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ABSTRACT

The overall objective of this project, "Strategies to Optimize Microbially-Mediated Mitigation of Greenhouse Gas Emissions from Landfill Cover Soils" was to develop effective, efficient, and economic methodologies by which microbial production of nitrous oxide can be minimized while also maximizing microbial consumption of methane in landfill cover soils. A combination of laboratory and field site experiments found that the addition of nitrogen and phenylacetylene stimulated in situ methane oxidation while minimizing nitrous oxide production. Molecular analyses also indicated that methane-oxidizing bacteria may play a significant role in not only removing methane, but in nitrous oxide production as well, although the contribution of ammonia-oxidizing archaea to nitrous oxide production can not be excluded at this time. Future efforts to control both methane and nitrous oxide emissions from landfills as well as from other environments (e.g., agricultural soils) should consider these issues. Finally, a methanotrophic biofiltration system was designed and modeled for the promotion of methanotrophic activity in local methane “hotspots” such as landfills. Model results as well as economic analyses of these biofilters indicate that the use of methanotrophic biofilters for controlling methane emissions is technically feasible, and provided either the costs of biofilter construction and operation are reduced or the value of CO_2 credits is increased, can also be economically attractive.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

The overall objective of this project, "Strategies to Optimize Microbially-Mediated Mitigation of Greenhouse Gas Emissions from Landfill Cover Soils" was to develop effective, efficient, and economic methodologies by which microbial production of nitrous oxide can be minimized while also maximizing microbial consumption of methane in landfill cover soils. These issues are coupled as attempts to enhance microbial consumption of methane typically stimulates biogenic nitrous oxide production. As methane and nitrous oxide are both potent greenhouse gases, with global warming potentials approximately 25 and 296 times that of carbon dioxide, respectively, it is imperative holistic strategies be developed that uncouple methane consumption and nitrous oxide production.

To consider these issues, the effects of a matrix nutrient amendments on methane oxidation and nitrous oxide production as well as microbial community structure and activity were examined by (1) constructing soil microcosms using landfill cover soils and (2) performing an 18 month pilot-scale demonstration of the most promising amendments at the King Highway Landfill in Kalamazoo Michigan.

In both the microcosm studies and field demonstration project, it was found that the addition of ammonium in the presence of phenylacetylene stimulated methane oxidation but inhibited nitrous oxide production. In the microcosm studies, molecular analyses indicated that Type II methanotrophs predominated, 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 observed in the microcosms. In the field demonstration project, the addition of NH_4^+ and NO_3^- caused overall methanotrophic diversity to decrease, with a significant reduction in the presence of Type I methanotrophs. The simultaneous addition of phenylacetylene caused methanotrophic diversity to increase, with greater presence of Type I methanotrophs. Clone libraries of the archaeal *amoA* gene showed that the addition of nitrogen increased AOAs affiliated with Crenarchaeal group 1.1b, while they decreased with the simultaneous addition of phenylacetylene.

Several pure cultures of methanotrophs were investigated to examine the relative importance of methanotrophic mediated N_2O production. Five out of six Type II methanotrophic strains produced 32 - 342 ppmv of N_2O, which are equivalent to 0.16-1.7% of the conversion rate of NH_4^+-N to N_2O-N, while two Type I strains did not produce detectable amount of N_2O. Collectively, these results suggest that the addition of phenylacetylene with NH_4^+ and NO_3^- reduces N_2O production by selectively inhibiting archaeal ammonia-oxidizers and/or Type II methanotrophs.

Finally, methanotrophic biotrickling filtration system was designed and modeled for the promotion of methanotrophic activity in local methane “hotspots” such as landfills. Model results indicate that assuming the global average atmospheric concentration of methane, 1.7 ppmv, methane removal is ineffective using these methanotrophic biofilters as the methane concentration is too low to enable cell survival. If the concentration is increased to 500-6000 ppmv, however, similar to that found above landfills, 4.98 – 35.7 tons of methane can be removed per biofilter per year assuming biotrickling filters of typical size (3.66 m in diameter

and 11.5 m in height). Using reported ranges of capital, operational, and maintenance costs, the cost of the equivalent ton of CO_2 removal using these systems is $90 – $910 ($2,070 – $20,900 per ton of methane), depending on the influent concentration of methane and if heating is required. The use of methanotrophic biofilters for controlling methane emissions is technically feasible, and provided either the costs of biofilter construction and operation are reduced or the value of CO_2 credits is increased, can also be economically attractive.

1. INTRODUCTION

Methane, a major greenhouse gas, has a global warming potential (GWP) ~25 times higher than that of CO_2 (IPCC, 2007). 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 (Qian, et al., 2002), as shown in Table 1.

Table 1. Typical constituents in municipal solid waste landfill gas (Qian, et al., 2002)

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 the USA emitted 132 Tg CO_2 equivalent of CH_4 from landfills (municipal solid waste and industrial landfills combined) (US EPA, 2007). 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 (US EPA, 2007). Global estimates of CH_4 emissions show that CH_4 emissions from landfills are 35-40 Tg $CH_4 \cdot yr^{-1}$, ~ 6-8 % of the total emission of CH_4 including both natural and anthropogenic sources (Fung, et al., 1991; Hein, et al., 1997; Lelieveld, et al., 1998b). A detailed list of sources of anthropogenic CH_4 is shown in Table 2 (US EPA, 2007).

Table 2. Recent trends of CH_4 emissions in the USA. Numbers in parentheses represent the percent of 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 (US EPA, 2007). Specifically, as shown in Table 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 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**

1.1. 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 (Vesilind and Pereira, 1980). 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 (Qian, et al., 2002). 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. Therefore, passive gas collection systems are relatively inexpensive but as it vents soil gas directly into the atmosphere, it can pose some environmental risk.

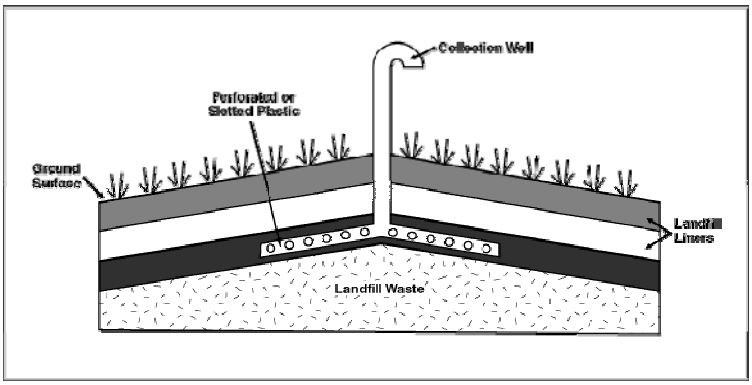


Figure 1. Passive gas collection system for release of landfill gas into the atmosphere (ATSDR, 2001)

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 (US EPA 1996, 1998). These rules require landfill gases to be collected and either flared or utilized at landfills that: 1) have a design capacity larger than 2.5 million metric tons and 2.5 million cubic meters, and; 2) 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 2.

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 (US EPA, 1999).

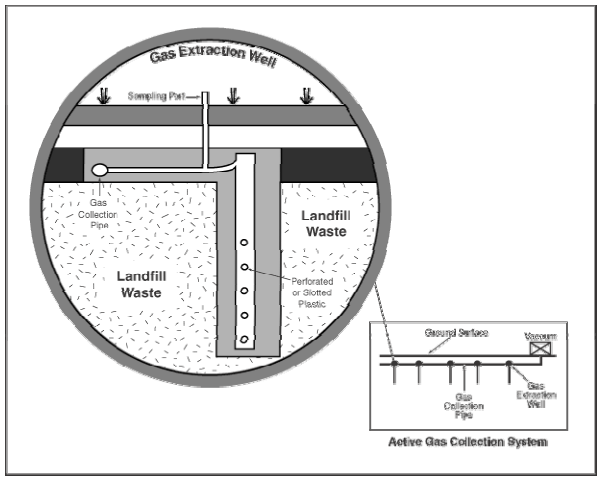


Figure 2. Active gas collection system for treatment of gas, e.g. energy generation, incineration (ATSDR, 2001)

It is estimated that as of 2004, more than 100,000 closed landfills exist in the USA (Sulflita, et al., 1992), as well as 1654 active landfills (Simmons, et al., 2006). 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 (Gebert, et al., 2004; Streese and Stegmann, 2003). 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.

1.2. 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 (Lelieveld, et al., 1998a). The major sink of atmospheric CH_4 is the reaction of CH_4 with OH• in the troposphere. In the troposphere CH_4 initially reacts with OH• to produce CH_3•(Ravishankara, 1988). CH_3• then further undergoes chemical reaction and produces CO, CO_2, and H_2O among other compounds. The OH• sink in the troposphere is reported to be responsible for ~510 $Tg(CH_4)\cdot yr^{-1}$ (IPCC, 2007, Lelieveld, et al., 1998a. 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 $Tg(CH_4)\cdot yr^{-1}$ (Lelieveld, et al., 1998a).

Another sink of CH_4 is via soil microbial activity. In soils, CH_4 can be oxidized by methanotrophs, are a group of bacteria that utilize CH_4 as its sole carbon and energy source. It has been estimated that anywhere from 10 to 100 % of the CH_4 generated in landfills is oxidized by these bacteria (Borjesson, et al., 2001; Chanton and Liptay, 2000; Chanton, et al., 1999; Czepial, et al., ,1996; Liptay, et al., 1998; Whalen, et al., 1990). Interestingly, there have been reports where landfills have acted as sinks of CH_4 rather than as sources (Bogner, et al., 1995, 1997). 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 (Bodelier, et al., 2000; De Visscher and van Cleemput, 2003; Mohanty, et al., 2006) 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 (IPCC, 2007). Thus, a strategy to mitigate one greenhouse gas, CH_4, could result in the production of a relatively more potent greenhouse gas, N_2O.

1.3. Physiology and phylogeny of methanotrophs

As mentioned above, methanotrophs are a group of bacteria that utilize CH_4 as their sole carbon and energy source. In the general methane oxidation pathway shown in Figure 3, methane is initially hydroxylated to methanol by the methane monooxygenase (MMO), which is further oxidized to formaldehyde by a periplasmic methanol dehydrogenase (MDH). In the catabolic pathway, formaldehyde is oxidized to CO_2 via formate by formaldehyde dehydrogenase (FalDH) and formate dehydrogenase (FDH), yielding reducing equivalents as either quinol or NADH. In the anabolic pathway, formaldehyde is incorporated into cell biomass via incorporation into either ribulose monophosphate (RuMP) or serine pathway, depending on the type of methanotroph. 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^- (Leak and Dalton, 1986).

Of particular importance here is the first step of CH_4 oxidation that 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 (Murrell, et al., 2000a; Semrau, et al., 2010`).

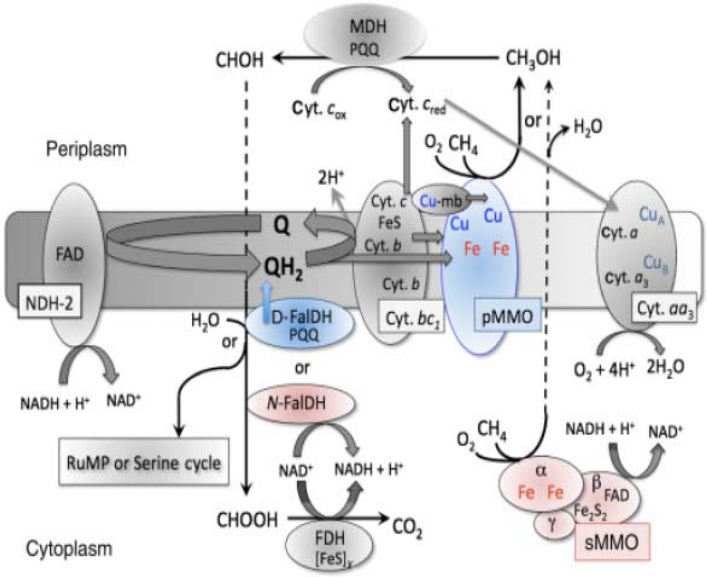


Figure 3. Proposed pathways of CH_4 oxidation in cells cultured under high and low Cu conditions. Proteins showing positive or negative Cu-regulation are shown in blue and red, respectively. Abbreviations: CytC, cytochrome *c*; D-FalDH, dye-linked/quinine-linked formaldehyde dehydrogenase; FDH, formaldehyde dehydrogenase; N-FlalDH, NAD(P)-linked formaldehyde dehydrogenase; NDH-2, type 2 NADH dehydrogenase; pMMO, membrane-associated or particulate methane monooxygenase; Q, ubiquinone; FAD, flavin adenine dinucleotide; MDH, methanol dehydrogenase; PQQ, pyroloquinoline quinine; sMMO, cytoplasmic or soluble methane monooxygenase; RuMP, ribulose monophosphate (Semrau et al., 2010).

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) (Semrau, et al., 2010). Recently, however, acidophilic bacteria belonging to the phylum Verrucomicrobia exhibiting CH_4 oxidation capabilities have been isolated (Dunfield, et al., 2007; Islam, et al., 2008; Pol, et al., 2007) 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 4.

Table 4. Characteristics of Type I and II methanotrophs and methanotrophic Verrucomicrobia

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* (Semrau, et al., 2010). Type I methanotrophs can further be divided into two different groups, Type Ia and Ib, where Type Ib methanotrophs are comprised of the moderate thermophiles, *Methylococcus*, *Methylocaldum* and *Methylothermus* whereas the remaining mesophilic 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* (Semrau, et al., 2010). In addition to the traditionally accepted Type I methanotrophs that belong to γ-Proteobacteria, filamentous microorganisms *Crenothrix polyspora* and *Clonothrix fusca* have also been reported to be methane-oxidizing γ-Proteobacteria (Semrau, et al., 2010; Stoecker, et al., 2006; Vigliotta, et al., 2007). Phylogenetic analysis based on 16s rRNA sequences of selected methanotrophs are shown in Figure 4.

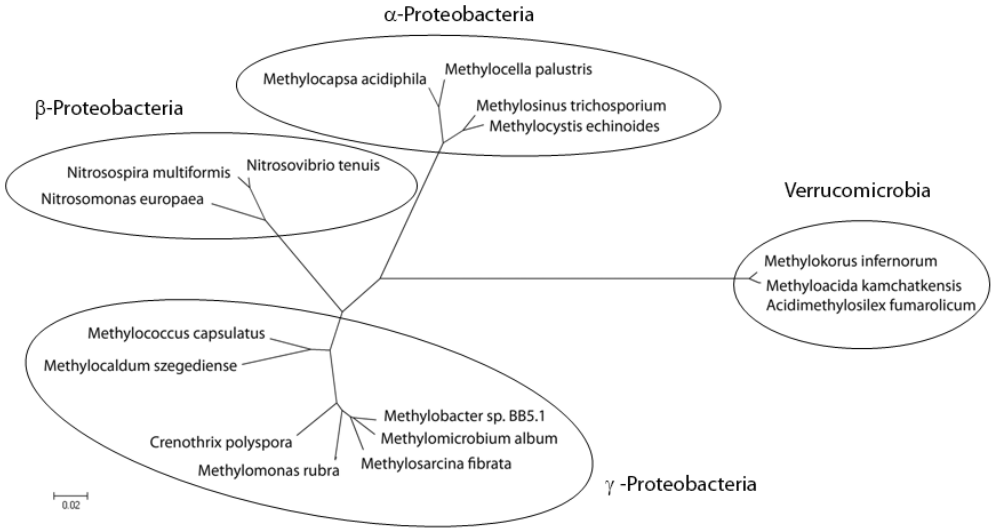


Figure 4. Phylogenetic tree constructed from 16s rRNA gene sequences using MEGA4 (Tamura, et al., 2007). The tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with 1304 positions of 16s rRNA. The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, et al., 2004) with the scales indicating 0.02 base substitutions per site (Semrau, et al., 2008).

The most significant difference found among Type I and II methanotrophs and methanotrophic Verrucomicrobia is the structure of intracytoplasmic membranes (ICM). All Type I methanotrophs that have been characterized to date possess stacks of vesicular disks, while most of the Type II methanotrophs possess 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. *Methylocella* has a vesicular membrane system connected to the cytoplasmic membrane, found on the periphery of the cytoplasm (Dedysh, et al., 2000, 2004; Dunfield, et al., ,2003; Semrau, et al., 2010), while *Methylocapsa* contain packed vesicular membranes aligned to only one side of the cell (Dedysh, et al., 2002). The methanotrophs belonging to the phylum Verrucomicrobia have yet another different membrane system, i.e., vesicular/tubular membranes or filled with polyhedral organelles ((Dunfield, et al., 2007; Islam, et al., 2008; Pol, et al., 2007. 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* (Pol, et al., 2007) or carboxysomes which are normally found in cyanobacteria and chemoautotrophs (Islam, et al., 2008).

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 (Bodrossy, et al., 1997; Semrau, et al., 2010). 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 (Dunfield, et al., 2007; Pol, et al., 2007). Additionally, in these studies, all the genes to form a complete Calvin-Benson cycle were identified (Dunfield, et al., 2007) or genes for Ribulose-1,5-bisphosphate carboxylase/oxylase (RuBisCO) was detected (Pol, et al., 2007). Unlike the RuMP and serine pathway where formaldehyde, a product of the oxidation of methanol shown in Figure 3, is utilized to produce biomass, the Calvin-Benson cycle utilizes CO_2. It may be that methanotrophic Verrucomicrobia utilize a mixture of pathways for carbon assimilation.

1.4. 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 5. Similar to ammonia monooxygenase genes in *Nitrosomonas europaea* (Chain, et al., 2003), some methanotrophs have been found to have multiple copies of pMMO genes (Ward, et al., 2004).

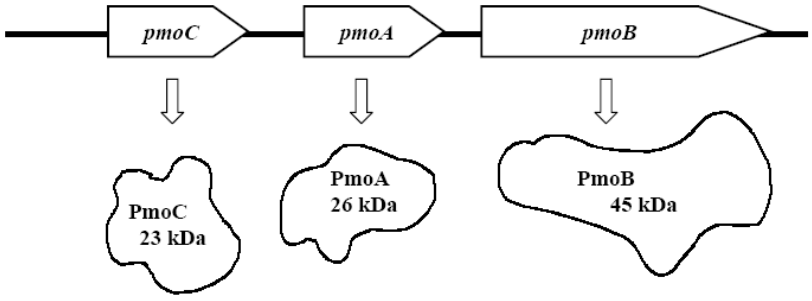


Figure 5. Gene cluster of pMMO (Murrell, et al., 2000b)

Genes that encode for sMMO are also clustered in the chromosome of methanotrophs (Murrell, et al., 2000b) as shown in Figure 6. 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 (Merkx and Lippard, 2002), but its function is still unclear.

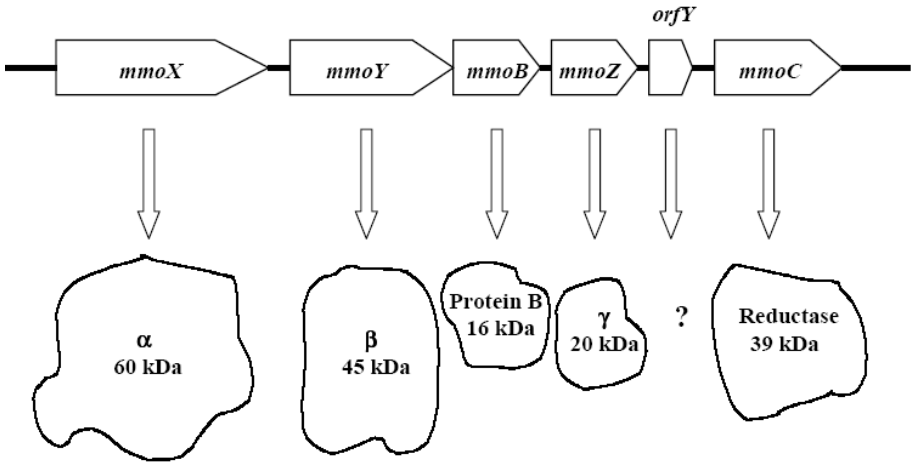


Figure 6. Gene cluster of sMMO (Murrell, et al., 2000b)

1.5 Kinetics of MMO

The kinetics of CH_4 oxidation by both MMOs have been reported as shown in Table 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 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](Han, et al., 1999); [b](Oldenhuis, et al., 1991); [c](Lontoh and Semrau, 1998)

1.6. Factors affecting methanotrophic community structure and activity

Little is known how different environmental conditions affect the distribution, numbers and activity of methanotrophs other than Type I and II strains. The following sections highlight the information collected to date.

1.6.1. Moisture Content

As discussed above, the possibility that Type II methanotrophs may dominate over time as they may turnover methane more quickly is intriguing, but one must be aware that several other factors have been shown to affect methanotrophic community composition and activity, including moisture content, with the general conclusion that methanotrophic activity has some optimal moisture content.

Specifically, high moisture contents have been shown to limit methanotrophic activity, likely due to limited diffusion of CH_4 and air (Bender and Conrad, 1995; Czepial, et al., 1996; Jones and Nedwell, 1993; Whalen and Reeburgh, 1990). Not all studies agree, however, as high moisture contents (>50%) can actually stabilize CH_4 consumption rates, possibly by inducing methanotrophic growth, by lowering dissolved oxygen concentrations, or by making bioavailable other organic compounds that may facilitate methanogenic activity deep in the soil column (Benstead and King, 1997; West and Schmidt, 1998). At low moisture contents where diffusion is not the rate-limiting step on CH_4 availability, methanotrophic activity has also been observed to be inhibited, likely due to increased osmotic stress and/or desiccation (Czepial, et al., 1996, Conrad, 1996; Jäckel, et al., 2001; Schnell and King, 1996).

From these conflicting findings, it is still not possible to clearly identify the mechanism(s) by which moisture content affects methanotrophic activity. It is possible that the conflicting results reported may be due to different communities with different activity (i.e., different MMO expression) having a competitive advantage under different wetting regimes. Such a hypothesis is supported from the findings of Henckel, et al. (2001). In this study, drainage of rice field soils revealed that Type I and II methanotrophs were differentially affected by reducing water content. Specifically it was discovered that Type I methanotrophs were more diverse after 8 days of drainage, but not evenly distributed vertically whereas Type II cells were still present throughout soils cores, but their composition was not drastically changed. Such a finding suggests that Type I methanotrophs may be more adaptable to changing environmental conditions, as indicated by other studies where methane and oxygen were varied (Henckel, et al., 2000a). Auman, et al., 2000).

1.6.2. Temperature

From the limited number of phylogenetic studies done to date on the effect of temperature on methanotrophic communities it appears that Type I methanotrophs dominate at low temperatures in biofilters (Gebert, et al., 2003, 2004), and all characterized psychrophilic methanotrophs to date are within the γ-*Proteobacteria*. This conclusion is supported by more recent studies of methanotrophic communities in landfill soils, where it was found that Type I signals were more dominant at 10º C than 20º C using PLFA analyses (Börjesson, et al., 2004). It has been well-documented, however, that temperature changes typically have little effect on overall methanotrophic activity in soils, with Q_{10} values typically between 1-2 (Börjesson et al., 2004; Crill, et al., 1994; King and Adamsen, 1992; Roslev et al., 1997; Whalen et al., 1990). Such low values are attributed to slow mass transfer of methane (Dunfield, 2007), although occasionally higher Q_{10} values are reported in soils that may have higher gas diffusivity (Christophersen, et al., 2000; MacDonald, et al., 1997) or exposed to methane concentrations greater than 10,000 ppmv (De Visscher, et al., 2001). At temperature extremes, however, i.e., <10 ºC and >40 ºC, methane oxidation is significantly limited in forest and landfill cover soil samples (Boeckx and Van Cleemput, 1996; Boeckx, et al, 1996; Christophersen, et al., 2000; Czepial, et al., 1996; Whalen and Reeburgh, 1996), likely due to inhibition of mesophilic methanotrophs.

1.6.3. Inorganic nitrogen

In general, NH_4^+ is found to inhibit CH_4 oxidation, whereas NO_3^- is found to have little influence (Boeckx and Van Cleemput, 1996). However, many conflicting results about the influence of inorganic N on CH_4 oxidation can be found in the literature.

King and Schnell (1994a, b) investigated the influence of the CH_4 concentration on NH_4^+ inhibition of CH_4 oxidation, both in the field and in batch cultures of *Methymicrobium album* BG8 (Type I) and *Methylosinus trichosporium* OB3b (Type II). In both cases, inhibition initially increased progressively with increasing CH_4 concentration from 1.7 to 100 ppm in the presence of 500 μM of ammonium, although the extent of inhibition decreased with CH_4 concentrations above 250 ppm. The increase of the inhibition was attributed to an increased formation of NO_2^- from NH_4^+ oxidation. The counter-intuitive findings of increased inhibition of CH_4 oxidation as CH_4 concentration increased can occur as the oxidation of CH_4 generates NADH + H^+, which can stimulate NH_4^+ oxidation. However, in the field no NO_2^- was observed. This could indicate that endogenously produced NO_2^- is a stronger inhibitor of CH_4 oxidation than exogenous NO_2^- (Schnell and King, 1994). Conversely, Boeckx et al. (1996) found that the first-order reaction rate constant of CH_4 oxidation in a landfill cover soil decreased as amounts of added NH_4^+ increased. Similarly, Hutsch (1998) reported application of 40 mg N kg^{-1} caused a strong inhibition of CH_4 oxidation up to 96%. Other studies, however, found that addition of ammonia actually enhanced methanotrophic population size and/or activity (Bender and Conrad, 1995; Hilger, et al., 2000; Krüger, et al., 2001), especially when added after a brief exposure to CH_4 (DeVisscher et al., 1999; 2001). Bender and Conrad (1995) also found that the methanotrophic activity was induced by the presence of NH_4^+ and the optimum concentration of NH_4^+ was 12-61 mM in the soil water phase at 100 μl of CH_4 l^{-1}. Mohanty and colleagues (2006) have shown that the addition of ammonia to rice paddy soil and forest soils selectively stimulated the growth of Type I methanotrophs. Similarly, the addition of urea also allowed Type I methanotrophs to outcompete Type II methanotrophs in landfill soils (Noll et al., 2008). These results suggest that fertilization of environments dominated by Type I methanotrophs will have little effect on CH_4 uptake, but those dominated by Type II methanotrophs could affect negatively due to changes of the methanotrophic community composition (Mohanty et al., 2006).

Combined these findings of both inhibition and enhancement of methanotrophic activity with the addition of ammonia make it difficult to interpret these data and develop an over-arching theory. It has been speculated that these contradictory findings may be due to either relief during nitrogen limitation in some situations which leads to community shifts or coupling of methane oxidation with nitrogen assimilation which leads to increased intracellular competition and consumption of reducing equivalents (Bodelier and Laanbroek, 2004). These findings are particularly important for high methane environments such as landfill cover soils, where the molar ratio of methane to nitrogen is high. At high methanotrophic activity, nitrogen limitation will likely be very significant (De Visscher et al., 1999). To circumvent this problem, nitrogen source amendment has been proposed, but such additions can increase nitrous oxide emissions, which is the third most important greenhouse gas after CO_2 and CH_4 (Majumdar, 2003).

1.6.4. Copper

Perhaps the most important factor controlling methanotrophic activity is the copper:biomass ratio. The general effect of copper on methanotrophic activity, specifically on the relative expression of sMMO and pMMO has been well known for some time (Dalton, 2005; Dalton et al. 1983, 1984; Scott, et al., 1981; Stanly et al, 1983). Initial studies from H. Dalton's group showed that it was "possible to manipulate the environmental growth conditions so one form of the enzyme (e.g. MMO) will predominate in the cell" (Dalton et al., 1983). In cells expressing pMMO, copper has also been shown to control expression up to 55 fold and to alter substrate affinity and specificity (Choi, et al., 2003; Lontoh and Semrau, 1998 Lontoh, 2000). Whole-cell methane oxidation by *Methylomicrobium album* BG8 (Type I, can only express pMMO) and *Methylosinus trichosporium* OB3b, (Type II, capable of expressing both sMMO and pMMO) were both enhanced with the addition of copper, but: (1) *Methylomicrobium album* BG8 had higher (~2x) pseudo-first order rates (V_{max}/K_s) of CH_4 oxidation than *Methylosinus trichosporium* OB3b at all copper concentrations examined, and; (2) *Methylosinus trichosporium* OB3b expressing pMMO had a higher affinity and pseudo-first order rates of CH_4 oxidation rates than when expressing sMMO (Lontoh and Semrau, 1998; Lontoh, 2000). As a result, cells expressing pMMO appear to have a competitive advantage over cells expressing sMMO for turning over CH_4 at low concentrations. Conversely, at high CH_4 concentrations, methanotrophic communities should preferentially express sMMO as the turnover of CH_4 is faster, thus allowing those cells capable of expressing sMMO to grow more rapidly. The importance of copper appears to be due to the large amount of copper found in active purifications of pMMO that are believed to be involved in the oxidation and/or electron transport from the *in vivo* reductant to O_2 (Balasubramanian and Rosenzweig, 2007; Basu, et al., 2003; Choi, et al., 2003, 2005; DiSpirito, et al.. 2004; Fu, et al., 2003; Nguyen, et al., 1994, 1996, 1998; Semrau, et al., 1995 ; Zahn and DiSpirito, 1996).

1.7. Biogenic N_2O production

N_2O has a strong potential for infrared adsorption, with a global warming potential ~298 times greater than CO_2 (IPCC, 2007). N_2O is produced by both natural and anthropogenic sources, and the primary anthropogenic source is agricultural soil in the USA.

Over the past several decades, the annual input of biologically reactive nitrogen has increased by a variety of anthropogenic activities, primarily agricultural practices and activities, including the use of synthetic and organic fertilizers, production of nitrogen-fixing crops, cultivation of high organic content soils, and the application of livestock manure to croplands and pasture. This surplus of nitrogen has stimulated natural microbial activity, the largest source of N_2O accounting for over 60% of total emission in US (USEPA, 2009). Although the mechanism of N_2O production is not completely understood, microbial production of N_2O is generally considered to be achieved through three different processes (Figure 7); (1) a by-product of nitrification by ammonia-oxidizing bacteria, (2) denitrification and (3) nitrifier denitrification, as well as possibly by (4) anaerobic ammonia oxidation and (5) nitrification by ammonia-oxidizing archaea.

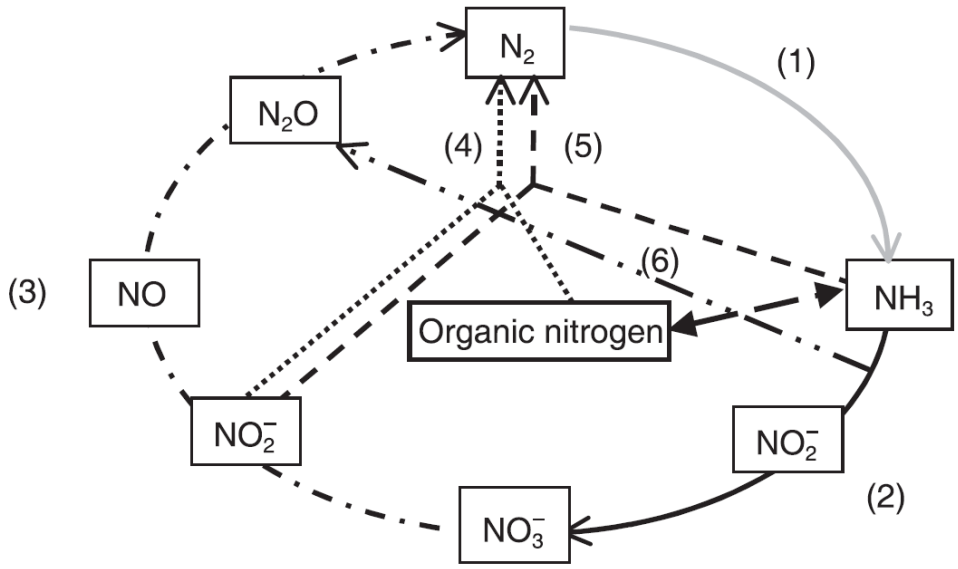


Figure 7. Microbiol processes in the nitrogen cycle. (1) Nitrogen fixation, (2) bacterial nitrification, archaeal nitrification and heterotrophic nitrification, (3) aerobic and anaerobic bacterial denitrification, nitrifier denitrification, fungal denitrification and archaeal denitrification, (4) and (5) co-denitrification (by fungi), (5) anammox and (6) N_2O production during nitrification (ammonia oxidation). (Hayatsu et al., 2008)

1.7.1. Nitrification

*A*mmonia- and methane- oxidizing bacteria produce N_2O during nitrification, specifically during the oxidation of hydroxylamine (NH_2OH) to nitrite (NO_2^-) (Arp and Stein, 2003). The best characterized metabolic pathway for oxidation of ammonia to nitrite is performed by ammonia-oxidizing bacteria. These organisms utilize ammonia to generate energy and fix CO_2 for carbon via the Calvin-Benson-Bassham (CBB) cycle. Their activity is stimulated by the addition of the ammonia, producing NO_2^- via oxidation of hydroxylamine. NO_2^- produced stimulates other processes in the nitrogen cycle, and these cells are also known to form N_2O as a by-product of the oxidation of hydroxylamine to NO_2^- (Hooper and Terry, 1979) as well as possibly indirectly through the abiotic decomposition of unstable intermediates (Ritchie and Nicholas, 1972).

Recently, the archaeal counterpart of autotrophic ammonia-oxidizing bacteria has been gaining interest as it appears that their abundance exceeds that of ammonia-oxidizing bacteria in soils (Leininger et al., 2006). However, the ability/inability of ammonia-oxidizing archaea to produce N_2O is not known, nor is their general activity *in situ*.

Holmes and colleagues (1995) have shown that genes encoding particulate methane monooxygenase and bacterial ammonia monooxygenase share high sequence identity, suggesting both microbial groups are evolutionarily related. Moreover, the similarities in size and structure of NH_4^+ and CH_4 allow for the co-oxidation of NH_4 by methanotrophs. Methane-oxidizing bacteria have been reported to be capable of producing N_2O (Yoshinari, 1985; Mandernack et al., 2000; Sutka et al., 2003). Methanotrophs oxidize ammonia to NO_2^- through a co-metabolic process, and also reduce NO_2^- to N_2O presumably in a fashion similar to ammonia oxidizers (Nyerges and Stein, 2009). However, this metabolism is less well characterized than that for ammonia oxidizers. These microorganisms, ammonia-oxidizing bacteria, ammonia-oxidizing

archaea, and methanotrophs, all occupy the same general environmental niche, i.e., and thus may play significant roles in N_2O production.

1.7.2. Denitrification

N_2O can also be produced and consumed by various microorganisms including heterotrophic denitrifying bacteria, archaea, and some fungi (Barnard, 2005). In this case, N_2O is produced and consumed by the step-wise reduction of nitrate (NO_3^-) to N_2 (Sutka et al., 2006) as shown in Figure 7. Since denitrification is a sequence of reductive reactions, this process is thought to occur mostly in anaerobic environments. For this reason, the potential for N_2O production from denitrification process is highest in water-saturated or anoxic zones which contain large concentrations of organic carbon and NO_3^-.

There has been substantial debate about the contribution of nitrification and denitrification to the emission of N_2O. The introduction of multi-isotope signature methodologies has widened our knowledge of mechanisms of biological N_2O production and the global N_2O budget, and the analysis of intramolecular distribution of ^{15}N in N_2O, i.e., site preference, showed that the site preferences of ~33 ‰ and ~0 ‰ are characteristic of nitrification and denitrification, respectively, which could offer additional information to apportion sources and sinks of N_2O more in detail in future studies (Perez et al., 2001; Stein and Yung, 2003; Sutka et al., 2006). Since there is no influence of nitrogen sources on site preferences, it could be used to distinguish N_2O production from nitrification and denitrification.

1.7.3. Nitrifier denitrification

Nitrifier-denitrification is another process by which ammonia-oxidizing bacteria produce N_2O, and, presumably, ammonia-oxidizing archaea. Ammonia-oxidizing bacteria have been shown to produce N_2O when NO_2^- is used as an alternative electron acceptor under anoxic conditions, i.e., nitrifier denitrification, where NO_2^- is reduced to NO or N_2O as in the classical heterotrophic denitrification pathway (Goreau et al., 1980; Poth and Focht, 1985; Arp and Stein, 2003). 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, the process was thought to occur only in O_2-limiting conditions (Goreau et al., 1980, Poth and Focht, 1985), but recently it has been shown that it can occur at atmospheric levels of O_2 (Shaw et al., 2006). However, the enzymes involved, the phylogenetic breadth, and metabolic purpose still remain unclear.

1.7.4. Anaerobic ammonia oxidation

Anaerobic ammonia oxidation, or anammox is a novel biotechnology for nitrogen removal. After identifying new organisms capable of oxidizing ammonium using nitrite as the electron acceptor (Strous et al., 1999), anammox is getting considerable attention for application in wastewater treatment plants as well as its role in the oceanic nitrogen cycle (Thamdrup and Dalsgaard, 2002; Kuypers et al., 2003). Anammox bacteria have been found to be metabolically flexible, exhibiting alternative metabolic pathways, e.g., subsequent reduction of nitrate to ammonia via nitrite, followed by the conversion of ammonium and nitrite to N_2 through the anammox pathway, allowing anammox bacteria to overcome ammonia limitations to growth. Anammox bacteria are also a potential source of N_2O production by nitric oxide detoxification (Kartal et al., 2007).

1.7.5. Ammonia oxidizing Crenarchaeota

While anammox continues to be explored as a 'new' process in the global nitrogen cycle, a novel group of aerobic ammonia oxidizing nitrifiers has recently been identified. These organisms were first postulated from the detection of a unique ammonia monooxygenase (AMO) gene apparently from uncultivated Crenarchaeota (Venter et al., 2004), suggesting the genetic capacity of archaea to perform ammonia oxidation. Traditionally, organisms responsible for major geochemical processes have been determined by characterizing physiological features of environmental isolates. Therefore, it has been assumed that all autotrophic ammonia oxidizers are bacteria because of the inability to cultivate ammonia-oxidizing archaea (AOA). Venter and colleagues (2004), however, demonstrated that some archaea may carry *amoA* genes using the high-throughput shotgun sequencing of Sagasso Sea water samples, as the first indication of possible crenarchaeal involvement in the nitrogen cycle. A year later, Schelper and colleagues (2005) identified a Sagasso Sea-like *amoA* homolog from soil-derived fosmids, indicating there may be ammonia-oxidizing archaea in soils. Archaeal ammonia oxidation was subsequently verified by the cultivation of an ammonia-oxidizing crenarchaeon – designated *Nitrosopumilus maritimus* – from the rocky substratum of a tropical saltwater aquarium tank (Konneke et al., 2005) that oxidizes ammonia to nitrite, apparently using a pathway very much like that known in ammonia-oxidizing bacteria. All archaeal *amoA* genes amplified from various sources show a high degree of sequence similarity (>73% on the amino acid level, and >66% on the DNA level), suggesting that this gene may be an ideal marker for this new player in ammonia oxidation. Recently, ammonia-oxidizing archaea have been demonstrated to predominate among ammonia-oxidizing prokaryotes in soils (Leininger et al., 2006) as well as in oceans (Wuchter et al., 2006). Furthermore, inhibition experiments have shown that crenarchaeal AMO (and AOA nitrification) is sensitive to AOB inhibitory compounds such as acetylene (Offre et al., 2009), suggesting that phenylacetylene, proposed as a selective inhibitor to distinguish between the activities of the ammonia oxygenase and the methane monooxygenases (Lontoh et al., 2000; Lee et al., 2009), may also be selectively effective on the activity of ammonia oxidizing archaea in landfills.

Phylogenetic analyses of AOA partial *amoA* and 16S rRNA gene sequences have revealed that there are distinct lineages that appear to prefer different habitats. Most of the known AOA marine sequences group within the 1.1a lineage, while some fresh water sediment and most soil sequences cluster in group 1.1b (Ochsenreiter et al., 2003; Francis et al., 2005; Nicol and Schleper, 2006; Prosser and Nicol, 2008). It is possible that the different groups of AOAs prefer environments with different amounts of ammonium, e.g., open ocean, marine water columns, where typical ammonium concentrations are <0.03~1 μM (Konneke et al., 2005; Wuchter et al., 2006;, Beman et al., 2008) are dominated by Group 1.1a, while Group 1.1b are found in many N-rich environments, e.g., bioreactors and agricultural soils. Furthermore, increased *amoA* expression by AOAs has been found in soils after addition of 10 mM NH_4Cl (Treusch et al., 2005) suggesting the expression of archaeal *amoA* gene, like bacterial *amoA* genes (Arp et al., 2002), may be induced by ammonia. Since agricultural soils and estuaries receiving agricultural run-off typically are usually reported to be higher ammonium concentrations, the ammonia-oxidizing archaea in group 1.1b lineage may have adapted to such environments, while the group 1.1a thrives in marine or oligotrophic ecosystems. Previous studies also have shown that most archaeal 16S rRNA and *amoA* gene sequences derived from agricultural soils were placed within the group 1.1b lineage (Nicol et al., 2008; Tourna et al., 2008).

There are several other critical questions that should be addressed regarding AOA-driven N_2O production including; (1) what is the fraction of ammonia oxidation due to ammonia oxidizing bacteria versus its archaeal counterpart in terrestrial ecosystems?; (2) what is the pathway of ammonia oxidation in AOA?; (3) do AOAs undergo the nitrifier denitrification; and (4) what are the physiological characteristics of AOAs, e.g., substrate affinity, growth rates, and potential capacity for N_2O production?

1.7.6. Methanotrophic-mediated nitrous oxide generation

Interestingly, methanotrophs have also been reported to be capable of producing N_2O (Mandernack, et al., 2000; Sutka, et al., 2003; Yoshinari, 1985). 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* (Yoshinari, 1985). In another study using landfill cover soils, however, methanotrophs were suggested to be directly linked to N_2O production via nitrification (Mandernack, et al., 2000). Methanotrophs can also be involved in N_2O production via cross-feeding of denitrifying through metabolite excretion (Amaral, et al., 1995). 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 (Amaral, et al., 1995). 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.

1.8. Biofiltration of methane

Recently, the capability of methanotrophs to consume methane and the utilization of this metabolic process in greenhouse gas reduction has been studied due to growing concerns over global warming. Global population increase and consequent rise in consumption of energy and waste generation have led to a large increase in the anthropogenic contribution to atmospheric methane emissions, which now accounts for ~ 60% of 600 Tg CH_4 emitted each year to the atmosphere (Fung et al., 1991). As a consequence, atmospheric methane concentrations have more than doubled from 695 ppb to 1.7 ppm in the period spanning from 1800 to 1981 (Etheridge et al., 1998). In response to this, many attempts have been made to reduce emission of methane from these major sources, e.g., installation of methane vents in modern sanitation landfills. Many of these devices have been successful in suppressing further increases in atmospheric methane concentration, but significant amounts of methane are still being emitted (IPCC, 2007). In response, recent research has considered methods to not only reduce methane emissions (Melse and van der Werf, 2005; Nikiema et al., 2005), but also to remove methane already in the atmosphere.

One such approach is the use of methanotrophs. Unfortunately, the average methane concentration in the atmosphere is too low to allow growth of most isolated laboratory methanotrophic strains. Most cultured methanotrophs have whole-cell half saturation constants (K_s) for methane of ~1- 10 μM (750 – 7500 ppmv assuming equilibrium between aqueous and gas phases at 30°C), and thus, the atmospheric methane concentration, 1.7 ppmv, results in negligible methane consumption rates. This is one of the reasons why past approaches for atmospheric methane removal have focused mainly on source reduction where methane is in very high concentrations (>7000 ppm) (Melse and Van der Werf, 2005; Nikiema et al., 2005). Here, we evaluated the feasibility of using biofiltration systems to accommodate methanotrophic

activity to remove methane from lower concentrations of methane (i.e., < 6000 ppm).

Biofiltration is an engineered biological treatment process that utilizes the metabolic activity of microorganisms attached onto a variety of packing materials to treat a wide range of organic and inorganic contaminants (Cohen, 2001). In a biofilter, contaminants in the gas phase diffuse into biofilms, a thin layer of a microbial consortium on the packing material, to be consumed by microorganisms therein. Biofilters are filled with a variety of packing materials made of natural and synthetic materials to provide the surface area for establishment of biofilms. In order to commercially apply this treatment system to removal of methane utilizing methane-degrading capability of methanotrophs, it is essential that both engineering and economic feasibilities are examined using model and pilot tests. Conditions that are specific to methanotrophs, e.g., copper concentration controlling expression of pMMO and sMMO, must also be considered in the model. Here the feasibility of methane removal via biofiltration was investigated through in silico analyses.

2. EXPERIMENTAL METHODS

2.1 Landfill soil microcosm experiments

2.1.1. 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 8. The soil was air-dried, sieved to exclude soil particles less than <2mm, homogenized, and stored at 4 ˚C in the dark. 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 (McBride, et al., 2004). 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

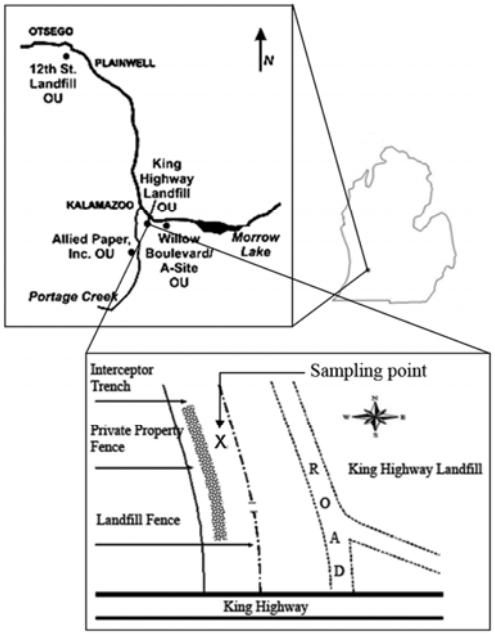


Figure 8. Location of King Highway Landfill and sampling site (http://www.kzooriver.com/kalamazoo)

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 (Chen and Ma, 2001). 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.

2.1.2. 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^+·(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^-·(kg soil)$^{-1}$ above background levels.

To examine the possibility of selectively inhibiting N_2O production by either nitrifiers 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 9. 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.

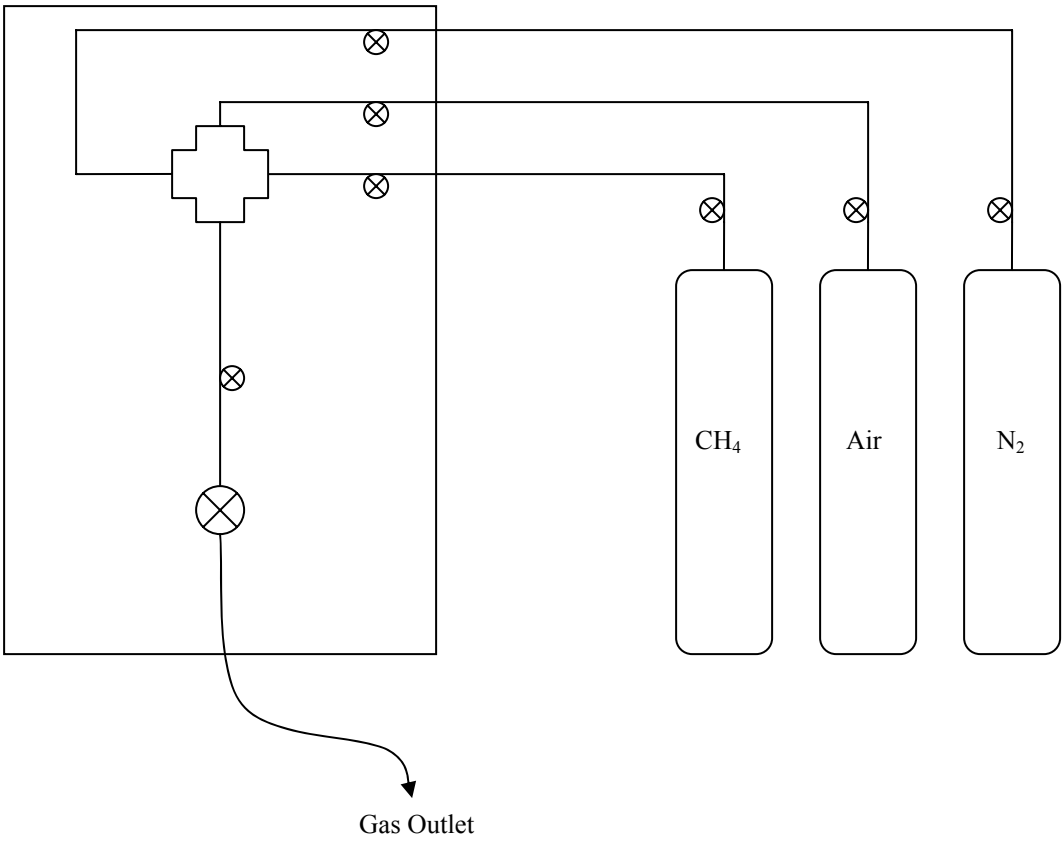


Figure 9. Schematic diagram of the gassing system for soil microcosms

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 assayed in triplicate.

2.1.3. 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.

2.2 Field study design, site and test plots

The study plots were located near Kalamazoo, Michigan, on the King Highway Landfill at the same location where soils were collected for microcosm work. The study design comprised 6 test plots having vertical well and gas sampling ports. Five different amendments were applied beginning November 2007 as outlined in Table 6. The stock amendment solution was prepared by adding 0.5 M of NH_4Cl and 0.25 M of KNO_3 in 1 liter of distilled water. For plot 4 and 7, 100 mg of 98% phenylacetylene was added in the same solution, and 4% (v/v) of methanol was added to increase the solubility of phenylacetylene. The resulting solution was sprayed to each plot by a pressurized vessel. For plot 2, 3, and 4, a canopy was installed in an attempt to control soil moisture content. The plots were separated by 2 m from each other to minimize any cross-influence between adjacent plots. Each treatment was applied by periodically wetting soils with either water or the stock amendment solutions. The first amendment was applied in November 2007 and repeated every two months for 8 months and then monthly until June 2009. The test plots were dismantled in July 2009, and six soil cores of 0.6~1.0-m depth from each test plot were collected

Table 6. Amendments applied to the study plots on the King Highway Landfill

Location	Amendments
Plot 2	No amendment under canopy
Plot 3	NH_4Cl/KNO_3 under canopy
Plot 4	NH_4Cl/C_8H_6 under canopy
Plot 5	No amendment no canopy
Plot 6	NH_4Cl/KNO_3 no canopy
Plot 7	NH_4Cl/ C_8H_6 no canopy

2.2.1 Landfill gas monitoring

Vertically-nested soil gas sampling probes were installed at 10 cm intervals between a depth below ground surface of 20 cm and 100 cm. Gas samples were collected through a thin tube connected to each probe at the time of each amendment addition. The soil gas samples were collected in 60 ml serum bottles sealed with butyl rubber stopppers by first purging the bottles with three system volumes using a 3-way valve and then filling the vials a final fourth time. To minimize any gas loss during transfer, the punctured butyl rubber stoppers were sealed with silicone adhesive after filling. Samples were then transported to the lab for analysis. CH_4 and O_2 was measured using a Buck Scientific model 910 gas chromatography equipped with a thermal conductivity detector. The oven temperature was programmed to start at 35 °C and then was increased to 218 °C at 25 °C per minute. This final temperature was maintained for 3 min. Helium was used as the carrier gas at a flow rate of 10 $ml \cdot min^{-1}$. For N_2O measurements, the technique described above was used, i.e., an HP 5890 series II gas chromatography equipped with a PoraPAK-Q column (0.53 mm I.D. x 25 m) and an electron capture detector was used. Oven, inlet, and detector temperatures were -10 °C, 125 °C, and 275 °C, respectively, with a gas flow rate of 56 $ml \cdot min^{-1}$. Nitrogen gas was used as both carrier and makeup gas.

2.2.2. Soil collection and analyses

On July 22, 2009, the field site was dismantled and one intact soil core, 1 m long and 5 cm in diameter was obtained from each treatment plot using a vehicle-mounted soil core sampler

(Model 6620DT, Geoprobe, Salina, KS). After extruding the soil cores from the test plots, the samples were transported to the lab on dry-ice for detailed geochemical and nucleic acid analyses. The soil cores were first fractionated into 2.5 cm thick layers. Each layer was then immediately sieved to exclude particles greater than 2 mm, placed in sterile 2 ml Eppendorf tubes and stored at -80 °C for subsequent molecular analysis of microbial community structure and activity. The rest of the soil from each soil layer was then air-dried, sieved in the same manner into 50 ml conical tubes, and then stored at -80 °C for geochemical analysis. The pH of the soil was measured after mixing fresh soil with 0.01 M $CaCl_2$ in 1:2 ratio (i.e., 5 g soil in 10 ml of 0.01 M $CaCl_2$), and shaking the soil slurry at 220 rpm for 30 min. Inorganic nitrogen, i.e., NH_4^+-N and (NO_3^-+ NO_2^-)-N was extracted using 30 g of air dried soil added to 60 ml solution of 2 M KCl. The slurry was shaken on an orbital shaker for 20 min at 220 rpm, and then filtered on Whatman #42 filter paper (Whatman Group, Middlesex, UK). The extract was collected and frozen for subsequent analysis of inorganic nitrogen. After thawing, NH_4^+ and NO_3^-+ NO_2^- were measured colorimetrically using a rapid flow analyzer (OI Analytical, College Station, TX). Since pMMO and sMMO are known to be regulated by the copper to biomass ratio, total and bioavailable copper was measured by using methods developed by McBride (2004). Briefly, 5 g of air-dried soil suspended in 12.5 ml of 0.01 M of $CaCl_2$ were heated at 90 °C for 30 min. The mixtures were then passed through #42 Whatman filter paper, and 10 ml of nitric acid added to prevent metal precipitation and microbial growth. To determine total copper concentration in each soil layer, 0.5 g air-dried soil samples were digested in 12 ml of Aqua regia (a mixture of concentrated nitric acid and concentrated hydrochloric acid in the ratio 1:3, respectively) first at 110 °C for 3 hours, and then at 60 °C for 3 hours. Nitric acid (2% vol·vol^{-1}) was added to adjust the total volume to 20 ml and filtered on #42 Whatman filter paper. Nitric acid and hydrochloric acid used for copper measurements were of trace metal grade (Fisher Scientific Inc., Pittsburgh, PA). Copper was then measured using inductively coupled plasma mass spectrometry (ELAN DRC-e; PerkinElmer, Waltham, MA). ^{63}Cu was used for measurement of copper. ^{71}Ga was added as an internal standard (PerkinElmer, Waltham, MA).

2.3. Nucleic acid extraction

2.3.1. DNA extraction

For molecular studies, DNA was extracted from (1) soil microcosm studies, and; (2) 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. For this purpose, functional genes that are unique or recognized as key components of CH_4 consumption or N_2O production, i.e., *pmoA*, *mmoX,* bacterial *amoA,* archaeal *amoA, nirK,* and *norB* were used. *pmoA* encodes the α subunit of pMMO and *amoA* encodes the α subunit of AMO. *pmoA* and *amoA* have been shown to be effective as a functional marker for studying methanotrophs and AOB/AOA in the environment (Dumont and Murrell, 2005a; Prosser and Nicol, 2008). *nirK* encodes the copper containing nitrite reductase, and *norB* encodes the nitric oxide reductase.

2.3.2. mRNA extraction

RNA from soils were extracted following previously developed methods with minor modifications (Han and Semrau, 2004). 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 (Chen,

et al., 2007), 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.

2.3.3. Expression of functional genes

PCR amplification was performed using specific primers for *pmoA*, *mmoX,* Bacterial *amoA,* and Archaeal *amoA* as shown in Table 7. PCR amplifications were performed using Biometra TPersonal thermal cycler system (Labrepco, Horsham, PA). Each PCR reaction consisted of 5 µl 10X PCR buffer (Invitrogen, Carlsbad, CA), 1.5 µl 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 1 µl 10 mM dNTP mixture (Invitrogen, Carlsbad, CA), 20 pmoles of each primer, 2.5 units Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 25 ng of DNA template. 1µl of 20 mg/ml bovine serum albumin was also added to reduce the inhibition of DNA polymerase (Kreader 1996). For *pmoA*, *mmoX*, and bacterial *amoA* gene amplification, the reaction mixture was denatured at 94°C for 5 min, followed by 36 cycles of 94°C for 1 min, annealing at 60°C for 1.5 min, and elongation at 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. Archaeal *amoA* gene was amplified by the following protocol: 95°C for 5 min for denaturation; 30 cycles consisting of 94°C for 45 s, 53°C for 60 s, and 72°C for 60 s; and 72°C for 15min. PCR products were analyzed via electrophoresis by loading 10 µl of the PCR mixture onto 1% (w/v) agarose gels and staining with ethidium bromide.

Table 7. Primers used for PCR amplification of functional genes of particulate methane monooxygenase (*pmoA*), soluble methane monooxygenase (*mmoX*), bacterial and archaeal ammonia monooxygenase (*amoA*), nitrite reductase (*nirK*), and nitric oxide reductase (*norB*).

Gene targeted	Primers	Sequence (5'-3')	Reference
pmoA	pmoA-A189 pmoA-mb661	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	Costello & Lidstrom (1999)
mmoX	mmoX206f mmoX886r	ATCGCBAARGAATAYGCSCG ACCCANGGCTCGACYTTGAA	Hutchens et al. (2004)
Bacterial *amoA*	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Rotthauwe, et al. (1997)
Archaeal *amoA*	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	Francis et al. (2005)
nirK	nirK1F nirK5R	GGRATGGTYCCSTGGCA GCCTCGATCAGRTTRTGG	Braker et al. (1998)
nirK	F1aCu R3Cu	ATCATGGTSCTGCCGCG GCCTCGATCAGRTTGTGGTT	Hallin and Lindgren (1999)
norB	norB1f norB8r	CGNGARTTYCTSGARCARCC CRTADGCVCCRWAGAAVGC	Casciotti and Ward (2005)

where, N = G, A, T, or C; Y = C or T; M = A or C; K = G or T; S=G or C; R = G or A; W=A or T; B= C or T or G; V= A or C or G; B= C or G or T

2.4. Microarray analysis

2.4.1.Microarray sample preparation

The concentration and purity levels of 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 (Stralis-Pavese, et al., 2004). 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. 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 (Bodrossy, et al., 2003; Stralis-Pavese, 2004). 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.

2.4.2.Description of probes

A detailed list of the sequences each probes are designed to target can be found in Appendix A. 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 (Horz, et al., 2005; Knief, et al., 2000; Nercessian, et al., 2005). 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 (Holmes, et al., 1999; Ogram, et al., 2006). "*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 which there is limited information.

2.5. Construction of archaeal *amoA* gene fragment libraries

A clone library of archaeal *amoA* amplicons obtained from ~25 - 27.5 cm depth of soil cores collected from each test plot at the field demonstration site was constructed to analyze the effect of different amendments on AOA community structure. Amplification products of the archaeal amoA gene from 3 separate reactions for each sample were pooled, and each PCR product was ligated into pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA), inserted into One Shot Top10 chemically competent E. coli (Invitrogen, Carlsbad, CA), and then screened for successful insertion via β-galactosidase activity as described by the protocols provided in the TOPO TA clone kit (Invitrogen, Carlsbad, CA). A total of 213 colonies was selected and sequenced by Beckman Coulter Genomics Inc. (Danver, MA). The sequence alignments were conducted with ClustalW implemented in MEGA 4.0 package (Tamura et al. 2007). Phylogenetic trees were constructed and molecular evolutionary analyses were performed using the neighbor-joining method, and distance and parsimony bootstrap analyses (100 replicates) were calculated using MEGA 4.0 (Tamura et al. 2007). The diversity of the archaeal *amoA* clone library was further investigated by rarefaction analysis. Rarefaction curves were produced by using the freeware program aRarefactWin 2.0 (available at http://www.uga.edu/strata/software/). Operational taxonomic units (OTUs) were defined as clones that exhibited 97% or greater sequence identity. To assess whether statistically significant changes in community structure were present, subsets of the AOA *amoA* gene sequences obtained from each core were analyzed by LIBSHUFF (Schloss et al., 2004). Sequences were first aligned in ClustalX, ver. 2.0.12, (Thompson, 1997) and used to construct a full distance matrix using DNADIST program from the PHYLIP package ver. 3.69 (Felsenstein, 1993), using the Jukes-Cantor correction for multiple substitutions. A P value of ≤ 0.025 indicates a statistically supported difference in microbial libraries.

2.6. Analysis of microbial diversity

Visualization of microarray and cloning 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 (Gebert, et al., 2008). 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. For ammonia-oxidizing archaea diversity in soil cores from different landfill test plots, the number of operational taxonomic units was used for the number of species. The indices used are as follows:

Simpson index of diversity (1/D)

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

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})

$$D_{Mn} = \frac{S}{\sqrt{N}} \qquad (2)$$

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)

$$E = \frac{1/D}{S} \qquad (3)$$

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

Berger-Parker index of dominance (d)

$$d = \frac{N_{\max}}{N} \qquad (4)$$

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 a decrease in diversity and increase of dominance by a limited number of species (Magguran, et al., 2004).

2.7. Statistical analysis of geochemical parameters in landfill test plots

A linear mixed-effects model implemented in SAS (PROC MIXED) was used to appropriately model the correlations between the repeated measures within a plot. In addition to the effect of plot, the month of measurement and the depth were used as predictors in the model. The estimated marginal means produced by the model for each plot were then compared. Since the mixed-effects model is a large and complex subject, a brief overview is provided here.

The normal linear model is given by the equation in matrix form:

$$y_i = X\beta + \varepsilon \quad (5)$$

$$\varepsilon \sim \mathrm{N_n}(0, \sigma^2 \mathrm{I}_n) \quad (6)$$

where, $\mathbf{y}=(y_1, y_2,\ldots, y_n)$ is the response vector; $\mathbf{X}$ is the model matrix, with typical row $x_i=(x_{1i}, x_{2i},\ldots, x_{pi})$; $\beta=(\beta_1, \beta_2,\ldots, \beta_p)$ is the vector of regression coefficients; $\varepsilon=(\varepsilon_1, \varepsilon_2,\ldots, \varepsilon_n)$ is the vector of errors; $\mathbf{N_n}$ represents the n-variable multivariate-normal distribution; is the error variance; $\mathbf{0}$ is an n×1 vector of zeros; and $\mathbf{I_n}$ is the order-n identity matrix. More specifically, ε is the random effect representing the influence of subject i on the repeated measures that is not captured by the observed covariates. The parameter σ^2 indicates the variance in the population distribution, and therefore the degree of heterogeneity of subjects.

The mixed effect models include additional random-effect terms, and are often appropriate for representing clustered and therefore dependent data, for example, when the data are collected hierarchically, when observations are taken on related individuals, or when data are gathered over time on the same individuals.

The linear mixed model is given by the equation in matrix form:

$$y_i = X\beta + Z_i b_i + \varepsilon \quad (7)$$

where, $\mathbf{Z_i}$ is the model for the random effects for observation in group i, and $\mathbf{b_i}$ is the vector of independently and identically distributed (IID) random effect coefficients of group i.
In probability theory and statistics, a sequence or other collection of random variables is "independent and identically distributed" if each random variable has the same probability distribution as the others and all are mutually independent.

2.8. Nitrous oxide production by methanotrophs

To estimate the contribution of methanotrophic activity on N_2O production, initial experiments examined the effects of growth condition on N_2O production using *Methylosinus trichosporium* OB3b grown in either NMS or AMS medium (Whittenbury, et al., 1970). Copper, as $CuSO_4 \cdot 5H_2O$ (JT Baker Chemical Co., Phillipsburg, NJ, Baker analyzed) was added at a concentration of 20 μM to induce pMMO expression, while separate systems with no added were prepared for the induction of sMMO expression. sMMO expression was verified by the naphthalene assay (Brusseau et al., 1990). Formate was prepared as 500 mM sodium formate stock solution. *M. trichosporium* OB3b was first grown to the late exponential phase (optical density at 600 nm [OD_{600}] of 0.7 - 0.8) in either 0.1X or 1X AMS with or without copper and with methane as the growth substrate. Cells were then diluted to an OD_{600} of 0.07~0.09 with the same pre-warmed media. Five milliliter aliquots of the diluted culture were then aseptically transferred into specially constructed 32.5 serum vials (Lee et al., 2006). The vials were capped with Teflon-coated butyl rubber stoppers (National Scientific Co., Duluth, GA) and crimp sealed with aluminum caps. Methane and air was added to the vials in a 1:2 ratio using the custom-

designed gassing apparatus as previously described (Lee et al., 2009). The vials were incubated at 30°C with shaking at 250 rpm. All conditions were performed in duplicate. Growth and N_2O production was monitored until the stationary phase was reached. Cell growth was monitored by measuring OD_{600}, while N_2O production was measured using GC-ECD as described earlier.

Subsequently further experiments were performed using *Methylomicrobium album* BG8, *Methylococcus capsulatus* Bath, *M. trichosporium* OB3b, *Methylocystis parvus* OBBP, *Methylocystis hirsuta* CSC1, *Methylocystis rosea* SV97, *Methylocystis* sp. strain M, and *Methylocystis* sp. strain SB2 grown in 1X ammonia mineral salt medium (AMS) with either 0 or 20 μM copper. Growth and N_2O production was monitored as described above.

2.9. Feasibility of atmospheric methane removal using methanotrophic biotrickling filters

The performance of the methane biotrickling filter was modeled using the methodology initially developed by Alonso et al (1997) for biotrickling filtration of volatile organics as shown in Figure 10. Several assumptions were made in the model development. First, it was assumed that the biofilm develops uniformly around identical spherical packing materials. Second, a two-phase system was assumed with the gas phase in direct contact with the biofilm, or solid phase. A cylindrical reactor was modeled as a one-dimensional system by assuming no concentration gradients in the gas phase in the radial direction. Since growth of the biofilm occurs over much longer time periods than either mass transfer or methane oxidation rates (Alonso et al., 1997), a quasi steady state was assumed in developing the equations characterizing biofilter performance. Furthermore, diffusion was assumed to be the only means of mass transfer through the biofilm and diffusion in axial direction was assumed to be negligible. Utilization of methane was modeled with Michaelis-Menten kinetics and methane was considered to be the sole growth limiting substrate. Despite its poor solubility in water (Fry et al., 1995) and low half saturation constant (van Bodegom et al., 2001) comparable to those of methane, oxygen was not considered to be a growth limiting factor in our model due to its abundance in the atmospheric air.

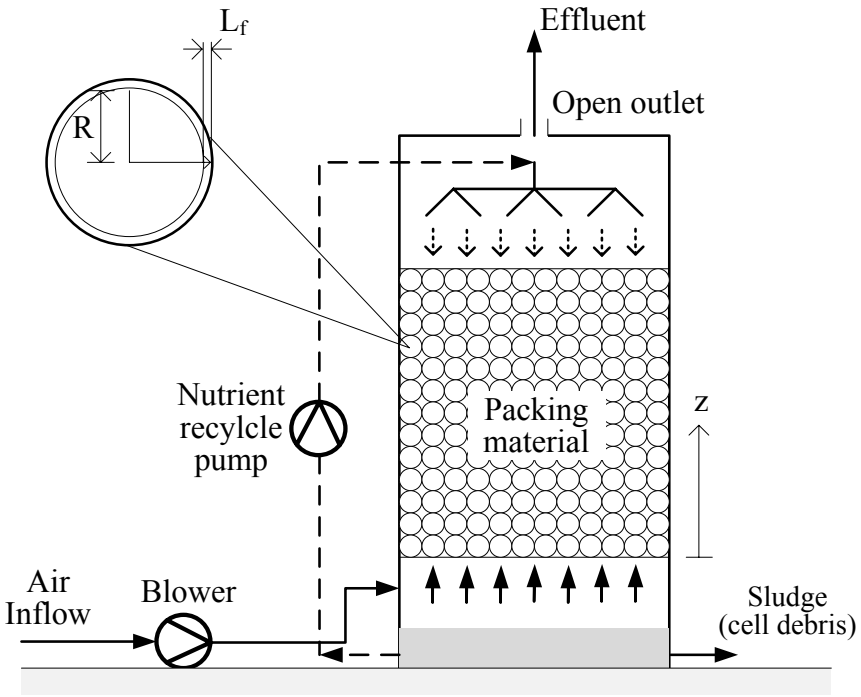


Figure 10. Schematics of the proposed methane biotrickling filter and the mathematical model

With these assumptions, a differential equation was developed describing mass transfer across the biofilm surrounding a spherical packing material. A spherical coordinate system was used with the point of origin at the center of the sphere. In this equation, methane diffusion into the biofilm was balanced with methane consumption by bacteria in the biofilm:

$$D_f\left[\frac{d^2C_f}{dr}+\frac{2}{r}\frac{dC_f}{dr}\right]=\frac{X_f V_{\max} C_f}{K_s+C_f} \qquad (8)$$

$$\left.\frac{dC_f}{dr}\right|_{r=R}=0 \qquad (9)$$

$$C_f\Big|_{r=R+L_f}=\frac{C_g}{H} \qquad (10)$$

where D_f is the diffusivity of methane in the biofilm, C_f is the methane concentration in the biofilm, r is the radial distance from the center of a spherical packing material. K_s is the half saturation constant, X_f is the bacterial density in the biofilm, V_{max} is the maximum rate of methane oxidation, R is the radius of the packing material, C_g is the concentration of methane in the gas phase, L_f is the biofilm thickness, and H is the Henry's constant for methane. Boundary conditions were defined from a no-flux condition at the surface of packing material ($r = R$) and concentration at the surface of biofilm equilibrated with methane concentration in gas phase at the respective axial position of the packing material.

The biotrickling filter was assumed to behave as an ideal plug flow reactor. The rate of methane

removal from the gas phase is the same as the volumetric transfer into biofilm. Therefore, the mass balance equation in the biofilter can be written as:

$$\frac{dC_g}{dz} = -\frac{Ja_f}{u_0} = -\frac{J_f R_g T a_f}{P M_v u_o} \qquad (11)$$

where z is the distance from the air inlet, J is the flux of methane out of the gas phase, J_f is the flux of methane into the biofilm, a_f is biofilm-gas interfacial surface area per unit bed volume, u_o is approach velocity of gas, R_g is the universal gas constant, T is temperature, P is system pressure, and M_v is molecular weight of methane.

The biofilm thickness, expressed as L_f in the above equations is not a constant value, since the population of methanotrophic bacteria in the biofilm changes over time upon growth and decay of microorganisms dictated by local methane concentrations. The biofilm thickness can be expressed as:

$$\frac{dL_f}{dt} X_f = \left(D_f \frac{dC_f}{dr}\bigg|_{r=R+L_f} \right) Y - L_f X_f b \qquad (12)$$

where Y is the yield coefficient and b is the specific combined shear/decay coefficient, which is a sum of the specific decay (death) coefficient and the specific shear rate. The specific decay coefficient is a constant property and the specific shear rate is a function of biofilm thickness (Rittman, 1982). The specific combined shear/decay coefficient can be written as:

$$b = b_s^0 \left(\frac{\varepsilon_0}{\varepsilon_f} \right)^2 + b_d \qquad (13)$$

where b_s^0 is the default shear rate coefficient, ε_0 is the clean packed-bed porosity, ε_f is the porosity of the bed with biofilm, and b_d is the specific decay coefficient.

a_f, the biofilm-gas interfacial surface area per unit bed volume can be calculated from geometrical relationships as done by Alonso et al (1997). If it is assumed that the biofilm develops uniformly around packing materials, the volume of the biofilm at the contact points must be excluded from the biofilm-gas interfacial surface area. Then, a_f can be expressed as:

$$a_f = \frac{3(1-\varepsilon_0)}{2R} \left(1 + \frac{L_f}{R} \right) \left((2-n) \frac{L_f}{R} + 2 \right) \qquad (14)$$

where n is the number of packing materials in contact with a single packing material. Finally, the bed porosity with biofilm, ε_f, can be calculated in the same way:

$$\varepsilon_f = 1-(1-\varepsilon_0)\left[\left(1+\frac{L_f}{R}\right)^3 - \frac{n}{4}\left(\frac{L_f}{R}\right)^2\left(2\frac{L_f}{R}+3\right)\right] \qquad (15)$$

MATLAB was used to solve these equations to estimate overall methane removal by biofilters under a range of conditions, e.g., temperature, air flow rates, and methane concentration. Using the input parameters shown in Table 8, methane flux into the biofilm at each incremental depth level was calculated by solving Equation (8) with boundary conditions Equations (9) and (10) with the numerical solver function for boundary value problems in MATLAB. Then, with this flux profile incremental changes in concentration of methane along biofilter depth, $\frac{dC}{dz}$, were calculated. This information is used to acquire the concentration profile along the depth of the biofilter, which in turn, is used in determination of $\frac{dL_f}{dt}$, the incremental change of biofilm depth over time. The model was run until steady-state was established in the system, i.e., there is no more growth or decay in the biofilm. The amount of methane removed in the incremental time at the steady state is calculated by multiplying the difference between inlet and outlet concentration with the air throughput. This steady-state methane removal rate was multiplied to give the total per-month removal rate.

Table 8. Microbial and biofilter design parameters used in this study

Symbol	Parameter	Value	Reference
$V_{max\text{-}pMMO}$	Maximum rate of methane oxidation for pMMO-exressing cells (nmol·mg biomass^{-1}·s^{-1})	4.83	(Sipkema et al., 1998)
$K_{s\text{-}pMMO}$	Half-saturation constant of pMMO-expressing cells for methane (μM)	3.0	(Sipkema et al., 1998)
$V_{max\text{-}sMMO}$	Maximum rate of methane oxidation for sMMO-exressing cells (nmol·mg biomass^{-1}·s^{-1})	4.83	(Sipkema et al., 1998)
$K_{s\text{-}sMMO}$	Half-saturation constant of sMMO-expressing cells for methane (μM)	37	(Sipkema et al., 1998)
$V_{max\text{-}LR1}$	Maximum rate of methane oxidation for *Methylocystis* LR1 (nmol·mg biomass^{-1}·s^{-1})	0.125	(Dunfield et al., 1999)
$K_{s\text{-}LR1}$	Half-saturation constant *Methylocystis* LR1 for methane(μM)	0.305	(Dunfield et al., 1999)
b_d	Cell decay rate coefficient (1/d)	0.24	(Arcangeli and Arvin, 1999)
b_s^0	Default shear rate coefficient (1/d)	0.005	(Alonso et al., 1997)
X_f	Film bacterial density (mg/L)	17000	(Alonso et al., 1997)
D_f	CH_4 diffusivity in Biofilm (cm^2/s)	1.55E-05	(Hildebrand, 1969) (Alonso et al., 1997)
$L_{f,0}$	Initial biofilm depth (cm)	0.0042	(Alonso et al., 1997)
H	Henry's constant for methane ((mg/L)/(mg/L))	30	(Hartman, 1998)
ε_0	Empty bed porosity	0.34	(Alonso et al., 1997)
n	Number of spheres in contact with single sphere	10	(Alonso et al., 1997)

Microbial kinetics parameters of *M. trichosporium* OB3b (NCIMB 11131) expressing pMMO and sMMO (Sipkema et al., 1998) were used as input parameters for our study. We have also used the mathematical model to evaluate the possibility of using high affinity methanotrophs with parameters obtained by Dunfield et al. (1999). For the diffusivity constant of methane, we used the value reported by Knief et al. (2005). Characteristic parameters for biofilm development were acquired from Alonso et al (1997). Since the optimal temperature for growth and metabolism of *M. trichosporium* OB3b is 30°C (Lee et al., 2006) and preliminary model results (data not shown) showed that performance increased with temperature, an operating temperature of 30°C was selected for our analyses. To make cost-benefit analysis more convenient and credible, sizing of our reactor was based on an existing biofiltration system at Grupo Cydsa in Mexico, 3.66 m in diameter and 11.5 m in height (Cox and Deshusses, 2002).

For cost-benefit analysis, estimates for capital and operating costs from Cox and Deshusses (2002) were used in conjunction with the performance estimates from the modeling results. Since the scale of the reactor in the model was identical to the Grupo Cydsa biofilter, it was assumed that the capital and operating cost of the reactor would be approximately the same as the costs associated with this system. The cost per metric ton of methane removed was then calculated by dividing the annualized total cost by the predicted biofilter performance. This was converted to cost per CO_2 equivalence considering that methane has a global warming potential 25 times that of CO_2.

3. RESULTS AND DISCUSSION

3.1. 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 (USDA, 1993). 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.

3.2. Methane consumption and Nitrous oxide production in soil microcosms

3.2.1. Results

3.2.1.1. Microcosms incubated with 20 % CH_4, 10 % O_2, and 15 % H_2O. As can be seen in Figure 11, 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).

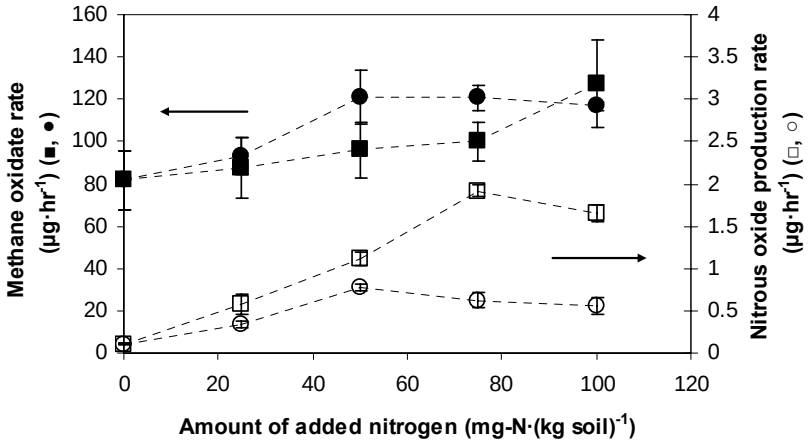


Figure 11. Effect of inorganic nitrogen on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of NH_4^+ on CH_4 consumption, ●: effect of NO_3^- on CH_4 consumption, □: effect of NH_4^+ on N_2O production, ○: effect of NO_3^- on N_2O production

As shown in Figure 12, 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.

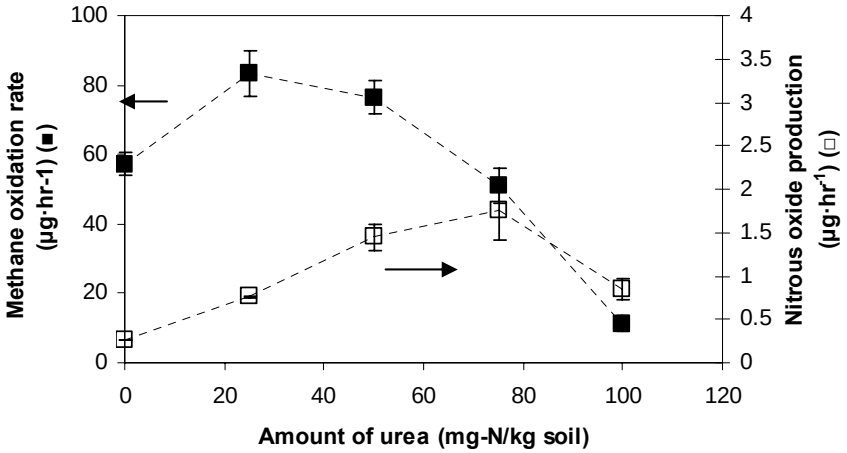


Figure 12. Effect of urea on CH_4 oxidation and N_2O production production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of urea on CH_4 consumption, □: effect of urea on N_2O production.

As shown in Figure 13, 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 $\mu g \cdot hr^{-1}$ while at 30 % moisture content, CH_4 oxidation rates were 51 ± 5 $\mu g \cdot hr^{-1}$. Compared to the CH_4 oxidation rate observed at 15 % moisture content, (82 ± 8 $\mu g \cdot hr^{-1}$), 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} $\mu g \cdot hr^{-1}$) was significantly lower than that measured at 15 % moisture content (0.14 ± 0.01$\mu g \cdot hr^{-1}$) at a 99 % confidence level. The rate of N_2O production at 30 % moisture content was 0.096 ± 0.012 $\mu g \cdot hr^{-1}$, significantly lower than the rate at 15 % moisture content at a 90 % confidence level.

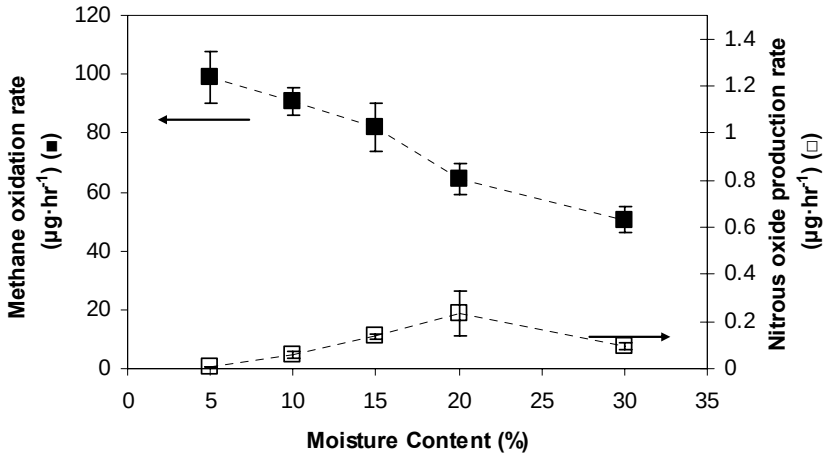


Figure 13. Effect of moisture content on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and varying moisture content. ■: effect of moisture content on CH_4 consumption, □: effect of moisture content on N_2O production.

As shown in Figure 14, 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 $\mu g \cdot hr^{-1}$), 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.

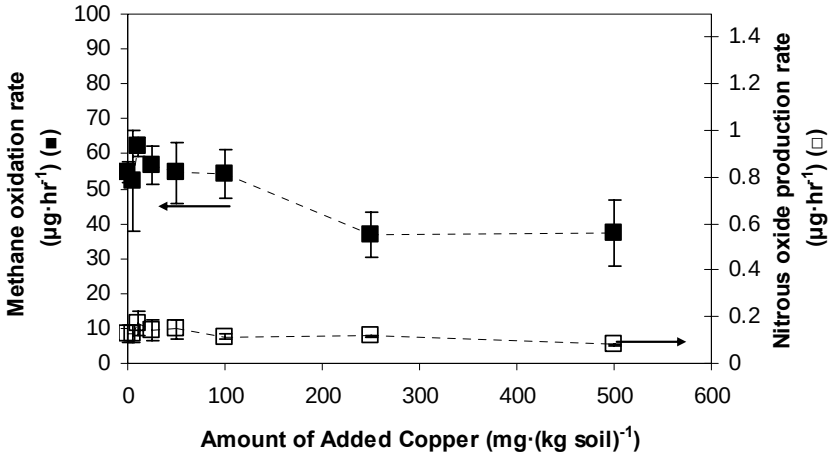


Figure 14. Effect of copper on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of copper on CH_4 consumption, □: effect of copper on N_2O production.

As shown in Figure 15, 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 µg·hr^{-1}, N_2O production rate when soil was amended with no organic carbon, to 0.40 ± 0.13 µg·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.

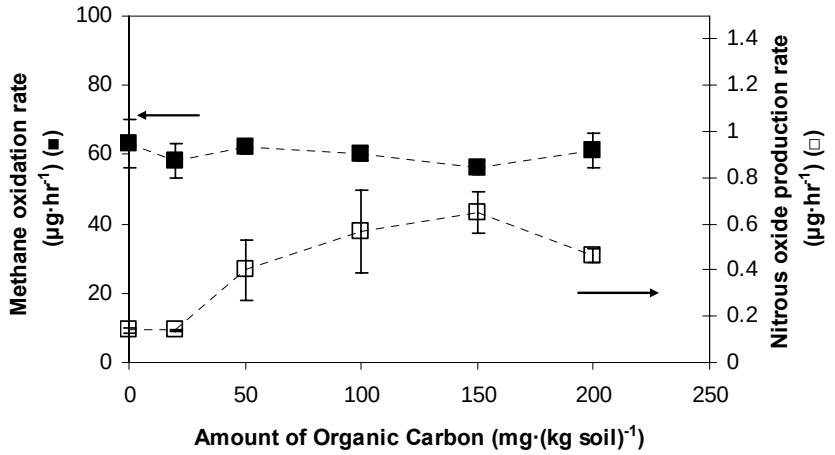


Figure 15. Effect of organic carbon on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 15 % moisture content. ■: effect of organic carbon on CH_4 consumption, □: effect of organic carbon on N_2O production.

3.2.1.2. 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 (Lontoh, et al., 2000). 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)$^{-1}$.

As can be seen in Figure 16, 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.

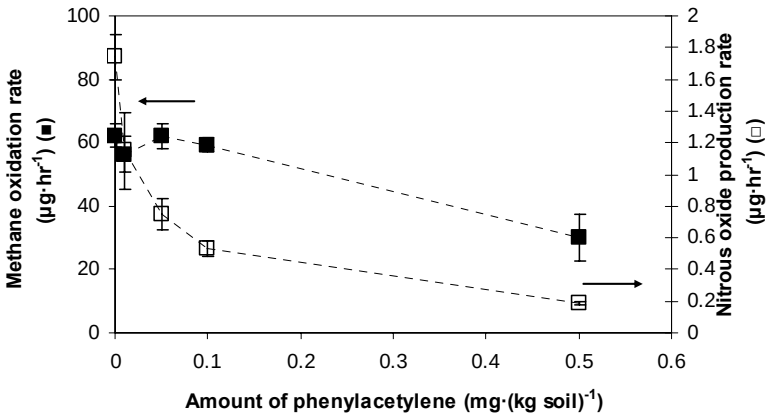


Figure 16. Effect of phenylacetylene on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 25 mg-N NH_4^+·(kg soil)$^{-1}$. ■: effect of phenylacetylene on CH_4 consumption, □: effect of phenylacetylene on N_2O production.

The applicability of a different specific inhibitor, i.e., chlorate, for N_2O producers through denitrification was also investigated (Kucera, 2006). 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 17, 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.

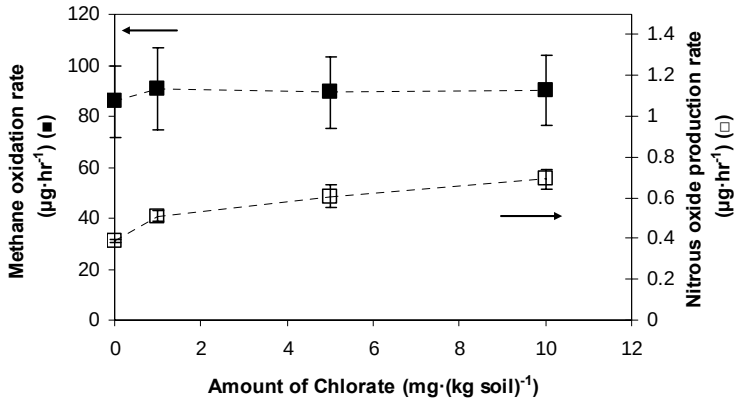


Figure 17. Effect of chlorate on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 25 mg-N NO_3^-·(kg soil)$^{-1}$. ■: effect of chlorate on CH_4 consumption, □: effect of chlorate on N_2O production.

3.2.1.3. 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 16), 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 18, 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)$^{-1}$.

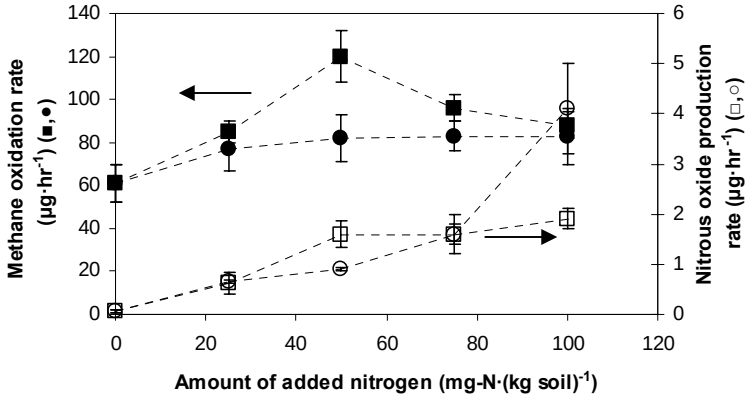


Figure 18. Effect of inorganic nitrogen on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of NH_4^+ on CH_4 consumption, ●: effect of NO_3^- on CH_4 consumption, □: effect of NH_4^+ on N_2O production, ○: effect 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 19, 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)$^{-1}$.

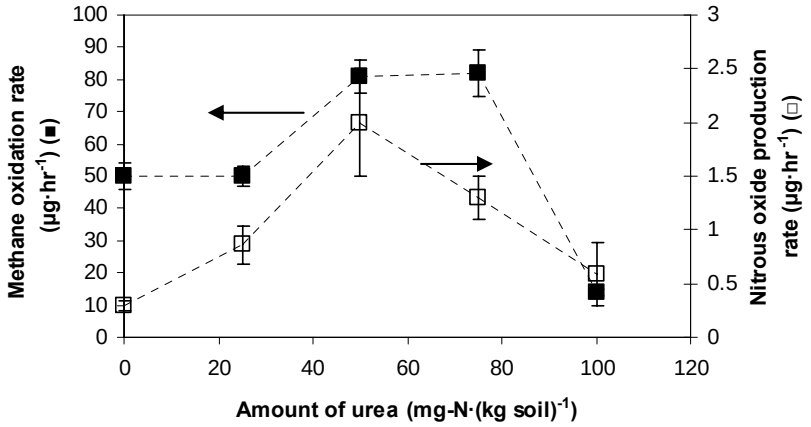


Figure 19. Effect of urea on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of urea on CH_4 consumption, □: effect of urea on N_2O production.

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 20, 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).

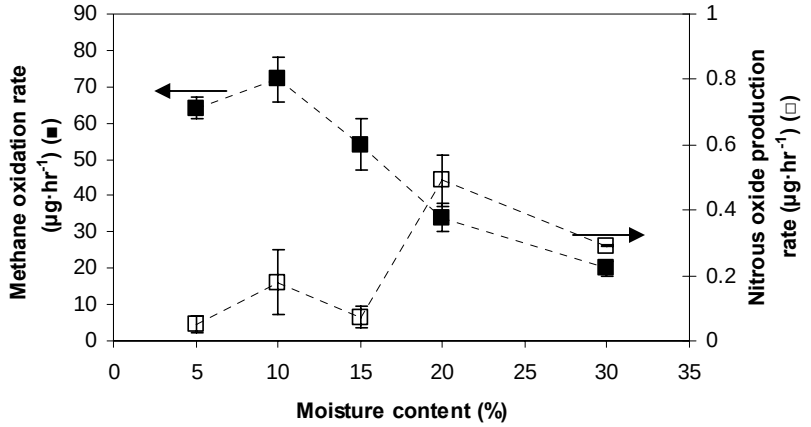


Figure 20. Effect of moisture content on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, varying moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of moisture content on CH_4 consumption, □: effect of moisture content on N_2O production.

As shown in Figure 21, addition of copper did not have any significant effect on CH_4 oxidation. Also, copper did not affect N_2O production even at 500 mg·(kg soil)$^{-1}$.

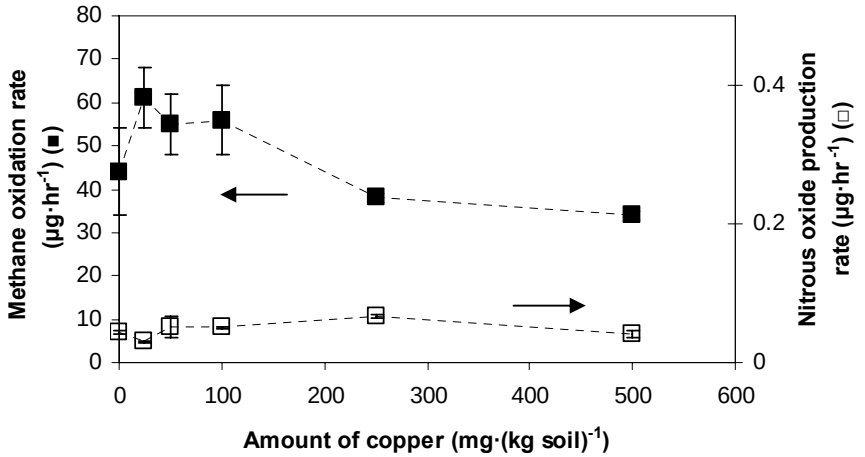


Figure 21. Effect of copper on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 mg·(kg soil)$^{-1}$ phenylacetylene. ■: effect of copper on CH_4 consumption, effect of copper on N_2O production.

As shown in Figure 22, 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 %).

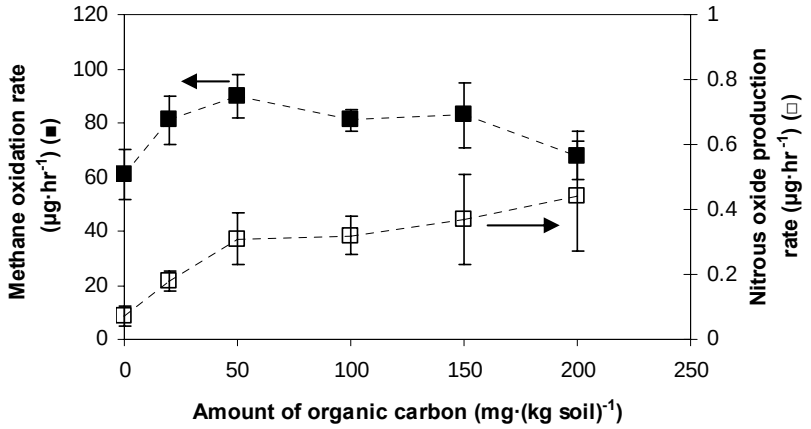


Figure 22. Effect of organic carbon on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, 15 % moisture content, and 0.1 $mg \cdot (kg\ soil)^{-1}$ phenylacetylene. ■: effect of copper on CH_4 consumption, □: effect of copper on N_2O production.

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 %).

3.2.1.4. 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 23, addition of 15 mg-N $NO_3^- \cdot (kg\ soil)^{-1}$ with 5% moisture content stimulated methane oxidation from 71 ± 4 $\mu g \cdot hr^{-1}$ found in the absence of any amendments at 5% moisture content to 100 ± 2 $\mu g \cdot hr^{-1}$ (significant at a 99% confidence interval).

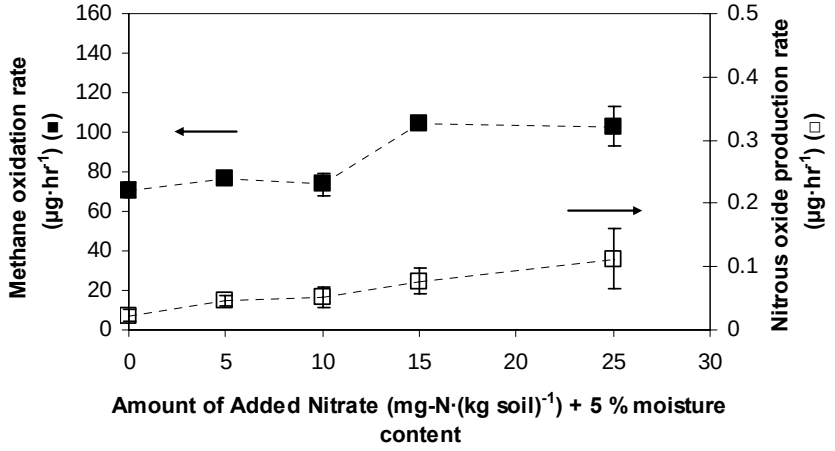


Figure 23. Effect of NO_3^- on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. ■: effect of NO_3^- on CH_4 consumption, □: effect of NO_3^- on N_2O production.

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 24), 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.

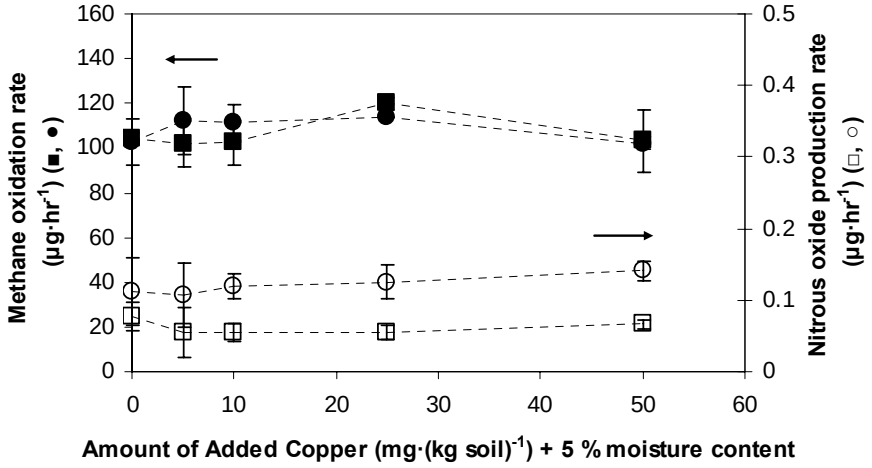


Figure 24. Effect of NO_3^- and copper on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. Effect of NO_3^- and copper on CH_4 oxidation (■,●) and N_2O production (□,○). Squares represent 15 mg-N NO_3^-·(kg soil)$^{-1}$, circles represent 25 mg-N NO_3^-·(kg soil)$^{-1}$.

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 24).

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 25). 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).

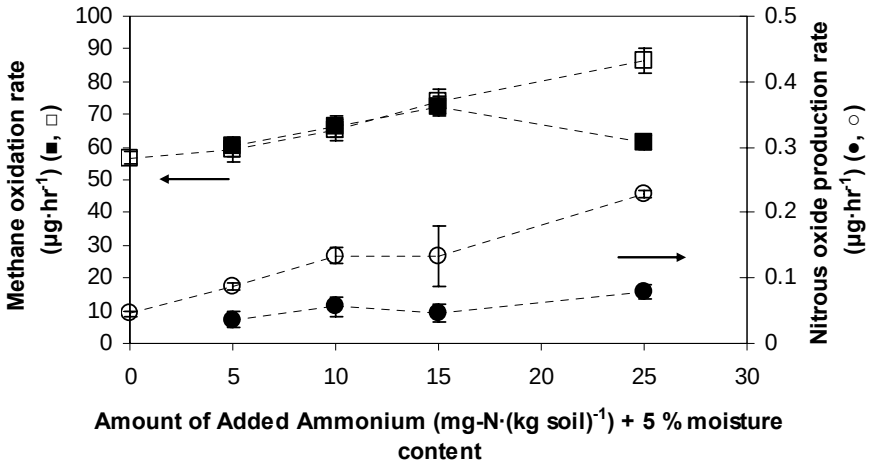


Figure 25. Effect of NH_4^+ with and without phenylacetylene on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. Effect of ammonium and 5% moisture content with (■,●) and without (□,○) 0.1 mg phenylacetylene·(kg soil)$^{-1}$ on CH_4 oxidation,(squares) and N_2O 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 25). 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 26.

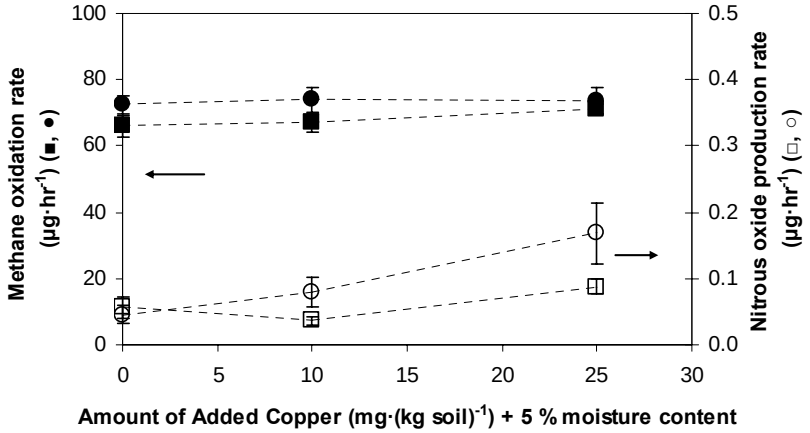


Figure 26. Effect of NH_4^+ and copper with 0.1 mg·(kg soil)$^{-1}$ on CH_4 oxidation and N_2O production under 20 % CH_4, 10 % O_2, and 5 % moisture content. Effect of NH_4^+ and copper on CH_4 oxidation (■,●) and N_2O production (□,○). Squares represent 10 mg-N NH_4^+·(kg soil)$^{-1}$, circles represent 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.

3.2.2. 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 11). 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 (Dunfield and Knowles, 1995; King and Schnell, 1994a, 1998). Alternatively, since methanotrophs can oxidize NH_4^+ to NO_2^-, inhibition due to the produced NO_2^- may occur (King and Schnell, 1994b). 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 (De Visscher and van Cleemput, 2007; Gulledge and Schimel, 1998). 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 (King and Schnell, 1998). 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^+. Although the production NO_2^- via MMO activity could have been 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, the effect of NO_3^- on CH_4 oxidation were similar to the effect 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 11). 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 (Hilger, et al., 2000). 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 perhaps due to utilization of the added NO_3^- by denitrifiers.

The addition of urea had stimulatory effects on CH_4 oxidation but also on N_2O production (Figure 12). However, unlike NH_4^+ and NO_3^-, when the amount of urea added to the soils was increased to 100 mg-N$\cdot(\text{kg soil})^{-1}$, urea inhibited CH_4 oxidation.

Moisture content was an important parameter in both CH_4 oxidation and N_2O production (Figure 13). 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 (Murrell, et al., 2000b), 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 14). 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 microbes leading to a decrease in N_2O production while CH_4 oxidation was not affected when 0.1 mg·(kg soil)$^{-1}$ of phenylacetylene was added (Figure 16). 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 17).

The effect of varying amendments, i.e., inorganic nitrogen, urea, moisture content, copper, and organic carbon, in the presence of 0.1 mg·(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·(kg soil)$^{-1}$ of phenylacetylene, the addition of NH_4^+ had a stimulatory effect on both CH_4 oxidation and N_2O production (Figure 18). 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 is the predominant pathway (Batemen and Baggs, 2005; Bollmann and Conrad, 1998; Khalil, et al., 2004). 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 (Bodelier and Frenzel, 1999; Megraw and Knowles, 1989). 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·(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 (Mandernack, et al., 2000) with ammonia-oxidizing bacteria having less significant role (Yoshinari, et al., 1985). 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.

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 9. 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 9 are % changes relative to the baseline conditions.

$$Effect\ on\ CH_4\ oxidation = \frac{CH_4\ oxidation\ rate\ (with\ amendments)}{CH_4\ oxidation\ rate\ (baseline)} \tag{16}$$

$$Effect\ on\ N_2O\ production = \frac{N_2O\ production\ rate\ (with\ amendments)}{N_2O\ production\ rate\ (baseline)} \tag{17}$$

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 9. 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 10. Similar to the aforementioned approach, the ratios of CH_4 production rates and N_2O production rates relative to rates observed at baseline conditions, (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 10. 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 11. 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 11. 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 12, 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 (Rinne, et al., 2005). 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 12. 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**

3.3. Effect of amendments on microbial community structure and gene expression in soil microcosms

3.3.1. Results f effect of amendments on methanotrophic community structure and gene expression

3.3.1.1. DNA analyses of the presence of methanotrophs, nitrifiers, and denitrifiers. PCR amplification of genes known to be involved in the oxidation of methane and nitrous oxide production were extracted from the soils used for microarray analyses. *pmoA* (encoding for the α-subunit of the particulate methane monooxygenase) was observed in all treatments (Figure 27A), as was *mmoX* (encoding for the α-subunit of the sMMO hydroxylase – Figure 27B). As can be seen in Figure 27C and D, evidence of both bacterial and archaeal ammonia oxidizers was found from PCR amplification of *amoA* (encoding for the α-subunit of the ammonia monooxygenase). Quantitative analyses are not possible with these amplifications, although stronger bands were observed from *amoA* amplification from bacterial ammonia oxidizers in microcosms incubated in the presence of ammonia as compared to no amendments (Figure 27C, lanes 1 and 2). The combined addition of phenylacetylene and ammonia reduced, but did not eliminate the band intensity of the *amoA* PCR product from ammonia-oxidizing bacteria (Figure 2C, lane 3). Similarly, while *amoA* from ammonia-oxidizing archaea was easily amplified from microcosms either with no amendments, or with the addition of ammonium, no amplification of archaeal *amoA* was found when phenylacetylene was also added (Figure 27D, lane 3). No PCR products from the amplification of *nirK*, encoding for the copper-containing nitrite reductase, were found using two different primer sets, one for general amplification of *nirK* from environmental samples, NirK1F and NirK5R (Braker and Tiedje, 2003) and another found to be more specific for ammonia-oxidizing bacteria, F1aCu and R3Cu (Hallin and Lindgren, 1999) (data not shown). Finally, no PCR products were observed from the amplification of *norB*, encoding for nitrate reductase (data not shown).

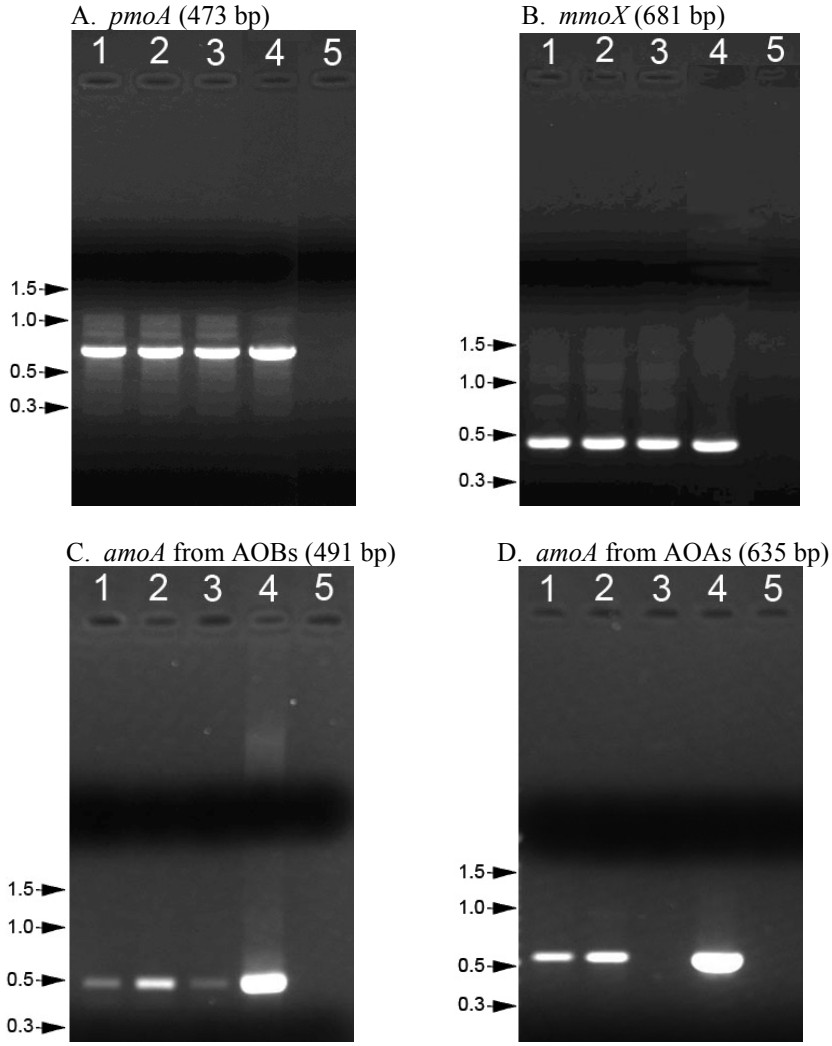


Figure 27. PCR amplification of fragments of: (A) *pmoA*(473 bp) ; (B) *mmoX* (681 bp); (C) *amoA* of ammonia oxidizing bacteria (491 bp), and (D) *amoA* of ammonia-oxidizing archaea (635 bp). Lane 1 - microcosms incubated with 20 % CH_4, 10 % O_2, and 5 % moisture content; Lane 2 – microcosms incubated with 20 % CH_4, 10 % O_2, 5 % moisture content, and 15 mg-N NH_4^+·(kg soil)$^{-1}$; Lane 3 – microcosms incubated with 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg soil)$^{-1}$, and 0.1 mg phenylacetylene·(kg soil)$^{-1}$. Lane 4 - positive control using either chromosomal DNA of *M. trichosporium* OB3b (4A and 4B); chromosomal DNA of *N. europaea* (4C), or; archaeal *amoA* clone from activated sludge; (4D). Lane 5 - negative control (water). Sizes of molecular standards (in kbp) are indicated to the left of each figure.

3.3.1.2. Microarray Analyses. 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., (1) 20 % CH_4, 10 % O_2, 5 % moisture content, (2) 20 % CH_4, 10 % O_2, 5 % moisture content with 15 mg-N NH_4^+·(kg soil)$^{-1}$, and (3) 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 the first 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 27. A complete list of probes used for the DNA microarray and the information on the targets can be found in Appendix A.

As shown in Figure 28, 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 $NH_4^+ \cdot (\text{kg soil})^{-1}$, 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 $NH_4^+ \cdot (\text{kg soil})^{-1}$. Signals from Mm531 (*Methylomonas*) and Ia193 (general Type I) and Msi423 (*Methylosinus*) showed a decrease in intensity.

With the addition of 15 mg-N $NH_4^+ \cdot (\text{kg soil})^{-1}$ and 0.1 mg $C_8H_6 \cdot (\text{kg soil})^{-1}$, 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.

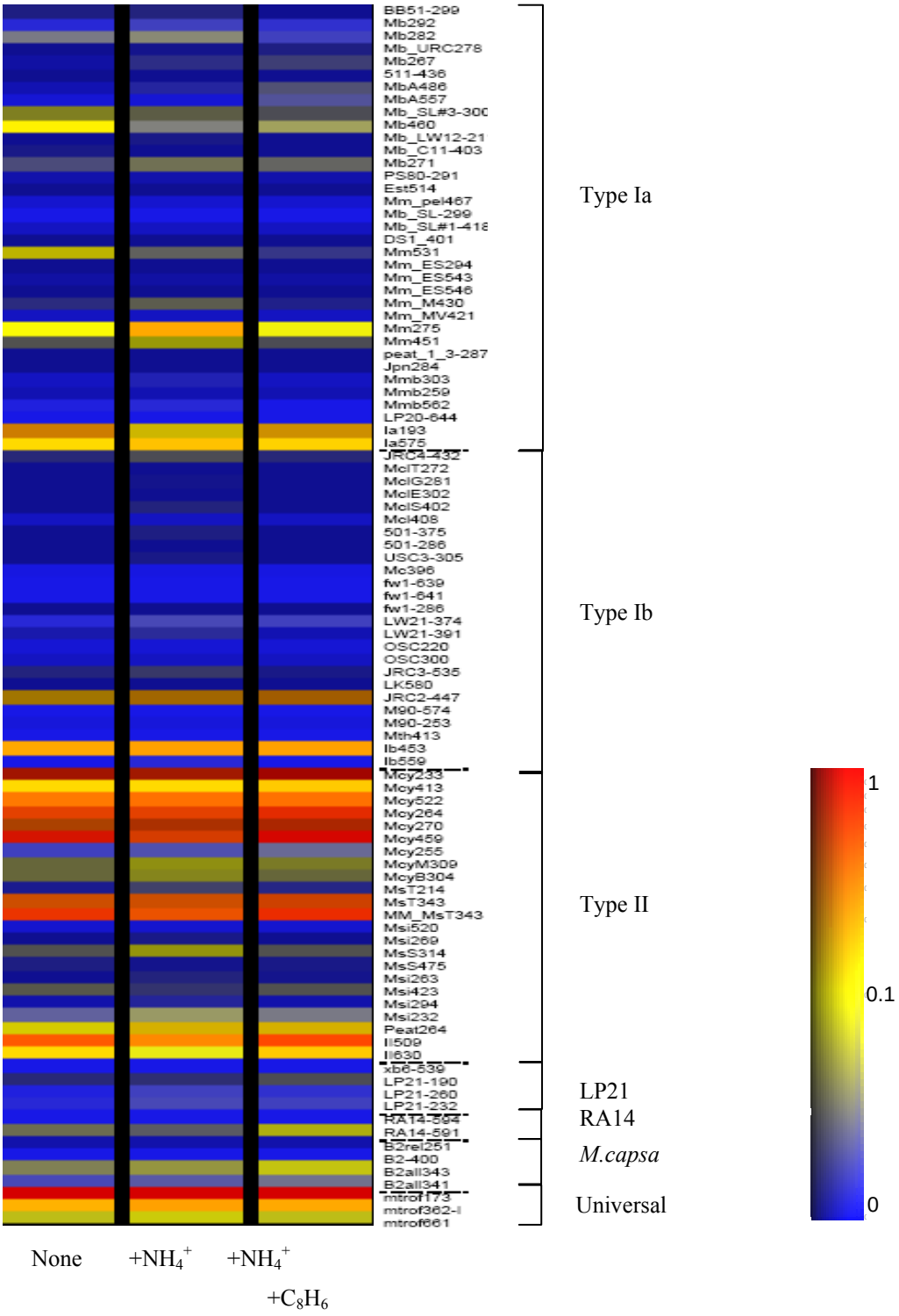


Figure 28. *pmoA* based DNA microarray. Relative signal intensities are shown as color spectrum with 1 being the maximum achievable signal for each probe. Labels on the bottom indicate; None: 20 % CH_4, 10 % O_2, 5 % moisture content, $+NH_4^+$: 20 % CH_4, 10 % O_2, 5 % moisture content, 15 mg-N NH_4^+·(kg 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)$^{-1}$.

3.3.1.3. 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 29A) but not *mmoX* (Figure 29B), indicating pMMO was the primary MMO being expressed *in situ*. Interestingly, *amoA* transcripts from either ammonia-oxidizing bacteria or archaea were not detected, nor were any transcripts of *nirK* or *norB* observed under any condition(data not shown).

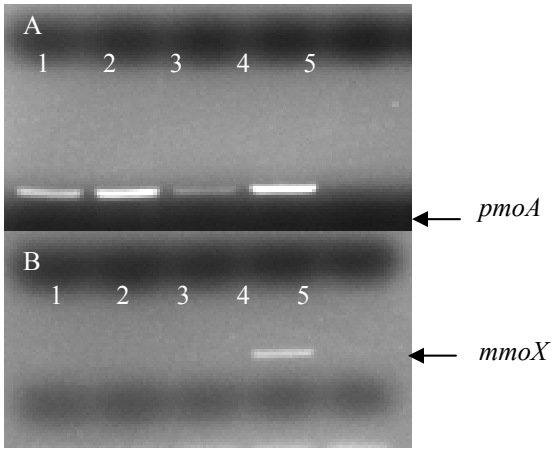


Figure 29. PCR amplification of cDNA prepared from mRNA extracted from soils incubated under i) 20 % CH_4, 10 % O_2, and 5 % moisture content (lanes 1), ii) 20 % CH_4, 10 % O_2, 5 % moisture content, and 15 mg-N NH_4^+·(kg soil)$^{-1}$ (lanes 2), and iii) 20 % CH_4, 10 % O_2, 5 % moisture content, 15 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*, and B) *mmoX*. Lanes 4 are positive controls using mRNA extracted from either pMMO- or sMMO-expressing *Methylosinus trichosporium* OB3b (A and B). Lanes 5 are negative controls with no nucleic acids added.

3.3.2. Discussion of DNA and mRNA analyses in soil microcosms

Overall, the soils used for microcosm experiments were predominated by the genera *Methylocystis*, which has been found in relatively large numbers in other landfills (Bodrossy, et al., 2003; Cebron, et al., 2007; Stralis-Pavese et al., 2004). Generally, Type I methanotrophs are suggested to be more competitive in nutrient-rich environments (Cebron, et al., 2007). 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 29, *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* (Chen, et al., 2007. 2008). 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^+. 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 microbes 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 microbes, respectively, although N_2O production rates of ammonia-oxidizing microbes, particularly that of AOAs can exceed that of methanotrophs (Yoshinari, et al., 1985), the size of the methanotrophic population could have been responsible for a large amount of N_2O produced.

3.4. Biogeochemistry changes in response to different amendments at King Highway Landfill

Different geochemical parameters can have different effects on methane oxidation and nitrous oxide production. In laboratory microcosms study using landfill cover soils, as described above, it was found that relatively dry soils (5% moisture content) along with 15 mg NH_4^+ (kg soil)$^{-1}$ and 0.1 mg phenylacetylene (kg soil)$^{-1}$ substantially enhanced methane oxidation while minimizing N_2O production. Here, the long-term effects of six amendments identified from this previous study, (1) control, i.e., no amendments; (2) addition of 0.5 M of NH_4Cl + 0.25 M of KNO_3 and; (3) addition of 0.5 M of NH_4Cl + 0.25 M of KNO_3 + 0.01% (w/v) phenylacetylene, each in the presence/absence of canopy, on the physico-chemical properties of soils as well as

landfill gases were investigated. Soil samples collected from the long term geochemical application site were analyzed for pH, inorganic nitrogen content (NH_4^+-N and NO_3^--N+NO_2^--N), bioavailable and total copper concentration. Since the amendments included inorganic nitrogen sources, the level of NH_4^+ and NO_3^--N+NO_2^--N were important parameters to investigate the long term effects of amendment on CH_4 oxidation and N_2O production as well as on microbial activity. Landfill gases, specifically, CH_4, N_2O and O_2, were periodically monitored during the field scale trials. Statistical analyses were performed to examine for statistically significant differences in soil physicochemical properties among the plots.

3.4.1. Results of amendments at King Highway Landfill

3.4.1.1. Vertical gradients of pH, inorganic nitrogen and metals. As noted above, the composition of the landfill soil was found to be ~93% sand, with the remainder a mixture of silt and clay, and was classified as sand based on standard USDA soil texture classification analyses (Soil Survey Division Staff, 1993). The soil pH varied between 7.2 and 7.5 over the entire depth with no clear trend (Figure 30). Inorganic nitrogen as ammonium and nitrate/nitrite associated with soils from each core was also measured (Figure 31). To determine if there were any significant differences among plots, statistical analyses were performed using the mixed models procedure (PROC MIXED) of SAS ver 9.2 (SAS Institute, NC) (Figure 32). Since the soil layer close to the surface has higher potential to exchange with the atmosphere, data from 20~40 cm of depth were used for the statistical analysis. Furthermore, the amendments applied in this research, i.e., NH_4^+, NO_3^- and phenylacetylene, mainly target methanotrophs and ammonia oxidizers, and they show much higher activities in aerobic regions. The ammonium content in plots 3 and 4 were significantly higher than all the other plots, including plots 6 and 7 which were also amended with the same amount of NH_4^+, but without a canopy. This suggests that a canopy inhibited biological activity related to NH_4^+ uptake or oxidation, possibly due to altered water content. On the other hand, the NH_4^+ content in plots 6 and 7 was lower than plot 5 even though plots 6 and 7 were amended with NH_4^+ and NO_3^-, suggesting amendment of NH_4^+ and NO_3^- enhanced biological activity in the absence of canopy. There was no significant difference in NH_4^+ concentration by the addition of phenylacetylene. Unlike NH_4^+ content, analysis of NO_3^- + NO_2^- concentration showed significantly lower concentrations of NO_3^- + NO_2^- in plot 2 and plot 7 than in other plots. Bioavailable and total copper and iron were also measured but no significant differences between plots were observed (Figures 33 and 34).

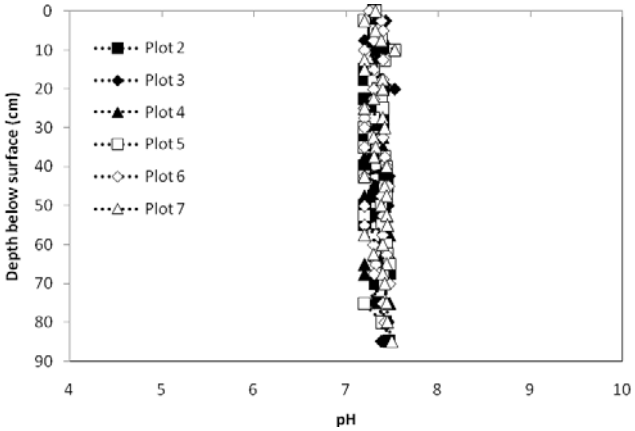
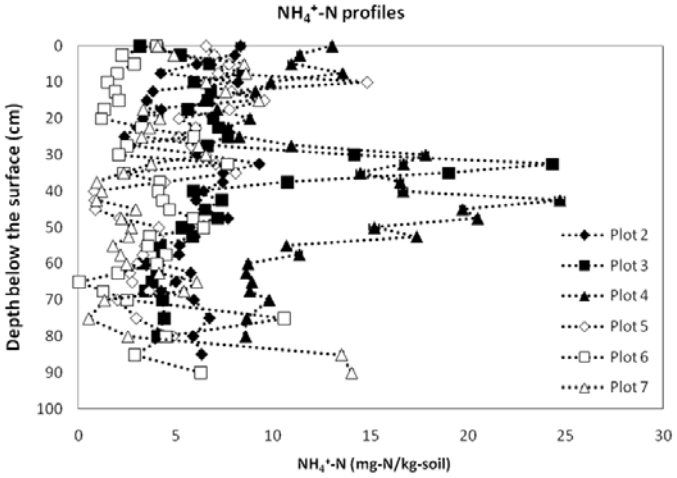


Figure 30. Depth profiles of soil pH of each plot. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

A



B

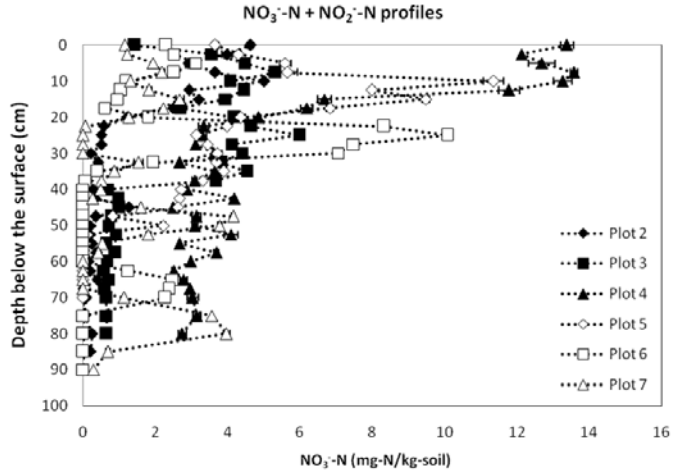


Figure 31. Depth profiles of NH_4^+ (A) and NO_3^-+NO_2^- (B) concentration of each plot. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

	2	3	4	5	6	7
2		3*	4*	NS	NS	NS
3			NS	3*	3*	3*
4				4*	4*	4*
5					5‡	5‡
6						NS
7						

NH_4^+ concentration

	2	3	4	5	6	7
2		3*	4*	5*	6*	NS
3			NS	NS	NS	3*
4				NS	NS	4†
5					NS	5†
6						6*
7						

NO_3^-+NO_2^- concentration

Figure 32. Significance of difference in NH_4^+ and NO_3^-+NO_2^- concentration among plots. Numbers in the box indicate plot with higher concentration. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene. *P < 0.01; †P < 0.05; ‡P < 0.10; NS: not significant (P > 0.10).

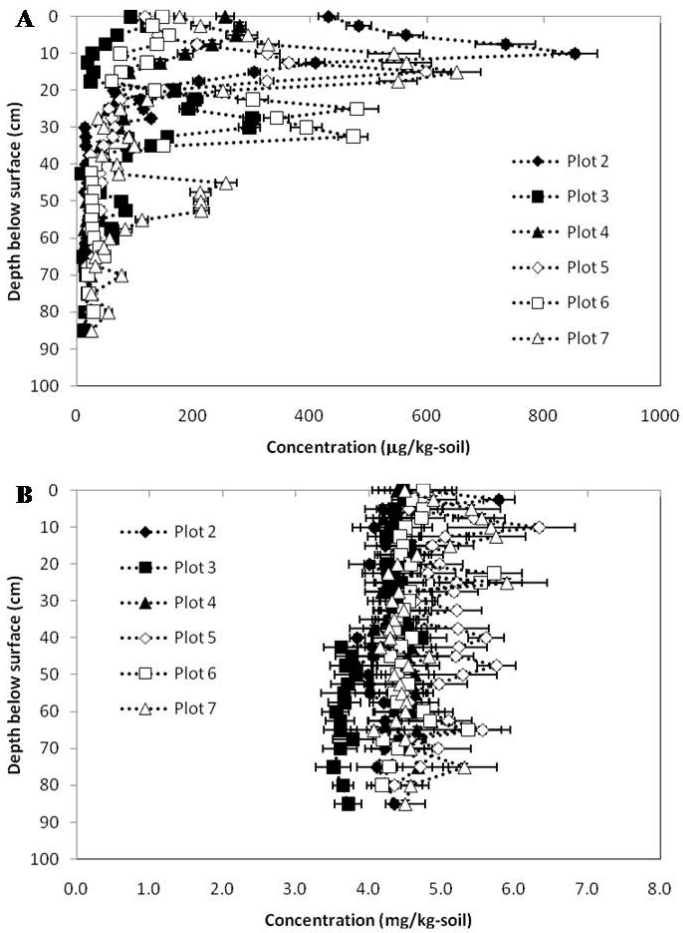


Figure 33. Vertical profiles of bioavailable copper (A) and iron (B). Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

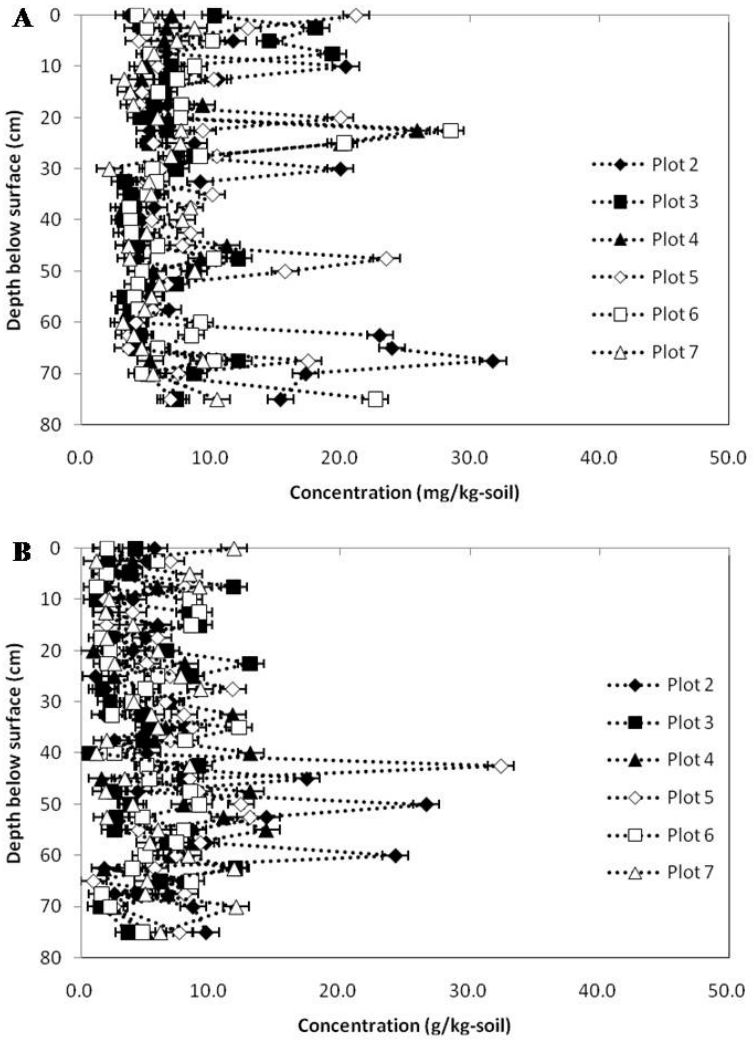


Figure 34. Vertical profiles of total copper (A) and iron (B). Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

From these results, it appears that any shifts in microbial activity and/or community structure that occurred (see below) are due to differences in nitrogen and/or phenylacetylene.

3.4.1.2. Monitoring of vertical landfill soil gas profiles. Soil gas samples were collected from 20 cm to a depth of 100 cm with an interval of 10 cm. N_2O profiles in each amendment plot from November 2007 to July 2009 are shown in Figure 35. The profiles showed significant spatial and seasonal variations with values varying from 0 to 92 ppmv. There were distict seasonal emission patterns, with higher production during the summer or fall and little N_2O detected during the winter or spring. CH_4 also showed strong seasonal pattern, i.e., higher methanotrophic activity in warmer temperatures, as evidenced by higher CH_4 concentration during the winter and spring than summer or fall (Figure 36). Both seasonal patterns of N_2O production and CH_4 oxidation suggest that both gases are regulated by microbial activities which are strongly dependent on temperature (Figure 37). O_2 profiles showed some variation (Figure 38), but there were no specific trends thoughout the field test.

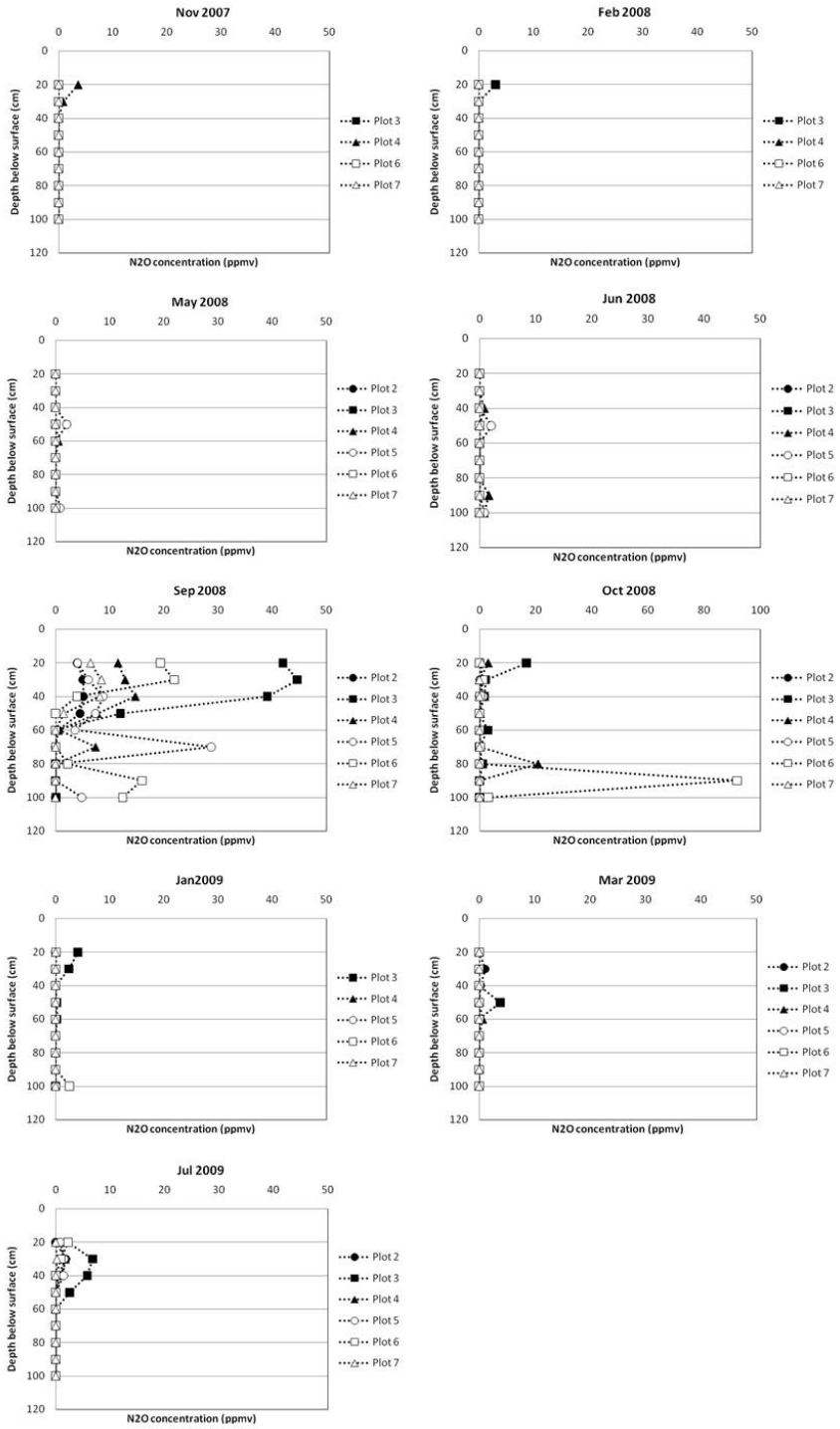


Figure 35. Nitrous oxide production from each plot from November 2007 to July 2009. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

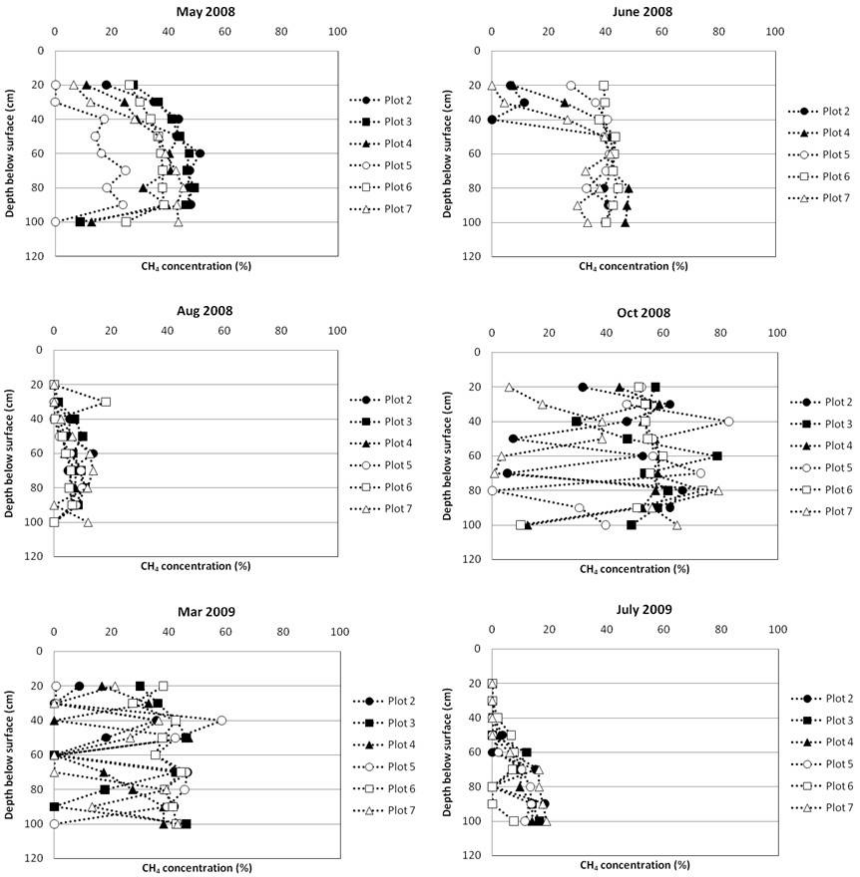


Figure 36. Depth profiles of methane concentration of each plot from May 2008 to July 2009. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

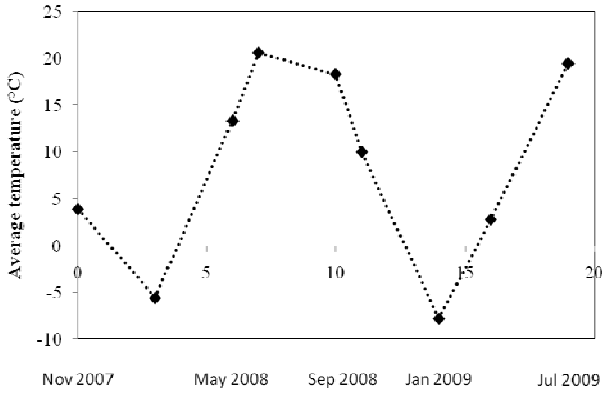


Figure 37. Average temperature in Kalamazoo, MI during the field application (Monthly weather summary. National Weather Service. 02 June 2010 <http://www.nws.noaa.gov/>).

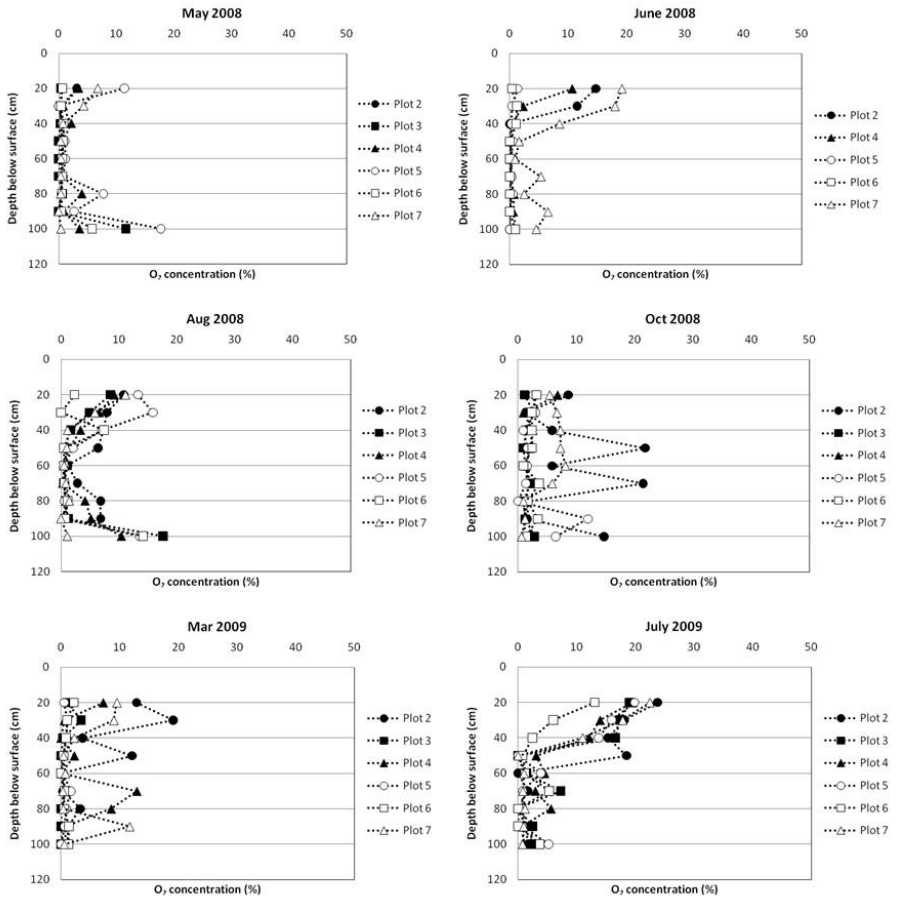


Figure 38. Depth profiles of oxygen concentration of each plot from May 2008 to July 2009. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

The same statistical analysis performed in the previous section on soil geochemical properties was done using the mixed models procedure (PROC MIXED) of SAS ver 9.2 (SAS Institute, NC) to test for statistically significant differences in N_2O and CH_4 concentrations among the different plots (Figure 39). N_2O productions from plot 3 and 6 were higher than those from plot 2 and 5, respectively, suggesting that nitrogen amendments enhanced N_2O production. N_2O production from plot 3 and 6 were also higher than plot 4 and 7, respectively, suggesting that phenylacetylene repressed N_2O production. Comparison between plot 3 and 6 revealed that a canopy enhanced N_2O production. Plot 7, which were amended with nitrogen and phenylacetylene, showed significantly lower CH_4 concentrations than plots 5 and 6, which were either not amended or amended with nitrogen, respectively. This suggests that phenylacetylene might help methanotrophs to outcompete other microbial groups for limiting nutrients. Also, plot 3, which was amended with nitrogen under a canopy, showed higher CH_4 concentration than plot 7, suggesting there might be an inhibitory effect of a canopy on CH_4 oxidation. There was no significant difference in O_2 concentration, indicating that O_2 was dominated by diffusion rather than microbial consumption.

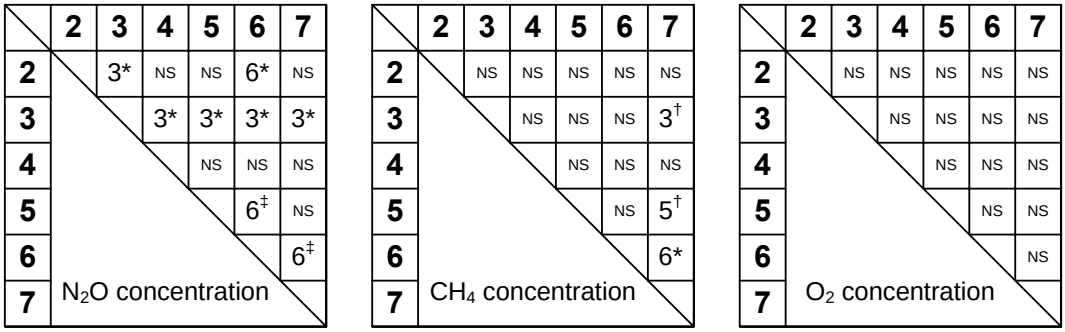


Figure 39. Significance of differences in nitrous oxide, methane and oxygen concentrations among plots. Numbers in the box indicate plot with higher concentration. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene. *P < 0.01; †P < 0.05; ‡P < 0.10; NS: not significant (P > 0.10).

3.4.1.3. Effects of amendments on microbial community structure and activity. DNA and mRNA were extracted from soil samples selected based on the landfill gas data and the soil physic-chemical properties to investigate the long-term effects of geochemical parameters on microbial community and their activities. For this purpose, functional genes that are unique or recognized as key components of CH_4 consumption or N_2O production, i.e., *pmoA*, *mmoX,* bacterial *amoA,* archaeal *amoA, nirK,* and *norB* were used. *pmoA* encodes the α subunit of pMMO and *amoA* encodes the α subunit of AMO. *pmoA* and *amoA* have been shown to be effective as a functional marker for studying methanotrophs and AOB/AOAs in the environment (Dumont and Murrell, 2005a; Prosser and Nicol, 2008). *nirK* encodes the copper containing nitrite reductase, and *norB* encodes the nitric oxide reductase.

The relative recovery efficiency of DNA and RNA from difference species in environmental samples is currently unknown and can be site-specific. To optimize the extraction method for the yield of bacterial and archaeal DNA from the King Highway landfill soils, three different bead beating times, i.e., 30 sec, 120 sec and 10 min, were tested. Figure 40 demonstrates the influence of bead beating times on bacterial and archaeal DNA extraction yield, represented by *pmoA* gene and archaeal *amoA* gene amplification, respectively. DNA extracted by bead beating for 10 min yielded the highest band intensities for *pmoA* gene while those for archaeal *amoA* gene was observed at 120 sec. From these results, it was decided that, for amplification of bacterial genes of interest (*pmoA*, bacterial *amoA*, *nirK*, and *norB*), bead beating times of 10 minutes would be used, and for amplification of archaeal genes of interest (archaeal *amoA*), a bead beating time of 120 sec would be used.

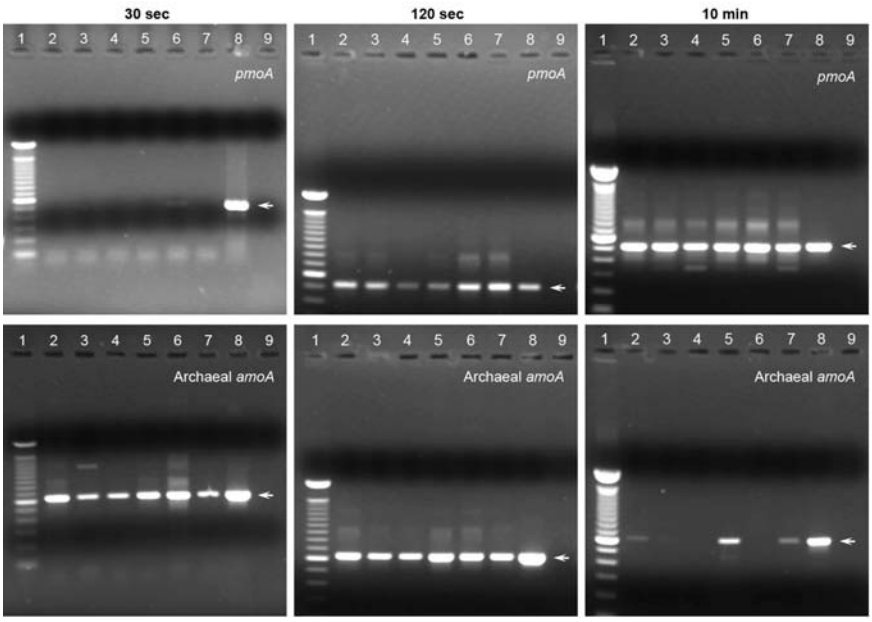


Figure 40. PCR amplification of DNA extracted from 25~27.5 cm depth of each soil core using three different bead beating times. Two different primer sets targeting pmoA and archaeal amoA genes were used. Lane 1: 100 bp DNA ladder; lane 2~7: plot 2~7, respectively; land 8: positive controls using either chromosomal DNA extracted from *Methylosinus trichosporium* OB3b for *pmoA*, archaeal *amoA* clone for archaeal *amoA* (courtesy of Dr. Craig S. Criddle). The arrow indicates the target fragment.

Microbial genomic DNA was extracted from 0.5 g of soil samples obtained from 25~27.5 cm depth of each soil core. Qualitative analysis was achieved by PCR assays targeting specific functional genes involved in the carbon/nitrogen cycle as described earlier (Figure 41). Methane oxidizing bacteria, especially those possessing particulate methane monooxygenase, were abundant in every plot as evidence by amplification of *pmoA*, while those possessing soluble methane monooxygenase was detected more in plots without a canopy as evidenced by amplification of *mmoX*. Interestingly, archaeal ammonia oxidizers were significantly more abundant than bacterial counterpart in every plot. Only multiple weak bands and smears were detected from the amplification of the *norB* gene, encoding for nitric oxide reductase, suggesting that the primer set and/or amplification conditions allowed for non-specific DNA amplification. Genes encoding copper-containing nitrite reductases, *nirK*, were detected in every plot. Both *norB* and *nirK* genes have been retrieved from denitrifying bacteria (Braker and Tiedje, 2003), and cultured AOB including *Nitrosomanas* and *Nitrosococcus* spp. (Casciotti and Ward, 2001; 2005). Since no bacterial *amoA* genes were detected in any plot, the presence of *nirK* genes may indicate those genes originated from denitrifying bacteria.

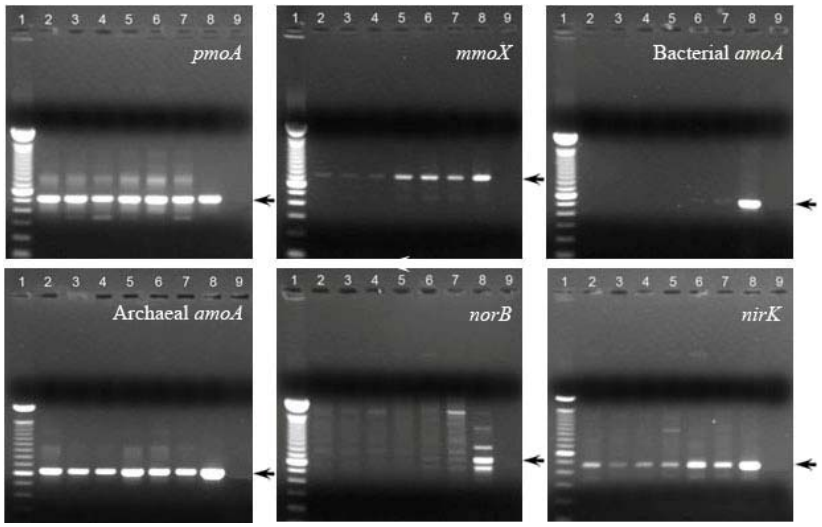


Figure 41. PCR amplification of DNA extracted from 25~27.5 cm depth of each soil core targeting specific functional genes. Lane 1: 100 bp DNA ladder; lane 2~7: plot 2~7, respectively; land 8: positive controls using either chromosomal DNA extracted from *Methylosinus trichosporium* OB3b for *pmoA* and *mmoX*, chromosomal DNA extracted from *Nitrosomonas europaea* for bacterial *amoA*, archaeal *amoA* clone for archaeal *amoA* (courtesy of Dr. Craig S. Criddle), chromosomal DNA extracted from *Achromobacter cycloclastes* for *norB* and *nirK*. The arrow indicates the expected size of the PCR product.

To examine the expression of functional genes, mRNA was extracted from soils from each soil core, and PCR assay was performed (Figure 42). Transcripts of *pmoA* were detected in all conditions but *mmoX* transcription products were not observed in any amendment. Transcripts of archaeal *amoA* were detected from plot 6. It appears that ammonia oxidizing archaea may be sensitive to phenylacetylene, but that cannot be conclusively proven at this point, largely due to the inability to cultivate these organisms in pure culture. No transcripts of bacterial *amoA*, *norB* and *nirK* were detected.

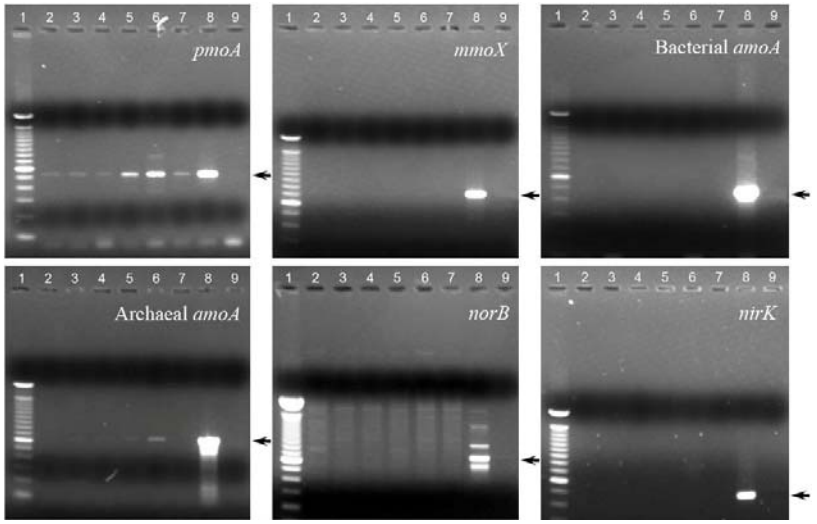


Figure 42. PCR amplification of cDNA reverse transcribed from mRNA extracted from 25~27.5 cm depth of each soil core targeting specific functional genes. Lane 1: 100 bp DNA ladder; lane 2~7: plot 2~7, respectively; land 8: positive controls using either chromosomal DNA extracted from *Methylosinus trichosporium* OB3b for *pmoA* and *mmoX*, chromosomal DNA extracted from *Nitrosomonas europaea* for bacterial *amoA*, archaeal *amoA* clone for archaeal *amoA* (courtesy of Dr. Craig S. Criddle), chromosomal DNA extracted from *Achromobacter cycloclastes* for *norB* and *nirK*. The arrow indicates the target fragment.

It has been shown that the AOA are prevalent in various terrestrial ecosystems (Leininger et al., 2006; He et al., 2007; Nicol et al., 2008) as well as in marine systems (Wuchter et al., 2006). Ammonia oxidizing archaea were abundant in the landfill cover soil than their bacterial counterpart, as shown in the PCR analysis on DNA extracted from each soil core (Figure 41). The level of transcripts of *amoA* from ammonia oxidizing archaea, however, was much lower than those of *pmoA* from methane oxidizing bacteria. It may be that the mRNA extraction efficiency of archaea is different from that of bacteria like DNA. Further optimization for archaeal RNA extraction may be required. It should be noted that, due to the intrinsic limitations of any PCR assay, a negative result may not always signify the absence of a gene. The composition of the isolated DNA is dependent on the efficiency of lysis, and some bacterial or microbial groups are far more difficult to lyse than others, as shown in this study as well as by others (More et al., 1994). Furthermore, the efficiency of cell lysis, efficiency and degree of nucleic acid purification and size of the isolated nucleic acids are crucial to the success of subsequent PCR methodologies but can be reduced when dealing with environmental samples such as soils.

pmoA gene PCR amplicons from each of the samples were hybridized against a microarray containing 175 probes encompassing the known breadth of methanotrophic diversity (Appendix A). As shown in Figure 43, Type II methanotrophs, especially *Methylocystis* populations (detected by probes McyB304, Mcy255, Mcy459, Mcy264, Mcy270, Mcy413, Mcy522, Mcy233, and McyM309, and broad specificity Type II-specific probes II509 and II630) dominated the methanotrophic community in plot 5, where no amendments were added. Hybridizations to *Methylosinus* sequences, in contrast, were relatively weak in plot 5. Probes targeting *Methylocapsa* and related species, B2all343 and B2all341, showed relatively high abundances in the control plot. Type Ia probes also revealed a generally high abundance of the *Methylobacter, Methylomonas, Methylomicrobium,* and *Methylosarcina* genera. Type Ib probes, targeting *Methylococcus, Methylothermus, Methylocaldum*, and related, uncultivated clades, were also abundant, but relatively less than other genera.

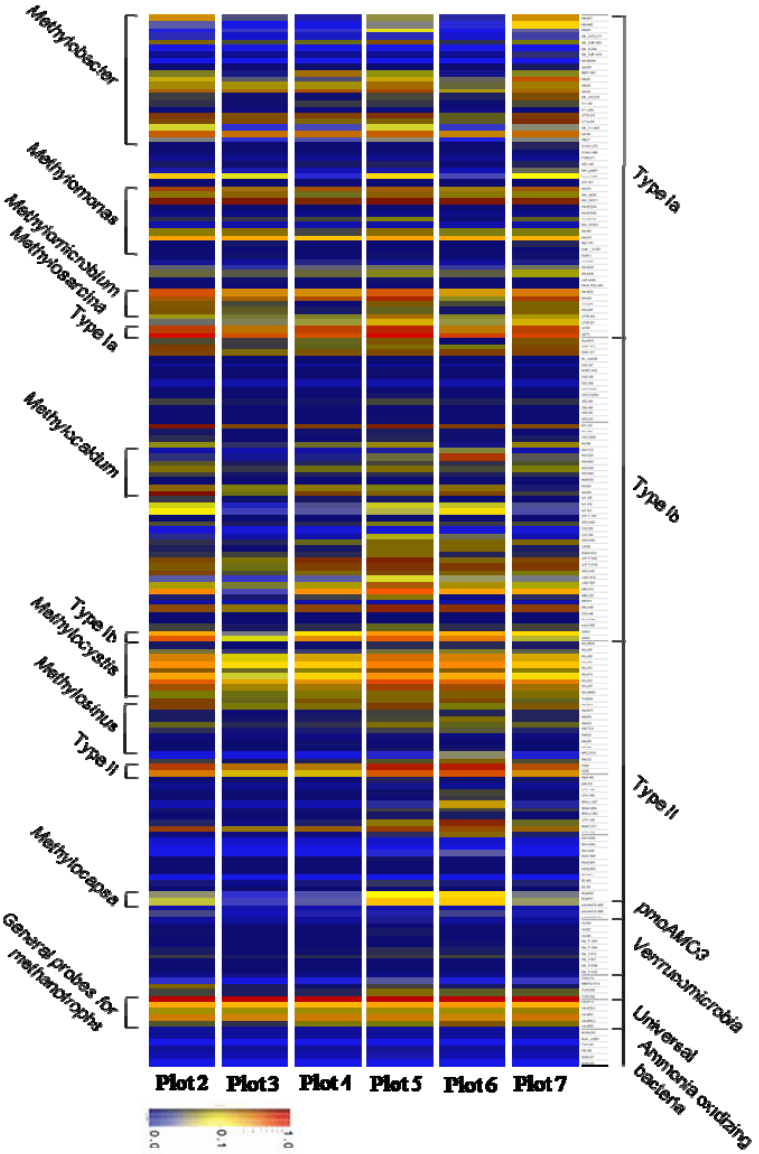


Figure 43. Summary of results for the *pmoA*-based methanotrophic community analysis using microarrays. Color coding bar on the right side represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates that 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

When comparing plot 2 and 5 with plot 3 and 6, respectively, with the addition of NH_4Cl and KNO_3 regardless of the presence or absence of a canopy, a decrease in probe intensity compared to that observed in each control plot (plot 2 and plot 5) was found for most probes except *Methylocaldum* and *Methylocystis sp.* Comparing plot 6 with 7, with the addition of phenylacetylene along with NH_4Cl and KNO_3 in the absence of a canopy, signals from most of Type Ia-probes, more specifically, 20 out of 21 probes targeting *Methylobacter*, 6 probes out of 11 targeting *Methylomonas*, 5 out of 6 probes targeting *Methylomicrobium*, and both probes targeting *Methylosarcina*, significantly increased, i.e., an increase in relative signal intensity

greater than 50% compared to that observed in soils with nitrogen only treatment. Similarly, comparing plot 3 with 4, a general increase of Type I probe was observed with the addition of NH_4Cl and KNO_3 together with phenylacetylene in the presence of a canopy. Inversely, comparing plot 6 with 7, most Type II probes showed a general decrease in intensity in response to the addition of phenylacetylene with NH_4Cl and KNO_3 in the absence of a canopy as compared to the plot amended with nitrogen alone. However, in the presence of a canopy, Type II probes showed a general increase with the addition of phenylacetylene comparing plot 3 with 4. Comparing plot 2, 3, and 4 with 5, 6, and 7, respectively, general decrease of both Type I and Type II targeting probes was observed in response to the presence of a canopy.

Table 13 shows the indices describing methanotrophic community diversity, richness, evenness and dominance in response to various amendments. Interestingly, comparing plot 2 and 5 with 3 and 6, respectively, the addition of NH_4Cl and KNO_3 reduced methanotrophic diversity as compared to each control plot both in the presence and absence of a canopy, but had only a small effect on richness and evenness. The methanotrophic community also exhibited more dominance with the addition of NH_4Cl and KNO_3, likely due to reduced presence of Type I methanotrophs based on the comparison of plot 2 and 5 with plot 3 and 6, respectively. Interestingly, comparing plot 2 and 5 with plot 4 and 7, respectively, the simultaneous addition of phenylacetylene with NH_4Cl and KNO_3 resulted in diversity and dominance indices similar to that of each control plot regardless of the presence or absence of a canopy. Evenness indices also increased to the greatest extent in response to phenylacetylene as shown from the comparison of plot 3 and 4 with plot 6 and 7.

Table 13. Diversity and richness estimates for methanotrophic community analysis. Plot 2: canopy, plot 3: canopy, NH_4Cl and KNO_3, plot 4: canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5: no amendment, plot 6: NH_4Cl and KNO_3, and plot 7: NH_4Cl, KNO_3 and phenylacetylene.

Plot	Simpson index of diversity	Menhinick index of richness	Simpson index of evenness	Berger-Parker index of dominance
2	47	19	0.60	0.05
3	35	19	0.64	0.09
4	42	18	0.69	0.05
5	53	20	0.59	0.04
6	48	21	0.60	0.05
7	53	22	0.65	0.04

The structure of the AOA communities in each soil core were examined via phylogenetic analysis of 213 archaeal *amoA* sequences collected from the six test plots. Between 12 to 27 AOA *amoA* OTUs were detected from each plot (Table 14). Rarefaction analysis was applied to evaluate whether the numbers of AOA sequences of each plot were sufficient to estimate diversity within the clone library. The calculated rarefaction curves were not fully saturated, but showed a slight tendency to saturation, indicating that an increasing number of clones would have revealed further diversity, especially more on the plot 7 which was amended with phenylacetylene, NH_4Cl and KNO_3 (Figure 44).

Table 14 summarizes the data obtained by the application of diversity and richness estimates to the obtained AOA *amoA* sequence data. All the sequences fell into two distinct groups, Group 1.1a (marine/sediment cluster) and 1.1b (soil/sediment cluster) as found by others (Ochsenreiter

et al. 2003; Francis et al. 2005; Nicol and Schleper 2006; Prosser and Nicol 2008). The sequences in each group share 46 to 99% identity at the nucleotide level, and 83 to 100% similarity and 76 to 100% identity at the amino acid level. The two groups share 46 to 74% identity at the nucleotide level, and 82 to 91% similarity and 75 to 94% identity at the amino acid level. Similarity and identity percentages were calculated using MatGAT 2.02 with BLOSUM50 scoring matrix. Of the 15 OTUs from plot 5, where no amendments were added, 5 OTUs fell into Group 1.1a lineage and 10 OTUs fell into 1.1b lineage. Increased availability of nitrogen as NH_4Cl and KNO_3, however, altered the AOA community structure regardless of the presence or absence of a canopy as shown from plot 3 and 6. Interestingly, OTUs affiliated with the Group 1.1a archaea disappeared in response to the addition of NH_4^+ and NO_3^-, while more Group 1.1b-related OTUs were recovered. Diversity estimates (*H'*) indicate that the Group 1.1a in the plot 2 and 5 were more diverse (*H'*=1.0~2.0) than in other plots (plot 3, 4, 6, and 7) receiving nitrogen or nitrogen along with phenylacetylene (*H'*=0.6 or 0). The number of OTUs of Group 1.1b increased from 3 to 10 in the presence of a canopy, and from 10 to 27 in the absence of a canopy when NH_4^+ and NO_3^- were added. Meanwhile, comparing plot 2 with plot 3, the number of OTUs of Group 1.1a decreased from 9 to 3 in the presence of a canopy, and it disappeared in the absence of a canopy as shown by a comparison of plot 5 with plot 6, when NH_4^+ and NO_3^- were added. The responses on the simultaneous addition of phenylacetylene together with NH_4Cl and KNO_3 were varied depending on the presence or absence of a canopy. Comparing plot 3 with plot 4, in the presence of a canopy, the number of OTUs of Group 1.1a decreased to 1, and the number of OTUs of Group 1.1b slightly increased to 11. The Shannon's biodiversity index (*H'*) of Group 1.1a decreased from 0.6 to 0, while that of Group 1.1b did not change. In the absence of a canopy, i.e., plot 7, on the other hand, 3 new OTUs affiliated with Group 1.1a were observed, and the number of OTUs in Group 1.1b lineage decreased by almost half, from 27 to 14, in comparison with that of the plot with NH_4^+ and NO_3^- amendments, i.e., plot 6. The Shannon's biodiversity index (*H'*) of Group 1.1b also decreased from 3.1 to 2.3, and that of Group 1.1a increased to 0.5. Regardless of the presence or absence of a canopy, community richness and evenness indices showed similar trends to the diversity index in the absence of a canopy. Those of group 1.1a decreased as nitrogen was added, and increased as phenylacetylene along with nitrogen was added, and vice versa for group 1.1b. Dominance indices showed that, unlike group 1.1b, group 1.1a was dominated by one clone in every plots, similar to the clone (GeneBank accession number: ABS52840) reported previously (Tourna et al. 2008).

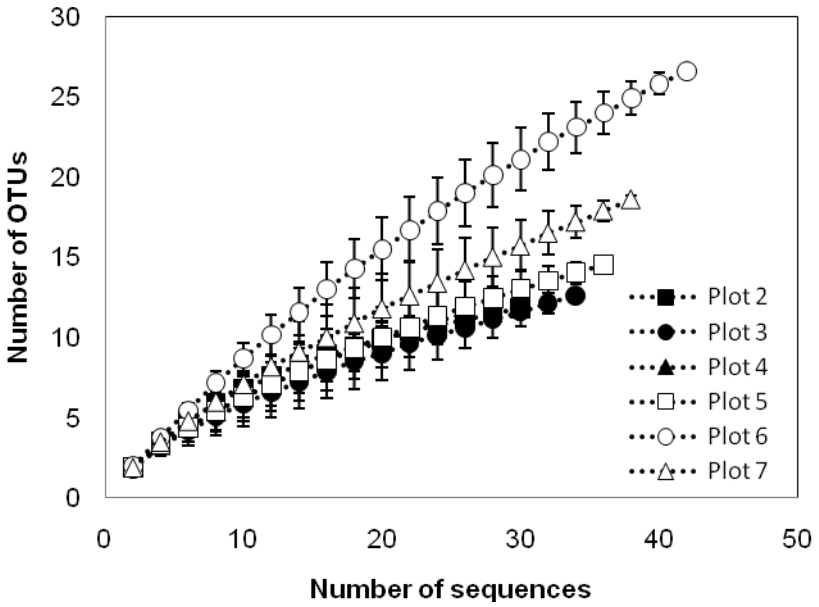


Figure 44. Rarefaction analyses of AOA *amoA* genes. OTUs were defined as those with ≥97% nucleotide sequence identity. Error bars represent 95% confidence limits. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

Table 14. Diversity and richness estimates for archaeal *amoA* gene libraries generated from King Highway landfill plots with added nitrogen and phenylacetylene. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3, plot 4 - canopy, NH_4Cl, KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3, and plot 7 – NH_4Cl, KNO_3 and phenylacetylene.

Plot	# of clones	Observed OTUs[a]			Shannon index (*H'*)		Simpson index of diversity (1/D)		Menhinick index of richness (D_{Mn})		Simpson index of evenness (E)		Berger-Parker index of dominance (d)	
		1.1a	1.1b	Total	1.1a	1.1b	1.1a	1.1b	1.1a	1.1b	1.1a	1.1b	1.1a	1.1b
2	31	9	3	12	2.0	1.1	6.9	3.0	1.7	1.7	0.8	1	0.2	0.3
3	35	2	10	12	0.4	2.0	1.5	5.3	0.8	2.2	0.7	0.5	0.9	0.4
4	27	1	10	11	0.0	2.0	1.0	5.3	1.0	2.2	1	0.5	1.0	0.4
5	35	5	8	13	1.0	1.6	2.0	4.9	1.3	1.8	0.4	0.6	0.7	0.5
6	38	0	22	22	N/A	2.7	N/A	18.1	N/A	3.6	N/A	0.8	N/A	0.2
7	35	3	12	15	0.5	2.1	1.4	10.9	0.8	2.6	0.5	0.9	0.8	0.2

[a]based on the similarity of 97% between sequences; N/A: not available

Libshuff analysis was employed to assess gross differences in archaeal ammonia oxidizers populations in response to amendments, as represented by the clone libraries (Table 15). The analysis showed that there were apparent variations between libraries from each plot indicating that the amendments affected the AOA community structure.

Table 15. Pairwise Libshuff comparisons of each archaeal amoA gene sequence libraries.

	P value for comparison of heterologous library (Y) with X					
	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	Plot 7
Plot 2		**0.002**	**0.002**			
Plot 3	0.085		0.836			
Plot 4	**0.004**	**0.016**				
Plot 5					**0.002**	0.815
Plot 6				**0.002**		**0.015**
Plot 7				**0.035**	**0.002**	

The consensus for Libshuff analyses is that if either X/Y or Y/X has a *P* value of <0.025, the two communities are significantly different (Schloss, 2004). Boldface values indicate significant P values.

3.4.2. Discussion of effect of amendments at King Highway Landfill

Long term evaluation of continuous landfill gas monitoring showed that application of NH_4^+ and NO_3^- together with phenylacetylene effectively enhanced CH_4 oxidation and simultaneously repressed N_2O production. Microarray and clone libraries analyses were performed to determine what effect these amendments had on the microbial community, specifically methane and ammonia-oxidizing microorganisms, respectively. Regardless of amendment, i.e., NH_4^+ and NO_3^-, or NH_4^+, NO_3^- and phenylacetylene, the soils were dominated by Type II methanotrophs, particularly the genus *Methylocystis*, which have been commonly found in large numbers in other landfills (Bodrossy et al. 2003; Stralis-Pavese et al. 2004; Cebron et al. 2007; Lee et al. 2009). With the addition of NH_4^+ and NO_3^-, N_2O production was enhanced, but there was no significant change in soil CH_4 concentration from the control plot at a depth between 20 to 40 cm, regardless of the presence of a canopy. Microarray results showed that the addition of nitrogen caused a reduction in the diversity of the methanotrophic community as compared to the control plot particularly reducing the presence of Type I methanotrophs. This suggests that either the activity of some methanotrophs (e.g., Type I methanotrophs), was inhibited by the addition of NH_4^+ and NO_3^-, (e.g., through competitive inhibition of CH_4 oxidation by the pMMO or through the production of toxic products such as NO_2^-) or the growth of other methanotrophs, (e.g., Type II methanotrophs), was preferentially stimulated, allowing these cells to dominate the methanotrophic community.

When phenylacetylene was added in conjunction with NH_4^+ and NO_3^- in the absence of a canopy, CH_4 oxidation was enhanced as evidenced by lower soil CH_4 concentrations, and the microarray analyses showed that signals of Type I methanotrophs increased, while the addition of NH_4^+ and NO_3^- alone led to decreased signals of these cells from microarray assays. Type I methanotrophs are believed to be more competitive in nutrient-rich environments (Graham *et al*. 1993; Bodelier

et al. 2000; Mohanty et al. 2006; Cebron et al. 2007), but they may be able to thrive best when nitrogen is added together with phenylacetylene as phenylacetylene may inhibit other microbial groups competing for the added nitrogen, e.g., ammonia-oxidizing microorganisms. Meanwhile, statistical analysis showed that CH_4 oxidation was not enhanced in the presence of a canopy when phenylacetylene was added in conjunction with NH_4^+ and NO_3^-. Since a canopy was installed to reduce soil moisture content, this may be due to that the modified moisture content caused an inhibitory effect on methanotrophic activity.

PCR analyses both on DNA and cDNA showed that the bacterial ammonia-oxidizers, which have earlier been considered to be major important contributors to *in situ* N_2O production (Jiang and Bakken 1999; Arp and Stein 2003), were not detected, while their archaeal counterparts were more abundant in every plot. Furthermore, the increase in AOA diversity as well as the shift in AOA community structure in response to nitrogen amendments suggested that AOAs, especially crenarchaeota affiliated with Group 1.1b, might contribute to N_2O production in this landfill cover soil. When phenylacetylene was added together with NH_4^+ and NO_3^-, N_2O production was repressed, and the level of expression of the archaeal *amoA* gene was reduced. Offre and his colleagues (2009) have also shown that archaeal nitrification can also be inhibited by acetylene, suggesting that phenylacetylene, a selective inhibitor known to preferentially inhibit AMO activity in ammonia-oxidizing bacteria might also act as an inhibitor of AMO activity in ammonia-oxidizing archaea and thus reduce the ability of these cells to produce N_2O from NH_4^+.

The effect of ammonia on the ammonia-oxidizing archaea community is intriguing. It is known that varying levels of ammonia can select for different groups of bacterial ammonia-oxidizers, with *Nitrosospira* 3a dominating at low ammonia concentrations (<76 μg NH_4^+-N(g-so il)$^{-1}$) while *Nitrosospira* cluster 3b more commonly found when incubated with 1 mg-N(g-soil)$^{-1}$ of urea, which can be rapidly converted to ammonia (Webster et al. 2005). It has also been suggested that AOAs and AOBs may prefer different niches, with AOAs predominating at very low ammonia levels (e.g., the growth threshold concentration of 'Candidatus *Nitrosopumilus maritimus*' strain SCM1 can be as low as 10-20 nM ammonium which is more than 100-fold lower than the minimum concentration required for growth (>1 μM at neutral pH) of cultivated AOBs) (Martens-Habbena et al. 2009). It is interesting to note that most of the archaeal *amoA* gene sequences affiliated with the group 1.1a have been obtained in low ammonium-containing environments such as the open ocean, and marine water columns, where typical ammonium concentrations are <0.03~1 μM (Konneke et al. 2005; Wuchter et al. 2006;, Beman et al. 2008), while most archaeal *amoA* genes detected from terrestrial environments, such as agricultural soils and the estuaries receiving agricultural run-off, where nitrogen content was reported to be as high as 7 - 9 mg/g-soil^{-1} (Nicol et al. 2003) or 0.28 – 3.91 mg/g-soil^{-1} (Nicol et al., 2005), were clustered within the group 1.1b (Nicol et al. 2003; Ochsenreiter et al. 2003; Nicol et al. 2005; Simon et al. 2005).

Even though PCR analysis showed high abundance of AOAs, the level of transcripts of *amoA* from AOAs was much lower than those of *pmoA* from methanotrophs. It may be that the mRNA extraction efficiency of archaea is different from that of bacteria like DNA. Further optimization for archaeal RNA extraction may be required.

In the earlier microcosm study, the results from microbial community structure analyses were different than that found in situ. PCR analyses in soil microcosms indicated the presence of *amoA* from ammonia oxidizing bacteria as well as archaea, and that the presence of the key genes associated with these cells was reduced with the addition of phenylacetylene. Furthermore, the methanotrophic community in the soil microcosms was far less diverse than that from the present study. Since the microcosm soil samples were first air-dried and stored at 4 °C in the dark for several months before analyses, the discrepancy may be due to variability in desiccation tolerance among different microbial groups. For example, ammonia oxidizing archaea may outcompete its bacterial counterpart in *in-situ* conditions, but it may be less successful after rewetting and reincubation. It has been reported that natural populations of ammonia oxidizing bacteria can survive for more than 3 months in air-dried soils, especially for the strains capable of producing extracellular polymeric substances (Allison and Prosser, 1991). Regarding methanotrophic community diversity, it can be suggested that there may be different desiccation tolerances among different methanotrophic species as well.

3.5. Methanotrophic-mediated nitrous oxide production

3.5.1. Results

3.5.1.1. Effects of nitrogen on methanotrophic production of nitrous oxide. The first experiments, aimed to investigate the effects of growth condition on N_2O production, were performed using *Methylosinus trichosporium* OB3b. The extent of N_2O production was dependent on growth conditions (Table 16), and more specifically, N_2O production was dependent on the concentration of ammonia. N_2O was detected up to 331 ppmv when *Methylosinus trichosporium* OB3b was growing on AMS in the presence of copper, while no N_2O was observed on AMS with reduced amount of ammonium (0.1X AMS). A small amount of N_2O, up to 27 ppmv, was observed from *M. trichosporium* OB3b grown in NMS only in the presence of copper. It is also interesting to note the addition of 1X AMS medium resulted in reduced growth of *M. trichosporium* OB3b as compared to 0.1X AMS, most likely due to oxidation of ammonia by the MMO, resulting in inhibition of methane oxidation.

Interestingly, the production of N_2O dramatically diminished from 331 to 34 ppmv when 20 mM of formate was added as sodium formate to AMS media. MMO utilizes two reducing equivalents to split the O-O bonds of dioxygen, and most of the reducing power required for the MMO activity is produced by the further oxidation of methanol to carbon dioxide. However, if the intermediates produced by MMO, such as methanol, cannot be metabolized to recycle reducing equivalents, intracellular NADH may become rate limiting. Therefore, exogenous formate usually acts as a readily available source of reducing equivalents during whole-cell assays of methanotrophs. Since NADH is consumed to oxidize NH_4^+ by MMO, the addition of formate was assumed to enhance N_2O production, which was the opposite of that observed. This is likely due to detrimental by-products of ammonium oxidation, such as H^+ or NO_2^-. It can also be speculated that the supply of reducing equivalents may help prevent the accumulation of potent precursors for N_2O such as hydroxylamine. The absence of copper seemed to inhibit N_2O production from both NMS and AMS and is discussed in more detail below. In the presence of acetylene, which has been known as an inhibitor of methane monooxygenase, N_2O was not detected in any media condition. This may be because MMOs act as a key enzyme in N_2O production, or because growth rates were poor when methanol was provided as a sole carbon source.

Table 16. N_2O production by *M. trichosporium* OB3b on various growth conditions. N.G.: no growth, N.D.: not detected

Media	Cu (20 uM)	Formate (20 mM)	Acetylene (1% by vol)	Carbon source	Grow rate (hr^{-1})	Max OD_{600}	Max N_2O
NMS				CH_4	0.055	0.54	N.D.
NMS			+	CH_4	N.G.		N.D.
NMS	+			CH_4	0.045	0.60	27 ppmv
NMS	+		+	CH_4	N.G.		N.D.
NMS		+		CH_4	0.070	0.58	N.D.
NMS		+	+	CH_4	N.G.		N.D.
NMS	+	+		CH_4	0.090	0.66	N.D.
NMS	+	+	+	CH_4	N.G.		N.D.
NMS				MeOH	0.086	0.17	N.D.
NMS			+	MeOH	N.G.		N.D.
NMS	+			MeOH	0.071	0.19	N.D.
NMS	+		+	MeOH	0.087	0.26	N.D.
NMS		+		MeOH	N.G.		N.D.
NMS		+	+	MeOH	0.053	0.18	N.D.
NMS	+	+		MeOH	0.028	0.24	N.D.
NMS	+	+	+	MeOH	0.030	0.25	N.D.
AMS				CH_4	0.042	0.60	119 ppmv
AMS			+	CH_4	N.G.		N.D.
AMS	+			CH_4	0.053	0.73	331 ppmv
AMS	+		+	CH_4	N.G.		N.D.
AMS		+		CH_4	0.077	0.52	30 ppm
AMS		+	+	CH_4	N.G.		
AMS	+	+		CH_4	0.046	0.38	34 ppm
AMS	+	+	+	CH_4	N.G		
AMS				MeOH	N.G.		N.D.
AMS			+	MeOH	N.G.		N.D.
AMS	+			MeOH	N.G.		N.D.
AMS	+		+	MeOH	N.G.		N.D.
AMS		+		MeOH	N.G.		N.D.
AMS		+	+	MeOH	N.G.		N.D.
AMS	+	+		MeOH	N.G.		N.D.
AMS	+	+	+	MeOH	N.G.		N.D.
0.1xAMS				CH_4	0.087	0.48	N.D.
0.1xAMS			+	CH_4	N.G.		N.D.
0.1xAMS	+			CH_4	0.078	0.56	N.D.
0.1xAMS	+		+	CH_4	N.G.		N.D.
0.1xAMS		+		CH_4	0.077	0.55	N.D.
0.1xAMS		+	+	CH_4	N.G.		N.D.
0.1xAMS	+	+		CH_4	0.052	0.39	N.D.
0.1xAMS	+	+	+	CH_4	N.G.		N.D.
0.1xAMS				MeOH	N.G.		N.D.
0.1xAMS			+	MeOH	N.G.		N.D.
0.1xAMS	+			MeOH	0.033	0.145	N.D.
0.1xAMS	+		+	MeOH	0.037	0.190	N.D.
0.1xAMS		+		MeOH	N.G.		N.D.
0.1xAMS		+	+	MeOH	N.G.		N.D.
0.1xAMS	+	+		MeOH	N.G.		N.D.
0.1xAMS	+	+	+	MeOH	0.030	0.210	N.D

Based on the results of the growth condition that caused enhanced N_2O production, i.e., 1X AMS with 20 μM of copper, other cultures were tested for N_2O production, and significant amounts of N_2O were detected from all the Type II strains including *Methylocystis parvus* OBBP, *Methylocystis hirsuta* CSC1, *Methylocystis rosea* SV97 and *Methylocystis sp.* SB2, except

Methylocystis sp. strain M (Table 17). The amount of N_2O produced varied from 32 to 343 ppmv, which is equivalent to 0.16% to 1.7% conversion rate of NH_4^+-N to NO_2^--N. Since the conversion rates of nitrifier or denitrifier pure strains have been reported to range from 0.1 to 3% depending on the growth condition (Blackmer et al., 1980; Kester et al., 1997; Shrestha et al., 2001), the results reported here suggest the N_2O production mediated by some methanotrophs can be comparably significant in terrestrial ecosystem. Interestingly, N_2O seems to be produced only by Type II methanotrophs and not by Type I methanotrophs, but this should be verified with analyses of more methanotrophs, particularly Type I strains. N_2O uptake experiments were performed for each strain to ensure that the results with no N_2O detected are not false negative. At the mid-exponential phase of growth, 100 ppmv of N_2O was injected, but no decrease was observed for over three days (data not shown).

Table 17. Methanotrophic mediated nitrous oxide production under pMMO-expressing conditions, i.e., AMS with 20 μM of copper.

Strain	Type	Growth rate (hr^{-1})	Max OD_{600}	Max N_2O (ppmv)
Methylomicrobium album BG8	I	0.054 (0.004)	0.725 (0.01)	ND
Methylococcus capsulatus Bath	I	0.138 (0.007)	0.810 (0.02)	ND
Methylosinus trichosporium OB3b	II	0.088 (0.003)	0.880 (0.04)	343.7 (7.1)
Methylocystis parvus OBBP	II	0.123 (0.004)	0.935 (0.02)	32.3 (2.9)
Methylocystis hirsuta CSC1	II	0.069 (0.001)	0.925 (0.04)	156.5 (2.8)
Methylocystis rosea SV97	II	0.071 (0.002)	0.700 (0.04)	233.1 (9.4)
Methylocystis sp. strain M	II	0.103 (0.008)	0.975 (0.04)	ND
Methylocystis sp. strain SB2	II	0.135 (0.005)	0.870 (0.04)	36.3 (1.2)

Numbers in parenthesis represent range of duplicate samples; ND: not detected.

3.5.1.2. Effect of copper on nitrous production by methanotrophs. Five methanotrophic strains, *Methylosinus trichosporium* OB3b, *Methylocystis parvus* OBBP, *Methylocystis hirsuta* CSC1, *Methylocystis rosea* SV97, and *Methylocystis* sp. strain SB2 shown to produce nitrous oxide (Table 17) were exposed to different concentrations of copper, 0 and 20 μM, to investigate the effects of copper on the production of N_2O. Of these, only *Methylosinus trichosporium* OB3b and *Methylocystis hirsuta* CSC1 have been shown to express both particulate and soluble MMO. The other strains possessing only pMMO, i.e., *Methylocystis parvus* OBBP, *Methylocystis rosea* SV97, and *Methylocystis* sp. strain SB2, showed significantly slower growth rate and lower maximum OD_{600} in the absence of copper (Figure 45). Therefore, it was expected that the strains possessing both MMOs would produce more N_2O than the other strains possessing only pMMO. Interestingly, however, the trend of the amount of N_2O was not the same with the growth pattern, i.e., the two strains possessing sMMO, *Methylosinus trichosporium* OB3b and *Methylocystis hirsuta* CSC1, showed the highest growth rates and maximum cell densities, but the amounts of N_2O produced was not the highest in the absence of copper. Rather, *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP produced significantly reduced amounts of N_2O and the other strains produced similar (*Methylocystis rosea* SV97) or more N_2O (*Methylocystis hirsuta* CSC1 and *Methylocystis* sp. strain SB2) (Figure 46).

In cells expressing pMMO, copper has been known not only to control expression but also to alter substrate affinity and specificity (Lontoh and Semrau, 1998). Therefore, these results may mean that either (1) copper decreased pMMO activity to different degrees in different organisms, i.e., more for *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP and less for

Methylocystis rosea SV97, *Methylocystis hirsuta* CSC1, and *Methylocystis* sp. strain SB2; or (2) N_2O production may not be dependent on the activity of MMOs, i.e., unlike CH_4 oxidation, the rate-limiting step for N_2O production may not be the first reaction, i.e., ammonia oxidation.

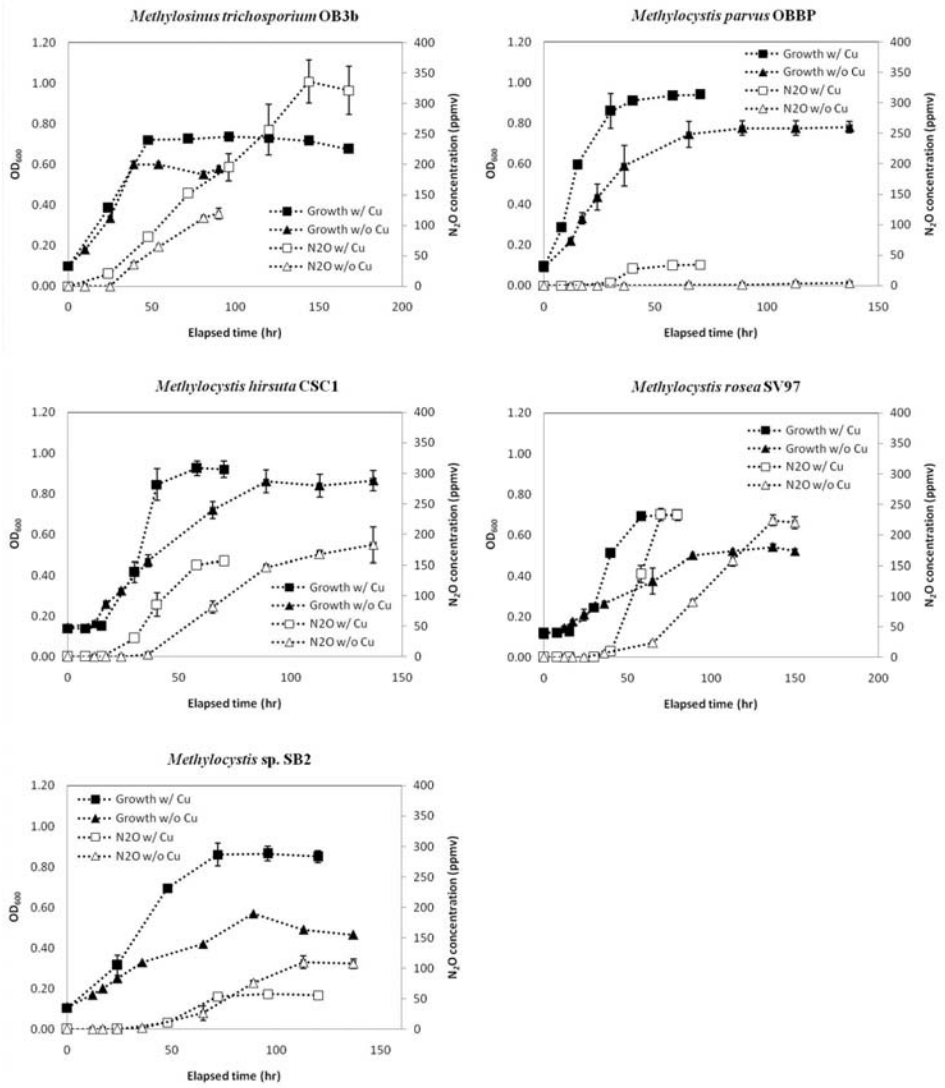


Figure 45. Growth and N_2O production of *Methylosinus trichosporium* OB3b^T, *Methylocystis* sp. SB2, *Methylocystis rosea* SV97^T, *Methylocystis parvus* OBBP^T, *Methylocystis hirsuta* CSC1^T when growing on AMS in the presence/absence of 20 μM of copper.

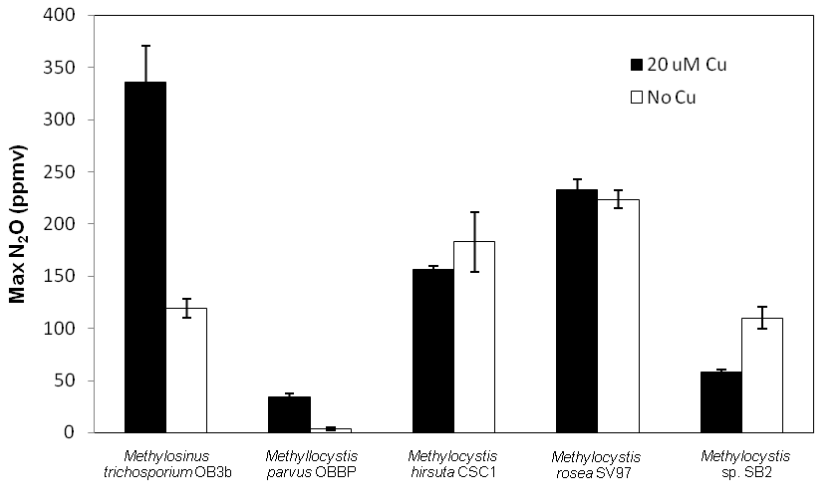


Figure 46. Methanotrophic mediated N_2O production in the presence/absence of 20 μM of copper.

3.5.1.3. Inhibition of methanotrophic production of nitrous oxide by phenylacetylene The effect of phenylacetylene on N_2O production was examined next. Phenylacetylene is known to act as a differential inhibitor of ammonium monooxygenase (AMO) of AOBs, sMMO, and pMMO (Lontoh et al., 2000). Since whole cell AMO activity in *Nitrosomonas europaea* was completely inhibited at phenylacetylene concentrations greater than 1 μM while MMO activity was not (Lontoh et al., 2000), phenylacetylene was proposed as a selective inhibitor in this study. To verify the effect of phenylacetylene on methanotrophic N_2O production, two strains, *Methylosinus trichosporium* OB3b and *Methylocystis sp.* SB2 were examined. Phenylacetylene was added from a 10 mM stock solution to the appropriate final concentrations ranging from 0.1 to 100 μM in the presence of 20 μM of copper. Figure 47 and Table 18 represent a demonstration of differential inhibition of N_2O production by phenylacetylene in *Methylosinus trichosporium* OB3b and *Methylocystis* sp. SB2. Growth rates and N_2O production was sensitive to phenylacetylene at concentrations greater than 10 μM. No growth was observed at the concentration of 50 μM or higher. In the case of *Methylosinus trichosporium* OB3b, the growth rate was reduced by 40%, and N_2O production was also repressed by 40%, from 347.7 (± 7.1) to 200.6 (± 5.9) ppmv, although the presence of phenylacetylene up to 10 μM did not affect the maximum culture density, with the OD values of 0.82~0.87 observed being similar to that observed in the absence of phenylacetylene, 0.88 (± 0.04). For *Methylocystis* sp. strain SB2, N_2O production decreased by 60% when 10 μM of phenylacetylene was added, but the maximum cell density increased up to 0.7 and the growth rate was reduced only by 20 %. This suggests that the reduced N_2O production may be partially due to reduced microbial growth, but the degree of inhibition by phenylacetylene on N_2O production may vary from species to species.

Table 18. Effect of phenylacetylene on growth rate and N_2O production of the cells growing on AMS in the presence of 20 μM of copper. Values in parenthesis indicate standard deviation of duplicate samples

Strain	C_8H_6 (μM)	Growth rate (hr^{-1})	Max OD_{600}	Max N_2O (ppmv)
M. trichosporium OB3b	0	0.088 (0.003)	0.88 (0.04)	343.7 (7.1)
	0.1	0.086 (0.002)	0.87 (0.01)	273.6 (35.1)
	1	0.071 (0.003)	0.87 (0.03)	307.6 (0.1)
	10	0.052 (0.004)	0.82 (0.01)	200.6 (5.9)
	50	No growth	-	-
	100	No growth	-	-
Methylocystis sp. SB2	0	0.135 (0.005)	0.87 (0.04)	36.3 (1.2)
	0.1	0.121 (0.004)	0.87 (0.02)	37.5 (2.8)
	1	0.114 (0.003)	0.85 (0.01)	35.2 (5.6)
	10	0.108 (0.007)	0.70 (0.02)	12.4 (0.1)
	50	No growth	-	-
	100	No growth	-	-

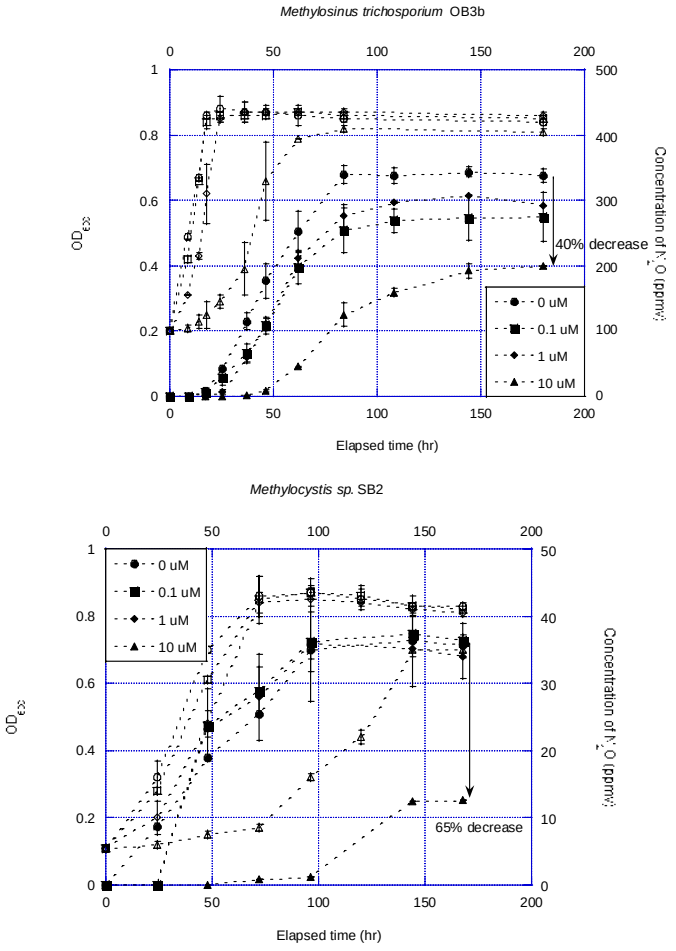


Figure 47. Effect of phenylacetylene on N_2O production
(open: optical density at 600 nm; closed: N_2O concentration)

3.5.1.4. Methanotrophic mediated nitrous oxide production via denitrification. Since N_2O was also occasionally produced in small quantities by *Methylosinus trichosporium* OB3b when grown on liquid media supplemented with nitrate as a sole nitrogen source (Table 16), it was speculated that N_2O might have been produced via denitrification. To test this hypothesis, *Methylosinus trichosporium* OB3b was incubated with various concentrations of nitrate and

nitrite. A similar amount of N_2O, 51 ppmv, was produced from this microbe growing on with 10 mM nitrate, but it may be due to ammonia as a by-product of assimilation. Nitrite has been shown to be toxic to methanotrophs (Schnell and King, 1995), and 10 mM of nitrite was toxic enough to inhibit the growth as shown in Figure 48. Although growth was not inhibited by the amounts of nitrite less than 1 mM, no significant amount of N_2O was observed, suggesting denitrification is not a significant pathway by which methanotrophs produce N_2O.

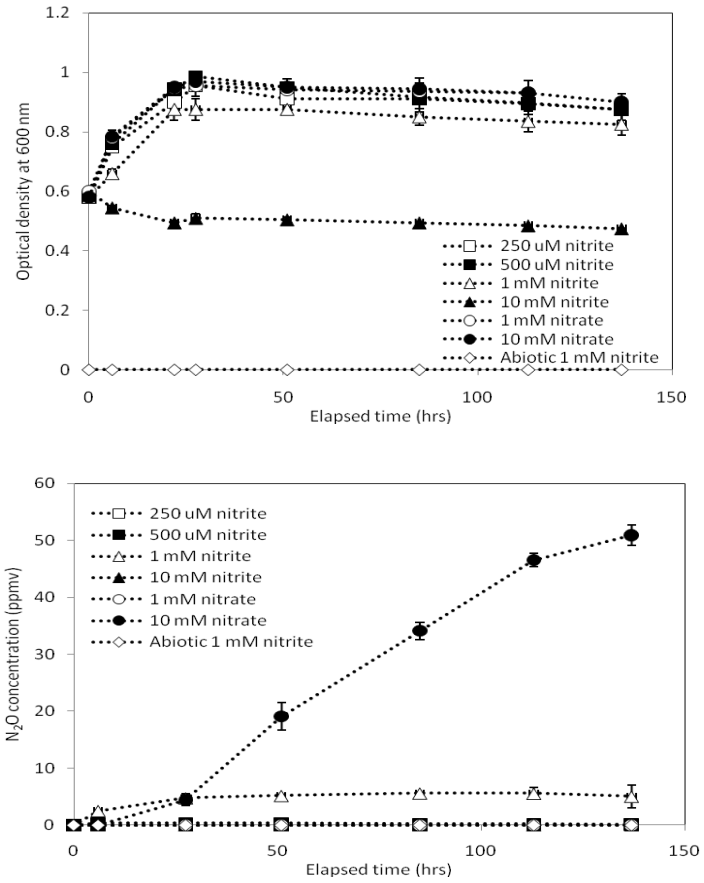


Figure 48. N_2O production by *Methylosinus trichosporium* OB3b in mineral salt media supplemented with various concentration of nitrite and nitrate.

3.5.2. Discussion

Methanotrophic mediated N_2O production, compared with autotrophic nitrification or denitrification, has not been well characterized and required additional investigation. The results presented here, however, show that several methanotrophs produce as much N_2O as ammonia oxidizers or denitrifiers. The conversion rate of NH_4^+-N to NO_2^--N ranged from 0.16% to 1.7%. Since the conversion rates of nitrifier or denitrifier pure strains have been reported to range from 0.1 to 3% depending on the growth conditions (Blackmer et al., 1980; Kester et al., 1997; Shrestha et al., 2001), the results reported here suggest the N_2O production mediated by methanotrophs can be significant in terrestrial ecosystems. Interestingly, five out of six Type II methanotrophic strains were able to produce significant amount of N_2O when growing on ammonium mineral salt media, while the two tested Type I strains did not produce detectable amount of N_2O. This result is consistent with the result of the microarray analysis reported in earlier, suggesting Type II methanotrophs may be more responsible for the N_2O production in the terrestrial ecosystem. The effect of copper on N_2O production was different on each strain,

i.e., *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP produced significantly reduced amounts of N_2O in the absence of copper, while similar or slightly higher amount of N_2O was produced from *Methylocystis hirsuta* CSC1, *Methylocystis rosea* SV97 and *Methylocystis* sp. SB2 for the same condition, suggesting either that (1) copper may have decreased pMMO activity to different degrees; or (2) methanotrophic-mediated N_2O production may not be dependent on the activity of MMO. The effect of phenylacetylene on the methanotrophic mediated N_2O production was also investigated, but the reduced N_2O production may be partly due to the reduced cell growth, and the degree of inhibition may vary from species to species.

3.6. Feasibility of Atmospheric Methane Removal Using Methanotrophic Biotrickling Filters

3.6.1. Results

3.6.1.1. Biofilter design and modeling. With the mathematical model using MATLAB, methane removal was estimated under a wide range of conditions, including different methane concentrations, pMMO- *vs* sMMO-expressing cells, and temperature. First, however, different inlet air velocities were tested with a fixed methane concentration, 1,000 ppmv, for both sMMO- and pMMO-expressing cells to determine the most appropriate inlet air velocity to be used in further analysis. As can be seen in Figure 49, the methane removal rate for pMMO-expressing cells was constant above an inlet air velocity of ~0.3 m/s, while sMMO-expressing cells failed to reach steady state under any assumed inlet velocities at this concentration of methane. An inlet velocity of 0.5 m/s (equal to a flow rate of 5.26 m^3/s) was thus used in further analyses.

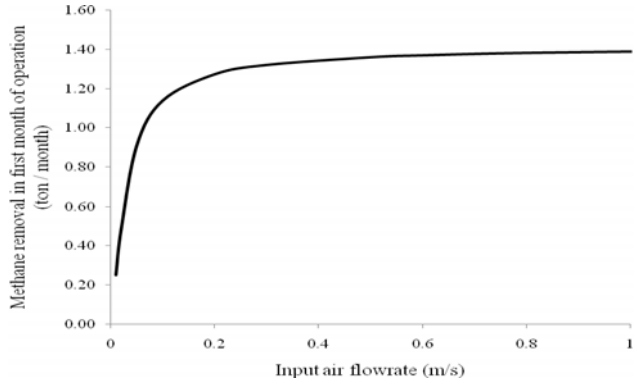


Figure 49. Optimization of input air flowrate for removal of atmospheric methane using methanotrophic biofilters composed of *M. trichosporium* OB3b expressing pMMO

Input methane concentrations were then varied from average atmospheric concentration, 1.7 ppmv, to 6,000 ppmv to evaluate the feasibility of methane biofiltration for cells expressing either sMMO or pMMO in locations with varying methane concentrations. The highest concentration, 6,000 ppmv, was based on the highest atmospheric methane concentrations measured in the vicinity of landfills by Carman and Vincent (1998). It was found that methane removal using biotrickling filtration is not feasible if the global average atmospheric methane concentration, 1.7 ppmv, is assumed. This concentration is too low to support cell growth for either sMMO- or pMMO-expressing cells such that complete washout (i.e., death) of the biofilm occurs in less than a month (data not shown). As shown in Figure 50, however, above 500 ppmv

both biofilm thickness and methane removal by pMMO-expressing cells achieved steady state within the first two months of operation, while sMMO-expressing cells achieved steady state at methane concentrations above 3,000 ppmv. Furthermore, as seen in Figure 50, sMMO-expressing biofilters consumed less methane than pMMO-expressing cells over the entire concentration range tested. Given these findings, only pMMO-expressing cells will be discussed in detail further.

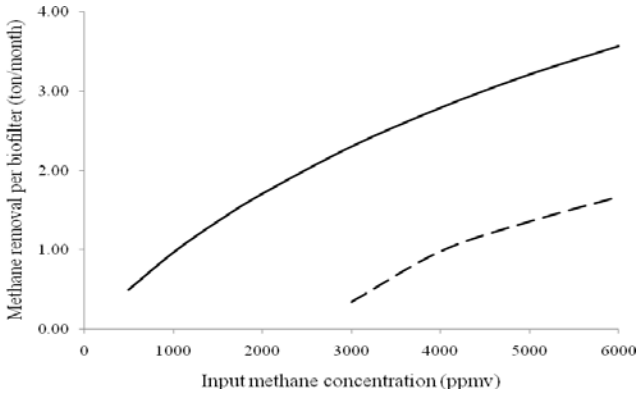


Figure 50. Steady state methane removal rate for *M. trichosporium* OB3b as a function of input methane concentration in methanotrophic biofilters (—: pMMO-expressing cells, - - -: sMMO-expressing cells)

From numerical simulations, if a methanotrophic biofilter expressing pMMO is placed in an environment with a local methane concentration of 500 ppmv, 498 kg of methane is removed per month after steady state is reached. The methane degradation rate increased to 3.57 tons of methane per month when the local atmospheric concentration of methane was raised to 6,000 ppmv (Figure 50). If pMMO-expressing biofilters are operated for 10 months of year with two months downtime for maintenance, we estimate that up to 35.7 tons of methane per year can be removed at 6,000 ppmv methane. For the entire concentration range tested, the steady-state biofilm depth did not exceed 1.4 x 10^{-2} cm at the inlet end, where the cells exhibit the fastest growth (Figure 51).

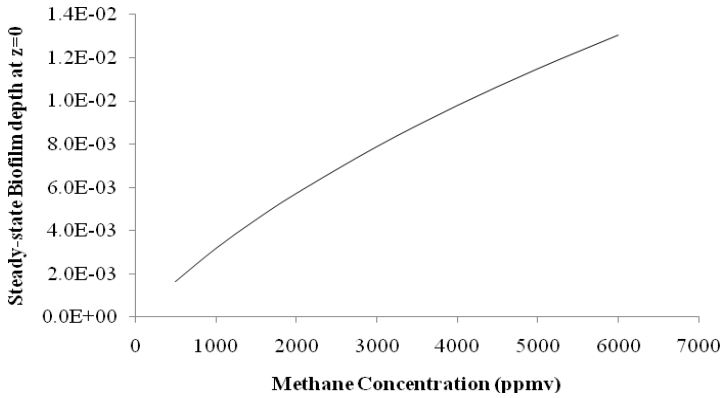


Figure 51, Biofilm depth at the inlet end (z=0) after steady-state is established.

3.6.1.2. Economic analysis. These estimates of biotrickling filter performance were then used to determine the unit cost of methane removal. As the methanotrophic biotrickling filter proposed

here has the same dimensions and composition and will be operated under similar conditions as a well-characterized biofilter at the Grupo Cydsa facility (Cox and Deshusses, 2002), we based our cost estimates on the financial data provided in the literature for this system. The costs were adjusted for inflation by using the cumulative inflation rate between 2002 and 2008 of 19%. Collectively, the capital cost of our proposed biofilter would be approximately $595,000.

The proposed biofiltration system requires electricity for three purposes: (1) to operate blowers, (2) to recycle nutrient medium, and (3) to maintain optimal temperature in the reactor. The electrical demand for blower operation is estimated to be 216 MWh per year based on the data presented by Gabriel and Deshusses (2003). The air inflow rate in our biotrickling filter is 10% larger but it was reported that blowers with capacity ranging from 17,000 to 68,000 m^3/h do not have more than 10% variation in their electrical demand (Gabriel et al., 2004). Reported electricity requirements for nutrient recycling in specific biofilters are 0.64 MWh per year for a flow rate of 1 m^3/h (Gabriel and Deshusses, 2003). Thus, it is estimated that electrical demand for nutrient recycling will be 11 MWh per year for our proposed system. Finally, in some situations, both the influent air and nutrient medium may require heating to provide the optimal conditions of methanotrophic growth. If it is assumed that heating from 20°C to 30°C is required and that the heat capacities of water and air are 4.18 and 1.003 kJ/kg/K, respectively, it is estimated that an additional 485 MWh per year is required per biofilter for heating. Collectively, it is estimated that total energy requirements vary from 227 to 712 MWh per year, dependent on heating requirements. Using an average cost of electricity of 6.16 ¢/kWh for industrial use (EIA, 2007), total electricity costs due to heating will range from $14,000 to $44,000 per year per biofilter.

Assuming that our biofilter has a lifetime of 20 years, and the discount factor is 3% (the average inflation rate for the past 5 years), the annualized capital cost of a methanotrophic biofilter is calculated to be ~ $40,000 per biofilter per year, with the total annualized cost varying between $74,000 and $104,000 per biofilter per year, depending on heating costs. When compared to the amount of methane removed as a function of influent methane concentration, the costs vary from $2,070 to $20,900 per ton of methane removed or $75 to $860 per equivalent ton of CO_2 (Figure52), depending on the methane concentration and if heating is required.

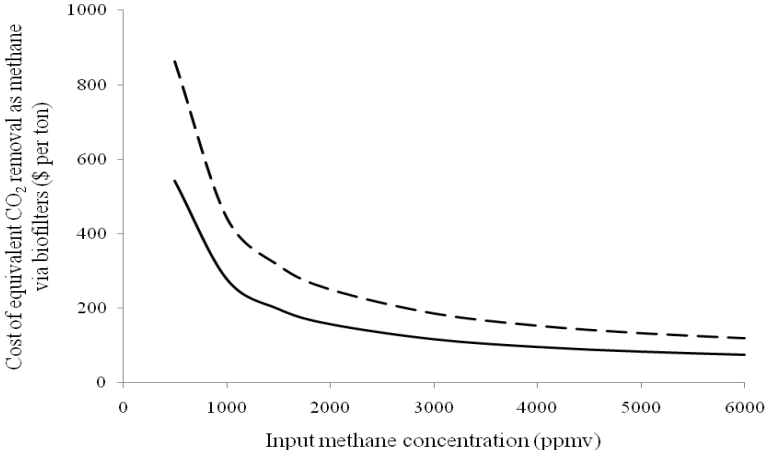


Figure 52. Cost of equivalent CO_2 removal as methane using methanotrophic biofilters composed of *M. trichosporium* OB3b expressing pMMO when heating is either unnecessary (—) or necessary (- - -)

As of November, 2010, 1 ton of CO_2 was traded at ~ €15 or $20 (www.Co2prices.eu). Our estimated costs of removal of an equivalent amount of CO_2 as methane are approximately 3.8- to 43-fold higher. The CO_2 market, however, has been unstable and there is the possibility that CO_2 prices might rise again. At some point in the future, the price of CO_2 may exceed the cost of methane removal using the biofiltration systems described here, but it is unknown when this might occur.

3.6.2. Discussion

From the model analysis, removal of methane at a significant rate is feasible by pMMO-expressing biofilters at concentrations greater than 500 ppmv and that these systems significantly outperform sMMO-expressing systems. Methane removal with this biotrickling filtration, however, is not economically feasible at current stage as the estimated cost of removal exceeds the current market price of CO_2 emissions in the European carbon market. The price of CO_2 emission, however, is subject to change as the carbon trading market is growing, and it may be possible to reduce the cost of building and operating these biotrickling filters if they are mass-produced.

Another issue that will determine success of greenhouse gas removal with these trickling biofilters is the carbon footprint associated with electricity generation. Carbon dioxide emitted during generation of the power required for operating the biofilter must be considered in conjunction with methane removal rates and costs when considering the feasibility of these biofilters for removal of greenhouse gases from the atmosphere. Carbon footprints of electricity generation range from 5 to 1,000 g/kWh depending on the methods of generation (Parliamentary Office of Science and Technology, 2006). Therefore, the carbon footprint of electricity used in removal of methane by our proposed biofilters will be 3.56–712 tons of CO_2 per year if heating is necessary and 1.13–227 tons of CO_2 per year if it is not. Considering that the expected removal rate is 4.98–35.7 tons of methane per year per biofilter, equivalent to 115–821 tons of CO_2 per year (assuming influent methane concentrations ranging from 500 to 6,000 ppmv), it will be difficult to have net carbon removal if electricity is supplied by conventional fossil fuel power plants (>500 g CO_2 emitted/kWh). If energy can be supplied from a low carbon energy source, e.g., wind power (<10 g CO_2 emitted/kWh), however, net removal of carbon using these biofilters will be feasible by a wide margin regardless of the electricity requirement for heating.

Biofilters and synthetic packing materials, in general, are known to have very long lifetimes of at least 10 years (van Groenestijn and Kraakman, 2005). However, clogging due to uncontrolled accumulation of biomass is a recurring problem in the operation of biofiltration that deteriorates biofilter function over time (Alonso et al., 1997; Cox and Deshusses, 2002). For the removal of methane, however, clogging is not a significant issue due to the low concentrations of the growth substrate, methane, even at the elevated concentrations assumed here. In our modeling studies, even at the highest atmospheric methane concentrations used in the study, e.g., 6,000 ppmv, steady-state biofilm thickness did not exceed 1.3×10^{-2} cm. Therefore, it is expected that the use of biofilters for atmospheric methane removal will require less maintenance due to clogging.

The major difficulty in removal of atmospheric methane, as mentioned earlier, is the extremely low atmospheric concentration of methane. This restricts use of these biofilters, even those expressing pMMO to local “hot spots” of methane such as that found above landfills and in

concentrated animal feeding operations (factory farms). This is due to the relatively low affinity for methane assumed here as compared to the global average atmospheric concentration. Here, the K_s value by the type strain *M. trichosporium* OB3b expressing pMMO used was 3 μM, equivalent to an atmospheric concentration of 2,170 ppmv.

It should be noted that much of the work on methanotrophs has focused on bacteria isolated from high-methane environments, including *M. trichosporium* OB3b, and as such, it is not surprising that these cells have relatively low affinities for methane. More recently, high-affinity methanotrophs with very low half-saturation constants (40.5 to 134.5 ppmv) have been found to exist in some environments, e.g., organic agricultural soils, with such cells able to consume methane at concentrations as low as 1.7 ppmv (Dunfield et al., 1999; Bull et al., 2000; Dunfield and Conrad, 2000; Knief et al., 2005). Their maximum capacity to degrade methane, however, was found to be very limited (Dunfield and Conrad, 2000). Using the average V_{max} and K_s values (0.125 nmol mg biomass^{-1} s^{-1} and 0.305 μM, respectively) obtained by Dunfield and Conrad (2000) for *Methylocystis* strain LR1, less than 6 kg per month was estimated to be removed under optimal conditions (i.e., an influent methane concentration of 6,000 ppmv, negligible cell death rates and an initial biofilm thickness of 42 μm). It should be noted that even under these conditions, the system failed to achieve steady state, i.e., the biofilm was continuously degraded as cell loss through shearing was greater than cell growth. Therefore, their direct application in engineering practices for removal of atmospheric methane seems infeasible at this time, although their existence suggests the possibility of engineering other methanotrophic strains to have higher affinities toward methane while maintaining high turnover rates, i.e., reduce K_s while maintaining a high V_{max} value. If the pMMO of a strain such as *M. trichosporium* OB3b could be modified to reduce the half-saturation constant to 100 nM (equivalent to 70 ppmv in air assuming equilibrium) and V_{max} is assumed to remain at 4.83 nmol mg biomass^{-1} s^{-1}, our model predicts that it is possible remove 0.385 ton of methane per month per biofilter at a methane concentration of 100 ppmv without the cells being washed out (as was the case with *M. trichosporium* OB3b expressing pMMO at the same methane concentration). Such a scenario, although potentially feasible, would require more extensive consideration on whole-cell metabolic fluxes of carbon and energy to ensure that relatively simple modifications of the pMMO would be stably maintained.

4. CONCLUSIONS

4.1. Soil microcosms

The effects of various amendments, moisture content, copper, different forms of nitrogen (NH_4^+, NO_3^-, and urea), organic carbon, and selective inhibitors phenylacetylene and chlorate, on methane oxidation and nitrous production were examined by constructing soil microcosms using landfill cover soils collected from King Highway Landfill. It was found that the addition of inorganic nitrogen stimulated both methane oxidation and nitrous oxide production. Moisture content, when lowered to 5-10 %, had a stimulatory effect on methane oxidation while inhibiting nitrous oxide production. The addition of phenylacetylene showed inhibition of nitrous oxide production while not affecting methane oxidation. When soils were maintained at low moisture content, 5 %, and amended with 15 mg-N NH_4^+·(kg soil)$^{-1}$ in conjunction with 0.1 mg C_8H_6·(kg soil)$^{-1}$, methane oxidation was stimulated but nitrous oxide 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 nitrous oxide 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* of ammonia-oxidizing bacteria 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 nitrous oxide when NH_4^+ was added.

Such findings indicate that inorganic nitrogen and moisture content are both important variables that should be considered when maximizing methane consumption in landfill cover soils and that the concurrent addition of phenylacetylene can reduce emissions of nitrous oxide. Finally, based on these studies, it appears that methanotrophs expressing pMMO can make substantial amounts of nitrous oxide.

4.2. Field site demonstration of the effect of nitrogen and phenylacetylene on greenhouse gas emissions and microbial community structure and activity.

It should be noted, however, that the soil microcosm work was performed in batch scales that may not reflect to the actual landfill conditions. To address this issue, a field demonstration was performed at the King Highway Landfill. From the field demonstration plots, it was found that (1) biological activities related to NH_4^+ uptake or oxidation may be inhibited by modified water content created by the presence of a canopy, and; (2) the addition of NH_4^+ and NO_3^- enhanced biological activity in the absence of a canopy. pH, bioavailable and total copper were also measured but no significant difference was found between plots. Nitrous oxide profiles, however, indicated that nitrogen amendments stimulated nitrous oxide production while phenylacetylene repressed its production. Methane concentration analyses suggest that phenylacetylene might help methanotrophs outcompete other microbial groups for limiting nutrients, while there might be an inhibitory effect of a canopy on CH_4 oxidation. There was no significant difference in O_2 concentration, indicating that O_2 was dominated by diffusion rather than microbial consumption.

Given increased concerns over greenhouse gas emissions, the effect of long-term application of NH_4^+ and NO_3^- with and without phenylacetylene on greenhouse gas emissions from landfill was determined. The addition of NH_4^+ and NO_3^- increased nitrous oxide production, but had no effect on methane concentrations *in situ* regardless of the presence or absence of a canopy. The simultaneous addition of phenylacetylene reduced nitrous oxide production both in the absence and presence of a canopy, but enhanced methane oxidation only in the absence of a canopy. Methanotrophic community diversity decreased in response to addition of NH_4^+ and NO_3^- but increased with the concurrent addition of phenylacetylene regardless of a presence of a canopy. Furthermore, the presence and activity of AOAs was also greatest in the plot amended with NH_4^+ and NO_3^- and reduced with the concurrent addition of phenylacetylene. As different AOA groups were stimulated and repressed with the addition of nitrogen and phenylacetylene, it may be that specific groups of methanotrophs and AOAs contribute more significantly to nitrous oxide production than others, i.e., it may be that Type II methanotrophs and Group 1.1b AOAs produce more N_2O from NH_3 than other methanotrophs and AOAs. Further work should be done to

determine if nitrous oxide production varies between AOA groups.

In the previous soil microcosm studies, the results of microbial community structure in these microcosms were different from that found at the field demonstration site. PCR analyses of soil cores taken from the field demonstration plots showed the presence of *amoA* from ammonia oxidizing bacteria as well as archaea, and that the presence of these genes was reduced with the addition of phenylacetylene. Furthermore, the methanotrophic community in the soil microcosms was far less diverse than that from the field demonstration. As the microcosm soil samples were first air-dried and stored at 4 °C in the dark for several months before analyses, the discrepancy may be due to variability in desiccation tolerance among different microbial groups.

4.3 Methanotrophic-mediated nitrous oxide production

Methanotrophic mediated nitrous oxide production, compared with autotrophic nitrification or denitrification, has not been well characterized and required additional investigation. The results reported here, however, show that several methanotrophs produce as much nitrous oxide as ammonia oxidizers or denitrifiers. The conversion rate of NH_4^+-N to NO_2^--N ranged from 0.16% to 1.7%. Since the conversion rates of nitrifier or denitrifier pure strains have been reported to range from 0.1 to 3% depending on the growth conditions (Blackmer et al., 1980; Kester et al., 1997; Shrestha et al., 2001), the results reported here suggest the nitrous oxide production mediated by methanotrophs can be significant in terrestrial ecosystems. Interestingly, five out of six Type II methanotrophic strains were able to produce significant amount of nitrous oxide when growing on ammonium mineral salt media, while the two tested Type I strains did not produce detectable amount of nitrous oxide. This result is consistent with microarray analyses, suggesting Type II methanotrophs may be more responsible for the nitrous oxide production in the terrestrial ecosystem. Effect of copper on nitrous oxide production was different on each strain, i.e., *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP produced significantly reduced amount of nitrous oxide in the absence of copper, while similar or slightly higher amount of nitrous oxide was produced from *Methylocystis hirsuta* CSC1, *Methylocystis rosea* SV97 and *Methylocystis* sp. SB2 for the same condition, suggesting either that (1) copper may have decreased pMMO activity to different degrees; or (2) methanotrophic-mediated N_2O production may not be dependent on the activity of MMO. The effect of phenylacetylene on the methanotrophic mediated nitrous oxide production was also investigated, but the reduced nitrous oxide production may be partly due to the reduced cell growth, but the degree of inhibition may vary from species to species.

4.4. Biofiltration of atmospheric methane

A thorough assessment was made of the feasibility of employing biofilters loaded with methanotrophic community for removal of methane from the atmosphere. A design of such a biofilter was developed from the existing biofiltration systems used for removal of organic/inorganic pollutants and our current knowledge on methanotrophs. Model results indicated that such biofiltration systems were able to remove methane from the atmosphere, provided that they are located in local “hotspots” of methane (> 500 ppmv) in vicinity of major anthropogenic sources and that pMMO expression condition is induced. The consistent supply of copper, therefore, is thought to be essential in operating these biofilters. Another important consideration was the carbon footprint associated with the electricity. As electricity requirement is not negligible in operating these biofilters, overall removal of carbon dioxide equivalence was

found to be obtainable only when renewable resources are used for power generation. Considering the estimated costs associated with this biofiltration system, removal of the greenhouse gas using methanotrophic biofiltration is not profitable with current technology. It is possible, however, that these systems may become economically attractive in the near future with improved biofilter designs based on enhanced knowledge on methanotrophic community coupled with cost reduction via mass production and increased value of future carbon dioxide credits.

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APPENDIX A: List of oligonucleotide probe sets used in microarrays.

Melting temperatures were calculated using the nearest neighbour method.

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*	CCGTTACCGTCTGCCTTTCG	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*	TTGTGGTGGCGTTACCGT	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*	ATGGTAATGACCTGGCTGACTTG	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	CACGGTCTGCGTTCTGGC	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)	GGCATTGCTGATGTCGATTGCCG	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*	ATTCTCGGGCGTGACCTTCTGC	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 A (continued)

75 Mcy255	O Mcy255	*M.cystis B (parvus/echinoides/* strain M)	GGCGTCGCAGGCTTTCTGG	19	68.4	62.3	
76 McyM309	P McyM309	*M.cystis strain M and related*	GGTTCTGGGCCTGATGATCGG	21	61.9	61.0	
77 McyB304	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**TTCGGCGCGAC**CT**T**CGC**	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* (?)	AGCGCTTCCGTCTGCCGAT	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**C**AGA**TG**A**TCCCGTCGGC	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**G**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)