outcome on growth of methanotroph expressing either form of MMO, i.e., pMMO or sMMO, could be when growth-substrate CH_4 was present along with co-metabolite NMOCs, i.e., vinyl chloride, *trans*-dichloroethylene, and trichloroethylene. Here, it was shown that even at low concentrations of NMOCs, growth of both pMMO-expressing and sMMO-expressing cells were inhibited by NMOCs. At high concentrations of NMOCs, due to the different substrate range of MMOs, pMMO-expressing cells were less affected by the presence of NMOCs compared to sMMO-expressing cells. As pMMO has lower pseudo-first-order rates (V_{max}/K_s) for chlorinated solvents compared to sMMO, sMMO-expressing cells were expected to bind and degrade chlorinated solvents more efficiently than pMMO-expressing cells. Consequently, sMMO-expressing cells were less efficient in binding and utilizing its growth-substrate CH_4 than pMMO-expressing cells especially when the concentrations of chlorinated solvents were increased evidenced by the growth rates. Also, at low concentrations of NMOCs, sMMO-expressing cells were more efficient in degrading NMOCs relative to

pMMO-expressing cells due to the broad substrate range of sMMO. However, when concentrations of NMOCs were increased, pMMO-expressing cells were more efficient in degrading NMOCs compared to sMMO-expressing cells. Because sMMO can bind NMOCs more rapidly compared to pMMO, if elevated levels of NMOCs were present along with CH_4, the negative impact on growth probably resulted in less degradation of the NMOCs.

When these observations are extrapolated into landfill cover soils where emissions of CH_4 and NMOCs are observed, it can be expected that CH_4 consumption can be affected by the presence of NMOCs and the type of MMO the methanotrophic community is expressing. Therefore, it is important to understand the composition of landfill gases emitted in landfills and the type of MMO being expressed *in situ*.

A simple model, based on Michaelis-Menten kinetic results, that can make predictions on how a system could function in terms of CH_4 consumption and NMOC degradation was developed and validated using *M .trichosporium* OB3b expressing either pMMO or sMMO in the presence of growth substrate CH_4 and co-metabolites vinyl chloride, *trans*-dichloroethylene, and trichloroethylene. Based on the observations, the dimensionless number Δ can provide preliminary assessment on how the methanotrophic system would behave when growth-substrate CH_4 was present with co-metabolites NMOCs. As both CH_4 and NMOCs are important compounds that could either affect global warming or human health, a predictive tool such as the dimensionless number Δ can assist in determining what form of the enzyme the system should be expressing in order to mitigate both CH_4 and NMOCs.

CHAPTER 4. Effect of amendments on CH_4 oxidation and N_2O production

4.1. Introduction

Methane is a potent greenhouse gas and landfills are one of the major anthropogenic sources of CH_4 in the US. Based on the capacity and the composition of landfill gas, some landfills are required to install gas collection systems. However, as not all landfills fall into this category, there are landfills which freely release CH_4 into the atmosphere posing great risk to global warming. Therefore, in the landfills where it is not required to capture the landfill gas, stimulation of microbial consumption of CH_4 can be an alternative solution in mitigating greenhouse emissions in landfill cover soils as microorganisms responsible for consuming CH_4, methanotrophs, oxidize CH_4 to produce either cell material or CO_2, a less potent greenhouse gas. Microbial CH_4 consumption in landfill cover soils can be affected by various geochemical parameters, e.g., moisture content or nutrient limitation. However, the geochemical parameters that can affect methanotrophic activity can also have impacts on other microorganisms *in situ*, specifically microorganisms that can produce N_2O, another potent greenhouse gas. Therefore, the objective of this chapter was to investigate the effects of selected geochemical parameters on CH_4 oxidation and N_2O production. Also, the applicability of selective inhibitors that could selectively inhibit N_2O production while not affecting CH_4

oxidation was examined. Finally, the methanotrophic community composition was investigated in conjunction with the studies looking at the effects of geochemical parameters on CH_4 oxidation and N_2O production.

Different geochemical parameters can have different effects on both CH_4 oxidation and N_2O production. Most studies so far have, however, focused on the effect of such parameters on either CH_4 oxidation or N_2O production. Thus, although particular conditions have been shown to favor CH_4 oxidation in certain soil system and repress N_2O production in another, such conditions may not be appropriate for the same community in the same soil. Numerous factors have been evaluated to assess its effect on CH_4 oxidation and N_2O production. In this chapter, soil microcosms were constructed using landfill cover soil and provided varying amount of nitrogen, copper, moisture content, organic carbon in the form of humic acids, and specific inhibitors of N_2O producers. A complete list of the parameters that was tested in these microcosm studies is shown in Table 4-1.

Table 4-1. List of geochemical parameters tested or to be tested for microcosm studies

Parameter	Range	Significance
Moisture content	5-30 %	• Variable mass transfer of CH_4 and O_2? • Variable osmotic stress on microbial community?
Copper	0-500 $mg \cdot kg^{-1}$	• Effect on pMMO/sMMO expression and activity? • Stimulation of nitrous oxide reductase and/or AMO?
Ammonium	0-100 $mg\text{-}N \cdot kg^{-1}$	• Nitrogen limitation relief?
Nitrate	0-100 $mg\text{-}N \cdot kg^{-1}$	• Nitrogen limitation relief? • Stimulation of denitrifiers?
Urea	0-100 $mg\text{-}N \cdot kg^{-1}$	• Competitive inhibition of MMO? • Nitrogen limitation relief?
Organic carbon	20-200 $mg \cdot kg^{-1}$	• Stimulation of denitrifiers due to availability of alternative carbon sources?
Phenylacetylene	0-10 $mg \cdot kg^{-1}$	• Selectively inhibit AMO?
Chlorate	0-250 $mg \cdot kg^{-1}$	• Selectively inhibit denitrifiers?

Methanotrophs are capable of utilizing both NH_4^+ and NO_3^- as a nitrogen source (179). Interestingly, MMO is capable of oxidizing NH_4^+/NH_3 to NO_2^- (45) resulting in inhibition of CH_4 oxidation in conditions where CH_4 and NH_4^+ are both present. The effect of NH_4^+ on CH_4 oxidation has been well studied but so far has given variable results. In some cases, NH_4^+ had inhibitory effects on CH_4 oxidation (58, 99, 173) whereas in other cases, NH_4^+ stimulated CH_4 oxidation (12, 17, 47). Although the mechanism for inhibition of CH_4 oxidation by NH_4^+ is attributed to competitive inhibition (58, 100, 101), it can also be due to the toxic effects of the end product, i.e., NO_2^-, of NH_4^+ oxidation by MMO. Since methanotrophs are capable of oxidizing NH_4^+ to NO_2^- via hydroxylamine (45), when active methanotrophs are exposed to NH_4^+, it can result in accumulation of NO_2^- which can affect the activity of methanotrophs (58, 101, 151). Additionally, hydroxylamine, the intermediate product of oxidation of NH_4^+ by MMO has been shown to be an inhibitor of CH_4 metabolism (91).

Stimulation of CH_4 oxidation due to ammonium is generally attributed to nitrogen relief. Although some methanotrophs can fix N_2 when nitrogen is depleted, such a process is energy intensive. Thus, when a methanotrophic community is deprived of inorganic nitrogen and are forced to utilize N_2, addition of nitrogenous fertilizers could indeed relieve the methanotrophs of nitrogen limitation (14). In some cases where both stimulation and inhibition of CH_4 oxidation due to addition of NH_4^+ was observed, it was attributed to the CH_4/NH_4^+ ratio (27). It was suggested that when this ratio was high, stimulation could occur, whereas when this ratio was low inhibition could take place.

In general, when NO_3^- is applied to soils, inhibitory effects only occur when the concentration of NO_3^- is high, i.e., at concentrations when other salts such as NaCl have

similar inhibitory effects (58, 136). At such concentrations, the inhibitory effects are likely due to a decrease in pH (58) and/or osmotic effects (15). Stimulation (47) and no effect at low NO_3^- concentrations (58, 136) on CH_4 oxidation have been also observed. Furthermore, it has been reported that methanotrophs can be selectively enriched by varying the concentration of NO_3^- (72). Specifically, this study showed that *Methylosinus trichosporium* OB3b, a type II methanotroph, can outcompete *Methylomicrobium album* BG8, a type I methanotroph, in NO_3^- limiting environments.

In another study, enrichment of landfill soil using varying concentrations of nitrate minimal salt (NMS) media for isolation of methanotrophs lead to selective enrichment of methanotrophs. At 1x NMS, only Type I methanotrophs were enriched whereas at 0.2x NMS, Type II methanotrophs were predominant (180). Although these results could be due to the different concentration of other nutrients in the media, when juxtaposed to the former study, NO_3^- appears be an important controlling factor in methanotrophic community structure and activity.

The concentration of CH_4 and possibly O_2 play a role in dictating the rate of CH_4 oxidation. In general, MMO activity or CH_4 oxidation rate can be modeled using simple Michaelis-Menten kinetics, i.e.,

$$Rate\ of\ CH_4\ oxidation = \frac{V_{max} \times [S]}{[S] + K_s}$$

V_{max}: Maximum velocity
S: Concentration of substrate, CH_4
K_s: Concentration of substrate at half of V_{max},
i.e., Michaelis-Menten constant

In a system where diverse methanotrophs exist, different concentrations of CH_4 may favor certain groups of methanotrophs. For example, as shown in Table 1-5 (p.18),

for methanotrophs expressing pMMO, the kinetics for CH_4 oxidation differs for *Methylomicrobium album* BG8 and *Methylosinus trichosporium* OB3b with *M.album* BG8 having significantly higher pseudo-first order rates, suggesting that *M. album* BG8 would predominate under pMMO-expressing conditions. One should note, however, that methanotrophs are obligate aerobes. Since oxidation of CH_4 is dependent on both CH_4 and O_2, the overall rate of CH_4 oxidation can be affected by O_2 levels as follows (134).

$$Rate\ of\ CH_4\ oxidation = V_{\max} \times \frac{S_{CH4}}{S_{CH4} + K_{s,CH4}} \times \frac{S_{O2}}{S_{O2} + K_{s,O2}}$$

V_{max}: Maximum velocity
S: Concentration of substrate, CH_4 or O_2
K_s: Michaelis-Menten constants for CH_4 or O_2

According to this equation, the rate of CH_4 oxidation is controlled by both CH_4 and O_2 concentrations. Thus, until both CH_4 and O_2 concentrations have reached a point where CH_4 oxidation is at maximum, increasing concentrations of CH_4 and/or O_2 may enhance CH_4 oxidation.

Moisture content is another parameter that can affect CH_4 oxidation and N_2O production in soils since moisture content in soils can either limit or enhance microbial activities. Generally, when microorganisms experience low moisture content, their activities can be inhibited by the decrease of intracellular water potential which leads to the hydration reduction and inhibition of enzymatic activity (158). Also, low moisture contents can limit diffusional transfer of substrates from soil particles. Conversely, when microorganisms experience high moisture contents, activities can also decrease because transfer of gaseous substrates in the atmosphere, i.e., CH_4 and O_2, into the soil system can

be limited. As diffusion of O_2 into the soil system becomes limited, anoxic areas will increase (154) and affect aerobic CH_4 oxidation. Therefore, it was hypothesized that at low moisture content, CH_4 oxidation could be stimulated due the increased diffusion of gases, CH_4 and O_2.

As previously mentioned, copper is known to regulate the expression of MMOs in methanotrophs that can express either form of MMO, pMMO and sMMO (132). Also, copper is necessary to express pMMO for methanotrophs that can only express pMMO. Thus, availability of copper can affect the expression of which form of MMO will be expressed in single cells and possibly the entire community. Depending on which form of MMO is expressed different kinetics can be expected. *M. trichosporium* OB3b which is capable of expressing either form of MMO has been shown to possess different Michaelis-Menten kinetics for pMMO and sMMO as shown in Table 4-2.

Table 4-2. Michaelis-Menten kinetics for CH_4 oxdiation of *M. trichosporium* OB3b expressing either sMMO or pMMO

	Copper (μM)	V_{max} (nmol·min^{-1}·mg protein^{-1})	K_s (μM)	V_{max}/K_s (ml·(min·mg protein)$^{-1}$)	Reference
sMMO	0	726*	92	7.9	(138)
pMMO	2.5	300	62	4.9	(122)
	5	177	28	6.3	
	10	110	14	7.9	
	20	82	8.3	9.9	

* Assumed 50% of cells were protein

As shown in Table 4-2, as the concentration of copper increased, the affinity of CH_4 also increased (122). Thus, copper can be important when trying to stimulate CH_4 oxidation in soils. Some studies have tried adding copper into soils with the intention of stimulating CH_4 consumption. These studies resulted in either no effect (46) or stimulation at low concentrations and inhibition at higher concentrations (12, 150).

Humic acids are heterogeneous high-molecular-weight organic materials that may affect speciation and distribution of metals. Specifically, it has been shown that whole-cell sMMO activity of *M. trichosporium* OB3b in the presence of humic-chelated copper decreased with increasing copper to biomass ratios indicating that humic compounds reduced bioavailability of copper (131). It was hypothesized that as humic acid concentrations increased, copper was bound onto the functional groups on the humic acid reducing copper bioavailability. Therefore, addition of humic acids could be effective in terms of making copper or other metals that could be at levels where it poses toxic effects to methanotrophs less bioavailable.

Finally, amendments that stimulated CH_4 oxidation while decreasing N_2O production compared to conditions where no amendments were applied were selected for further investigation of the methanotrophic community structure. Understanding whether changes in CH_4 oxidation are due to shifts in the methanotrophic community structure or change in number of active methanotrophs will provide important information on how to manage a methanotrophic community in landfill cover soils.

4.2. Results

4.2.1. Basic landfill cover soil properties

The composition of the landfill cover soil was determined to be 93 % sand with the remainder being a mixture of silt and clay, and was classified as sand based on standard USDA soil texture classification analyses (157). Soil pH was found to be 7.1 (± 0.1) and moisture content of the soil at the time of sampling was 9.3 ± 0.5 %. Inorganic N, i.e., NH_4^+ and NO_3^-+NO_2^- was 16.0 ± 0.2 and 7.5 ± 0.1 mg-N·(kg soil)$^{-1}$, respectively. Bioavailable and total copper was measured to be 1.3 ± 0.01 and 23 ± 0.2 mg copper·(kg soil)$^{-1}$, respectively.

4.2.2. Soil microcosms incubated at 20 % CH_4, 10 % O_2, and 15 % H_2O

In order to understand CH_4 consumption and N_2O production behaviors, microcosms were constructed using soils collected from King Highway Landfill were used. The soil microcosms were incubated under 20 % CH_4 and 10 % O_2 and 15 % moisture content unless otherwise stated.

As can be seen in Figure 4-1, addition of at least 50 mg-N NO_3^-·(kg soil)$^{-1}$ was found to be necessary to significantly enhance CH_4 oxidation rates above baseline conditions (i.e., increase from 82 ± 14 to 120 ± 13 μg·hr^{-1}, significant at a 95% confidence level). At least 100 mg-N NH_4^+·(kg soil)$^{-1}$ was necessary to substantially increase CH_4 oxidation rates (to 130 ± 21 from 82 ± 14 μg·hr^{-1}, significant at a 90% confidence level). Nitrous oxide production, however, was significantly stimulated by the addition of as little as 25 mg-N·(kg soil)$^{-1}$ of either NH_4^+ or NO_3^-. Specifically, the

rates of N_2O production in the presence of 25 mg-N·(kg soil)$^{-1}$, 0.57 ± 0.12 and 0.34 ± 0.04 μg·hr^{-1} for NH_4^+ and NO_3^- additions, respectively, were both significantly different at a 95 % confidence level from the rate measured in the absence of any amendment (0.10 ± 0.01 μg·hr^{-1}). If 100 mg-N·(kg soil)$^{-1}$ was added, N_2O production rates increased even more to 1.7 ± 0.1 and 0.6 ± 0.1μg·hr^{-1}, for NH_4^+ and NO_3^- respectively (such increases were found to be significantly different at 99 and 95% confidence intervals, respectively).

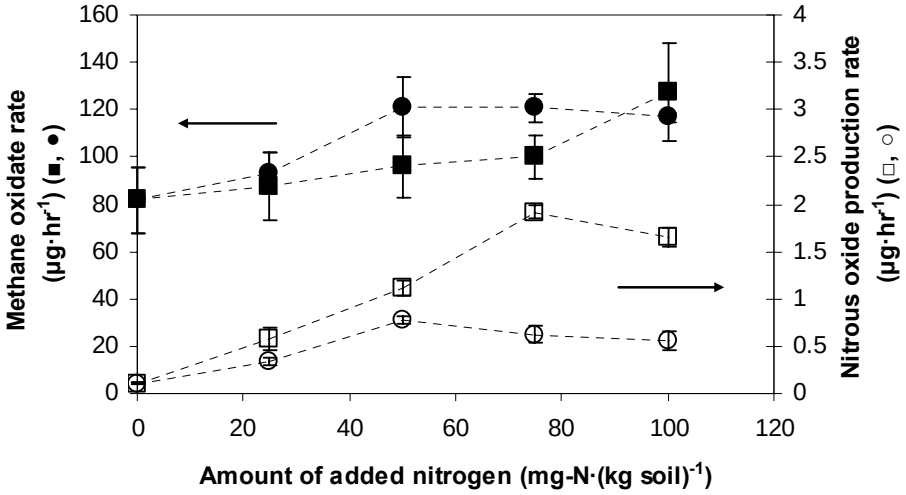

Figure 4-1. Effect of inorganic nitrogen on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of NH_4^+ on CH_4 consumption, ●: effect of NO_3^- on CH_4 consumption, □: effect of NH_4^+ on N_2O production, ○: effect of NO_3^- on N_2O production

As shown in Figure 4-2, addition of organic nitrogen, urea, resulted in stimulatory effect on CH_4 oxidation up to 50 mg-N·(kg soil)$^{-1}$ by increasing CH_4 oxidation rates from 57 ± 3 to 84 ± 7 and 76 ± 5 μg·hr^{-1} when 25 and 50 mg-N urea·(kg soil)$^{-1}$ was added, respectively. The increase in CH_4 oxidations due to the addition of 25 and 50 mg-N urea·(kg soil)$^{-1}$ were both significant at 99 %. However, excessive amounts of urea appeared to have inhibitory effects on CH_4 consumption evidenced by a decrease in CH_4 oxidation rates from 57 ± 3 μg·hr^{-1}, the CH_4 oxidation rate when no urea was

added, to 11 ± 2 μg·hr^{-1}, when 100 mg-N urea·(kg soil)$^{-1}$ was applied, significant at 99 %. Nitrous oxide production was also enhanced by the addition of urea, even as small as 25 mg-N urea·(kg soil)$^{-1}$, an increase from 0.64 ± 0.02 to 2.1 ± 0.3 μg·hr^{-1} which was significant at 99 % confidence level.

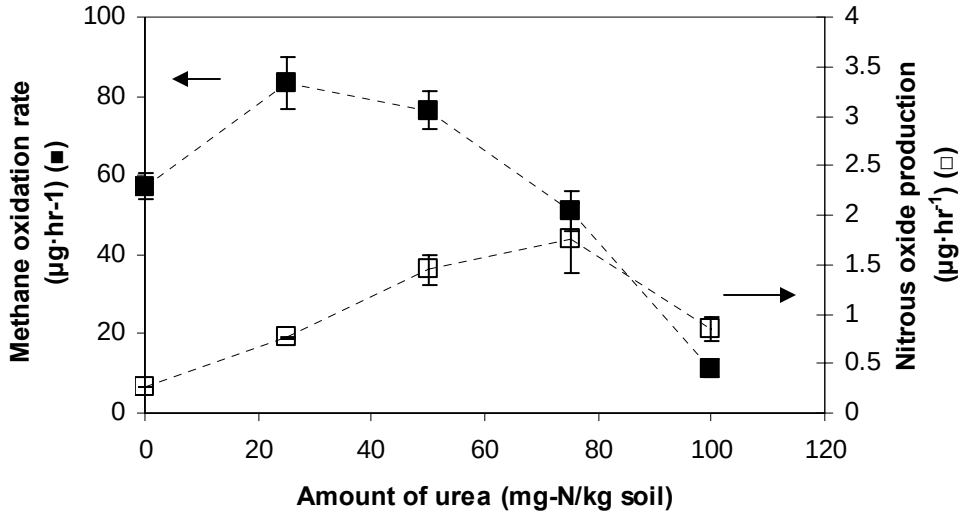

Figure 4-2. Effect of urea on CH_4 oxidation and N_2O production production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of urea on CH_4 consumption, □: effect of urea on N_2O production.

As shown in Figure 4-3, the highest CH_4 oxidation rates were observed at 5 % moisture content, with CH_4 oxidation rates decreasing as moisture content increased. At 5 % moisture content, CH_4 oxidation rates were 99 ± 9 μg·hr^{-1} while at 30 % moisture content, CH_4 oxidation rates were 51 ± 5 μg·hr^{-1}. Compared to the CH_4 oxidation rate observed at 15 % moisture content, (82 ± 8 μg·hr^{-1}), i.e., the baseline condition, CH_4 oxidation rates at 5 and 30 % moisture content were significantly different at 90 % and 99 % confidence levels, respectively. Interestingly, N_2O production rates increased with increasing moisture content up to 20 % but then decreased slightly when the moisture

content was increased to 30%. The N_2O production rate at 5 % moisture content ((9.0 ± 0.3) × 10^{-3} μg·hr^{-1}) was significantly lower than that measured at 15 % moisture content (0.14 ± 0.01μg·hr^{-1}) at a 99 % confidence level. The rate of N_2O production at 30 % moisture content was 0.096 ± 0.012 μg·hr^{-1}, significantly lower than the rate at 15 % moisture content at a 90 % confidence level.

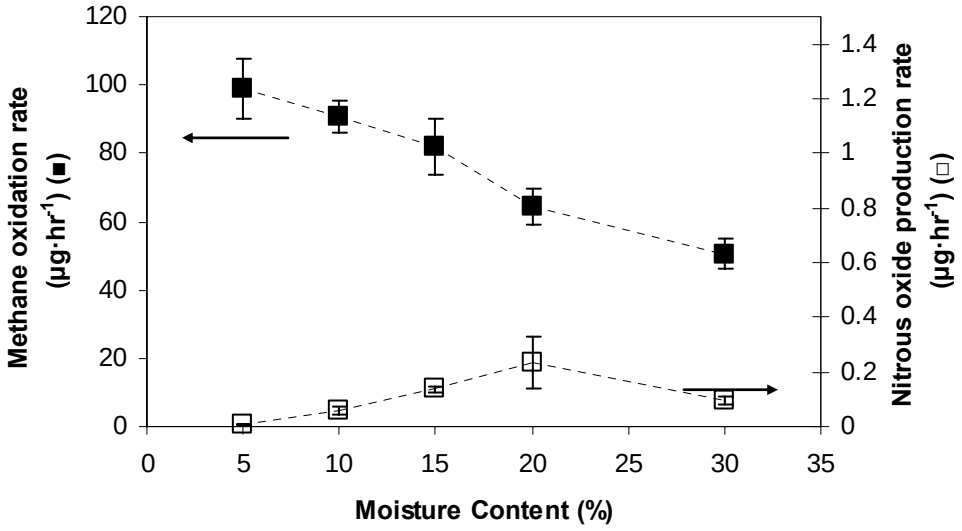

Figure 4-3. Effect of moisture content on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and varying moisture content. ■: effect of moisture content on CH_4 consumption, □: effect of moisture content on N_2O production.

As shown in Figure 4-4, addition of copper appeared to have little effect on CH_4 oxidation when soils were amended with as much as 100 mg copper·(kg soil)$^{-1}$. When 250 mg·(kg soil)$^{-1}$ of copper was added, however, CH_4 oxidation rates were ~70 % of the rate observed when soils were amended with no copper (i.e., a decrease from 55 ± 3 to 37 ± 7 μg·hr^{-1}), and this difference was significantly different at a 95% confidence interval. Increasing the amount of added copper did not result in any further decrease of measured CH_4 oxidation rates. N_2O production rates, however, were not affected when as much as 250 mg copper·(kg soil)$^{-1}$ was added. At 500 mg copper·(kg soil)$^{-1}$ N_2O production rates

did decrease to ~60 % of the rates observed when no copper was added (i.e., a drop to 0.08 ± 0.004 from 0.13 ± 0.04 $\mu g \cdot hr^{-1}$), and this was significantly different at a 95 % confidence interval.

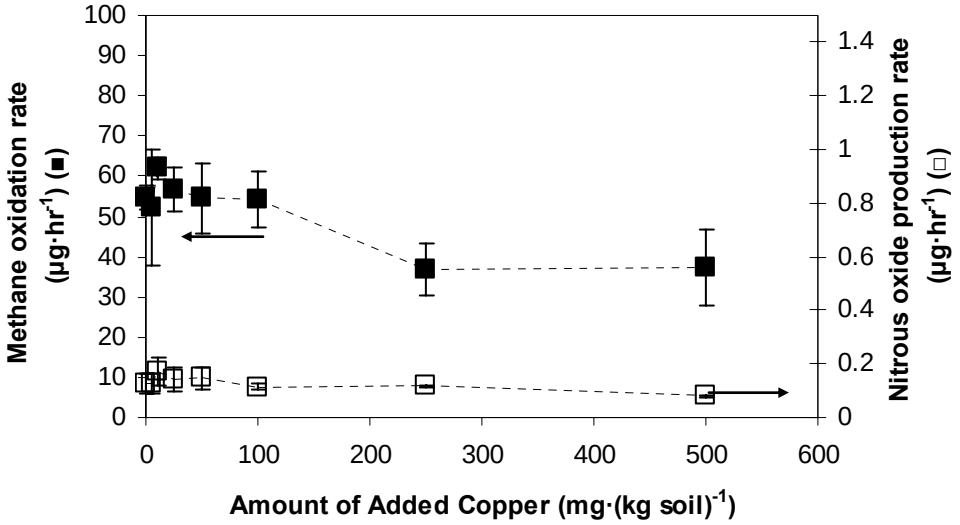

Figure 4-4. Effect of copper on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of copper on CH_4 consumption, □: effect of copper on N_2O production.

As shown in Figure 4-5, addition of organic carbon in the form of humic acid did not have any discernible effect on CH_4 oxidation in the amounts tested 20-200 mg·(kg soil)$^{-1}$. Nitrous oxide production, however, was stimulated with increased amounts of organic carbon beyond 50 mg·(kg soil)$^{-1}$ from 0.14 ± 0.01 $\mu g \cdot hr^{-1}$, N_2O production rate when soil was amended with no organic carbon, to 0.40 ± 0.13 $\mu g \cdot hr^{-1}$, N_2O production rate when 50 mg·(kg soil)$^{-1}$ of organic carbon was introduced to the soil, which was significant at 90 % confidence level.

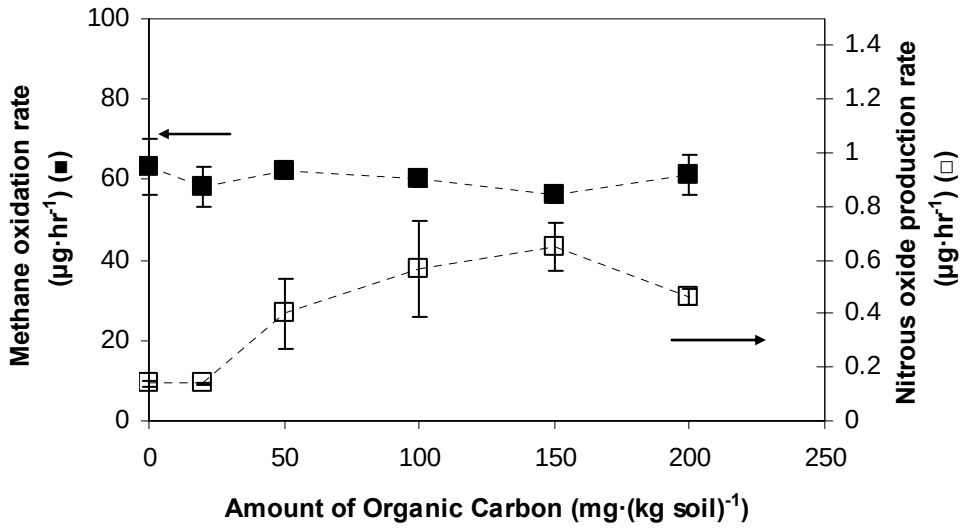

Figure 4-5. Effect of organic carbon on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of organic carbon on CH_4 consumption, □: effect of organic carbon on N_2O production.

4.2.3. Effect of specific inhibitors on CH_4 consumption and N_2O production

Phenylacetylene, a specific inhibitor of MMO and AMO was provided in a subset of microcosms to investigate its usefulness to selectively inhibit N_2O production. It has been shown that AMO expressing ammonia-oxidizing bacteria are completely inhibited at concentrations of phenylacetylene two orders of magnitude than methanotrophs expressing either sMMO or pMMO (120). Thus, the effect of phenylacetylene was determined in soils incubated under 20 % CH_4, 10 % O_2, 15 % moisture content, and 25 mg-N·(kg soil)$^{-1}$.

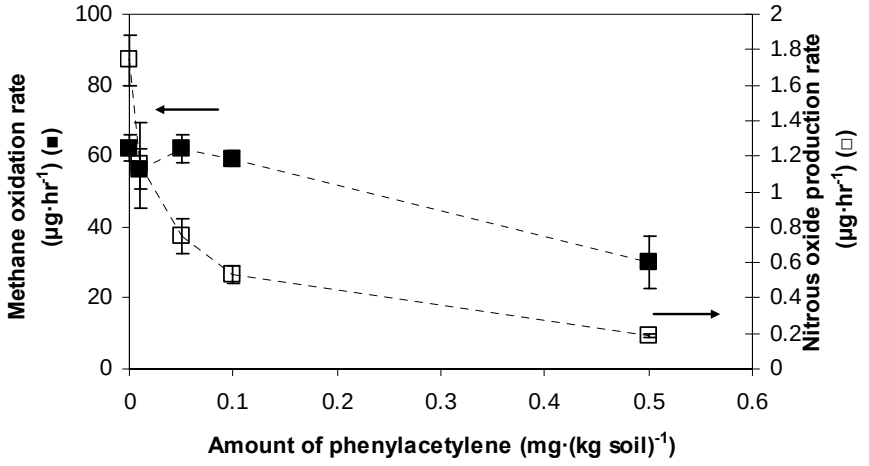

Figure 4-6. Effect of phenylacetylene on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 25 mg-N NH_4^+·(kg soil)$^{-1}$. ■: effect of phenylacetylene on CH_4 consumption, □: effect of phenylacetylene on N_2O production.

As can be seen in Figure 4-6, 0.01-0.1 mg phenylacetylene ·(kg soil)$^{-1}$ did not inhibit CH_4 oxidation. At 0.5 mg·(kg soil)$^{-1}$ of phenylacetylene, however, CH_4 oxidation rates decreased by approximately half compared to CH_4 oxidation rates observed in the absence of phenylacetylene (i.e., 30 ± 7 and 62 ± 4 µg·hr^{-1}, respectively), significant at a 95 % confidence level. Nitrous oxide production rates were reduced, however, with the addition of as little as 0.01 mg phenylacetylene ·(kg soil)$^{-1}$, and rates decreased with increasing addition of phenylacetylene. If 0.1 mg phenylacetylene·(kg soil)$^{-1}$ was added, the rate of N_2O production decreased ~70%, to 0.5 ± 0.1 µg·hr^{-1} as compared to rate measured in the absence of phenylacetylene (1.7 ± 0.1 µg·hr^{-1}). This difference, significant at a 99 % confidence level, was presumably through selective inhibition of ammonia-oxidizing bacteria.

The applicability of a different specific inhibitor, i.e., chlorate, for N_2O producers through denitrification was also investigated (108). Chlorate was added in the range of 1-10 mg·(kg soil)$^{-1}$ along with 25 mg-N NO_3^-·(kg soil)$^{-1}$ under 20 % CH_4, 10 % O_2, and

15 % moisture content. As shown in Figure 4-7, addition of chlorate in the range tested did not have any discernible effect on CH_4 oxidation. Adding as little as 1 mg ClO_3^-·(kg soil)$^{-1}$, however, stimulated N_2O production. Specifically, the N_2O production rate at 1 mg ClO_3^-·(kg soil)$^{-1}$ slightly increased from 0.39 ± 0.01 to 0.51 ± 0.03 μg·hr^{-1} found in the absence of any added chlorate. This difference, although minimal, was significant at a 95 % confidence level.

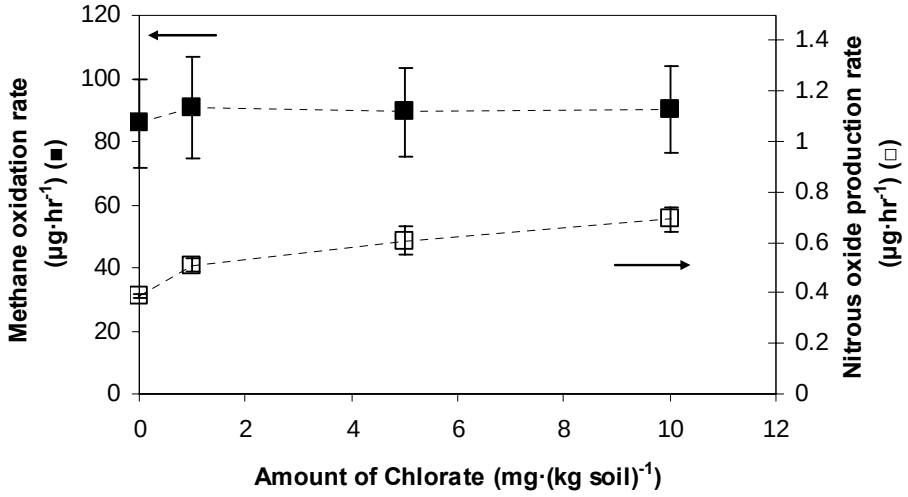

Figure 4-7. Effect of chlorate on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 25 mg-N NO_3^-·(kg soil)$^{-1}$. ■: effect of chlorate on CH_4 consumption, □: effect of chlorate on N_2O production.

4.2.4. Soil microcosms incubated at 20 % CH_4, 10 % O_2, and 15 % H_2O with 0.1 mg·(kg soil)$^{-1}$ phenylacetylene

As 0.1 mg·(kg soil)$^{-1}$ of phenylacetylene was deemed effective in selectively inhibiting N_2O production while not affecting CH_4 oxidation (Figure 4-6), 0.1 mg·(kg soil)$^{-1}$ of phenylacetylene was used along with other geochemical parameters tested, i.e., varying amounts of ammonium, nitrate, urea, moisture content, copper, and organic

carbon. Chlorate was not tested further due to the stimulation of N_2O production as shown in the previously.

To further consider the impact of phenylacetylene, a fixed amount of 0.1 mg phenylacetylene·(kg soil)$^{-1}$ was added while NH_4^+ was amended up to 100 mg-N NH_4^+·(kg soil)$^{-1}$. As shown in Figure 4-8, both CH_4 oxidation and N_2O production rates increased with NH_4^+ concentrations up to 50 mg-N NH_4^+·(kg soil)$^{-1}$. At 50 mg-N NH_4^+·(kg soil)$^{-1}$, the rate of CH_4 oxidation increased from 61 ± 9 μg·hr^{-1} in the absence of any added NH_4^+ to 120 ± 12 μg·hr^{-1} (significant at a 95 % confidence level). Furthermore, at this NH_4^+ level, N_2O production rate increased to 1.6 ± 0.3 μg·hr^{-1} from 0.072 ± 0.033 μg·hr^{-1} when no NH_4^+ was added (significant at a 95 % confidence level). Above 50 mg-N NH_4^+·(kg soil)$^{-1}$, however, the rate of CH_4 oxidation was observed to decrease from the maximum observed at 50 mg-N NH_4^+·(kg soil)$^{-1}$, and such a difference was significant at a 95 % confidence level. The rate of N_2O production increased slightly as NH_4^+ concentrations were increased to 100 mg-N NH_4^+·(kg soil)$^{-1}$, although this increase was not significantly different from that measured in the presence of 50 mg-N NH_4^+·(kg soil)$^{-1}$.

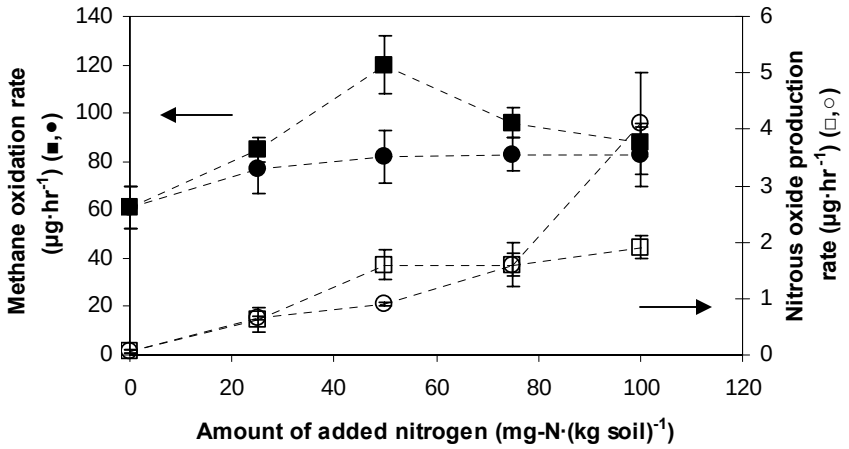

Figure 4-8. Effect of inorganic nitrogen on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of NH_4^+ on CH_4 consumption, ●: effect of NO_3^- on CH_4 consumption, □: effect of NH_4^+ on N_2O production, ○: effect of NO_3^- on N_2O production.

The addition of NO_3^- elevated CH_4 oxidation rates even with the smallest amount tested. In the absence of NO_3^-, the measured CH_4 oxidation rate was 61 ± 9 µg·hr^{-1}, but this increased with 25 mg-N NO_3^-·(kg soil)$^{-1}$ to 77 ± 10 µg·hr^{-1} (significant at a 95 % confidence level). However, additional amounts of NO_3^- did not further stimulate CH_4 oxidation. The addition of NO_3^- also increased N_2O production rates. Nitrous oxide production rates increased from 0.072 ± 0.033 µg·hr^{-1}, in the absence of additional NO_3^-, to 0.64 ± 0.03 µg·hr^{-1} with the addition of 25 mg-N NO_3^-·(kg soil)$^{-1}$ which was the smallest amount of NO_3^- tested. The increase was significant at 95 % confidence level.

As shown in Figure 4-9, the addition of urea elevated CH_4 oxidation when 50-75 mg-N urea·(kg soil)$^{-1}$ while inhibition of CH_4 consumption occurred when 100 mg-N urea·(kg soil)$^{-1}$ was applied. The addition of urea resulted in increase in N_2O production rate with increasing amounts of urea up to 50 mg-N urea·(kg soil)$^{-1}$.

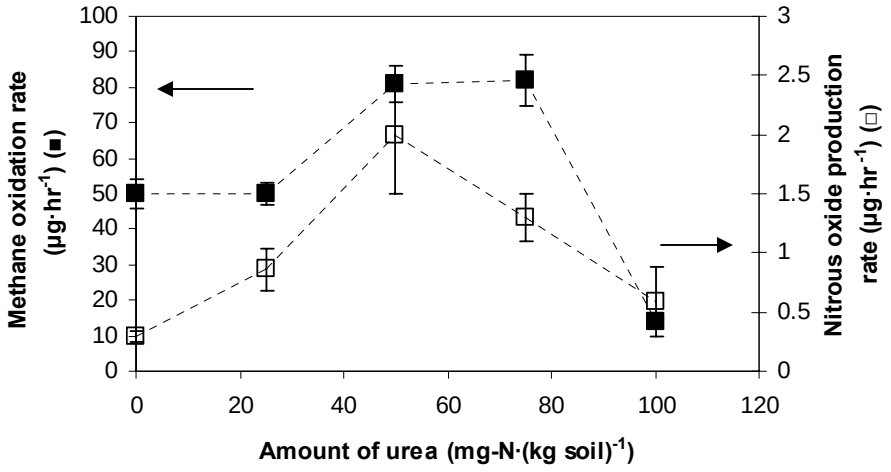

Figure 4-9. Effect of urea on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of urea on CH_4 consumption, □: effect of urea on N_2O production.

An increase of N_2O production rates from 0.29 ± 0.05 to 2.0 ± 0.5 µg·hr^{-1} was observed when 0 and 50 mg-N urea·(kg soil)$^{-1}$ was applied, respectively (significant at 99 % confidence interval). However, excessive amounts of urea above 75 mg-N urea·(kg soil)$^{-1}$ resulted in a decrease in N_2O production rates with increasing amounts of urea.

As shown in Figure 4-10, increased moisture content, i.e., 20-30 % moisture content, resulted in decrease in CH_4 oxidation. Methane oxidation rates decreased from 54 ± 7 to 20 ± 2 µg·hr^{-1} when moisture content was increased from 15 % to 30 %, respectively (significant at 99 % confidence interval). Meanwhile, N_2O production rates were increased at higher moisture content, i.e., 20-30 % moisture content. Nitrous oxide production rates were 0.29 ± 0.002 µg·hr^{-1}, an increase from 0.072 ± 0.033 µg·hr^{-1}, when moisture content was 30 and 15 %, respectively (significant at 99 % confidence interval).

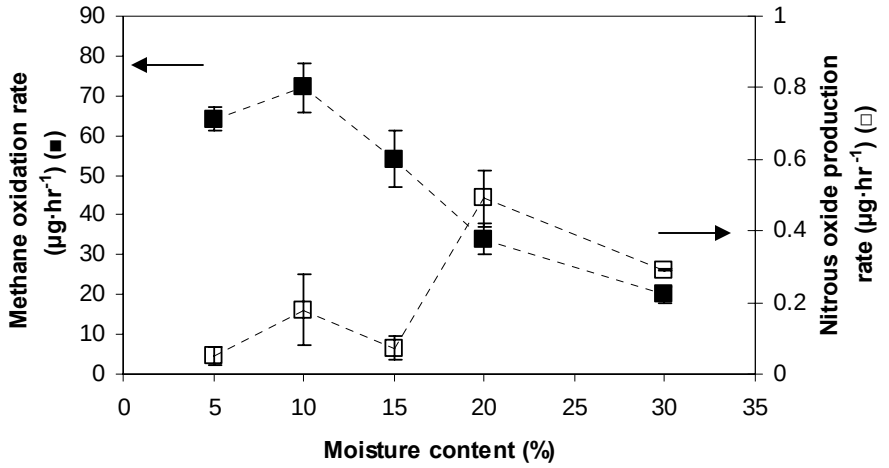

Figure 4-10. Effect of moisture content on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, varying moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of moisture content on CH_4 consumption, □: effect of moisture content on N_2O production.

As shown in Figure 4-11, addition of copper did not have significant effect on CH_4 oxidation. Also, copper did not affect N_2O production even at 500 mg·(kg soil)$^{-1}$.

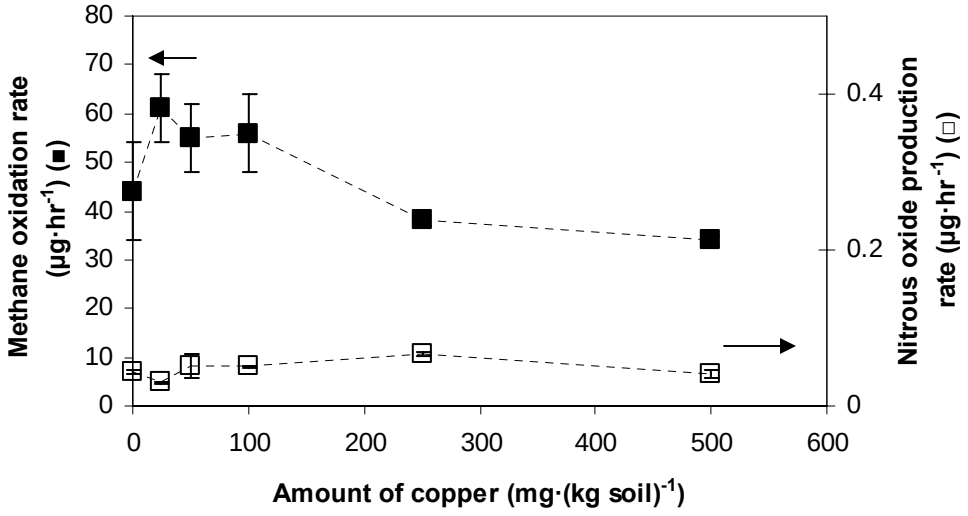

Figure 4-11. Effect of copper on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of copper on CH_4 consumption, □: effect of copper on N_2O production.

As shown in Figure 4-12, addition of organic carbon had interesting effects on CH_4 oxidation. The presence of additional organic carbon elevated the CH_4 oxidation rates from 61 ± 9 to 81-90 $\mu g \cdot hr^{-1}$ when 20-150 $mg \cdot (kg\ soil)^{-1}$ of organic carbon was added (significant at 99 %).

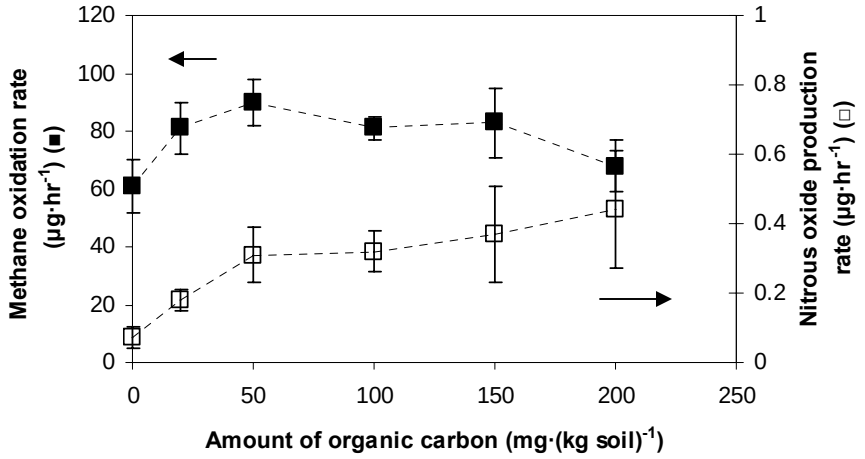

Figure 4-12. Effect of organic carbon on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 $mg \cdot (kg\ soil)^{-1}$ phenylacetylene. ■: effect of copper on CH_4 consumption, □: effect of copper on N_2O production.

Nitrous oxide production rates increased with increasing amounts of organic carbon. Even with the addition of 20 $mg \cdot (kg\ soil)^{-1}$ of organic carbon, N_2O production rates were increased from 0.072 ± 0.033 to 0.18 ± 0.03 $\mu g \cdot hr^{-1}$ (significant at 99 %).

4.2.5. Soil microcosms incubated at 20 % CH_4, 10 % O_2, and 5 % H_2O- effects of multiple geochemical parameters

To examine the collective effect of multiple amendments, conditions that stimulated methane oxidation, i.e., 5 % moisture content, addition of varying amounts of copper, and either ammonium or nitrate, were combined. Copper was tested to further examine possible weak positive effects on methane oxidation.

As shown in Figure 4-13, addition of 15 mg-N NO_3^-·(kg soil)$^{-1}$ with 5% moisture content stimulated methane oxidation from 71 ± 4 μg·hr^{-1} found in the absence of any amendments at 5% moisture content to 100 ± 2 μg·hr^{-1} (significant at a 99% confidence interval).

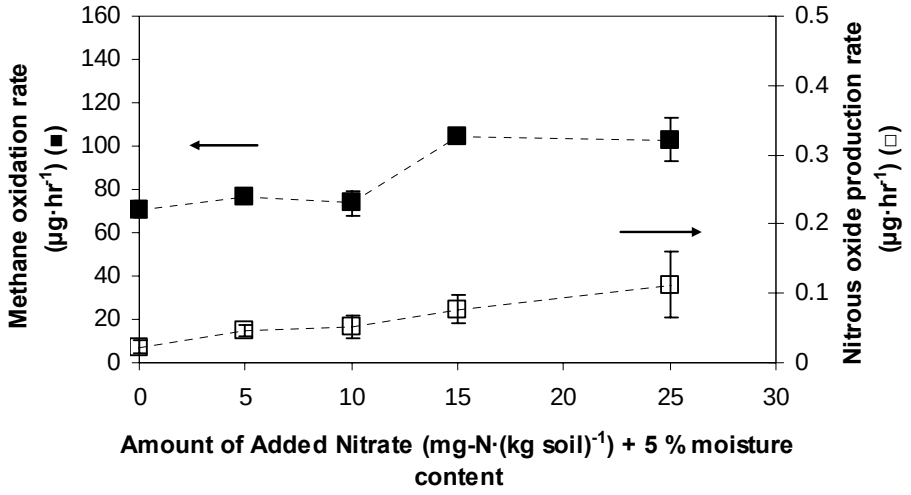

Figure 4-13. Effect of NO_3^- on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. ■: effect of NO_3^- on CH_4 consumption, □: effect of NO_3^- on N_2O production.

Increasing the amount of nitrate to 25 mg-N·(kg soil)$^{-1}$ was not observed to result in any significant difference in methane oxidation rates from that measured with 15 mg-N·(kg soil)$^{-1}$. A slight increase in nitrous oxide production was observed with the addition of 15 mg-N NO_3^-·(kg soil)$^{-1}$ (0.077 ± 0.021 μg·hr^{-1}) as compared to when without nitrate addition at 5% moisture content (0.023 ± 0.010 μg·hr^{-1}), different at a 90 % confidence interval. When 25 mg-N NO_3^-·(kg soil)$^{-1}$ was added, although the average rate of nitrous oxide production increased to 0.11 ± 0.05 μg·hr^{-1} from 0.077 ± 0.021 μg·hr^{-1} measured in the presence of 15 mg-N NO_3^-·(kg soil)$^{-1}$, variability in measurements made this difference statistically insignificant.

When either 15 or 25 mg-N NO_3^-·(kg soil)$^{-1}$, was added along with 5-25 mg copper·(kg soil)$^{-1}$ with 5 % moisture content (Figure 4-14), the results were similar to what was observed when these amendments were examined independently, i.e., no synergistic effects on methane oxidation between the combination of nitrate, copper, and low moisture content were observed.

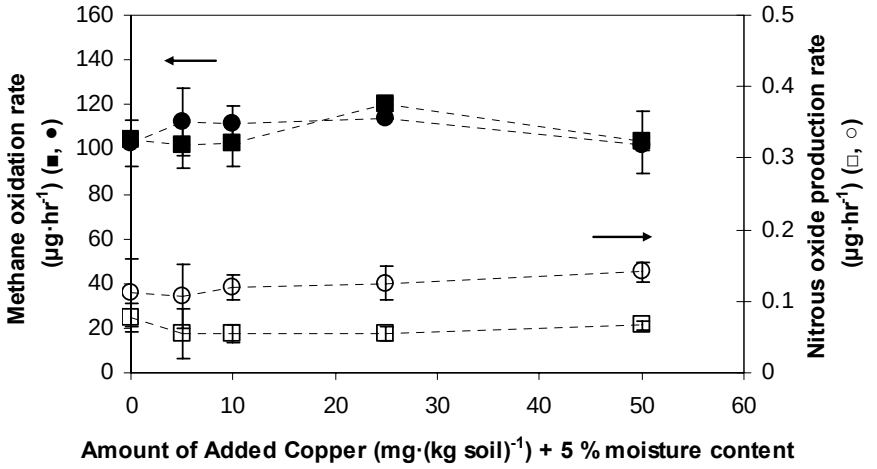

Figure 4-14. Effect of NO_3^- and copper on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. Effect of NO_3^- and copper on CH_4 oxidation (■,●) and N_2O production (□,○). Squares represent 15 mg-N NO_3^-·(kg soil)$^{-1}$, circles represent 25 mg-N NO_3^-·(kg soil)$^{-1}$.

A slight increase in nitrous oxide production was observed when nitrate levels were increased from 15 to 25 mg-N·(kg soil)$^{-1}$, although this was not significantly different when these levels of nitrate were added in the absence of copper with 5 % moisture content (Figure 4-13).

Similar experiments were performed with the addition of ammonium in soil microcosms with 5% moisture content, but with the addition of 0.1 mg phenylacetylene·(kg soil)$^{-1}$ in one series to examine the impact of this inhibitor on the rates of methane consumption and N_2O production (Figure 4-15). When phenylacetylene was not added, the rates of both methane oxidation and N_2O production increased with

increasing amounts of ammonium. Specifically, when either 10, 15, or 25 mg-N NH_4^+·(kg soil)$^{-1}$ were added in the presence of 5% moisture, methane oxidation rates increased from 57 ± 2 to 65 ± 3, 74 ± 4, and 87 ± 4 μg·hr^{-1}, respectively (significantly different at 95, 99, and 99.9 % confidence levels). Similarly, N_2O production rates increased with increasing amounts of ammonium in the absence of phenylacetylene. The addition of as little as 5 mg-N NH_4^+·(kg soil)$^{-1}$ without phenylacetylene significantly increased nitrous oxide production rates as compared to that observed in microcosms with 5% moisture content and no added ammonium (i.e., an increase from 0.045 ± 0.004 μg·hr^{-1} to 0.087 ± 0.005 μg·hr^{-1}, significant at a 95 % confidence level).

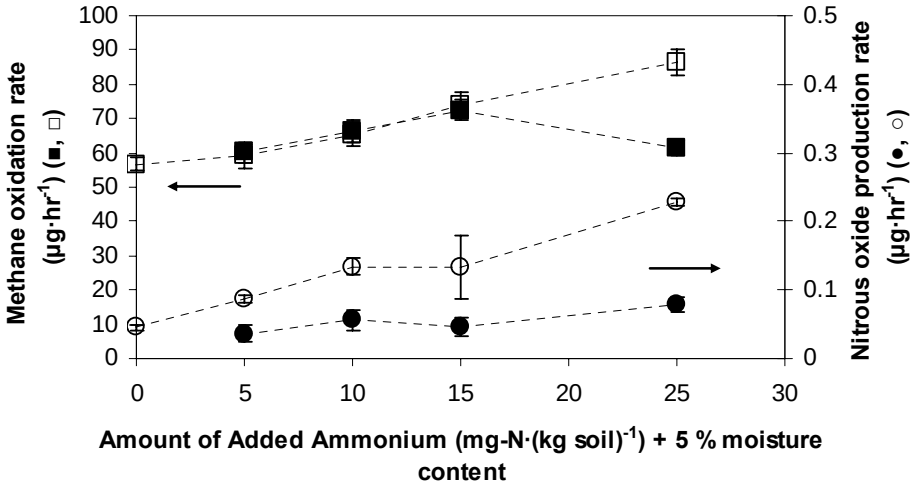

Figure 4-15. Effect of NH_4^+ with and without phenylacetylene on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. Effect of ammonium and 5% moisture content with (■,●) and without (□,○) 0.1 mg phenylacetylene·(kg soil)$^{-1}$ on CH_4 oxidation,(squares) and N_2O production (circles).

When 0.1 mg phenylacetylene·(kg soil)$^{-1}$ was added simultaneously with varying amounts of ammonium, methane oxidation rates increased slightly when up to 15 mg-N NH_4^+·(kg soil)$^{-1}$ was added (significant at a 95% confidence level), but the rates were not

significantly different from those measured with equal amounts of ammonium in the absence of phenylacetylene at 5 % moisture content (Figure 4-15). At higher levels of ammonium (25 mg-N NH_4^+·(kg soil)$^{-1}$) with phenylacetylene and relatively dry soils, methane oxidation rates were observed to decrease and were indistinguishable from that found in the presence of 5 mg-N NH_4^+·(kg soil)$^{-1}$. Furthermore, this was ~70% of the rate of methane oxidation measured in the absence of phenylacetylene with an equal amount of ammonium at 5 % moisture content (significant at 99.9 % confidence level). Nitrous oxide production was relatively stable with amounts of ammonium up to 15 mg-N NH_4^+·(kg soil)$^{-1}$ in the presence of phenylacetylene. In the presence of 25 mg-N NH_4^+·(kg soil)$^{-1}$, however, N_2O production rates increased from that observed in the presence of 15 mg-N NH_4^+·(kg soil)$^{-1}$, and such an increase was significant at a 90 % confidence level. Nitrous oxide production rates at all amounts of ammonium in the presence of 0.1 mg phenylacetylene·(kg soil)$^{-1}$ were ~60-70 % lower than the rates measured in microcosms with equal amounts of ammonium but in the absence of phenylacetylene, and such differences were significant at least a 95% confidence level.

To further examine the effects of phenylacetylene on the rates of methane oxidation and N_2O production, copper was combined with ammonium in the presence of 0.1 mg phenylacetylene·(kg soil)$^{-1}$. When 10 or 25 mg copper·(kg soil)$^{-1}$ was combined with either 10 or 15 mg-N NH_4^+·(kg soil)$^{-1}$, little effect was observed on methane oxidation rates as shown in Figure 4-16.

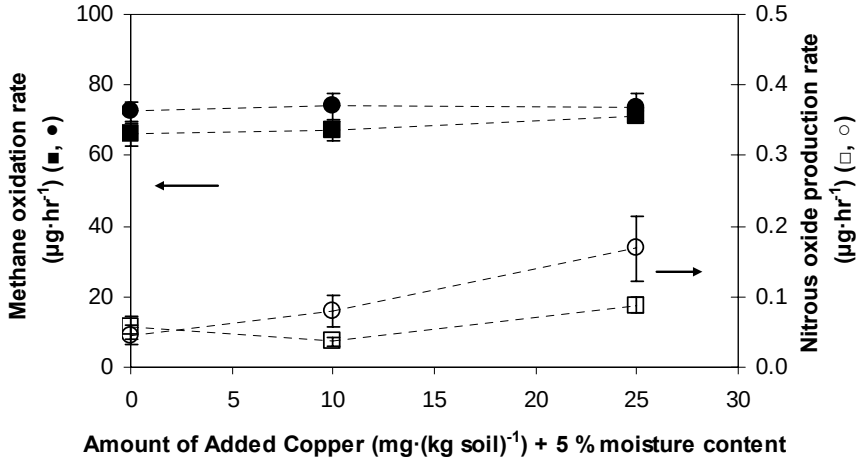

Figure 4-16. Effect of NH_4^+ and copper with 0.1 mg·(kg soil)$^{-1}$ on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. Effect of NH_4^+ and copper on CH_4 oxidation (■,●) and N_2O production (□,○). Squares represent 10 mg-N NH_4^+·(kg soil)$^{-1}$, circles represent 15 mg-N NH_4^+·(kg soil)$^{-1}$.

Nitrous oxide production rates were also not significantly affected by the addition of 10 mg copper·(kg soil)$^{-1}$ with either 10 or 15 mg-N NH_4^+·(kg soil)$^{-1}$ in the presence of 0.1 mg phenylacetylene·(kg soil)$^{-1}$. However, when 25 mg copper·(kg soil)$^{-1}$ was added along with 15 mg-N NH_4^+·(kg soil)$^{-1}$, the N_2O production rate, 0.17 ± 0.05 µg·hr^{-1}, increased significantly compared to the production rate observed when 10 mg copper·(kg soil)$^{-1}$ was combined with 15 mg-N NH_4^+·(kg soil)$^{-1}$, 0.080 ± 0.02 µg·hr^{-1}, as well as the rate measured when 25 mg copper·(kg soil)$^{-1}$ was combined with 10 mg-N NH_4^+·(kg soil)$^{-1}$, 0.09 ± 0.01 µg·hr^{-1}. These increases were found to be significant at 95 and 99 % confidence levels, respectively.

4.2.6. Discussion of microcosm studies

Methanotrophic activity can be affected by various geochemical parameters such as availability of different sources of nitrogen, e.g., NH_4^+, NO_3^-, or urea, moisture content,

organic carbon, and copper. However, when such geochemical parameters are applied to soils in order to stimulate methanotrophic activity, it can also affect the activities of other members of the soil microbial community, e.g., ammonia-oxidizing bacteria or denitrifiers. The addition of inorganic nitrogen, i.e., NH_4^+ and NO_3^-, stimulated CH_4 oxidation when incubated under 20 % CH_4 and 10 % O_2, possibly due to relief of nitrogen sources (Figure 4-1). However, such amendments also stimulated the activity of N_2O producers resulting in increases in N_2O production rates.

Generally, inhibition of CH_4 oxidation due to NH_4^+ is attributed to competitive binding of NH_4^+ to MMO, the enzyme that carries out the first step of CH_4 oxidation in methanotrophs (58, 100, 101). Alternatively, since methanotrophs can oxidize NH_4^+ to NO_2^-, inhibition due to the produced NO_2^- may occur (99). However, it appears that the addition of NH_4^+ did not result in any significant inhibitory effects in CH_4 oxidation. Based on this observation, the CH_4 mixing ratio, 20 %, could have been high enough to outcompete NH_4^+/NH_3 in binding to MMOs. If so, it could have at least reduced the effects of competitive inhibition of MMOs by NH_4^+/NH_3. Consequently, production of NO_2^-, a possible inhibitory anion to methanotrophs, via MMO activity could have also been reduced.

As NH_4^+ exists in an ionic form, when soils are amended with NH_4^+, counter-ions are introduced along with NH_4^+. Some studies have suggested the possible inhibitory effect of a counter-ion, Cl^- on CH_4 oxidation (48, 73). In this study, as NH_4^+ was added in the form of NH_4Cl, the proposed inhibition of methanotrophic activity by Cl^- could have occurred. Another mechanism that was proposed was the differential effects of added anions on desorption of NH_4^+ in soils increasing the amount of NH_4^+ in the aqueous phase

(101). Although the effects of Cl^- cannot be discarded, based on the stimulatory effects of the addition of NH_4^+ on CH_4 oxidation, it appears that such effects were minimal.

Nitrous oxide production, however, was also stimulated by the addition of NH_4^+ possibly due to the stimulation of ammonia-oxidizing bacteria. Although the production NO_2^- via MMO activity could have reduced by the high CH_4 mixing ratio, 20 %, it does not necessarily result in reduced activities of AMO. Therefore, if the added NH_4^+ were oxidized by MMO, it could explain the stimulation of N_2O production due to the addition of NH_4^+. Nitrous oxide production via denitrification could also have been triggered by the addition of NH_4^+. As NH_4^+ is subsequently oxidized to NO_2^- and NO_3^- by ammonia oxidizing bacteria and nitrite oxidizing bacteria, the final product of nitrification, NO_3^- could have been utilized by the denitrifying community.

Interestingly, effects of NO_3^- on CH_4 oxidation were similar to the effects observed with the addition of NH_4^+. The addition of NO_3^- also resulted in stimulation of CH_4 oxidation at 20 % CH_4 and 10 % O_2 (Figure 4-1). In previous studies, it was shown that approximately 100 mg-N $NO_3^-\cdot(\text{kg soil})^{-1}$ had no inhibitory effect on CH_4 oxidation (20, 136). In another study, 22 mg-N $NO_3^-\cdot(\text{kg soil})^{-1}$ resulted in stimulation of CH_4 oxidation relative to CH_4 oxidation observed when soils were amended with equivalent amounts of KCl to consider the effects of counter-ions (83). Based on these studies, it appears that the concentration range tested in this study should stimulate CH_4 oxidation at low NO_3^- and have no effects at high NO_3^-. In this study, the addition of NO_3^- stimulated CH_4 oxidation over the entire range tested. Similar to the effects of added NH_4^+ on CH_4 oxidation, it appears that NO_3^- relieved methanotrophs of N-limitation. The addition of

NO_3^- also stimulated N_2O production probably because the added NO_3^- was utilized by the denitrifying community.

The addition of urea had stimulatory effects on CH_4 oxidation but also on N_2O production (Figure 4-2). However, unlike NH_4^+ and NO_3^-, when amount of urea added to the soils increased to 100 mg-N·(kg soil)$^{-1}$, urea inhibited CH_4 oxidation.

Moisture content was an important parameter in both CH_4 oxidation and N_2O production. By controlling gas diffusivity, moisture content plays an important role in CH_4 consumption and N_2O production. Here in drier soils, i.e., 5-15 % moisture content, CH_4 oxidation rates were higher and N_2O production rates lower compared to that observed in wetter soils, i.e., 20-30 %. As methanotrophs rely on the availability of both CH_4 and O_2, lower moisture contents allow for greater diffusion of these substrates such that higher CH_4 oxidation rates can be expected in drier soils. The low N_2O production rates observed at drier soils could be attributed to the greater availability of O_2. However, it is expected that if the soils are extremely dry, < 5 % for this particular soil, the dryness of the soils could stress the microbial community in general.

The addition of copper and organic carbon did not have significant impact on CH_4 oxidation. As copper is known to regulate the expression of MMO in methanotrophs that are capable of expressing either form of MMO (132), it was hypothesized that addition of copper could shift the expression of MMO, i.e., if there exist sMMO expressing methanotrophs in the soil, addition of copper would change the form of MMO that is being expressed to pMMO, thus resulting in changes in CH_4 oxidation rates. However, addition of copper did not have any effect on CH_4 oxidation below 100 mg Cu·(kg soil)$^{-1}$ (Figure 4-4). This could be because that the majority if not all of the methanotrophs in the

soil were expressing pMMO and the amount of copper in the background was already sufficient to achieve maximum CH_4 oxidation rates by such cells.

To investigate the possibility of selectively inhibiting N_2O production either through nitrification or denitrification, phenylacetylene and chlorate were tested. Phenylacetylene appeared to be able to selectively inhibit ammonia-oxidizing bacteria leading to a decrease in N_2O production while CH_4 oxidation was not affected when 0.1 $mg \cdot (kg\ soil)^{-1}$ of phenylacetylene was added (Figure 4-6). However, addition chlorate was not effective in inhibiting N_2O production. Rather, stimulation of N_2O production was observed after soils were amended with chlorate (Figure 4-7).

The effect of varying amendments, i.e., inorganic nitrogen, urea, moisture content, copper, and organic carbon, in the presence of 0.1 $mg \cdot (kg\ soil)^{-1}$ of phenylacetylene was also examined. Overall, the effect of all the amendments that were tested was similar to what was observed in the absence of phenylacetylene. Thus, it appears that the role of nitrification in the production of N_2O induced by the changes in NO_3^-, moisture content, copper, and organic carbon could be minor. Interestingly, even with 0.1 $mg \cdot (kg\ soil)^{-1}$ of phenylacetylene, the addition of NH_4^+ had a stimulatory effect on both CH_4 oxidation and N_2O production (Figure 4-8). This could presumably be because either the excessive amount of added NH_4^+ allowed for greater binding to AMO, reducing phenylacetylene inhibition and/or the increase in amount of NH_4^+ resulted in giving NH_4^+ competitiveness to bind to MMOs. Therefore, when applying NH_4^+ along with phenylacetylene to stimulate CH_4 oxidation while inhibiting N_2O production, the amount of NH_4^+ added should be limited to enable effective inhibition of ammonia-oxidizing bacteria and stimulation of methanotrophs.

Nitrous oxide production in soils has been attributed to both nitrification and denitrification depending on the environmental conditions, e.g., availability of O_2. Generally, in conditions where O_2 is readily available, nitrification has been noted to be the primary producer of N_2O, while where O_2 is not readily available, denitrification (9, 23, 98). However, these studies did not attempt to discriminate between nitrification via ammonia-oxidizing bacteria and methanotrophs. Thus, the contribution of methanotrophs on N_2O production via nitrification was not assessed. Interestingly, there have been reports that nitrification in rice plant rhizosphere and humisols were carried out primarily by methanotrophs rather than ammonia-oxidizing bacteria (13, 127). If King Highway Landfill cover soils showed similar behaviors, i.e., nitrification being primarily due to methanotrophs, then the N_2O production via nitrification could be attributed to the methanotrophs.

As expected when NO_3^- was added along with phenylacetylene, the effects on CH_4 oxidation and N_2O production did not change compared to what was observed in the absence of phenylacetylene. The addition of urea along with 0.1 $mg\cdot(kg\ soil)^{-1}$ of phenylacetylene also did not have differential effects on CH_4 oxidation and N_2O production rates compared to the effects observed without the addition of phenylacetylene. Here, increasing amounts of urea probably lead to increased production of NH_3, a product of urea hydrolysis, which subsequently resulted in stimulation of N_2O production. As seen with the addition of NH_4^+, by increasing the amounts of urea, it probably overwhelmed the amounts of phenylacetylene added such that N_2O production initiated by nitrification occurred.

The effect of moisture content in the presence of 0.1 mg·(kg soil)$^{-1}$ of phenylacetylene on CH_4 oxidation and N_2O production did not differ from the effects in the absence of phenylacetylene. Drier soils, i.e., 5-15 % moisture contents, consumed more CH_4 while producing N_2O at a slower rate compared to wetter soils. The addition of copper and organic carbon in the presence of phenylacetylene resulted in similar effects on CH_4 oxidation and N_2O production compared to conditions in the absence of phenylacetylene.

To investigate if synergistic effects could occur when multiple geochemical parameters were applied, combinations of 5 % moisture content, varying amounts of copper, and varying amounts of either NH_4^+ (with and without phenylacetylene) or NO_3^- was considered. It was found that providing relatively dry soils (5% moisture content) along with either 15 mg NO_3^-·(kg soil)$^{-1}$ or 15 mg NH_4^+ ·(kg soil)$^{-1}$ and 0.1 mg phenylacetylene·(kg soil)$^{-1}$ provided the greatest stimulation of CH_4 oxidation while minimizing any effect on N_2O production. Specifically, addition of 15 mg NO_3^-·(kg soil)$^{-1}$ in soils with 5% moisture content increased CH_4 oxidation rates by 48% as compared to no addition of nitrate with 5% moisture. Nitrous oxide production rates, however, increased by over 2-fold, offsetting the reduction of global warming potential associated with reduced CH_4 emissions. If 15 mg NH_4^+ ·(kg soil)$^{-1}$ and 0.1 mg phenylacetylene·(kg soil)$^{-1}$were added, CH_4 oxidation rates increased by ~28% as compared to microcosms with no added ammonium or phenylacetylene and 5% moisture, yet N_2O production rates were not affected. As such, these conditions were the most appropriate of the combinations tested for manipulation of the microbial community present in the landfill cover soils at this site for mitigation of greenhouse gas emissions. Specifically, these

finding suggest that methanotrophic activity is limited by nitrogen at this site. Studies have been performed to indentify who is responsible for the N_2O produced in soils. Some have suggested that methanotrophs produce N_2O (125) with ammonia-oxidizing bacteria having less significant role (182). Here, based on the findings, it appears that microorganisms which were stimulated by the addition of NH_4^+ but were inhibited by phenylacetylene were the primary producers of N_2O, while in soils amended with NO_3^-, the denitrifiers were the primary producers of N_2O. As not all MMOs behave the same, N_2O production after the addition of NH_4^+ could be either by methanotrophs or ammonia-oxidizing bacteria that are sensitive to phenylacetylene.

In order to develop a method that could provide a more quantitative suggestion on the effects of amendments on CH_4 oxidation and N_2O production, relative changes in CH_4 oxidation rates and N_2O production was examined. A summary of the effects of amendments without phenylacetylene on CH_4 oxidation and N_2O production can be seen in Table 4-3. As a way to assess the effects, the ratios of the CH_4 oxidation rates and N_2O production rates under various conditions relative to those observed at 20 % CH_4, 10 % O_2, and 15 % moisture content, were calculated based on the average rates observed, as shown below. Here, the rates at baseline are that observed at 20 % CH_4, 10 % O_2, and 15 % moisture content. Values reported in Table 4-3 are % changes relative to the baseline conditions.

$$Effect\ on\ CH_4\ oxidation = \frac{CH_4\ oxidation\ rate\ (with\ amendments)}{CH_4\ oxidation\ rate\ (baseline)}$$

$$Effect\ on\ N_2O\ production = \frac{N_2O\ production\ rate\ (with\ amendments)}{N_2O\ production\ rate\ (baseline)}$$

Although CH_4 production rates and N_2O production rates observed in microcosms might not be representative of what may occur *in situ*, e.g., fluxes of CH_4 and N_2O, the rates were used to better predict what could happen *in situ*. In general, the addition of nitrogen, NH_4^+, NO_3^-, and urea all enhanced CH_4 oxidation but also lead to enhanced N_2O production. Therefore, the soils used could have been N-limited which could be relieved by the addition of nitrogen in order to stimulate CH_4 oxidation.

Table 4-3. Effects of amendments on CH_4 oxidation rates and N_2O production rates based on the average rates observed relative to rates observed at 20 % CH_4, 10 % O_2, and 15 % moisture content (baseline) (values are reported as % change).

Amendments	Concentration	CH_4 oxidation	N_2O production
NH_4^+ (mg-N·(kg soil)$^{-1}$)	25	**7**	**460**
	50	**18**	**1000**
	75	**22**	**1800**
	100	**56**	**1500**
NO_3^- (mg-N·(kg soil)$^{-1}$)	25	**14**	**230**
	50	**48**	**660**
	75	**48**	**510**
	100	**43**	**450**
Urea (mg-N·(kg soil)$^{-1}$)	25	**46**	**190**
	50	**33**	**460**
	75	**-11**	**580**
	100	**-80**	**220**
Moisture content (%)	5	**21**	**-93**
	10	**11**	**-57**
	20	**-21**	**72**
	30	**-38**	**-30**
Copper (mg·(kg soil)$^{-1}$)	5	**-4**	**0**
	10	**13**	**33**
	25	**4**	**13**
	50	**0**	**15**
	100	**-1**	**-12**
	250	**-33**	**-9**
	500	**-32**	**-38**
Org. Carbon (mg·(kg soil)$^{-1}$)	20	**-8**	**0**
	50	**-2**	**190**
	100	**-5**	**310**
	150	**-11**	**360**
	200	**-3**	**230**

A summary of the effects of amendments with phenylacetylene on CH_4 oxidation and N_2O production can be seen in Table 4-4. Similar to the aforementioned approach,

the ratios of CH_4 production rates and N_2O production rates relative to rates observed at baseline conditions, here it was 20 % CH_4, 10 % O_2, 0.1 mg C_8H_6·(kg soil)$^{-1}$, and 15 % moisture content, were calculated based on the average rates observed.

Table 4-4. Effects of amendments on CH_4 oxidation rates and N_2O production rates based on the average rates observed relative to rates observed at 20 % CH_4, 10 % O_2, 0.1 mg C_8H_6·(kg soil)$^{-1}$ and 15 % moisture content (baseline). (values are reported as % change).

Amendments	Concentration.	CH_4 oxidation	N_2O production
NH_4^+ (mg-N·(kg soil)$^{-1}$)	25	**39**	**760**
	50	**97**	**2100**
	75	**58**	**2100**
	100	**44**	**2500**
NO_3^- (mg-N·(kg soil)$^{-1}$)	25	**26**	**790**
	50	**34**	**1200**
	75	**36**	**2100**
	100	**36**	**5600**
Urea (mg-N·(kg soil)$^{-1}$)	25	**0**	**200**
	50	**62**	**590**
	75	**64**	**350**
	100	**-72**	**100**
Moisture content (%)	5	**33**	**-32**
	10	**0**	**150**
	20	**-37**	**580**
	30	**-63**	**300**
Copper (mg·(kg soil)$^{-1}$)	25	**39**	**-30**
	50	**25**	**19**
	100	**27**	**19**
	250	**-14**	**56**
	500	**-23**	**-7**
Org. Carbon (mg·(kg soil)$^{-1}$)	20	**33**	**150**
	50	**48**	**330**
	100	**33**	**340**
	150	**36**	**410**
	200	**11**	**510**

Similar to the effects of amendments on CH_4 oxidation and N_2O production in the absence of phenylacetylene, with the addition of phenylacetylene it appears that the addition of nitrogen could enhance CH_4 oxidation. However, N_2O production was also enhanced even with the addition of phenylacetylene.

A summary of the effects of selected amendments when moisture content was reduced to 5 % on CH_4 oxidation and N_2O production can be seen in Table 4-5. Similar

to the approaches used previously, to assess the effects on CH_4 oxidation and N_2O production, ratios of CH_4 production rates and N_2O production rates observed under various amendments relative to rates observed at baseline conditions, i.e., 20 % CH_4, 10 % O_2, and 5 % moisture content, were calculated based on the average rates observed.

When moisture contents were decreased to 5 % and the amounts of added nitrogen, NH_4^+ and NO_3^-, were reduced to 5-25 mg-N·(kg soil)$^{-1}$, the effects of phenylacetylene when added along with NH_4^+ were clear. Specifically, when NH_4^+ and phenylacetylene were added together, CH_4 oxidation was enhanced and N_2O production was unchanged from that without the addition of NH_4^+. Therefore, based on this assessment, it appears that small amounts of NH_4^+, 15 mg-N·(kg soil)$^{-1}$ with 0.1 mg C_8H_6·(kg soil)$^{-1}$ are the most effective amendments to reduce greenhouse gas net emission.

Table 4-5. Effects of selected amendments at reduced moisture contents on CH_4 oxidation rates and N_2O production rates based on the average rates observed relative to rates observed at 20 % CH_4, 10 % O_2, and 5 % moisture content (baseline). (values are reported as % change)

Amendments	Concentration	CH_4 oxidation	N_2O production
NH_4^+ (mg-N·(kg soil)$^{-1}$)	5	**4**	**93**
	10	**15**	**200**
	15	**31**	**200**
	25	**53**	**410**
NH_4^+ (mg-N·(kg soil)$^{-1}$) 0.1 mg C_8H_6·(kg soil)$^{-1}$	5	**6**	**-20**
	10	**17**	**24**
	15	**28**	**2**
	25	**8**	**76**
NO_3^- (mg-N·(kg soil)$^{-1}$)	5	**8**	**100**
	10	**5**	**120**
	15	**48**	**230**
	25	**46**	**390**

Here, the effects of each amendment on CH_4 oxidation and N_2O production were separately examined. i.e., both CH_4 oxidation and N_2O production were equally treated.

Based on this approach, the optimal amendments are 15 mg-N NH_4^+·(kg soil)$^{-1}$ along with 0.1 mg·(kg soil)$^{-1}$ phenylacetylene.

If CH_4 oxidation and N_2O production rates are weighted based on their GWP, CH_4 oxidation rates and N_2O production rates can be combined by referencing the rates on CO_2 basis which enables the determine what the effects of amendments are as a whole on greenhouse gas mitigation. Based on this approach, as shown in Table 4-6, the optimal amendments are 25 mg-N NH_4^+·(kg soil)$^{-1}$, 15 and 25 mg-N NO_3^-·(kg soil)$^{-1}$.

This approach is similar to that used in a previous study to assess the significance of N_2O emission in a landfill site (146). Although NO_3^- could potentially be an effective candidate, because it is vulnerable to leaching in pH neutral soils as a result of percolation, continuous application will be necessary making NH_4^+-based fertilization a more attractive option. Although N_2O has an order of magnitude higher GWP values compared to CH_4, because the amount of CH_4 being oxidized is much larger that the amount of N_2O being produced, calculations based on CH_4 oxidation rates and N_2O production rates weighted by GWP indicate that in this particular system, stimulation of CH_4 oxidation could be more important than trying to inhibit N_2O production.

Table 4-6. Effects of selected amendments at reduced moisture contents on CH_4 oxidation rates and N_2O production rates, collectively, based on the average rates observed relative to rates observed at 20 % CH_4, 10 % O_2, and 5 % moisture content (baseline). The rates were weighted by their GWP.

Amendments	Concentration	CH_4 oxidation- N_2O production
NH_4^+ (mg-N·(kg soil)$^{-1}$)	5	**3**
	10	**13**
	15	**29**
	25	**48**
NH_4^+ (mg-N·(kg soil)$^{-1}$) 0.1 mg C_8H_6·(kg soil)$^{-1}$	5	**7**
	10	**16**
	15	**28**
	25	**7**
NO_3^- (mg-N·(kg soil)$^{-1}$)	5	**7**
	10	**4**
	15	**47**
	25	**44**

4.2.7. Effect of amendments on methanotrophic community structure

DNA microarray analyses were performed to investigate the effect of selected amendments on the methanotrophic community structure during soil microcosm studies. DNA was collected from 3 different soil microcosms, i.e., i) 20 % CH_4, 10 % O_2, 5 % moisture content, ii) 20 % CH_4, 10 % O_2, 5 % moisture content with 15 mg-N NH_4^+·(kg soil)$^{-1}$, and iii) 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$ with 0.1 mg C_8H_6·(kg soil)$^{-1}$. The rationale for choosing these conditions for microarray analysis was to have one serve as a baseline, and others to consider the effect of NH_4^+ added individually and in combination with phenylacetylene as these were found to be the most promising amendments for reducing greenhouse gas emissions. Microarray results using DNA collected from soils are shown in Figure 4-17. A complete list of probes used for the DNA microarray and the information on the targets can be found in Appendix 1 (p.124). The probes used in this study was the third major updated version of the probes initially designed but yet to be published by the developers (19).

As shown in Figure 4-17, in soils incubated with no amendments of nitrogen and phenylacetylene, Type II methanotrophs dominated the methanotrophic community, particularly the genera *Methylocystis* (Mcy233, Mcy522, Mcy264, Mcy270, and Mcy459). Relatively weak signals were detected from probes targeting genera *Methylosinus* (MsS314, Msi423, and Msi232). Probes targeting Type Ia methanotrophs produced positive signals from probes Mb_SL#3-300 (*Methylobacter*), Mb460 (*Methylobacter*), Mm531 (*Methylomonas*) and Mm275 (*Methylomonas*) with probes targeting general Type Ia methanotrophs Ia193 and Ia575 yielding relatively strong signals compared to other probes that target Type Ia methanotrophs. Probes targeting

Type Ib methanotrophs (*Methylococcus, Methylothermus, Methylocaldum,* and related) showed no signals except from probe JRC2-447 (sequences closely related to Japanese Rice Cluster #2) and Ib453 (general Type Ib methanotrophs).

With the addition of 15 mg-N NH_4^+·(kg soil)$^{-1}$, an increase in signals were observed from probes Mm275 (*Methylomonas*) and Mm451 (*Methylomonas*). Here, an increase/decrease in the relative abundance was determined as 50 % increase/decrease in the relative signals compared to the relative signals observed from *pmoA* amplified from soils with no amendments. Signals from Mb282 (*Methylobacter*), Mb271 (*Methylobacter*) and Mm_M430 (*Methylomonas*), which were below detection limit (<0.05) in soils with no treatment, were positively detected in soils amended with 15 mg-N NH_4^+·(kg soil)$^{-1}$. Signals from Mm531 (*Methylomonas*) and Ia193 (general Type I) and Msi423 (*Methylosinus*) showed a decrease in intensity.

With the addition of 15 mg-N NH_4^+·(kg soil)$^{-1}$ and 0.1 mg C_8H_6·(kg soil)$^{-1}$, increases in signals were detected from only two probes compared to signals from soils with no treatment, JRC2-447 (Japanese rice cluster) and Mb271 (*Methylobacter*). The signal intensity from probes Mb_SL#3-300 (*Methylobacter*), Mm531 (*Methylomonas*) and Mm451 (*Methylomonas*) showed a decrease as compared to soils with no treatment.

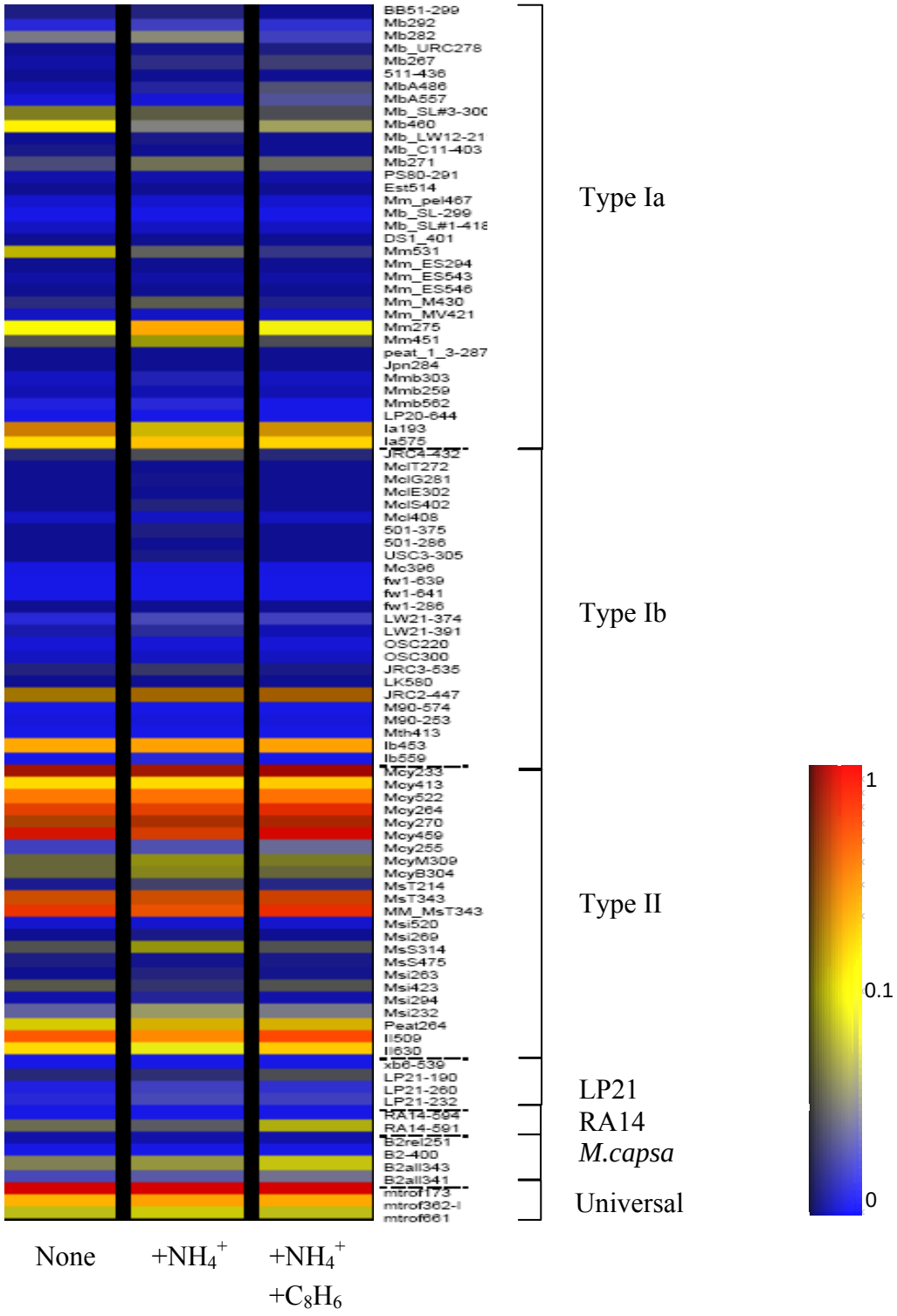

Figure 4-17. *pmoA* based DNA microarray. Relative signal intensities are shown as color spectrum with 1 being the maximum achievable signal for each probe. Labels on the bottom indicate; None: 20 % CH_4, 10 % O_2, 5 % moisture content, +NH_4^+: 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$, +NH_4^++C_8H_6: 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$ with 0.1 mg C_8H_6·(kg soil)$^{-1}$.

4.2.8. Expression of functional genes in soil microcosms

To examine the expression of functional genes, mRNA was extracted from soils incubated under i) 20 % CH_4, 10 % O_2, and 5 % moisture content, ii) 20 % CH_4, 10 % O_2, 5 % moisture content, and 15 mg-N NH_4^+·(kg soil)$^{-1}$, and iii) 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$, and 0.1 mg C_8H_6·(kg soil)$^{-1}$ while CH_4 was actively being consumed. Transcripts of *pmoA* were detected in all conditions and time points (Figure 4-18A) but not *mmoX* (Figure 4-18B), indicating pMMO was the primary MMO being expressed *in situ*. Interestingly, *amoA* transcripts were not detected (Figure 4-18C).

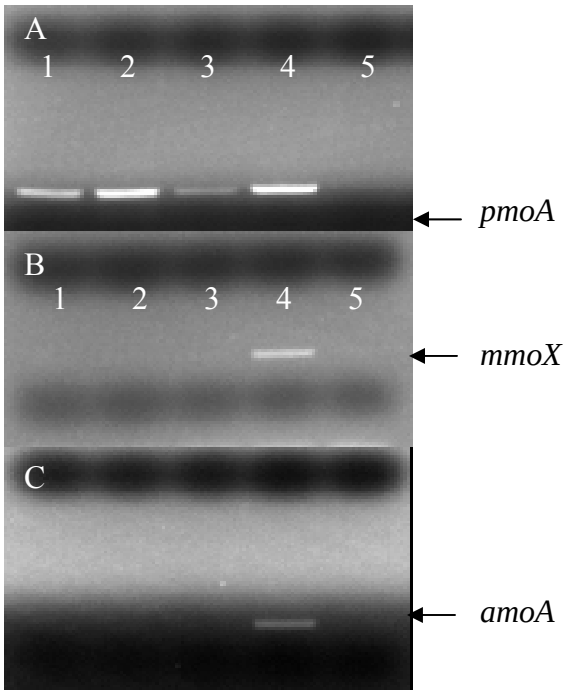

Figure 4-18. PCR amplification of cDNA prepared from mRNA extracted from soils incubated under i) 20 % CH_4, 10 % O_2, and 5 % moisture content (lanes 1), ii) 20 % CH_4, 10 % O_2, 5 % moisture content, and 15 mg-N NH_4^+·(kg soil)$^{-1}$ (lanes 2), and iii) 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$, and 0.1 mg C_8H_6·(kg soil)$^{-1}$ (lanes 3) while CH_4 was actively being consumed. A) *pmoA*, B) *mmoX*, and C) *amoA*. Lane 4s are positive controls using mRNA extracted from either pMMO- or sMMO-expressing *Methylosinus trichosporium* OB3b (A and B), and *Nitrosomonas europaea* (C). Lane 5s are negative controls with no nucleic acids added.

4.2.9. Discussion of microarray and reverse-transcription PCR

Overall, the soils used for microcosm experiments were predominated by the genera *Methylocystis*, which has been found in relatively large numbers in other landfills (19, 32, 161). Generally, Type I methanotrophs are suggested to be more competitive in nutrient-rich environments (32). However, with the addition of 15 mg-N NH_4^+·(kg soil)$^{-1}$, both Type I and II methanotrophs were affected. Although an increase in relative signal intensities were observed in specific probes targeting Type I methanotrophs, i.e., *Methylomonas* and sequences related to environmental sequences. Also, some *Methylobacter* and *Methylomonas* species were positively detected which were below detection limit (<0.05) in soils with no amendments. As *Methylobacter* and *Methylomonas* are Type I methanotrophs, it indicates that Type I methanotrophs were able to gain advantage with the addition of NH_4^+. Although signals from Ia193 (general Type I) decreased, it has been reported that the specific probe does not have full coverage of *Methylobacter* and *Methylomonas*. Therefore, the result obtained from Ia193 might not effectively reflect what occurred to Type I methanotrophs as a whole.

With the addition of 15 mg-N NH_4^+·(kg soil)$^{-1}$ and 0.1 mg phenylacetylene·(kg soil)$^{-1}$, signals for *Methylobacter* and *Methylomonas* decreased or were at the levels observed from soils with no treatment. As the microarray method used here can only provide information on relative abundance and not the actual quantity of specific taxa, the increases and decreases only reflect the changes in relative abundance. Therefore, the stimulation in CH_4 oxidation due to the addition of either 15 mg-N NH_4^+·(kg soil)$^{-1}$ or 15 mg-N NH_4^+·(kg soil)$^{-1}$ and 0.1 mg phenylacetylene·(kg soil)$^{-1}$, could be due to an increase in relative abundances of certain methanotrophs but cannot eliminate the possibility of

increase in the total size of the methanotrophic community. If a change in community structure is responsible for the stimulation of CH_4 oxidation, both Type I and Type II methanotrophs could have been responsible. Also, regarding the inhibition of N_2O production due the addition of 0.1 mg phenylacetylene·(kg soil)$^{-1}$, it appears that Type I methanotrophs, i.e., *Methylobacter* and *Methylomonas* related methanotrophs were responsible for producing N_2O as the increase in signals were observed for probes targeting these genera with the addition of NH_4^+ but were also affected by the simultaneous addition of C_8H_6.

To better understand the activity of methanotrophs and ammonia-oxidizing bacteria *in situ*, PCR was performed using cDNA synthesized from mRNA collected from soils incubated under three different conditions, under i) 20 % CH_4, 10 % O_2, and 5 % moisture content, ii) 20 % CH_4, 10 % O_2, 5 % moisture content, and 15 mg-N NH_4^+·(kg soil)$^{-1}$, and iii) 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$, and 0.1 mg C_8H_6·(kg soil)$^{-1}$ while CH_4 was actively being consumed. Here as shown in Figure 4-18, *pmoA* was being expressed in the soils and not *mmoX* for all three conditions indicating that only pMMO was being expressed with sMMO being expressed below detection limits if at all. In other previous studies where CH_4 was being actively consumed, similar results were obtained, where only the transcripts of *pmoA* were detected and not *mmoX* (40, 41). This finding could explain why the addition of copper to the soils did not affect CH_4 oxidation. Here, even without the addition of external copper, only the transcripts of *pmoA* were detected. Interestingly, the transcripts of *amoA* were not detected even with the addition of NH_4^+ (Figure 4-18C). It was initially expected to see transcripts of *amoA* when NH_4^+ was the sole amendment, as N_2O production rates

were increased at the particular condition suggesting a minimal role of ammonia-oxidizing bacteria in the production of N_2O via oxidation of NH_4^+. If *pmoA* and *amoA* transcripts are an indication of the size of methanotrophs and ammonia-oxidizing bacteria, respectively, although N_2O production rates of ammonia-oxidizing bacteria can exceed that of methanotrophs (182), the size of the methanotrophic population could have been responsible for a large amount of N_2O produced.

4.3. Conclusions

In this chapter, effects of various geochemical parameters, i.e., inorganic nitrogen, urea, moisture content, copper, organic carbon, and selective inhibitor, on CH_4 oxidation and N_2O production were examined. When amendments are sought to stimulate CH_4 oxidation in landfills or other sites that is to mitigate CH_4 emissions, one should take into account the possible consequences of such amendments. As landfill cover soils could contain complex microbial communities, an amendment to solve one problem, i.e., stimulation of CH_4 oxidation, could bring about another problem, i.e., stimulation of N_2O production. Therefore, holistic approaches should always be taken in landfill gas management.

Based on the findings, it appears that inorganic nitrogen and moisture content are all important variables that should be considered when managing landfills to mitigate greenhouse gas emissions. Although both forms of inorganic nitrogen, i.e., NH_4^+ and NO_3^-, could potentially be effective in mitigation of greenhouse gas emissions, because NO_3^- could be lost in soils from leaching in pH-neutral soils, NH_4^+ seems favorable. On the other hand, the mobility of NO_3^- could benefit the methanotrophic communities in deeper regions if the amendments are applied to the surface. Therefore, a combination of

NH_4^+ and NO_3^-, along with selective inhibitor phenylacetylene, could also be considered to stimulate CH_4 oxidation while inhibiting N_2O production *in situ*.

CHAPTER 5. Vertical community composition of methanotrophs *in situ*

5.1. Introduction

Methane is the product of anaerobic decomposition, as mentioned in Chapter 1, such that it is more likely to find large amounts of CH_4 in deeper parts of the landfill. As O_2 penetrates the landfill cover soils counter gradients of CH_4 and O_2 are observed, i.e., low CH_4 to O_2 ratios near the surface and high CH_4 to O_2 ratios in deeper regions. Although the two generally accepted types of methanotrophs, Type I (or more specifically Type Ia) and Type II methanotrophs, have been found to be able to coexist, some studies have suggested that the two types of methanotrophs may have certain preferences toward substrate availability. In CH_4-rich and O_2-limiting environments, Type II methanotrophs seem to outcompete Type Ia methanotrophs while Type Ia seem to outcompete Type II methanotrophs in CH_4-limiting and O_2-rich environments (3, 31, 80, 123, 161). In nitrogen and/or nutrient limiting environments, it appears that Type II methanotrophs have an advantage over Type Ia methanotrophs while in nitrogen rich environments Type Ia methanotrophs appear to predominate (17, 72, 129, 180). Therefore, even within methanotrophs, different geochemical parameters could exert a strong influence on the distribution and activity of methanotrophic community *in situ*. In this chapter, the vertical distribution of methanotrophic community composition was examined through the use of *pmoA*-based DNA microarray. It was expected to observe

Type I methanotrophs gain advantage where CH_4/O_2 is low (shallow regions) and Type II methanotrophs thrive where CH_4/O_2 is high (deeper regions).

Understanding the methanotrophic distribution *in situ* in landfill cover soils may enable one to better manage landfills to mitigate greenhouse gas emissions, e.g., should amendments such as NH_4^+ or NO_3^- be applied and if so, what depths should be targeted for application? As it has been suggested that Type Ia methanotrophs react quickly to changes in the surroundings (80), understanding where Type Ia methanotrophs exist and how they respond to amendments could be useful in mitigating CH_4 emissions. In this part, vertical community composition of methanotrophs in King Highway Landfill cover soil was examined by microarray analysis. Although 16s rRNA has been traditionally used to identify the members of the microbial community in the environment, *pmoA* was used as almost all known methanotrophs possess this gene except for certain exceptions, e.g., some *Methylocella* species, which are acidophilic, and not believed to be numerous in landfills (53).

5.2. Results

5.2.1. Vertical geochemical properties of the soil.

Vertical soil gas properties of the soil are shown in Figure 5-1. At this particular site, O_2 penetrated down to a depth of 90 cm although the amount of O_2 declined with increasing depth. The amount of CH_4 in the soil gas between depths of 60-100 cm was relatively stable around 50-60 % which then declined with shallower depths. At 20 cm, the shallowest depth where soil gas measurements were taken, CH_4 was present at 2.3 %.

Meanwhile, CO_2 in the soil gas was relatively stable between 20-29% in the depths of 50-100 cm but also declined with shallower depths.

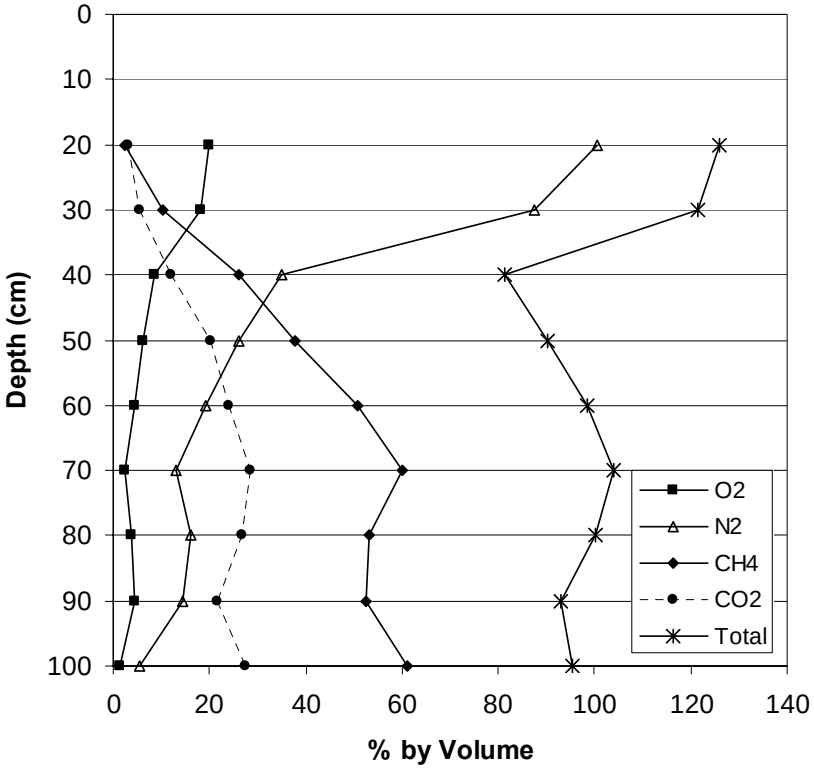

Figure 5-1. Vertical gas profile of King Highway Landfill measured in Feb 2007 (7)

The soil pH varied between 7.3 and 7.5 over the entire depth, i.e., 0-95 cm. Inorganic nitrogen, ammonium and nitrate, associated with the soils from core sample was measured as shown in Figure 5-2. Inorganic ammonium ranged between 0.3 and 29.4 mg-N·(kg soil)$^{-1}$. The inorganic ammonium content was generally low, i.e., between 0.3 and 2.1 mg-N·(kg soil)$^{-1}$, in shallow regions, i.e., 0-20 cm while inorganic ammonium was slightly higher at 5.7-10.9 mg-N·(kg soil)$^{-1}$ at depths of 20-50 cm. Higher amounts of inorganic ammonium, i.e., (>20 mg-N·(kg soil)$^{-1}$), was present in deeper regions of the

soil, i.e., below 50 cm, but no evident trend was seen as the amount of inorganic ammonium fluctuated below depths of 50 cm with values as low as 5.6 mg-N·(kg soil)$^{-1}$ and as high as 29 mg-N·(kg soil)$^{-1}$ was observed. Inorganic nitrate varied between 1.2 and 20.2 mg-N·(kg soil)$^{-1}$. The amount of nitrate was relatively stable at 22.5-62.5 cm depths between 1.6-6.4 mg-N·(kg soil)$^{-1}$. However, at other depths, above 22.5 cm and below 62.5 cm, the amount of nitrate fluctuated significantly, with as much as 20 mg-N·(kg soil)$^{-1}$ found.

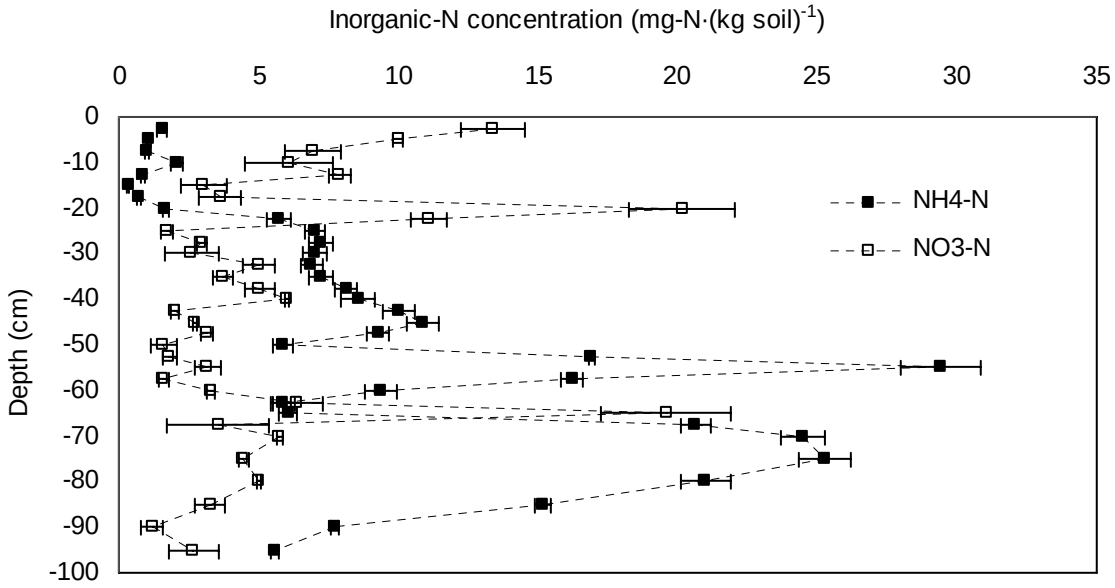

Figure 5-2. Vertical profile of inorganic ammonium and nitrate associated with King Highway Landfill core sample obtained in May 2007

The bioavailable and total copper were also measured as shown in Figure 5-3. Bioavailable copper was relatively higher at depths of 0-20 cm compared to deeper regions, ie., 80-230 µg·(kg soil)$^{-1}$ and 14-92 µg·(kg soil)$^{-1}$, respectively. The amount of total copper content fell in the range of 5-26 mg·(kg soil)$^{-1}$.

Bioavailable and total iron were measured as shown in Figure 5-4. Bioavailable iron was relatively stable, varying between 3.4-6.0 mg·(kg soil)$^{-1}$ while total iron content fluctuated between 2.3 and 14 g·(kg soil)$^{-1}$.

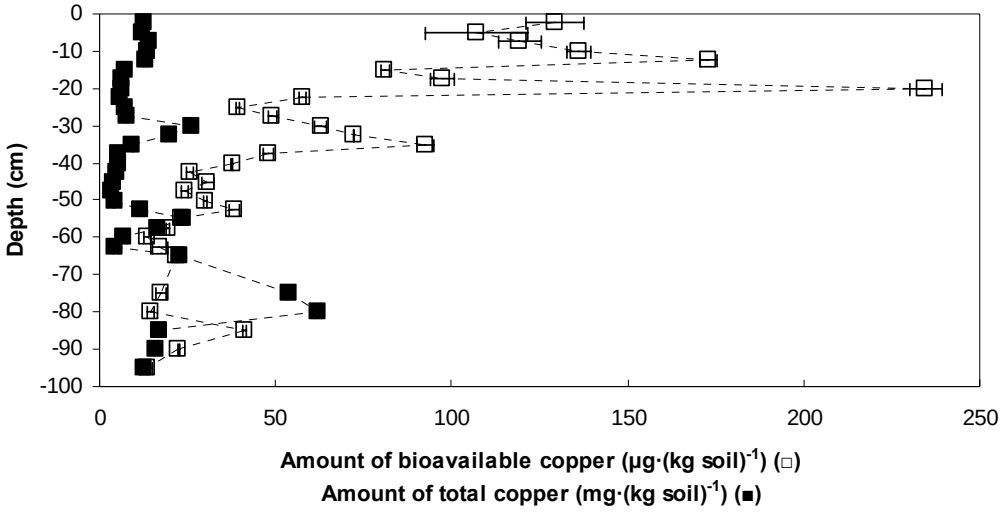

Figure 5-3. Vertical profile of bioavailable and total copper. □: bioavailable copper, ■: total copper. Error bars indicate standard deviations from measurements of samples of triplicates.

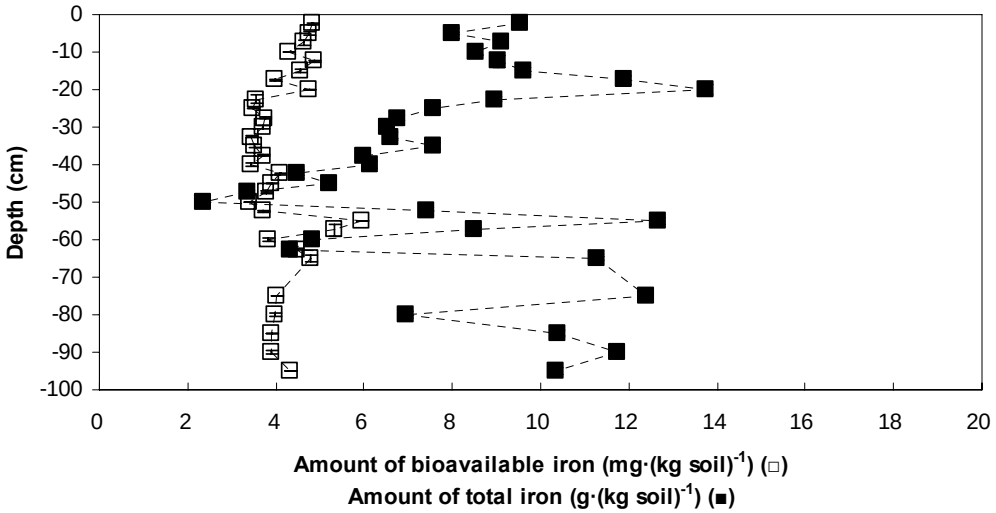

Figure 5-4. Vertical profile of bioavailable and total iron. □: bioavailable iron, ■: total iron. Error bars indicate standard deviations from measurements of samples of triplicates.

5.2.2. Methanotrophic community structure

The vertical community composition of methanotrophs in the landfill cover soil was assessed by diagnostic microarray targeting the functional gene *pmoA* (19, 161) as shown in Figure 5-5. A summary of the oligonucleotide probes used for microarray analysis is provided in Appendix 1 (p.124). In general, King Highway Landfill cover soil was predominated by Type II *pmoA* sequences, specifically *pmoA* sequences related to *Methylocyctis* genera, throughout the depth tested as evidenced by the high signal values obtained in probes Mcy233, Mcy413, Mcy522, Mcy264, Mcy270, and Mcy459. Although at lower intensities, probes specific to *Methylocystis parvus*, *Methylocystis echinoides*, and strain M (McyB304), and *Methylocystis* strain M and related (McyM309) further provided evidence that soils examined contained relatively abundant *Methylocystis*-related *pmoA* sequences. Sequences related to *Methylosinus*, another genera belonging to Type II methanotrophs, were mainly found from probes MsT343 and MM_MsT343, which are probes both intended to hybridize to *Methylosinus trichosporium* OB3b and related *pmoA* sequences. However, MsS314 was the only probe that target *Methylosinus sporium* that produced positive signals indicating that in these soils *Methylosinus*-related methanotrophs were mainly closely related to *Methylosinus trichosporium* OB3b.

Signals retrieved from probes Mb460, Mm531, Mm275, and Mm451 indicated the presence of Type I methanotrophs, *Methylobacter* and *Methylomonas* related methanotrophs throughout. However, results from probes BB51-302, Mb292, Mb282, and MmM430 indicated certain *Methylobacter* and *Methylomonas*-related methanotrophs were found mainly above 27.5 cm and not deeper than 32.5 cm. Mb_URC278, Mb267,

MbA486, and Mb271 indicated that certain *Methylobacter* related methanotrophs could be found at depths of 32.5-35 cm but not at deeper regions. No signals were obtained from probes targeting the genera *Methylomicrobium* and *Methylosarcina* at any depths tested. Probes targeting Type Ia methanotrophs in general Ia193 and Ia575 both showed positive results.

Based on the signals from probes,(JRC4-432, JRC2-447, USC3-305, LW21-374, JRC3-535 and MclS402) targeting specifically Type Ib related methanotrophs, i.e., *Methylothermus*, *Methylococcus*, and *Methylocaldum* related, these cells were not as abundant in King Highway Landfill soils as Type Ia methanotrophs, i.e., *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, and *Methylomonas* related methanotrophs, or Type II methanotrophs, i.e., *Methylocystis* and *Methylosinus* related. Japanese rice cluster #2 and #4 (probe JRC2-447 and JRC4-432, respectively) related sequences were also found throughout the depths tested. Weak signals, slightly above detection limit, were obtained from probes MclS402, 501-375, USC3-305, LW21-374, and JRC3-535, which were probes designed to target *Methylocaldum szegediense*, *Methylococcus* related, Upland Soil Cluster #3, LW21 group, and Japanese rice cluster #3, respectively. Although the signals were weak, they indicated that methanotrophs that possessed sequences related to such groups were only present down to 32.5-35 cm and not found at 42.5-45 cm.

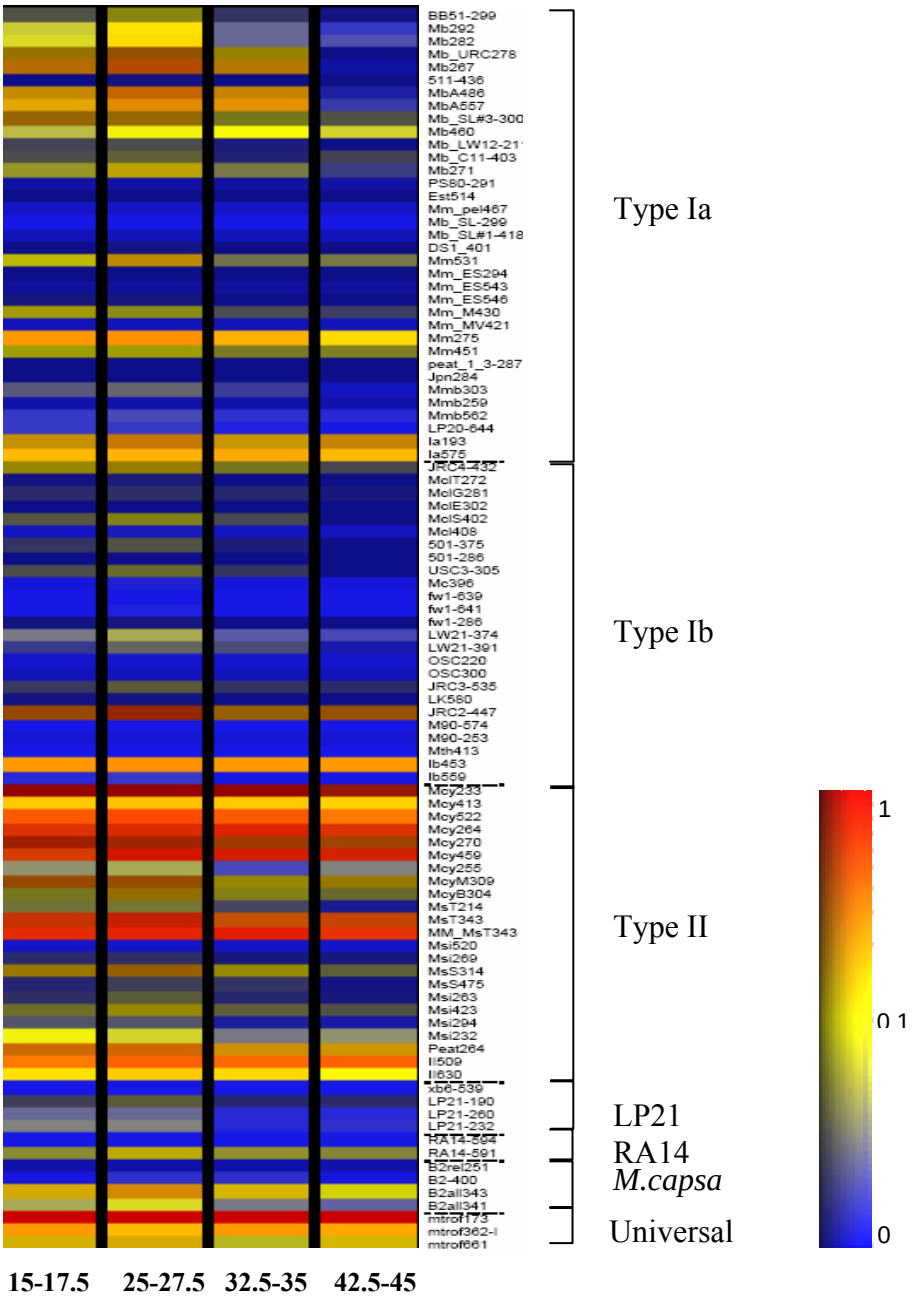

Figure 5-5. DNA microarray using *pmoA* prepared by primer set pmoA189-mb661. Relative signal intensites are shown as color spectrum with 1 being the maximum achievable signal for each probe. DNA was extracted from four different depths, 15-17.5, 25-27.5, 32.5-35, and 42.5-45 cm.

5.3. Discussion

Based on the vertical soil gas profile of King Highway Landfill cover soil (Figure 5-1), it is apparent that the system was not limited in O_2 for oxidation of CH_4. Inorganic nitrogen, (ammonium and nitrate), were present along with trace nutrients of copper and iron that are important for methanotrophic growth. When considering the results from Chapter 4.2.2, i.e., soil microcosm studies done at 20 % CH_4 and 10 % O_2 resembling the conditions seen at depths of approximately 40 cm, the amount of inorganic nitrogen may not be sufficient *in situ* since in soil microcosm studies, the addition of inorganic nitrogen stimulated CH_4 consumption. An alternative hypothesis is that the moisture content was not at optimum *in situ* as moisture content could have an impact on methanotrophic activity by affecting mass transfer of CH_4 and/or O_2.

Overall, Type II methanotrophs predominated the soils over the entire depths that were examined (15-17.5, 25-27.5, 32.5-35, and 42.5-45 cm). Specifically, in the soils tested, members of *Methylocystis* genera were the predominant components of the methanotrophic community. It is not surprising to observe *Methylocystis* species predominating this particular environment as *Methylocystis*-related methanotrophs have been found to be abundant in other landfill cover soils (70, 161). When the vertical profile of soil gas at this site comes into consideration, as shown in Figure 5-1, the predominance of *Methylocystis*-related methanotrophs was to be expected as the high concentrations of CH_4, as Type II methanotrophs, *Methylocystis* and *Methylosinus* genera are reported to have an advantage over the Type Ia methanotrophs, e.g., *Methylobacter* and *Methylomonas*, at high CH_4 to O_2 ratios (80, 161). However, as this analysis was based on the DNA extracted from the site, it cannot be used to infer that *Methylocystis*

were primarily responsible for CH_4 consumption in this soil. In a previous study using mRNA and/or ^{13}C-labelled CH_4 probing of DNA, active methanotrophs did not necessarily result in active expression of *pmoA/mmoX* (40). In the study, transcripts of 16S rRNA and *mxaF*, which encodes the subunit of methanol dehydrogenase, were found for *Methylocystis* but no transcripts of *pmoA* that belong to *Methylocystis* were detected. Therefore, although *Methylocystis* may have a major role in consuming CH_4 in King Highway Landfill cover soil, it could also be possible that instead of consuming CH_4, *Methylocystis* could be consuming methanol produced by other methanotrophs. Without further information on mRNA that is being expressed *in situ* or proteins being synthesized, it remains speculative on which groups of methanotrophs are doing what *in situ*.

Type Ia methanotrophs were also found in this soil but results from certain probes, BB51-302, Mb292, Mb282, , Mb_URC278, Mb267, MbA486, Mb271 and MmM430 showed that Type Ia methanotrophs were present down to as deep as 32.5-35 cm but not at 42.5-45 cm. These probes target *Methylobacter* and *Methylomonas* species. Interestingly, probes Mb460 (*Methylobacter*), Mm531, Mm275, and Mm451 (*Methylomonas*) showed that methanotrophs that are targeted by these probes were found throughout. Type I methanotrophs have been reported to prefer low CH_4 and high O_2 environments while Type II methanotrophs seemed to outcompete Type I methanotrophs in high CH_4 and low O_2 environments (3, 31, 80, 123). Here, it was shown that certain Type Ia methanotrophs were able to gain competitiveness against Type II methanotrophs at low CH_4 to O_2 ratios but there were yet other Type Ia methanotrophs that were able to exist at high CH_4 to O_2 ratios. Another variable that could have some effect in

determining which group of methanotrophs gain advantage over other methanotrophs is the amount of available nutrients. In several studies, it was reported that nutrient-rich environments favored Type I methanotrophs over Type II methanotrophs (26, 32, 33, 180). In this study, in order to minimize the effect of amount of available nutrients in interpreting the results, DNA was extracted from soils samples that contained relatively equal amounts of inorganic nitrogen, i.e., NH_4^+ and NO_3^-. Therefore, although it cannot be entirely ruled out, the effect of nutrients on selection of methanotrophs at least by inorganic nitrogen appear minimal.

When the DNA microarray results presented in this chapter are compared to those of Chapter 4.2.4, the abundance of *Methylocystis*-related methanotrophs was identical in both soils from microcosm studies and the soils obtained as a core from the site. However, the Type Ia methanotrophs, although not as abundant as Type II methanotrophs, were observed in the upper regions of the landfill cover soil while it was almost not detected in soils from microcosm studies. This is not so surprising since the soil used from microcosm studies were sampled from depths of 40-60 cm in the site. At such depths, based on the results for 42.5-45 cm, it appeared that Type Ia methanotrophs were in either very small numbers such that it was under the detection limit or might not have been present. The reason Type Ia methanotrophs were not detected in higher abundance in soil microcosm studies could be attributed to where the soil was sampled from.

The predominance of Type II methanotrophs with Type I methanotrophs being present only minimally in the methanotrophic community structure in soils used for microcosm studies could be explained by the vertical composition of methanotrophic community. As the soils used for microcosm studies were collected from a depth of 40-

60 cm, based on the vertical methanotrophic community structure where the majority of the *pmoA*-containing methanotrophs were affiliated with Type II methanotrophs (Figure 5-5), Type II methanotrophs can be expected to predominate in soils collected for microcosm studies.

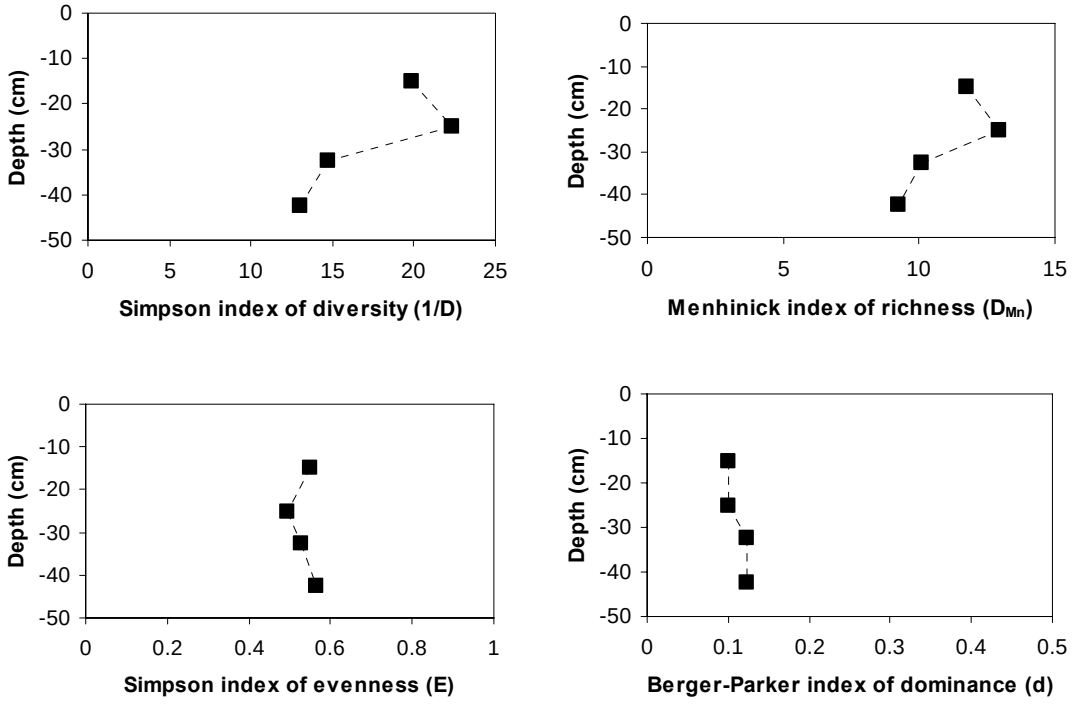

Figure 5-6. Indices of diversity, richness, evenness, and dominance in methanotrophic community composition in King Highway Landfill cover soil.

Indices for diversity, richness, evenness, and dominance in methanotrophic community composition in King Highway Landfill cover soil were calculated following the methods described earlier for DNA microanalysis performed using the same set of probes (70) as shown in Figure 5-6. The indices of diversity (1/D) and richness (D_{Mn}) reflect the loss of positive signals with increasing depth which was observed in the microarray analysis where loss of signals from probes targeting Type I methanotrophs

occurred. The indices for evenness (E) and dominance (d) were relative stable which can be attributed to the strong positive signals retrieved from probes targeting Type II methanotrophs. As Type II methanotrophs, specifically *Methylocystis*-related methanotrophs, dominated the soils at the depths examined, disappearance of Type I methanotrophs with increasing depths was not reflected in these indices, E and d.

5.4. Conclusions

In this chapter, DNA microarray was used to examine the vertical distribution of methanotrophic community composition. It was initially expected to see Type I methanotrophs gaining advantage where CH_4/O_2 was low (near surface) while Type II methanotrophs take advantage where CH_4/O_2 was high (deeper regions). Interestingly, the results showed that although Type I methanotrophs were more abundant where CH_4/O_2 was low, Type II methanotrophs, specifically *Methylocystis*-related methanotrophs, predominated the methanotrophic community at all depths examined. As Type I and II methanotrophs possess different pathways to assimilate carbon, such a difference could be the reason for giving an advantage to Type I methanotrophs where CH_4 is limited. Also, based on the DNA microarray results, not all Type I methanotrophs lost competitiveness with increasing CH_4/O_2. Interestingly, among the probes targeting Type I methanotrophs that were positively hybridized throughout, probes Mm275 and Mm451 increased in signal intensities with the addition of NH_4^+ in the microcosm studies (Chapter 4). If the Type I methanotrophs that are primarily responsible for the N_2O production are ones that can be targeted by probes Mm275 and Mm451, then the problem becomes more complicated as such methanotrophs could exist even at high CH_4/O_2 ratios.

Therefore, understanding the conditions that specifically inhibit the activities of such methanotrophs will become important.

CHAPTER 6. Conclusions and Future work

6.1. Conclusions

The objective of this study was to understand what factors affect CH_4 oxidation by methanotrophs. First, the effects of NMOCs on methanotrophic growth were examined. As NMOCs which are commonly found in landfill gas can bind to MMOs without providing any benefits to the methanotrophs, it was hypothesized that the presence of NMOCs will inhibit the methanotrophic growth. Also, as MMOs can be found in two different forms, pMMO and sMMO, with sMMO having a relatively broad substrate range, it was hypothesized that sMMO-expressing methanotrophs would be affected more significantly by the presence of NMOCs than pMMO-expressing methanotrophs. Furthermore, a dimensionless number was developed which can predict the inhibitory effects of NMOCs on methanotrophic growth. In Chapter 3, the effects of the presence of NMOCs on growth of methanotrophs expressing either form of MMO were examined using custom-designed vials enabling both measurement of growth and degradation of NMOCs. Consistent with the aforementioned hypothesis, with increasing amounts of NMOCs, growth of methanotrophs expressing either form of MMO were both affected. Also, sMMO-expressing cells were affected more than the pMMO-expressing cells. Dimensionless number, Δ, was effective in predicting the inhibitory effects of increasing amounts of multiple NMOCs on methanotrophic growth.

Second, the effects of amendments on CH_4 oxidation and N_2O production were examined by constructing soil microcosms using landfill cover soils collected from King Highway Landfill. The amendments tested were moisture content, copper, different forms of nitrogen (NH_4^+, NO_3^-, and urea), organic carbon, and selective inhibitors phenylacetylene and chlorate. In Chapter 4, it was shown that the addition of inorganic nitrogen could stimulate CH_4 oxidation but also N_2O production. Moisture content, when lowered to 5-10 %, had a stimulatory effect on CH_4 oxidation while inhibitory on N_2O production. The addition of phenylacetylene showed inhibition of N_2O production while not affecting CH_4 oxidation. When soils were maintained at low moisture content, 5 %, and amended with 15 mg-N $NH_4^+ \cdot (\text{kg soil})^{-1}$ in conjunction with 0.1 mg $C_8H_6 \cdot (\text{kg soil})^{-1}$, CH_4 oxidation was stimulated but N_2O production was inhibited. Methanotrophic community composition via DNA microarray analyses indicated that Type I methanotrophs increased in relative abundance with the addition of NH_4^+ but decreased with the addition of phenylacetylene. This indicated that a shift in methanotrophic community composition was responsible for the stimulation of N_2O production with the addition of NH_4^+, but could be reduced with the addition of phenylacetylene. Additionally, transcripts of functional genes were amplified to examine what gene was being expressed when NH_4^+ was added with and without phenylacetylene. Results showed that only *pmoA* was expressed, while *mmoX* and *amoA* were absent or below detection limits, indicating that the methanotrophs were mainly expressing *pmoA* and that pMMO-expressing methanotrophs and not ammonia-oxidizing bacteria were responsible for the production of N_2O when NH_4^+ was added.

In Chapter 5, the vertical distribution of the methanotrophic community composition was examined via DNA microarray analyses. Results showed that Type I methanotrophs were relatively more abundant in environments with low CH_4/O_2, in agreement with the literature, and Type II methanotrophs were abundant throughout. However, not all Type I methanotrophs were limited near the surface where CH_4/O_2 ratios were low. It was found that there were some methanotrophs within Type I methanotrophs that were able to exist where CH_4/O_2 ratios were high. Interestingly, ones that gained in relative abundance with the addition of NH_4^+ in soil microcosm studies were found in such high CH_4/O_2 ratios.

When managing landfills to mitigate greenhouse gas emissions, it will be important to come up with a strategy that can stimulate CH_4 oxidation and inhibit N_2O production. Based on the findings presented here, adding NH_4^+ in conjunction with phenylacetylene at relatively dry soils can stimulate CH_4 oxidation and inhibit N_2O production. This can be achieved by applying NH_4^+ and phenylacetylene to the soils while limiting any further addition of moisture content (H_2O), e.g., rain. In the future, it will be important to create designs which allow efficient drainage in landfill cover soils. Additionally, in landfills where emission of NMOCs is observed along with CH_4, understanding the availability of CH_4 and NMOCs *in situ* will also be important. Once such information is obtained, using the dimensionless number, Δ, developed here, the performance of methanotrophic activities can be predicted. In Chapter 4, it was shown that the addition of NH_4^+ increased the relative abundance of Type I methanotrophs and in Chapter 5, low CH_4/O_2 was important for a diverse and rich methanotrophic community composition. Therefore, if a diverse methanotrophic community composition is desired for maximum CH_4 oxidation, then addition of NH_4^+ to deeper parts of the landfill cover soils may be required.

Alternatively, establishing vegetation that has roots that penetrate deeper into the soil column could be worthwhile due to root-transport of O_2.

6.2. Future Work

The work presented here was performed in batch scales that may not reflect to the actual landfill conditions. As the rate of CH_4 migration upwards *in situ* and methanotroph population size is unknown at this time, it is difficult to determine what the ultimate capacity of CH_4 oxidation would be *in situ*, i.e., how much of the CH_4 generated in the soils would be prevented from being emitted to the atmosphere. Similarly, the extent of N_2O that enters the atmosphere is unknown *in situ*. Therefore, it would be interesting to relate the actual transport phenomena of CH_4 and N_2O to CH_4 oxidation rates and N_2O production/consumption rates, as well as to quantify the number of methanotrophs as a function of depth. By doing so, information on where CH_4 oxidation and N_2O production occurs and to what extent can be gathered. Such information could be useful in management of landfills. Additionally, studies using soil columns fed with ^{13}C-CH_4 could provide very interesting results, as stable isotopes allow separation of labeled nucleic acids or PLFA from the non-labeled, which provides information on the organisms that assimilate the labeled substrates (57). Although stable-isotope-labeled substrates such as ^{13}C-CH_4 and ^{15}N-NH_4^+ have not been used in column studies, it could enable molecular studies to understand the functionality of the active microbial community that is supported by CH_4. Therefore, such studies could not only provide knowledge on what could happen, i.e., the kinetics of CH_4 oxidation and N_2O production, but also on the active microbial community structure. Although stable-isotope-based experiments have been extensively used to study active methanotrophs in soils, the mixing ratio of CH_4 used has been very low, < 1 % CH_4 (33, 41), compared to what is observed *in situ*. As the amount of available CH_4 and possibly O_2 appears to have a significant role in controlling

the composition of methanotrophic community, the amounts of ^{13}C-CH_4 used for such experiments are not a realistic representation of what actually is occurring *in situ*. As a first step, labeled CH_4 can be introduced in the soil microcosm by the gassing system (Chapter 4) to allow a higher mixing ratio of CH_4.

One question of interest would consider what conditions induce sMMO activity *in situ*. Even though some methanotrophs are capable of utilizing sMMO for CH_4 oxidation in controlled laboratory condition, transcripts of *mmoX* have yet to be successfully amplified from soils that consume CH_4, as shown here and elsewhere (41).

Here, phenylacetylene and chlorate were examined for their applicability as selective inhibitors for either nitrifiers or denitrifiers. However, it appears that chlorate was not an effective inhibitor of denitrification. Among the possible candidates as a selective inhibitor of denitrifiers, hippuric acid and benzoic acid appear very interesting since these are naturally occurring compounds. Hippuric acid, which can be found in cattle urine, and benzoic acid, a breakdown compound of hippuric acid, have been shown to inhibit N_2O production via denitrification (105, 171). At this time, however, the mechanism of such inhibition is unclear. Therefore, it would be interesting to elucidate the inhibition mechanism and its selectiveness, i.e., what is the effect of hippuric acid and benzoic acid on CH_4 oxidation? This can be performed using the laboratory setup used for soil microcosm studies done in chapter 4. By doing so, CH_4 and O_2 mixing ratios found *in situ* could be achieved.

Here, the microorganisms responsible for the production of N_2O were determined by examining the changes in abundance and the transcripts of functional genes. As an addition to this molecular technique-based approach, use of N_2O isotopomers could give

an independent perspective on this matter. There have been studies on N_2O isotopomers in order to find distinctive patterns in N_2O produced by the different pathways (164, 165, 168). Isotopomers, or intramolecular distributions of ^{15}N, can come in two forms for N_2O; ^{15}NNO and $N^{15}NO$ (159). Previous studies have shown that different metabolic pathways using pure cultures produce different fractions of isotopomers (164, 165, 168). Also, isotopomer studies have been utilized in soil samples to identify the sources of N_2O, i.e., is the N_2O produced via nitrification or denitrification (177)? The isotopomer studies could provide further knowledge on what is responsible for the production of N_2O in different conditions and at what depths. Therefore, it could be a valuable resource in making decisions on what to do in order to reduce/eliminate N_2O production *in situ*. This can be achieved through the use of custom-designed vials (chapter 3) and the gassing system (chapter 4) such that desired CH_4/O_2 mixing ratios can be prepared while examining the growth of cells. Initially, the work can start by examining the N_2O isotopomers at different growth stages with model organisms to investigate if there are any differences in what stage of growth the cultures are in, and also the type of organism, e.g., *Methylomonas* vs *Methylocystis*. Once this is understood, experiments can be performed with multiple cultures to see if such N_2O isotopomers can be differentiated, eventually moving forward to complex systems.

The role of ammonia-oxidizing archaea, if any, in N_2O production should also be investigated. Here, the focus was on methanotrophs and ammonia-oxidizing bacteria. However, it has been reported that ammonia-oxidizing archaea may be more abundant than ammonia-oxidizing bacteria in soils (114). As only a limited number of cultures of

ammonia-oxidizing archaea have been isolated (104), it is still unknown what such microorganisms can do.

Finally, this work was based on a model methanotroph, *M. trichosporium* OB3b, and soils collected from one landfill cover. It would be interesting to see how different landfill cover soils respond to the same amendments. This could inform whether generalization of amendments can be made for management of different landfill sites. And whatever the outcome is, i.e., different or same responses, further study on what, e.g., methanotrophic community composition or geochemical property of the soils, dictates such behavior on CH_4 oxidation and N_2O production would give more insight to the processes involved. This can be done through similar approaches taken in Chapter 4.

Appendix

Appendix 1. List of oligonucleotide probe sets used. Melting temperatures were calculated using the nearest neighbour method. Updated from previous arrays (19, 161).

Order	Name	Alternative names	Intended specificity	Sequence 5' → 3'	L	GC%	T_m	MM
1	BB51-302	O_BB51-302, O_BB51-299	*Methylobacter*	CGGTTGTTTGTGTCTTAGGTCTG	23	47.8	57.2	
2	Mb292	Mb292	*Methylobacter*	CCGTTACCGTCTGCCTTTCCG	20	60.0	59.1	G/T T AT
3	Mb282	O_Mb282	*Methylobacter*	TTACCGTCTGCCTTTCGGC	19	57.9	58.6	
4	Mb_URC278	P_Mb_URC278	*Methylobacter*	GTTCCGTTACAGACTGCCTTTCGG	24	54.2	61.3	
5	Mb267	P_Mb267	*Methylobacter*	GCATGCTTGTGGTTCCGTTAC	21	52.4	58.1	
6	511-436	511-436	*Methylobacter*	GTTTTGATGCTGTCTGGCAG	20	50.0	55.5	A CC AA
7	MbA486	P_MbA486	*Methylobacter*	AGCATGACATTGACAGCGGTTGTT	24	45.8	61.6	
8	MbA557	P_MbA557	*Methylobacter*	CAATGGCATGATGTTCACTCTGGCT	25	48.0	61.5	
9	Mb_SL#3-300	P_Mb_SL#3-300	*Methylobacter*	GGCGCTGTTGTTTGTGTATTGGGT	24	50.0	62.2	
10	Mb460	Mb460	*Methylobacter*	GACAGTTACAGCGGTAATCGGTGG	24	54.2	60.9	
11	Mb_LW12-211	P_Mb_LW12-211	*Methylobacter*	CGTCTTTGGGTTACTGTTGTGCC	23	52.2	60.0	
12	Mb_C11-403	P_Mb_C11-403	*Methylobacter*	CAAACTTCATGCCTGGTGCTATCGT	25	48.0	61.4	
13	Mb271	Mb271	*Methylobacter*	TTGTGGTGGCGTTACCGGT	18	55.6	58.0	C T C
14	PS80-291	PS80-291	clone PS-80	ACCAATAGGCGCAACACTTAGT	22	45.5	58.3	
15	Est514	Est514	*Methylomicrobium*-related clones	AATTGGCCTATGGTTGCGCC	20	55.0	59.9	
16	Mm_pel467	Mm_pel467	*Methylomicrobium pelagicum*	ACTGCGGTAATCGATGGTTTGGC	23	52.2	61.6	
17	Mb_SL-299	Mb_SL-299	soda lake *Methylobacter* isolates and clones	GGGGTGCAACTCTGTGTATCTTAGG	25	52.0	60.5	T
18	Mb_SL#1-418	O_Mb_SL#1-418	soda lake *Methylobacter* isolates and clones	GCGATCGTATTAGACGTTATCCTGATG	27	44.4	58.6	
19	DS1_401	P_DS1_401	Deep sea cluster #1	GCGCGGTAGTTTGTGTTATGGCT	23	52.2	61.7	
20	Mm531	P_Mm531	*Methylomonas*	CTCCATTGCACGTGCCTGTAGA	22	54.5	60.7	
21	Mm_ES294	P_Mm_ES294	*Methylomonas*	CCAATCGGTGCAACAATTTCTGTAGT	26	42.3	59.8	
22	Mm_ES543	P_Mm_ES543	*Methylomonas*	GTGCCAGTTGAGTATAACGGCATGA	25	48.0	60.9	
23	Mm_ES546	P_Mm_ES546	*Methylomonas*	CCAGTTGAGTATAACGGCATGATGAT	26	42.3	58.7	
24	Mm_M430	P_Mm_M430	*Methylomonas*	TGGACGTGATTTTGATGTTGGGCAA	25	44.0	61.6	
25	Mm_MV421	P_Mm_MV421	*Methylomonas*	CTATCGTGCTGGATACAATCCTGATGT	27	44.4	60.0	
26	Mm275	Mm275	*Methylomonas*	GTGGTGGAGATACCGTTTGCC	21	57.1	59.2	A A
27	Mm451	P_Mm451	*Methylomonas*	CTGATGTTGGGTAACAGCATGACT	24	45.8	58.8	
28	peat_1_3-287	peat_1_3-287	*Mehtylomonas*-related peat clones	AACTGCCTTTAGGCGCTACC	20	55.0	58.6	
29	Jpn284	Jpn284	clone Jpn 07061	ACCGTATCGCATGGGGTG	18	61.1	58.0	
30	Mmb303	Mmb303	*Methylomicrobium album*	CAATGCTGGCTGTTCTGGGC	20	60.0	60.3	A C
31	Mmb259	P_Mmb259	*Methylomicrobium album* + Landfill *M.microbia*	CTGTTCAAGCAGTTGTGTGGTATCG	25	48.0	59.8	
32	Mmb562	O_Mmb562	*Mmb. album* and *Methylosarcina*	ATGGTAATGACCCTGGCTGACTTG	24	50.0	60.6	T
33	LP20-644	LP20-644	*Methylomicrobium*-related clones	GTACACTGCGTACTTTCGGTAA	22	45.5	56.0	
34	Ia193	O_Ia193	Type I a (*M.bacter-M.monas-M.microbium*)	GACTGGAAAGATAGACGTCTATGGG	25	48.0	57.8	T C G
35	Ia575	O_Ia575	Type I a (*M.bacter-M.monas-M.microbium-M.sarcina*)	TGGCTGACTTGCAAGGTTACCAC	23	52.2	61.3	A TC A/T T
36	JRC4-432	P_JRC4-432	Japanese rice cluster #4	GACGTTGTCCTGGCTCTGAG	20	60.0	58.3	
37	MclT272	P_MclT272	*Methylocaldum tepidum*	GGCTTGGGAGCGGTTCCG	18	72.2	61.9	
38	MclG281	P_MclG281	*Methylocaldum gracile*	AAAGTTCCGCAACCCCTGGG	20	60.0	61.5	
39	MclE302	P_MclE302	*Methylocaldum* E10	CGCAACCATGGCCGTTCTG	19	63.2	60.3	
40	MclS402	P_MclS402	*Methylocaldum szegediense*	GCGCTGTTGGTTCCGGGT	18	66.7	61.8	
41	Mcl408	Mcl408	*Methylocaldum*	GGTTCCGGGTGCGATTTTG	19	57.9	57.8	A A G A
42	501-375	P_501-375	*Methylococcus*-related marine and freshwater sediment clones	CTTCCCGGTGAACTTCGTGTTCC	23	56.5	61.3	
43	501-286	O_501-286	*Methylococcus*-related marine and freshwater sediment clones	GTCAGCCGTGGGGCGCCA	18	77.8	66.7	C
44	USC3-305	P_USC3-305	Upland soil cluster #3	CAGGGTCTGCGTTCTGGC	18	66.7	59.5	
45	Mc396	Mc396	*Methylococcus*	CCCTGCCTCGCTGGTGCC	18	77.8	64.4	C A
46	fw1-639	fw1-639	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	GAAGGGCACGCTGCGTACG	19	68.4	62.0	
47	fw1-641	O_fw1-641	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	AGGGCACGCTGCGTACGTT	19	63.2	63.3	
48	fw1-286	P_fw1-286	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGTCAACCGTGGGGCG	18	66.7	61.1	
49	LW21-374	P_LW21-374	LW21 group	CTACTTCCCGATCACCATGTGCT	23	52.2	60.2	
50	LW21-391	P_LW21-391	LW21 group	TGTGCTTCCCCTCGCAGATC	20	60.0	60.5	
51	OSC220	P_OSC220	Finnish organic soil clones and related	TCACCGTCGTACCTATCGTACTGG	24	54.2	60.8	
52	OSC300	P_OSC300	Finnish organic soil clones and related	GGCGCCACCGTATGTGTACTG	21	61.9	61.4	
53	JRC3-535	P_JRC3-535	Japanese Rice Cluster #3	CGTTCCACGTTCCGGTTGAG	20	60.0	59.3	
54	LK580	P_LK580	fw-1 group + Lake Konstanz sediment cluster	CCGACATCATTGGCTACAACTATGT	25	44.0	58.7	
55	JRC2-447	P_JRC2-447	Japanese Rice Cluster #2	CTGAGCACCAGCTACCTGTTCA	22	54.5	60.2	
56	M90-574	O_M90-574	*M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGCCGACCTGCTGGGTTA	20	60.0	62.2	
57	M90-253	O_M90-253	*M.coccus-M.caldum* related marine and freshwater sediment clones	GCTGCTGTACAGGCGTTCCTG	21	61.9	61.7	
58	Mth413	Mth413	*Methylothermus*	CACATGGCGATCTTTTTAGACGTTG	25	44.0	58.3	
59	Ib453	Ib453	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCAGCTACCTGTTCACCGC	20	65.0	61.7	T G
60	Ib559	Ib559	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCATGCTGATGTCGATTGCCG	22	59.1	62.5	C C C
61	DS3-446	P_DS3-446	Deep sea cluster #3	AGCTGTCTGGCAGTTTCCTGTTCA	24	50.0	62.5	
62	JR2-409	P_JR2-409	JR cluster #2 (California upland grassland soil)	TTATTCCCGGCGCTATCATGATCG	24	50.0	60.5	
63	JR2-468	P_JR2-468	JR cluster #2 (California upland grassland soil)	ACAGCCATAATTGGACCATTCTTCTG	26	42.3	59.2	
64	JR3-505	P_JR3-505	JR cluster #3 (California upland grassland soil)	TGTATCCTACCAATTGGCCTCATCTG	26	46.2	60.1	
65	JR3-593	P_JR3-593	JR cluster #3 (California upland grassland soil)	CTATCAGTATGTGCGGACAGGC	22	54.5	58.6	
66	Nc_oce426	Nc_oce426	*Nitrosococcus oceani*	CTTGGATGCCATGCTTGCGA	20	55.0	59.8	
67	USCG-225	P_USCG-225	Upland soil cluster Gamma	CTGACGCCGATCATGTGCAT	20	55.0	59.1	
68	USCG-225b	P_USCG-225b	Upland soil cluster Gamma	CTGACGCCGATCATGTGCATCA	22	54.5	61.2	
69	Mcy233	P_Mcy233	*Methylocystis*	ATTCTCGGCGTGACCTTCTGC	21	57.1	60.9	
70	Mcy413	O_Mcy413	*Methylocystis*	TTCCGGCGATCTGGCTTGACG	21	61.9	63.2	C C
71	Mcy522	O_Mcy522	*Methlocystis* A + peat clones	GGCGATTGCGGCGTTCCA	18	66.7	62.3	C
72	Mcy264	P_Mcy264	*Methylocystis*	CAGGCGTTCTGGTGGGTGAA	20	60.0	61.0	
73	Mcy270	P_Mcy270	*Methylocystis*	TTCTGGTGGGTGAACTTCCGTCT	23	52.2	61.8	
74	Mcy459	P_Mcy459	*Methylocystis*	GTGATCACGGCGATTGTTGGTTC	23	52.2	60.2	

Appendix 1. (continued)

75	Mcv255	O Mcy255	*M.cystis B (parvus/echinoides/* strain M)	GGCGTCGCAGGCTTTCTGG	19	68.4	62.3	
76	McvM309	P McyM309	*M.cystis strain M and related*	GGTTCTGGGCCTGATGATCGG	21	61.9	61.0	
77	McvB304	P McyB304	*M.cystis B (parvus/echinoides/* strain M)	CGTTTTCGCGGCTCTGGGC	19	68.4	62.7	
78	MsT214	P MsT214	*Methylosinus trichosporium* OB3b and rel.	TGGCCGACCGTGGTTCCG	18	72.2	63.5	
79	MsT343	P MsT343	*Methylosinus trichosporium* OB3b and rel.	TCAACCGCTACTGCAACTTCTGG	23	52.2	60.9	
80	MM MsT343	P MM MsT343	*Methylosinus trichosporium* OB3b and rel. - MM control probe!	TCAACCGCTACTTCAACTTCTGG	23	47.8	58.5	
81	Msi520	Msi520	*Methylosinus trichosporium*	GCGATCGCGGCTCTGCA	17	70.6	61.6	
82	Msi269	O Msi269	*Methylosinus trichosporium*	TCTTCTGGGAGAACTTCAAGCT**G**C	24	50.0	60.6	C
83	MsS314	P MsS314	*Methylosinus sporium*	GGTTCTGGGTCTGCTCATCGG	21	61.9	60.8	
84	MsS475	P MsS475	*Methylosinus sporium*	TGGTCGGCGCCCTGGGCT	18	77.8	68.3	
85	Msi263	P Msi263	*Methylosinus sporium* + 1 *Msi.trichosporium subclaster*	GGCGTTCCTGTGGGAGAACTTC	22	59.1	61.2	
86	Msi423	P Msi423	*Methylosinus*	CTGTGGCTGGACATCATCCTGC	22	59.1	61.4	
87	Msi294	O Msi294	*Methylosinus*	**G**TTCGGCGCGACC**TT**C**GC**	18	72.2	62.5	T AC TCT
88	Msi232	Msi232	*M.sinus* + most *M.cystis* -considered as additional type II probe	AT**C**CT**G**GGCGTGAC**C**TTC**GC**	20	65.0	63.3	T C G TG
89	Peat264	Peat264	peat clones	GGCGTTTTTCTGGGTCAACTTCC	23	52.2	60.3	
90	II509	O II509	Type II	CGAACAACTGGCCGGCGAT	19	63.2	61.7	
91	II630	O II630	Type II	**C**AT**GG**T**C**GA**G**CGCGGCAC	18	72.2	62.4	G CA G A
92	xb6-539	xb6-539	Novel *pmoA* copy of type II and related environmental clones	AGGCCGCCGA**G**GTCGA**C**	17	76.5	63.0	A T
93	LP21-190	LP21-190	Novel *pmoA* copy of type II and related environmental clones	ATCGACTTCAAGGATCGCCG	20	55.0	58.2	
94	LP21-260	O LP21-260	Novel *pmoA* copy of type II and related environmental clones	CGCAGTC**C**TTCTTCTGGACG	20	60.0	58.6	G
95	NMcv1-247	P NMcy1-247	Novel *pmoA* copy of *M.cystis #1* (?)	TCGACATCGTGCTGATGATCTCGG	24	54.2	62.1	
96	NMcv2-262	P NMcy2-262	Novel *pmoA* copy of *M.cystis #2* (?)	CAGTCCTTCTTCTGGCAGAAGTTCC	25	52.0	60.9	
97	NMsiT-271	P NMsiT-271	Novel *pmoA* copy of *M.sinus trichpsporium* (?)	ACCGCTTCCGTCTGCCGAT	19	63.2	62.9	
98	LP21-232	LP21-232	Novel *pmoA* copy of type II and related environmental clones	ATCGTCGCCATGTGCTTCGC	20	60.0	61.9	
99	RA14-594	O RA14-594	RA14 related clones	CCACAACGTTCGTACCTCGA	20	55.0	57.9	
100	RA14-591	P RA14-591	RA14 related clones	GGCTTCCACAACGTTCGTACCT	22	54.5	60.9	
101	Wsh1-566	P Wsh1-566	Watershed + flodded upland cluster 1	GCTCATGAGCTTGGCCGACATC	22	59.1	61.8	
102	Wsh2-491	P Wsh2-491	Watershed + flodded upland cluster 2	TCATTTGGCCAACCTCTCTCATTCC	25	48.0	60.9	
103	Wsh2-450	P Wsh2-450	Watershed + flodded upland cluster 2	CAAGAGCTGGATCATCACGATG	22	50.0	56.8	
104	B2rel251	O B2rel251	*Methylocapsa*-related clones	CCGCCGCGGCCCAGTATTA	19	68.4	63.4	
105	B2-400	B2-400	*Methylocapsa*	ACCTCTTTGGTCCCGGCTGC	20	65.0	63.4	
106	B2all343	B2all343	*Methylocapsa* and related clones	AACCG**C**TAC**AC**CAA**T**TTCTGGG**G**	23	52.2	61.2	A GT C A
107	B2all341	O B2all341	*Methylocapsa* and related clones	TCAACCGCTACACCAATTTCTGGG	24	50.0	61.1	
108	pmoAMO3-400	pmoAMO3-400	clone pmoA-MO3	A**C**CC**AGA**TG**A**TCCGTCGGC	20	65.0	62.6	G TTG T
109	ESR-579	P ESR-579	ESR (Eastern Snake River) cluster	GACCTGATCGGATTCGAGAACATC	24	50.0	58.5	
110	TUSC409	P TUSC409	Tropical Upland Soil Cluster #2	CGATCCCGGGCGCGATTC	18	72.2	61.8	
111	TUSC502	P TUSC502	Tropical Upland Soil Cluster #2	TCTTCTACTTCGGCAACTGGC	21	52.4	58.3	
112	mtrof173	mtrof173	Universal	GGbGACTGGGACTTCTGG	18	66.7	57.4	
113	mtrof362-I	mtrof362-I	Methanotrophs	TGGGGCTGGACCTACTTCC	19	63.2	59.5	
114	mtrof661	mtrof661	Methanotrophs	GGTAARGACGTTGCKCCGG	19	63.2	60.4	
115	mtrof662-I	mtrof662-I	Methanotrophs	GGTAAGGACGTTGCGCCGG	19	68.4	61.9	
116	mtrof656	mtrof656	Methanotrophs	ACCTTCGGTAAGGACGT	17	52.9	53.2	
117	NmNc533	NmNc533	*Nitrosomonas-Nitrosococcus*	**C**AACCCAT**T**TGCCAATCGTTGTAG	24	45.8	58.6	G C
118	Nsm eut381	Nsm eut381	*Nitrosomonas eutropha*	CCACTCAATTTTGTAACCCCAGGTAT	26	42.3	59.0	
119	PS5-226	PS5-226	*Nitrosomonas-Nitrosococcus* related clones	ACCCCGATTGTTGGGATGATGTA	23	47.8	59.9	
120	Pl6-306	Pl6-306	*Nitrosomonas-Nitrosococcus* related clones	GGCACTCTGTATCGTATGCCTGTTAG	26	50.0	60.5	
121	NsNv207	NsNv207	*Nitrosospira-Nitrosovibrio*	TCAATGGTGGCCGGT**G**G	17	64.7	58.5	T
122	NsNv363	NsNv363	*Nitrosospira-Nitrosovibrio*	**T**AC**T**C**G**TGGTC**G**CA**C**TA**C**CC	20	60.0	59.6	C G C A T T
123	Nit rel471	P Nit rel471	AOB related clones/probably methanotrophs	CGTTCGCGATGATGTTTGGTCC	22	54.5	60.1	
124	Nit rel223	Nit rel223	AOB related clones/probably methanotrophs	GTCACACCGATCGTAGAGGT	20	55.0	56.9	
125	ARC529	P ARC529	AOB related clones/probably methanotrophs	TAAGCAGCCGATGGTCGTGGAT	22	54.5	62.2	
126	Nit rel470	Nit rel470	AOB related clones/probably methanotrophs	CGATA**T**TCGGGGTATGGGCG	20	60.0	58.4	A
127	Nit rel351	Nit rel351	AOB related clones/probably methanotrophs	GTTTGCCTGGTACTGGTGGG	20	60.0	59.2	
128	Nit rel304	Nit rel304	AOB related clones/probably methanotrophs	CGCTCTGCATTCTGGCGCT	19	63.2	61.8	
129	M84P105-451	M84P105-451	environmental clones of uncertain identity	AACAGCCTGACTGTCACCAG	20	55.0	58.1	
130	WC306 54-385	WC306 54-385	environmental clones of uncertain identity	AACGAAGTACTGCCGGCAAC	20	55.0	59.2	
131	M84P22-514	M84P22-514	environmental clones of uncertain identity	AACTGGGCCTGGCTGGG	17	70.6	61.0	
132	gp23-454	gp23-454	environmental clones of uncertain identity	AACGCGCTGCTCACTGCG	18	66.7	62.3	
133	MR1-348	MR1-348	environmental clones of uncertain identity	AATCTTCGGTTGGCACGGCT	20	55.0	61.1	
134	gp619	P gp619	environmental clones of uncertain identity	CGGAATATCTGCGCATCATCGAGC	24	54.2	61.5	
135	gp391	gp391	environmental clones of uncertain identity	A**T**CTGGCCGGCGA**CCATG**	18	66.7	61.1	A TTGCC
136	gp2-581	gp2-581	environmental clones of uncertain identity	ACATGATCGGCTACGTGTATCCG	23	52.2	60.0	
137	RA21-466	RA21-466	clone RA21 - environmental clone of uncertain identity	CGGCGTTCTTGGCGGCAT	18	66.7	62.4	
138	hyaBp	hyaBp	spiking control (*hyaB* gene of E.coli)	GATTACGCGCATCGAAGGC	19	57.9	57.5	

N . O and P in front of the probe names indicates that the probes were added during the first, second and third major update of the array
MM indicates the mismatches in the most closely related sequences (their position is indicated by bold-underlined in the probe sequence)

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