

Integrated Laboratory and Fieldwork Exercises
for Controlling Greenhouse Gas Emissions from Landfills

by

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ABSTRACT

The global average surface temperature has increased dramatically, over 0.5 °C, in the last 150 years. It is the prevailing view that this temperature increase is closely related to the increase in the atmospheric concentration of greenhouse gases (GHGs). Among GHGs, methane (CH₄) is a very potent, second only to carbon dioxide (CO₂) as an anthropogenic contributor to global warming, representing 8.2% of total greenhouse gas emissions. The global warming potential of CH₄ is ~25 times that of CO₂. In the United States, landfills are the largest anthropogenic source of CH₄ emissions, but a considerable amount of CH₄ can be biologically oxidized by bacteria, i.e., methanotrophs, to CO₂. The addition of nitrogen is typically used to stimulate *in situ* methanotrophic activity, but it also has been known to stimulate other microorganisms capable of producing nitrous oxide (N₂O), which is ~290 times stronger than CO₂ in terms of global warming potential. Therefore, it is important to develop rational strategies to stimulate CH₄ oxidation, while simultaneously repressing N₂O production. Earlier work demonstrated that the addition of ammonium in the presence of phenylacetylene, a selective inhibitor of ammonia monooxygenase, soluble methane monooxygenase and particulate methane monooxygenase, stimulated CH₄ oxidation and also inhibited N₂O production. To transfer this technique from the laboratory to field applications, a pilot-scale demonstration was performed from November 2007 to July 2009 at a closed landfill in Kalamazoo, Michigan. The addition of NH₄⁺ and NO₃⁻ increased N₂O production, but had no effect on CH₄ concentrations *in situ*. The simultaneous addition of phenylacetylene reduced N₂O production and enhanced CH₄ oxidation. Multiple soil cores

were collected from the site, and microbial community analyses as well as physico-chemical analyses were performed to determine the specific microbial populations responsible for CH₄ consumption and N₂O production. Polymerase chain reaction (PCR) analyses showed that methanotrophs, especially those possessing particulate methane monooxygenase (pMMO), were more abundant than those possessing soluble methane monooxygenase, and, interestingly, archaeal ammonia-oxidizers were more abundant than their bacterial counterparts. After PCR amplification, microarray analysis was performed to detect changes in the methanotrophic community structure in response to different amendments. Microarray analyses showed that the addition of NH₄⁺ and NO₃⁻ caused the 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 a greater presence of Type I methanotrophs. Also, archaeal *amoA* gene clone libraries were constructed to examine the effects of the amendments on the ammonia oxidizing archaea (AOA) community structure. Clone libraries showed that the addition of NH₄⁺ and NO₃⁻ increased the presence of archaeal ammonia-oxidizers affiliated with Crenarchaeal Group 1.1b, while their presence decreased with the simultaneous addition of phenylacetylene. Several methanotrophs were investigated to examine their roles in N₂O production. Interestingly, several methanotrophs produced as much N₂O as ammonia oxidizers or denitrifiers. More specifically, five out of six Type II methanotrophic strains produced 32 - 342 ppmv of N₂O, which is equivalent to 0.16-1.7% of the conversion rate of NH₄⁺-N to N₂O-N, while two Type I strains did not produce detectable amount of N₂O. The effects of copper on N₂O production, i.e., some strains produced more N₂O in the absence of copper regardless of the presence of soluble methane monooxygenase (sMMO), suggested

that (1) the absence of copper had an inhibitory effect, but may be in various degree from species to species; or (2) methanotrophic-mediated N₂O production may not be controlled by the activity of pMMO. Collectively, these results suggest that the addition of phenylacetylene with NH₄⁺ and NO₃⁻ reduces N₂O production by selectively inhibiting archaeal ammonia-oxidizers and/or Type II methanotrophs. Such a possibility is intriguing as pure culture studies indicated that Type II methanotrophs produce more N₂O from ammonia than Type I methanotrophs, but it is currently unknown what the magnitude of N₂O production might be from archaeal ammonia-oxidizers. Once the major contributors on N₂O production are identified, we may be able to come up with a better strategy to mitigate *in situ* GHG emissions from a landfill.

CHAPTER 1. Introduction

CH₄ primarily produced through anaerobic decomposition of organic matter, is a potent greenhouse gas with a global warming potential (GWP) approximately 25 times that of carbon dioxide (CO₂) (IPCC, 2007). The atmospheric concentration of CH₄ has increased from a pre-industrial value of about 715 ppb in 1750 to 1774 ppb in 2005, with the increase largely due to anthropogenic activity (IPCC, 2007). Landfills have been reported to contribute ~37% of total anthropogenic CH₄ emissions in the US (IPCC, 2007), and it is believed that the mitigation of CH₄ emissions from a landfill is one of the most practical ways of stabilizing the CH₄ concentration in the atmosphere (Crutzen, 1991). N₂O, the third most important greenhouse gas next to CH₄, is produced by natural and human-related sources. Primary anthropogenic sources of N₂O include agricultural soil management, animal manure management, sewage treatment, and nitric acid production process. N₂O is also produced naturally from a wide variety of biological sources in soil, water, particularly microbial action in wet tropical forests (IPCC, 2007). N₂O reacts with ozone and is the main naturally occurring regulator of stratospheric ozone. It is also a major greenhouse gas having a global warming potential ~298 times greater than CO₂ over 100 year time frame (IPCC, 2007).

1.1 Methane as a greenhouse gas

The anthropogenic emissions of radiatively active trace gases and the resulting increase of the atmospheric concentration of those gases have raised concerns over the potential consequences for climate change. Wuebbles and Katherine (2002) reported that the global surface temperature has increased by over 0.5 °C in the last 150 years. It is the prevailing view that this temperature increase is closely related to the increase in the concentration of greenhouse gases (GHGs) (Allen et al., 2009; Meinshausen et al., 2009). Although many greenhouse gases, e.g., CO₂, CH₄, and N₂O, occur naturally in the atmosphere, human activities have changed their atmospheric concentrations. From the pre-industrial era (i.e., ~ 1750) to 2005, concentrations of CO₂, CH₄, and N₂O have increased globally by 36, 148, and 18%, respectively (IPCC, 2007). Among GHGs, CH₄ is a very potent greenhouse gas, second only to CO₂ as an anthropogenic contributor to global warming representing approximately 8.2% of total greenhouse gas emissions (EPA, 2009). Fortunately, a considerable amount of CH₄ can be biologically oxidized to CO₂ which has a much lower global warming potential than CH₄. An estimated 30 Tg of CH₄ from the atmosphere per year is reported to be removed by microbially-mediated oxidation in upland soils (Hanson and Hanson, 1996; Knief et al., 2003), and biological CH₄ oxidation is recognized to account for about 80% of global CH₄ consumption (Reeburgh et al., 1993).

1.2 Nitrous oxide as a greenhouse gas

N₂O is a greenhouse gas that interferes with the formation and destruction of stratospheric ozone (Crutzen, 1981). N₂O from soils is the main source of GHGs in

agriculture and contributed approximately 60% of the total anthropogenic emissions of N₂O in 2005, mainly associated with N fertilizers and manure applied to soils (IPCC, 2007). Concentrations of N₂O in the atmosphere have been increasing at a rate of 0.3% per year (Rasmussen and Khalil, 1986). The rise in atmospheric concentrations of N₂O is thought to result predominantly from microbial activity and not from anthropogenic emissions (Jiang and Bakken, 1999). In certain environments, such as agricultural fields, management practices could mitigate emissions of CO₂ and trace gases by stimulating and inhibiting various microbial processes (Robertson et al., 2000). Such processes include nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and chemodenitrification (Hayatsu et al., 2008). Mitigations of emissions by management in such environments depend on knowledge of the microbial processes responsible for N₂O production. To date, many studies have focused on defining the relative importance of nitrification and denitrification, but have not addressed the possibility of methanotrophic nitrification as a mechanism for the production of N₂O (Blackmer et al., 1980; Perez et al., 2001).

1.3 Landfills and the greenhouse effect

Landfill organic waste decomposes anaerobically, leading to the production of CH₄ and CO₂. Typical CH₄ mole fractions in landfill gas are 50-60%, with the remainder mostly CO₂ (Bogner et al., 1997). With such a composition, 88-92% of the total global warming potential of landfill gas is due to CH₄, and the remainder is due to CO₂. The contribution to the greenhouse effect is not the only environmental effect of landfill gas. Lateral migration of landfill gas can also lead to unexpected explosions. A well-

documented case of a dramatic landfill gas explosion was at the Skellingsked landfill, Denmark, where lateral migration during a period of decreasing barometric pressure following rain led to an explosion in a nearby house (Kjedsen and Fischer, 1995).

According to Crutzen (1991), mitigation of landfill CH₄ emissions is one of the most practical ways of stabilizing the CH₄ concentration in the atmosphere. The surest way to achieve this is by no longer landfilling any biodegradable materials. However, this may be a daunting task on a global scale (Reinhart and Townsend, 1998). Combustion of landfill gas for energy recovery is not always an economically viable option (Milke, 1998). Especially in small, old landfills, the amount of CH₄ produced is too small for energy recovery. However, part of the CH₄ that diffuses through the landfill cover will be biologically oxidized before reaching the atmosphere. Microbial CH₄ oxidation thus forms an alternative to landfill gas extraction for the mitigation of the CH₄ emission. Biofilters have also been proposed as an alternative to landfill cover soil CH₄ oxidation (Park et al., 2009; Philipoulos, 2009)

Bogner and Matthews (2003) estimated that global CH₄ emissions from landfills range from 16-57 Tg CH₄ year⁻¹. In the United States, 6.6 Tg CH₄ was emitted from landfills in 2007, which represents an increase of 4.3% since 1990, as well as the single most important anthropogenic source of CH₄ (Figure 1-1). The first attempt to quantify oxidation of CH₄ by landfill covers was made by Whalen et al. (1990). They estimated that half the CH₄ produced by U.S. landfills was oxidized in the cover soil, about 45 g m⁻² d⁻¹. A later study by Czepiel et al. (1996) indicated that only ~11% of produced CH₄ is oxidized in landfill cover soils, with peaks of 20-30% in summer months. In contrast, Kjedsen et al. (1997) found that CH₄ oxidation in landfills can be up

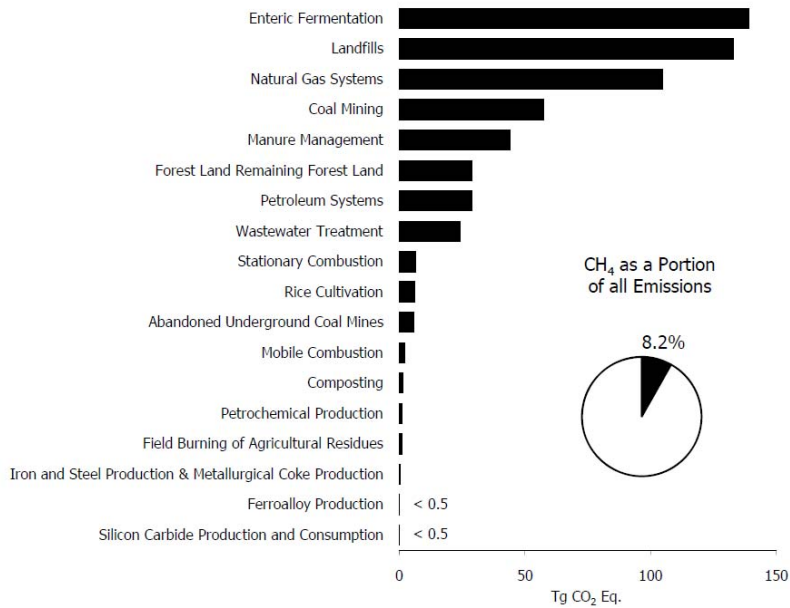


Figure 1-1. 2007 Sources of CH₄ emissions (IPCC, 2007).

to 100% of that produced from anaerobic organic decomposition. In the case of a 20-year-old landfill, a cover soil under ideal conditions can even oxidize all the CH₄ produced in winter (Christophersen et al., 2000). The fact that CH₄ fluxes out of landfills are generally larger in winter than in summer also indicates that microbial CH₄ oxidation in landfill cover soils can be substantial (Borjesson and Svensson, 1997b; Klusman et al., 2000).

In situ geochemical amendments to enhance microbial consumption of CH₄ in landfill soil have been proposed as a strategy to mitigate CH₄ emissions (Borjesson and Svensson, 1997a; Reinhart et al., 2002; Berge et al., 2005; Bogner et al., 2008). Ammonium is generally found to inhibit CH₄ oxidation, but small additions of NH₄⁺ can stimulate microbial consumption of CH₄ (Bender and Conrad, 1995; Lee et al., 2009). The addition of ammonium, however, also has been known to stimulate N₂O production, but when phenylacetylene was added in conjunction with ammonia, N₂O production was found to be selectively inhibited (Lee et al., 2009).

1.4 Methanotrophs

1.4.1 Physiology of methanotrophs

Methane-oxidizing bacteria, or methanotrophs, are a subset of a physiological group of bacteria known as methylotrophs. Methylotrophs can be defined as a group of bacteria which can grow on one-carbon compounds containing one or more carbons but not on compounds containing no carbon-carbon bonds. Methylotrophs that grow on CH₄ are usually known as methanotrophs. Methanotrophs are unique in their ability to utilize CH₄ as a sole carbon and energy source, and generally are obligately methanotrophic, growing only on CH₄ and methanol. The pathway of CH₄ oxidation is shown in Figure 1-2.

Methanotrophs oxidize CH₄ and methanol to formaldehyde, which can be subsequently assimilated to form intermediates of the central metabolic pathway. There are two pathways for carbon assimilation, each starting from formaldehyde, i.e. the ribulose monophosphate (RuMP) pathway (Figure 1-3), and the serine pathway (Figure 1-4). In the RuMP pathway, three molecules of formaldehyde are assimilated forming a three-carbon intermediate, while in the serine pathway, two molecules of formaldehyde and one molecule of carbon dioxide are utilized in each cycle forming a three-carbon intermediate of central metabolism. Methanotrophs are distinctly divided into two different physiological groups. One of the groups, commonly referred to as Type I, utilizes the RuMP pathway for primary carbon assimilation, while the other group, Type II methanotrophs utilizes the serine pathway. Interestingly, *Methylococcus capsulatus* Bath, Type I methanotroph, possesses low levels of both ribulose biphosphate

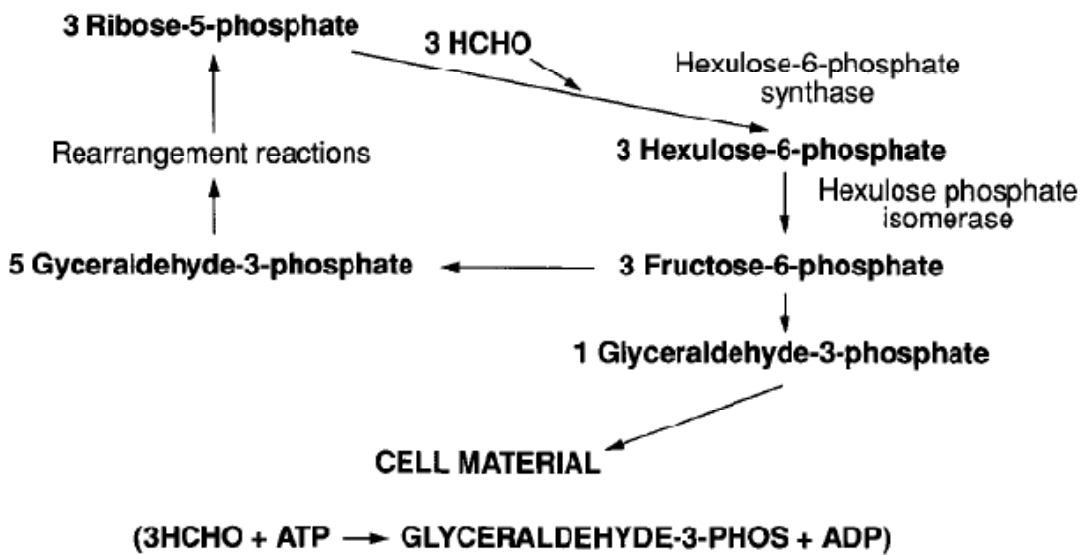


Figure 1-3. Ribulose monophosphate pathway for carbon assimilation by methanotrophic bacteria (Hanson and Hanson, 1996).

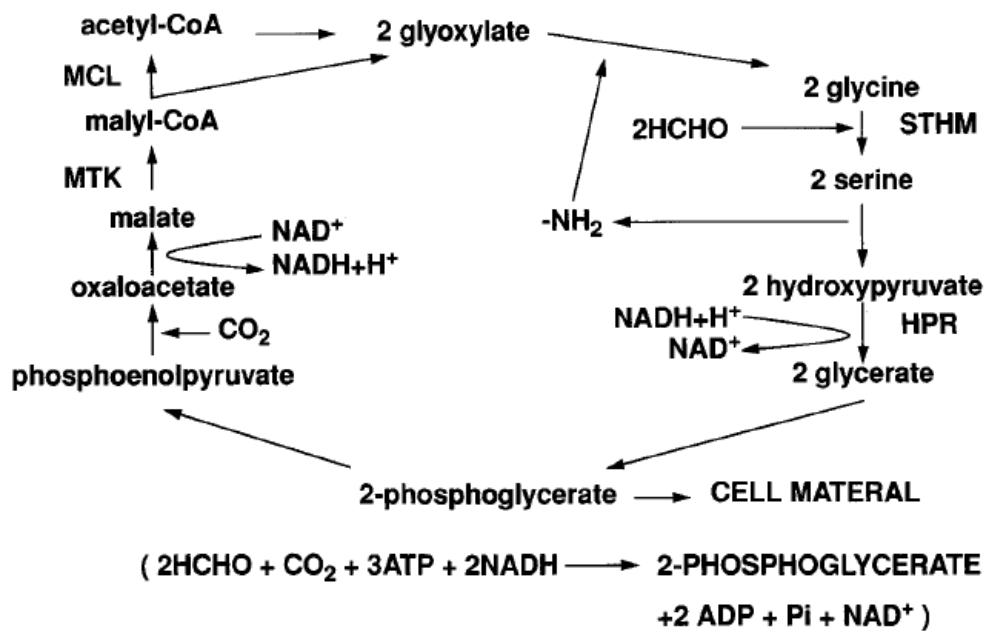


Figure 1-4. Serine pathway for carbon assimilation by methanotrophic bacteria (Hanson and Hanson, 1996).

1997). The current state of the taxonomy of methanotrophs is covered in detail in a recent paper by Semrau et al. (2010).

As mentioned above, methanotrophs are subdivided into two general groups, Type I and Type II. Type I methanotrophs are part of the γ -subdivision of the *Proteobacteria*. These organisms have intracytoplasmic membranes throughout the cell as bundles of vesicular disks, and use the RuMP pathway for formaldehyde assimilation. Signature phospholipid fatty acids are 14 and 16 carbon in length. Many of these strains are incapable of fixing N₂ although some Type I strains can do so (Auman et al., 2001). In the classic review of Hanson and Hanson (1996), the genera within the γ -*proteobacteria* included *Methylobacter*, *Methylococcus*, *Methylomicrobium*, and *Methylomonas* (Figure 1-5, Figure 1-6), but novel methanotrophs species and genera are still being discovered. For instance, *Methylosphaera hansonii*, a psychrophilic Type I methanotrophs was recently isolated from Antarctic sediments (Bowman, 1997), *Methylocaldum*, a group of moderately thermophilic methanotrophs most closely related to *Methylococcus thermophilus* (Bodrossy et al., 1997), was isolated from the effluent of an underground hot spring. *Methylohalobius*, a moderately halophilic methanotrophs was isolated from hypersaline lake (Heyer et al., 2005). *Methylosarcina*, another Type I methanotroph, which grew in irregularly shaped, refractile cell pockets that resembled sarcina-like clusters, was isolated from landfill soil (Wise et al., 2001). More ‘unusual’ filamentous methanotrophs containing a phylogenetically very unusual particulate methane monooxygenase have been discovered within the genera *Clonothrix* and *Crenothrix* (Stoecker et al., 2006; Vigliotta et al., 2007), but these are also classified into Type I methanotrophs, and phylogenetically a subset of the *Methylococcaceae* even

though the family *Crenotrichaceae* is validated (Op den Camp et al., 2009). As of present, 12 genera in total have been reported in the γ -subdivision of the *Proteobacteria*.

Type II methanotrophs are part of the α -subdivision of the *Proteobacteria* having intracytoplasmic membranes aligned along the periphery of the cell, utilization of the serine pathway for carbon assimilation, and signature phospholipid fatty acids of 18 carbons in length. These organisms typically can fix nitrogen. Initially, *Methylosinus*, and *Methylocystis* were the only genera within the α -*Proteobacteria* in the review of Hanson and Hanson (1996) (Figures 1-5, 1-6), but two more genera have been newly described including *Methylocella* and *Methylocapsa*. Strains within the new genera, *Methylocella* and *Methylocapsa*, were isolated from sphagnum peat bogs and acidic forest soils (Dedysh et al., 2000, 2002, 2004; Dunfield et al., 2003). These cells utilize the serine pathway for carbon assimilation, but are not considered to be classic Type II methanotrophs as they are within the *Beijerinckiaceae* family, and not *Methylocystaceae* as the other two genera *Methylosinus* and *Methylocystis* are.

More recently, three thermoacidophilic methanotrophs were isolated and placed in the *Verrucomicrobia* phylum, and represent the first non-*proteobacterial* methanotrophs ever isolated (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008; Semrau et al., 2008). These three strains were isolated from geothermal locations quite distant from each other, i.e., New Zealand, Italy and Russia, but they share remarkable 16s sequence similarity over 98% (Op den Camp et al., 2009). The genome analysis suggested that they seemed to have acquired numerous genes including those for enzymes of methylotrophic pathway via horizontal gene transfer, in particular from *Proteobacteria* (Hou et al., 2008).

Despite the broad phylogenetic diversity of methanotrophs, they have generally been considered to be obligate in nature, i.e., growing on one-carbon compounds such as CH₄ or methanol. The genome sequence of *Methylococcus capsulatus* (Bath) has shown that there is a “potential” for metabolic flexibility, with the presence of a genes encoding α -ketoglutarate dehydrogenase which is a key control point of the TCA cycle (Ward et al., 2004; Kelly et al., 2005), but there is no evidence yet for expression of this enzyme. Claims for the existence of facultative methanotrophs, i.e., methanotrophs capable of growing on multicarbon as well as one-carbon substrates, have a long history dating back over 30 years (Theisen and Murrell, 2005). The first claim for the existence of facultative methanotrophs dating back almost 30 years when Patt et al. isolated and characterized the strain, *Methylobacterium organophilum* (Patt et al., 1974; 1976). This strain was able to grow on glucose as well as on CH₄, but later the methane-oxidizing capacity was lost (Green et al., 1983). This was followed by *Methylobacterium ethanolicum*, but this strain was later shown to be actually a mixed culture of a methylotrophic in a strong syntrophic association with a *Xanthobacter* species (Lidstrom et al., 1983). More recently, the existence of a new facultative methanotroph, *Methylobacterium populi*, was proposed, but was refuted by others (Dedysh et al., 2004; Van Aken et al., 2004). Facultative methanotrophy, however, does occur as *Methylocella* has been isolated (Dedysh et al., 2005). *Methylocella* is the first fully authenticated Type II methanotroph, found to grow on multicarbon substrates but only expressing the soluble methane monooxygenase (sMMO). *Methylocella* species, especially, *M. silvestris*, can use various organic acids including acetate, pyruvate, succinate and malate as well as ethanol as the sole growth substrates. Other facultative methanotrophs, able to grow on acetate, have been reported

within the genus *Methylocystis*, specifically the mild acidophiles *Methylocystis* strain H2s, *Methylocystis heyeri* H2^T, the mesophile *Methylocystis echinoides* IMET10491^T (Belova et al., 2010) and *Methylocystis* sp. SB2 (Im et al., 2010). The precise mechanism and pathway by which this cell consumes multi-carbon substrates, however, is not understood yet.

The oxidation of CH₄ to CH₃OH is catalyzed by the methane monooxygenase (MMO). There are two types of MMO, one in the cytoplasmic or soluble fraction, i.e., the soluble MMO (sMMO), found in both Type I and II methanotrophs but is only known to be expressed by a small subset of methanotrophs. The other form expressed by most known methanotrophs is the membrane-bound, or particulate MMO (pMMO), located in the intracytoplasmic membranes, or particulate fraction. In methanotrophs having both forms of MMO, expression strongly depends on the copper to biomass ratio, i.e., sMMO is only expressed when the copper to biomass ratio is low. There is also evidence that copper inhibits the activity of sMMO (Green et al., 1985). The pMMO has been found in all known methanotrophs, with the exception of *Methylocella* species possessing only sMMO (Dedysh et al., 2005).

Despite largely being unable to grow on compounds other than CH₄ or methanol, methanotrophs can mediate a surprising number of reactions, including the oxidation of alkanes, alkenes, aromatic hydrocarbons, phenols and chlorinated hydrocarbons (Higgins et al., 1980; Yoon and Semrau, 2008). sMMO can catalyze a broader range of reactions than pMMO (Burrows et al., 1984). sMMO has been known to oxidize alkanes up to C-8 as well as ethers, cyclic alkanes, and aromatic hydrocarbons (Colby et al., 1977; Hou et al., 1979; Burrows et al., 1984), while pMMO can oxidize alkanes up to C-5, but is not

able to oxidize cyclic alkanes or aromatic compounds (Burrows et al., 1984). Some of these reactions, like trichloroethylene degradation, have been extensively studied for their potential use in bioremediation of polluted sites (Lee et al., 2006; Yoon and Semrau, 2008). pMMO-expressing cells survive more readily in the presence of mixtures of chlorinated ethens and actually degraded more, due to the greater specificity of pMMO for CH₄ and thus slower production of toxic by-products of chlorinated compounds, suggesting, despite the broader substrate range of sMMO, pMMO-expressing cells may be more responsible for the degradation of such pollutants *in situ* (Lee et al., 2006; Yoon and Semrau, 2008).

1.4.2 Factors affecting methanotrophic activity

1.4.2.1 Moisture content

Castro et al. (1994) studied atmospheric CH₄ oxidation in temperate forest soils. They found that the oxidation decreases linearly with water-filled pore space between values of 30 and 100% (v/v). At 100% (v/v), CH₄ oxidation rates were almost zero. Koschorrek and Conrad (1993) and Lessard et al. (1994) also found a strong negative correlation between the soil moisture content and CH₄ oxidation.

At low moisture levels (13% of water holding capacity), CH₄ oxidation rates were low as well due to water stress (Whalen and Reeburgh, 1996). This effect was clear when measurements on a desert soil were made (Striegl et al., 1992). During the first ten days following rain events, the CH₄ uptake averaged 1.87 mg CH₄ m⁻² d⁻¹, whereas the mean flux during dry periods averaged 0.30 mg CH₄ m⁻² d⁻¹ in winter and 0.74 mg CH₄ m⁻² d⁻¹ in the rest of the year.

Schnell and King (1996) quantified the influence of water stress in cultures and in soils. In cultures, the water stress was induced by adding sucrose or NaCl. In soils, it was induced either by adding NaCl or sucrose, or by drying the soil. In all cases the water stress was quantified along with the (osmotic or matric) water potential. In all cases an increase in water stress caused a decrease in CH₄ uptake.

The opposing effects of diffusion limitations of gaseous transport and water stress lead to the occurrence of an optimum moisture for CH₄ oxidation. Whalen and Reeburgh (1996) found that this optimum was at 20-30% of water holding capacity in the case of two boreal forest soils, and 50% of water holding capacity in the case of a bog soil. Methanotrophic community structure is also influenced by the moisture content (Henckel, et al., 2001). In this study, the drainage of rice paddy soils affects the methanotrophic structure, more specifically, stimulating the activity of Type I methanotrophs. On the other hand, Type II methanotrophs were found throughout the soil cores, but their composition did not show any significant difference to soil drainage. Such a finding suggests that Type I methanotrophs may be more adaptable to environmental change, as indicated by other studies where CH₄ and O₂ were varied (Auman et al., 2000; Henckel et al., 2000).

1.4.2.2 Temperature

Whalen and Reeburgh (1996) studied the influence of temperature on CH₄ oxidation in disturbed boreal soils in batch experiments, and found a linear increase of the oxidation rate between 4 °C and 28 °C in two boreal forest soils in Alaska. In this study, an optimal temperature between 25 °C and 30 °C was found for a boreal bog

sample. King and Adamsen (1992) also observed that the CH₄ oxidation by *Methylomonas rubra* was more strongly dependent on temperature at 10,000 ppm of CH₄ than at 100 ppm. At 100 ppm, there was little response to temperature, which was likely because mass transfer was rate limiting. In contrast, at 10,000 ppm, CH₄ oxidation was strongly dependent on temperature, indicating that enzymatic activity was likely rate-limiting. The temperature dependence was much less pronounced in soil cores than in pure cultures. The difference was attributed to diffusion limitations in the soil, especially for the vertical diffusion in the gas phase. From the limited number of phylogenetic studies done on the effects of temperature on methanotrophic communities, it appears that Type I methanotrophs dominate at low temperatures in biofilters (Gebert et al., 2003, 2004). This result is compatible with the recent studies of methanotrophic communities in landfill soils, where Type I methanotrophs were more dominant at 10 °C than 20 °C (Borjesson et al., 2004).

1.4.2.3 Inorganic N

In general, NH₄⁺ is found to inhibit CH₄ oxidation, whereas NO₃⁻ is found to have little influence (Boeckx and Van Cleemput, 1996). However, many conflicting results about the influence of inorganic N on CH₄ oxidation can be found in the literature.

King and Schnell (1994a; 1994b) investigated the influence of the CH₄ concentration on NH₄⁺ inhibition of CH₄ 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 CH₄ concentration from 1.7 to 100 ppm in the presence of 500 μM of ammonium, although

the extent of inhibition decreased with CH₄ concentrations above 250 ppm. The increase of the inhibition was attributed to an increased formation of NO₂⁻ from NH₄⁺ oxidation. The counter-intuitive findings of increased inhibition of CH₄ oxidation as CH₄ concentration increased may be attributed as the oxidation of CH₄ generates NADH + H⁺, which in turn stimulates NH₄⁺ oxidation. However, in the field no NO₂⁻ was observed. This could indicate that endogenously produced NO₂⁻ is a stronger inhibitor of CH₄ oxidation than exogenous NO₂⁻ (Schnell and King, 1994). Conversely, Boeckx et al. (1996) found that the first-order reaction rate constant of CH₄ oxidation in a landfill cover soil decreased as amounts of added NH₄⁺ increased. Similarly, Hutsch (1998) reported that the application of 40 mg N kg⁻¹ caused a strong inhibition of CH₄ oxidation up to 96%. Other studies, however, found that the 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 CH₄ (DeVisscher et al., 1999; 2001). Bender and Conrad (1995) also found that the methanotrophic activity was induced by the presence of NH₄⁺ and the optimum concentration of NH₄⁺ was 12-61 mM in the soil water phase at 100 µl of CH₄ L⁻¹. Mohanty and his 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 CH₄ uptake, but those dominated by Type II methanotrophs could be affected negatively due to changes of the methanotrophic community composition (Mohanty et al., 2006).

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 CH₄ 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 CH₄ environments such as landfill cover soils, where the molar ratio of CH₄ 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 amendments have been proposed, but such additions can increase emissions of N₂O, the third most important greenhouse gas after CO₂ and CH₄ (Majumdar, 2003).

1.5 N₂O production

N₂O has a strong potential for infrared adsorption and thus has been considered as the third most important greenhouse gas after CO₂ and CH₄. Over a 100 year time frame, it has a global warming potential ~298 times greater than CO₂ (IPCC, 2007). N₂O is produced by both natural and anthropogenic sources, and the primary anthropogenic source is agricultural soil in the US (Figure 1-7). Over the past several decades, the annual input of biologically reactive nitrogen has increased from a variety of anthropogenic sources, 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 N₂O accounting for over 60% of total emission in the US (EPA, 2009). The requirement for nitrogen in biological molecules leads to a diversity of mechanisms for assimilation. Except for nitrogen fixation, the majority of assimilatory mechanisms in bacteria require the uptake of either ammonia or nitrate. In addition to assimilation, nitrogenous compounds play important roles for energy generation. Key microbial enzymes in the nitrogen cycle are summarized in Table 1-1.

Although the mechanism of N₂O production is not completely understood, microbial production of N₂O is generally considered to be achieved through three different processes (Figure 1-8); (i) as a by-product of nitrification, (ii) nitrifier denitrification, and (iii) denitrification.

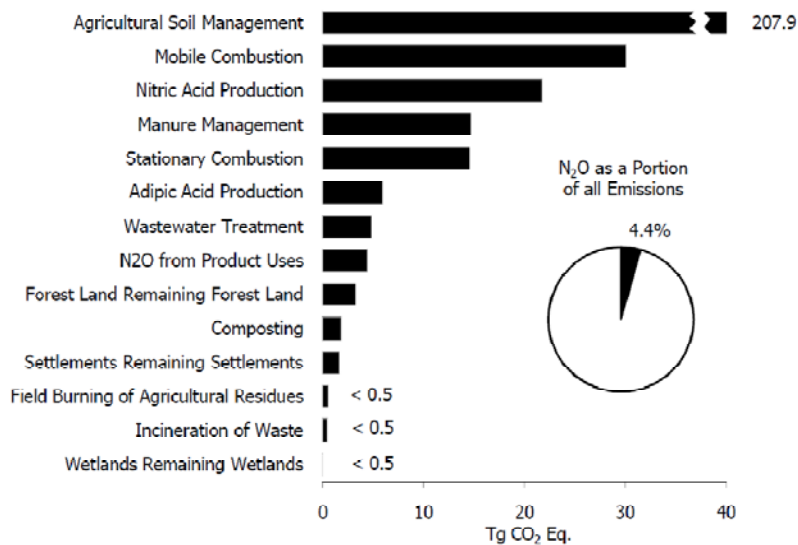


Figure 1-7. 2007 sources of N₂O emissions (EPA, 2009).

Table 1-1. Bacteria in the nitrogen cycle and their enzymes (Stein and Yung, 2003).

Bacterial group	Process	Main enzyme	Metabolism
Chemolithotrophic ammonia-oxidizers	NH ₃ oxidation to NO ₂ ⁻ via NH ₂ OH	AMO, HAO	dissim.
	NH ₂ OH oxidation to NO and N ₂ O	HAO or abiotic NIR	?
	NO ₂ ⁻ reduction to NO	NOR	?
	NO reduction to N ₂ O	?	?
Methane oxidizers	NH ₃ oxidation to NO ₂ ⁻ via NH ₂ OH	MMO, *HAO	fortuitous
	NH ₂ OH oxidation to NO and N ₂ O	*HAO or abiotic ?	?
	NO ₃ ⁻ reduction to NO ₂ ⁻	?	assim.
	NO ₂ ⁻ reduction to NH ₃	N ₂ ase	assim.
	N ₂ fixation to NH ₃	NIR	assim.
	NO ₂ ⁻ reduction to NO	NOR	?
Heterotrophic ammonia-oxidizers	NO reduction to N ₂ O		?
	NH ₃ oxidation to NO ₂ ⁻ via NH ₂ OH	AMO, HAO	fortuitous
Nitrite oxidizer	NO ₂ ⁻ oxidation to NO ₃ ⁻	nitrite oxidase	dissim.
	NO ₃ ⁻ reduction to NO ₂ ⁻ , NH ₃ , and N ₂ O	nitrite oxidase or ?	dissim.
	NO oxidation to NO ₂ ⁻ and NO ₃ ⁻	?	?
Dissimilatory denitrifiers	NO ₃ ⁻ reduction to NO ₂ ⁻	NAR, NAP	dissim.
	NO ₂ ⁻ reduction to NO	NIR	dissim.
	NO reduction to N ₂ O	NOR, NOS	dissim.
	N ₂ O reduction to N ₂	N ₂ OR	dissim.
	NO oxidation NO ₃ ⁻	?	?
	NO ₃ ⁻ reduction to NH ₃ via NO ₂ ⁻	NAR, NAS	dissim., assim.
Assimilatory denitrifiers	NO ₃ ⁻ reduction to NO ₂ ⁻	NAS	assim.
	NO ₂ ⁻ reduction to NH ₃	sirohaem NIR, hexahaem NIR	assim.
Fungal denitrifiers	NO ₂ ⁻ reduction to NO	Cu-NIR	?
Nitrogen fixers	N ₂ to NH ₃	nitrogenase	?
Anammox	NH ₃ and NO ₂ ⁻ to N ₂ via N ₂ H ₄ and NH ₂ OH	nitrite reductase hydrazine hydrolase, hydrazine oxidase	assim. dissim.

AMO: ammonia monooxygenase, HAO: hydroxylamine oxidoreductase, NIR: nitrite reductase, NOR: nitric oxide reductase, MMO: methane monooxygenase, NAR: membrane-bound nitrate reductase, NAP: periplasmic nitrite reductase, NOS: nitrous oxide reductase, *N₂ase: nitrogenase, N₂OR: nitrous oxide reductase. Assim. = assimilatory, dissim. = dissimilatory, fortuitous = co-metabolic.

*HAO gene was recently discovered in the *M. capsulatus* bath genome and its mRNA level increased in response to ammonia, but the actual form of the enzyme has not been purified yet. (Ward et al., 2004; Arp et al., 2007; Poret-Peterson et al., 2008).

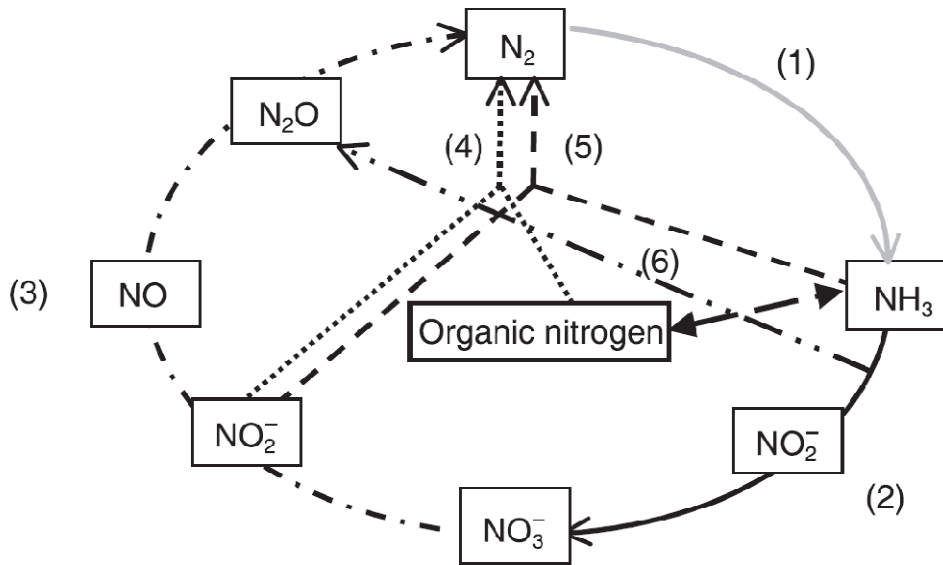


Figure 1-8. Microbiological processes in the nitrogen cycle. (1) Nitrogen fixation, (2) bacterial nitrification, archaeal nitrification and heterotrophic nitrification, (3) aerobic and anaerobic bacterial denitrification, nitrifier denitrification, fungal denitrification and archaeal denitrification, (4) and (5) co-denitrification (by fungi), (5) anammox and (6) N_2O production during nitrification (ammonia oxidation) (Hayatsu et al., 2008).

1.5.1 Nitrification

Ammonia- 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 to 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 the 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 that 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 oxidizer (Nyerges and Stein, 2009). However, this metabolic pathway is less well characterized than that for ammonia oxidizers. These microorganisms, ammonia oxidizing bacteria/archaea, and methanotrophs occupy the same environmental niche, i.e., aerobic zones, and thus may play significant roles in N_2O production in various environments.

1.5.2 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,

N₂O is produced and consumed by the step-wise reduction of nitrate (NO₃⁻) to N₂ (Sutka et al., 2006) as shown in Figure 1-8. Since denitrification is a sequence of reductive reactions, this process is thought to occur mostly in anaerobic environments. For this reason, the potential for N₂O production from denitrification process is highest in water-saturated or anoxic zones which contain large concentrations of organic carbon and NO₃⁻.

There has been substantial debate about the contribution of nitrification and denitrification to the emission of N₂O. The introduction of multi-isotope signature methodologies has widened our knowledge of mechanisms of biological N₂O production and the global N₂O budget, and the analysis of intramolecular distribution of ¹⁵N in N₂O, 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 N₂O 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 for this study providing supporting information to distinguish N₂O production from nitrification and denitrification.

1.5.3 Nitrifier denitrification

Nitrifier denitrification is another process that produces N₂O which can be attributed to autotrophic ammonia-oxidizing bacteria and, presumably, ammonia-oxidizing archaea. Ammonia-oxidizing bacteria has been shown to produce N₂O when NO₂⁻ is used as an alternative electron acceptor under anoxic conditions by a process termed nitrifier denitrification, where NO₂⁻ is reduced to NO or N₂O as in the classical heterotrophic denitrification pathway (Goreau et al., 1980; Poth and Focht, 1985; Wrage

et al., 2001; Arp and Stein, 2003). Studies using *Nitrosomonas* and *Nitrospira* 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.

1.5.4 Anammox

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 for 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 limitation (Kartal et al., 2007). Anammox bacteria are also a potential source of N_2O production by nitric oxide detoxification (Kartal et al., 2007).

1.5.5 Ammonia oxidizing Crenarchaeota

While anammox continues to be explored as a 'new' process in the global nitrogen cycle, a new 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. 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 is 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 $<0.03\sim 1\ \mu\text{M}$ (Konneke et al., 2005; Wuchter et al., 2006; Beman et al., 2008) are dominated by Group 1.1a, while Group 1.1b is 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 that 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 at 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 have also 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).

Recently, new phylum, Thaumarchaeota, has been proposed for the mesophilic crenarchaeota including ammonia oxidizing archaea. Originally, archaeal domain was divided into two major phyla, the Euryarchaeota and Crenarchaeota. At first, Crenarchaeota included only hyperthermophiles. However, recent discovery of mesophilic crenarchaeota and genome data analysis showed that they should be considered as a third archaeal phylum (Brochier-Armenet 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?

1.6 Previous study

In previous study (Lee et al., 2009), a matrix of soil microcosms was constructed using landfill cover soils obtained from the King Highway Landfill in Kalamazoo, Michigan and exposed to various geochemical parameters known to affect CH_4 consumption by methanotrophs while also minimizing microbial N_2O production. It was found that relatively dry soils (5% moisture content) along with $15 \text{ mg } NH_4^+ (\text{kg soil})^{-1}$ and $0.1 \text{ mg phenylacetylene } (\text{kg soil})^{-1}$ provided the greatest stimulation of CH_4 oxidation while minimizing N_2O production. Microarray analyses of *pmoA* showed that the methanotrophic community structure was dominated by Type II organisms, but Type I signals increased with the addition of ammonia. When phenylacetylene was added in

conjunction with ammonia, the methanotrophic community structure was more similar to that observed in the control. PCR analyses showed the presence of both bacterial and archaeal *amoA* was reduced with the addition of phenylacetylene. In order to transfer the findings to *in situ* application, a field demonstration was performed in this study at King Highway Landfill, in Kalamazoo, MI. The detailed set up will be discussed in the Chapter 2.1.

1.7 Goals and objectives

The primary goal of this thesis is to find a strategy to control greenhouse gases in landfill cover soils by selectively stimulating the *in situ* microbial community. The following specific objectives are addressed.

1.7.1 Objective 1: Understand the long-term effects of geochemical parameters on methane consumption and nitrous oxide production in landfill cover soils.

In previous laboratory microcosms study using landfill cover soils, it was shown 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 CH_4 oxidation (by as much as 50%) while minimizing N_2O production, as compared to controls with no amendments (Lee et al., 2009). Here, in order to transfer the

technique to *in situ* application, a pilot-scale demonstration was performed, i.e., six amendments identified from this previous study were applied to an array of field test plots located near Kalamazoo, Michigan, at the King Highway Landfill: (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 a canopy. The first amendment was applied in November 2007 and repeated every two months for 8 months and then monthly until June 2009. These amendments were added to determine the long-term efficacy of these amendments for minimizing greenhouse gas emissions from landfills, as well as to better characterize and quantify seasonal fluctuations in CH_4 and N_2O and the microbial communities regulating the emission of these greenhouse gases. Multiple soil cores were collected from the site, and microbial community analysis as well as physico-chemical analysis were performed to determine the microbial populations responsible for CH_4 consumption and N_2O production. More specifically, PCR

amplifications on the DNA and cDNA reverse-transcribed from mRNA extracted from each soil core have been performed. Also, DNA microarray analyses were performed and *amoA* gene fragment clone libraries were constructed to examine the long-term effects of the amendments on the methanotrophic and AOA community structure, respectively.

1.7.2 Objective 2: Verification and control of methanotrophic-mediated production of N₂O.

The oxidation of NH₄⁺ by methanotrophs has been referred to as methanotrophic nitrification, and there have been several observations of forest and grassland soils that consume CH₄ and simultaneously produce N₂O (Castro et al., 1994; Neff et al., 1994). Mandernack and his colleagues (2000) have shown that such methanotrophic-mediated N₂O production may be significant. However, the relative importance of methanotrophs in N₂O production in native soils or sediments is not well understood. Therefore, in order to better understand the environmental and microbial control of N₂O production in soils, laboratory incubation experiments using eight methanotrophs pure strains were performed.

CHAPTER 2. Materials and methods

2.1 Field study design, site and test plots

Previous research in our laboratory has found that phenylacetylene acts as a differential inhibitor of the ammonia monooxygenase (AMO) of ammonia-oxidizing bacteria, soluble monooxygenase (sMMO), and membrane-bound methane monooxygenase (pMMO) of methanotrophs (Lontoh et al., 2000). In a previous microcosm study, it was found that relatively dry soils (5% moisture content) along with $15 \text{ mg NH}_4^+ (\text{kg soil})^{-1}$ and $0.1 \text{ mg phenylacetylene (kg soil)}^{-1}$ provided the greatest stimulation of CH_4 oxidation while minimizing N_2O production (Lee et al., 2009). To transfer this technique from the laboratory to the field, a pilot-scale demonstration was carried out with periodic gas monitoring. The study plots were located near Kalamazoo, Michigan, at the King Highway Landfill (Figure 2-1). From 1987 to 1998, Georgia Pacific Inc., one of the leading manufacturers of tissue, packaging, paper, and pulp, used the landfill for disposal of dewatered paper-making residuals. The King Highway landfill is a mono-fill of paper residuals. The landfill covers 15 acres and consists of four cells. The total volume of residuals in the landfill is estimated at 282,000 cubic yards (EPA, 2007). 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 2-1. 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 plots 4 and 7, 100 mg of 98%

phenylacetylene was added to the same solution, and 4% (v/v) of methanol was added to increase the solubility of phenylacetylene. The resulting solution was sprayed on each plot by a pressurized vessel. For plots 2, 3, and 4, a canopy was installed in an attempt to control soil moisture content by preventing precipitation (Figure 2-2). The canopy was 1.5 m wide and 2 m high as shown in Figure 2-1. The frame material was PVC and it was covered with semi-transparent woven polypropylene sheet. The plots were 1 m by 1 m in size, 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 a soil core of 0.6~1.0-m depth from each test plot was collected.

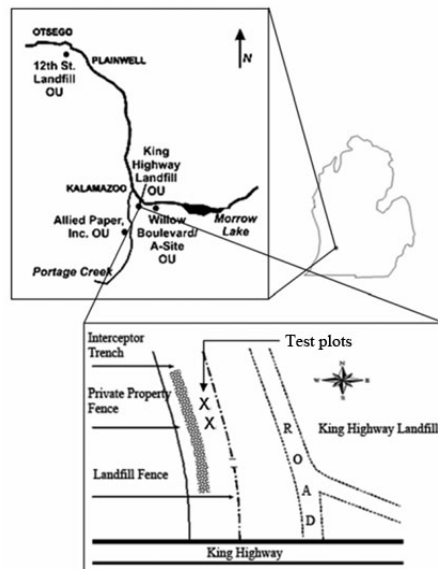


Figure 2-1. Location of King Highway landfill and test plots.

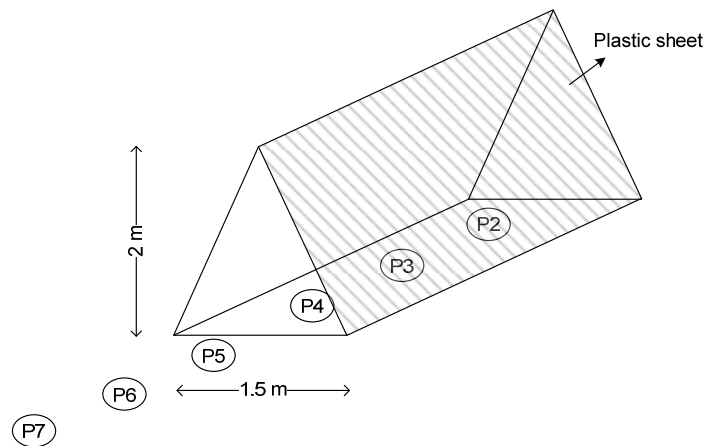


Figure 2-2. Schematic diagram of a canopy installed for plot 2, 3, and 4.

Table 2-1. Amendments applied to the study plots on the King Highway Landfill

Location	Amendments
Plot 2	No amendment under canopy
Plot 3	NH ₄ Cl/KNO ₃ under canopy
Plot 4	NH ₄ Cl/KNO ₃ /C ₈ H ₆ under canopy
Plot 5	No amendment no canopy
Plot 6	NH ₄ Cl/KNO ₃ no canopy
Plot 7	NH ₄ Cl/KNO ₃ /C ₈ H ₆ no canopy

2.2 Landfill gas monitoring

The vertically-nested soil gas sampling probes were installed between 20 cm and 100 cm depth and 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 stoppers 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. CH₄ and O₂ were measured using a Buck Scientific model 910 gas chromatograph (Buck Science Inc., East Norwalk, CT) 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. The final temperature was maintained for 3 min. Helium was used as the carrier gas at a flow rate of 10 mL·min⁻¹. For N₂O measurement, an HP 5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) 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·min⁻¹. Nitrogen gas was used as both the carrier and makeup gas. The oven temperature was set to -10 °C by injecting liquid nitrogen into the oven chamber using an automated cryogenic valve.

2.3 Statistical analysis

A linear mixed-effects model implemented in SAS (PROC MIXED) was used to determine the correlations between the repeated measures within a plot. In addition to the effects of treatment, the month and the depth of measurement 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 introduced as below.

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

$$y_i = X\beta + \varepsilon \quad (1)$$

$$\varepsilon \sim N_n(0, \sigma^2 I_n) \quad (2)$$

where $y=(y_1, y_2, \dots, y_n)$ is the response vector; X is the model matrix, with typical row $x_i=(x_{1i}, x_{2i}, \dots, x_{pi})$; $\beta=(\beta_1, \beta_2, \dots, \beta_p)$ is the vector of regression coefficients; $\varepsilon=(\varepsilon_1, \varepsilon_2, \dots, \varepsilon_n)$ is the vector of errors; N_n represents the n-variable multivariate-normal

distribution; σ is the error variance; 0 is an $n \times 1$ vector of zeros; and 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 effects 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 (Singer, 1998).

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

$$y_i = X\beta + Z_i b_i + \varepsilon \quad (3)$$

where Z_i is the model for the random effects for observation in group i , and 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 (Moore and McCabe, 2009).

2.4 Soil collection and analyses

On July 22, 2009, the field site was dismantled and an intact soil core, 1 m long and 5 cm in diameter, from each treatment was obtained using a vehicle-mounted soil core sampler (Model 6620DT, Geoprobe, Salina, KS). After extruding the soil cores from the test plots receiving different amendments, 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 CaCl₂ in 1:2 ratio (i.e., 5 g soil in 10 mL of 0.01 M CaCl₂), and shaking the soil slurry at 220 rpm for 30 min. Inorganic nitrogen, i.e., NH₄⁺-N and (NO₃⁻+ NO₂⁻)-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, NH₄⁺ and NO₃⁻+ NO₂⁻ 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 CaCl₂ 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). Soil pH was measured once, because it required relatively large amount of soil sample, 5 g, and also repeated measures of samples prepared independently generated consistent results. The other parameters including inorganic nitrogen contents and metal contents were prepared in duplicate and measured independently.

2.5 DNA extraction and PCR amplification

Soil core samples collected at a depth of 25~27.5 cm were used for microbial community analysis. This soil layer was chosen as the N_2O , CH_4 , and O_2 were monitored from 20 to 100 cm of depth, and soils at this depth have a higher potential to exchange gases with the atmosphere, i.e., O_2 and thus be a more favorable habitat for methanotrophs and ammonia-oxidizing microorganisms. Also, the layer was consistent in soil texture since there was sometimes debris such as rocks or concrete observed from the other soil layers. DNA was extracted from 0.5 g of soil using the UltraClean soil DNA extraction kit (MoBio, Solana, CA). To optimize the extraction method for the yield of bacterial and archaeal DNA, three different bead beating times, i.e., 30 sec, 120 sec and 10 min, were tested. PCR amplification was performed using specific primers for *pmoA*, *mmoX*, Bacterial *amoA*, and Archaeal *amoA* as shown in Table 2-2. PCR amplifications were performed using Biometra TPersonal thermal cycler system (Labrepc, Horsham,

PA). Each PCR reaction consisted of 5 μ L 10X PCR buffer (Invitrogen, Carlsbad, CA), 1.5 μ L 50 mM MgCl₂ (Invitrogen, Carlsbad, CA), 1 μ L 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. 1 μ L 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 μ L of the PCR mixture onto 1% (w/v) agarose gels and staining with ethidium bromide.

2.6 mRNA extraction and reverse transcription-polymerase chain reaction assays

RNA was extracted following previously developed procedures with minor modifications (Han and Semrau, 2004; Lee et al., 2009). Briefly, 0.5 g of soil samples collected from the same depth as in DNA extraction, i.e., 25~27.5 cm, was added to 1 mL of extraction buffer (0.2% CTAB, 1 mM DTT, 0.2 M sodium phosphate buffer, 0.1 M NaCl, 50 mM EDTA), with 1% β -mercaptoethanol and 1 g of 0.1 mm silica glass beads. Six 30 s cycles of bead beating were performed in a Mini-BeadbeaterTM (BioSpec

Table 2-2. Primers used for PCR amplification of functional genes of particulate methane monooxygenase (*pmoA*), soluble methane monooxygenase (*mmoX*), bacterial ammonia monooxygenase (*amoA*), archaeal ammonia monooxygenase (*amoA*), nitrite reductase (*nirK*), nitric oxide reductase (*norB*), and anammox 16s rRNA.

Gene targeted	Primers	Sequence (5'-3')	Reference
<i>pmoA</i>	pmoA-A189 pmoA-mb661	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	Costello & Lidstrom (1999)
<i>mmoX</i>	mmoX206f mmoX886r	ATCGCBAARGAATAYGCSCG ACCCANGGCTCGACYTTGAA	Hutchens et al. (2004)
Bacterial <i>amoA</i>	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Rotthauwe, et al. (1997)
Archaeal <i>amoA</i>	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	Francis et al. (2005)
<i>nirK</i>	nirK1F nirK5R	GGRATGGTYCCSTGGCA GCCTCGATCAGRTRRTGG	Braker et al. (1998)
<i>nirK</i>	F1aCu R3Cu	ATCATGGTCTGCCGCG GCCTCGATCAGRRTGTGGTT	Hallin and Lindgren (1999)
<i>norB</i>	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

products, Bartlesville, OK). The mixture was put on ice for 1 min between each bead-beating cycle. After cell lysis, the soil/bead suspension was sampled by piercing the bottom of the tube using a sterile 22 gauge needle, placing it on top of a sterile collection tube, and centrifuging at 2,500 rpm for 5 min using a swinging bucket centrifuge IEC Centra CL2 (International Equipment Co., Needham Heights, MA). One volume of 70% ethanol was then added to the collected buffer and total RNA extracted using the Qiagen RNeasy kit according to manufacturer's instructions (Valencia, CA). After being treated with RNase-free DNase I (Promega, Madison, WI) to remove any DNA contamination, DNase-treated RNA was then purified using the Qiagen RNeasy kit according to manufacturer's instructions (Valencia, CA, USA). After confirming the complete removal of DNA by PCR with the RNA samples as a template, RNA was then reverse

transcribed using SuperScript II reverse transcriptase with 250 ng of random primers (Invitrogen, Carlsbad, CA) according to manufacturer's instructions (Invitrogen, Carlsbad, CA) to obtain cDNA and stored at -20 °C until further analysis. PCR was carried out by the same procedure used for DNA amplification. 20 ng of cDNA was used as a template for PCR.

2.7 Construction of archaeal *amoA* gene fragment libraries

A clone library of archaeal *amoA* amplicons obtained from 25~27.5 cm depth of each soil core was constructed to analyze the effects 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 were selected and sequenced by Beckman Coulter Genomics (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.

2.8 Microarray sample preparation

Sample preparation and data analysis were carried out following previously described methods (Lee et al., 2009). Briefly, PCR amplification of the *pmoA* gene from DNA collected from soils was performed using the primer set pmoA189-mb661 with the T7 promoter site attached to the 5' end of primer mb661 to enable *in vitro* transcription of the PCR products via T7 RNA polymerase (Table 2-3). Triplicates of PCR reactions were then pooled and purified using Qiagen PCR Purification Kit (Qiagen, Valencia, CA). Methods for *in vitro* transcription and hybridization were used as described previously (Bodrossy et al., 2003; Stralis-Pavese et al., 2004). Results of individual microarray experiments were first normalized to positive control probe mtrof173, a general probe targeting methanotrophs, and then to the reference values determined individually for each probe and displayed using GeneSpring (Agilent, Palo Alto, CA).

Table 2-3. Primers used for amplification of *pmoA* for use in microarrays.

	Primers	Sequence (5'-3')	Reference
Forward	pmoA-A189	GGNGACTGGGACTTCTGG	Costello & Lidstrom (1999)
Reverse	pmoA-mb661	CCGGMGCAACGTCYTTACC	
5' end of reverse primer	T7-promotor site	TAATACGACTCACTATAG	Stralis-Pavese et al., (2004)

2.9 N₂O production by methanotrophs

To estimate the contribution of methanotrophic activity on N₂O production, *Methylobacterium 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 were grown in ammonia mineral salt media (AMS) media (Whittenbury, 1970). The first experiment, aimed to investigate the effects of growth condition on N₂O production, was performed using *Methylosinus trichosporium* OB3b. Copper, as CuSO₄·5H₂O (JT Baker Chemical, Phillipsburg, NJ, Baker analyzed) was added at a concentration of 20 μM for pMMO expression, while separate systems with no added were prepared for sMMO expression. sMMO expression was verified by the naphthalene assay (Brusseau et al., 1990). Formate was prepared as 500 mM sodium formate stock solution. Cells were first grown to the late exponential phase (optical density at 600 nm [OD₆₀₀] of 0.7 - 0.8) in either 0.1X or 1X AMS with or without copper and with CH₄ as the growth substrate. Cells were then diluted to an OD₆₀₀ of 0.07~0.09 with the same pre-warmed media. Five mL 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. CH₄ and air was added to the vials in a 1:2 ratio using the custom-designed gassing apparatus as previously described (Lee, 2008). Briefly, a pressure equivalent to 15 psi was removed via vacuum extraction and refilled with CH₄ using a series of three way valves to control the flow of CH₄. A schematic diagram of the gassing system is shown in Figure 2-3. The

vials were incubated at 30 °C with shaking at 250 rpm. All conditions were performed in duplicate. Growth and N₂O production was monitored until the stationary phase was reached. Cell growth was monitored by measuring OD₆₀₀, while N₂O production was measured using GC-ECD as described earlier (Lee et al., 2009).

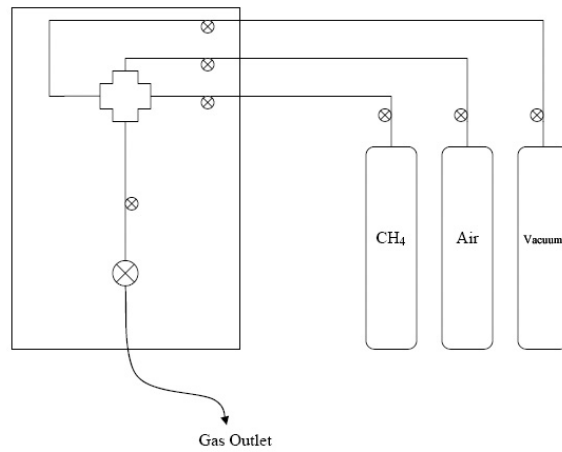


Figure 2-3. Schematic diagram of the gassing system to introduce desired concentration of CH₄ (Lee, 2008).

CHAPTER 3. Landfill biochemistry in response to different amendments

3.1 Introduction

Different geochemical parameters may have different effects on CH₄ oxidation and N₂O production. In previous laboratory microcosms study using landfill cover soils, it was shown that relatively dry soils (5% moisture content) along with 15 mg NH₄⁺ (kg soil)⁻¹ and 0.1 mg phenylacetylene (kg soil)⁻¹ substantially enhanced CH₄ oxidation (by as much as 50%) while minimizing N₂O production, as compared to controls with no amendments (Lee et al., 2009). 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 NH₄Cl + 0.25 M of KNO₃ and; (3) addition of 0.5 M of NH₄Cl + 0.25 M of KNO₃ + 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 (NH₄⁺-N and NO₃⁻-N+NO₂⁻-N), bioavailable and total copper concentration. Since the amendments included inorganic nitrogen sources, the level of NH₄⁺ and NO₃⁻-N+NO₂⁻-N were important parameters to measure to investigate the long-term effects of amendments on CH₄ oxidation and N₂O production as well as on microbial activity. Landfill gases, specifically, CH₄, N₂O and O₂, were periodically monitored during the

field scale trials. Statistical analyses were performed to determine statistically significant differences in soil physicochemical properties among the plots.

3.2 Results and discussion

3.2.1 Vertical gradients of pH, inorganic nitrogen and metals

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 3-1). Inorganic nitrogen, such as ammonium and nitrate/nitrite associated with soils from each core was measured (Figure 3-2). To determine if there was any significant difference among plots, the statistical analysis was performed by using the mixed models procedure (PROC MIXED) of SAS ver 9.2 (SAS Institute, NC) (Figure 3-3). Since the soil layer close to the surface has a higher potential to exchange with the atmosphere, data from 20~40 cm below the ground surface 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 that in plot 5 even though plots 6 and 7 were amended with NH_4^+ and NO_3^- , suggesting that the amendment of NH_4^+ and

NO_3^- enhanced biological activity in the absence of the canopy. There was no significant difference in NH_4^+ concentration by the addition of phenylacetylene. Unlike NH_4^+ content, analysis of $\text{NO}_3^- + \text{NO}_2^-$ concentration showed significantly lower concentrations of $\text{NO}_3^- + \text{NO}_2^-$ in plot 2 and plot 7 than in the other plots. Bioavailable and total copper and iron were also measured but no significant differences among the plots were observed (Figures 3-4, 3-5).

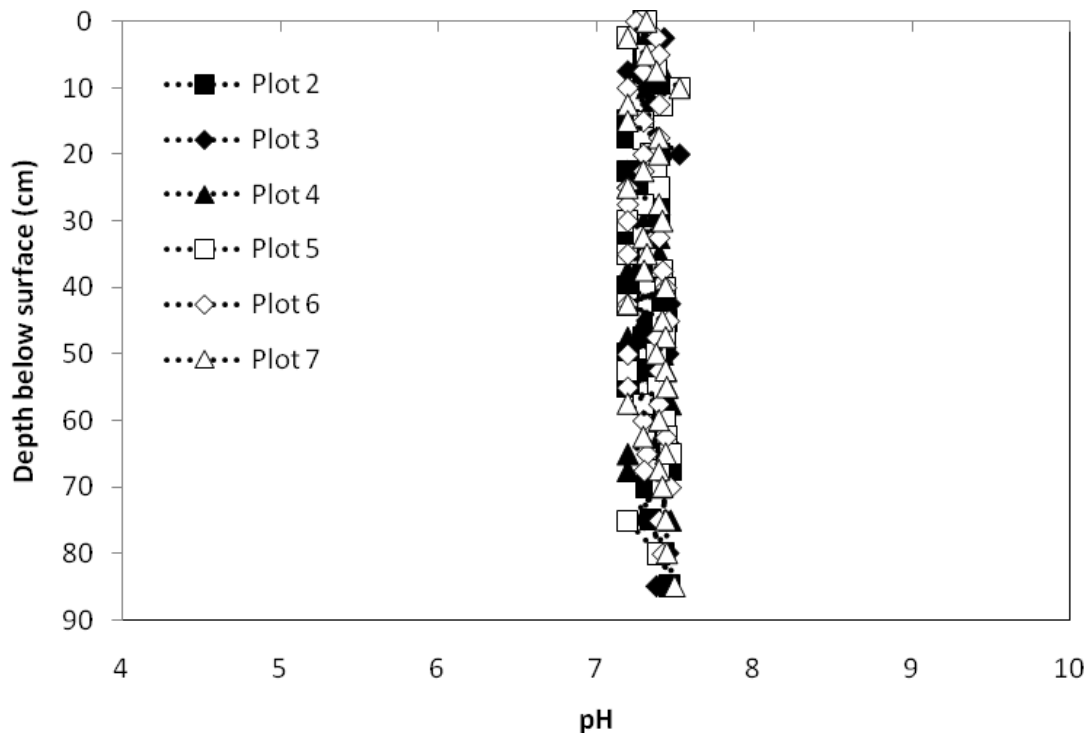


Figure 3-1. Depth profiles of soil pH of each plot. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3 , plot 4 - canopy, NH_4Cl , KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3 , and plot 7 – NH_4Cl , KNO_3 and phenylacetylene.

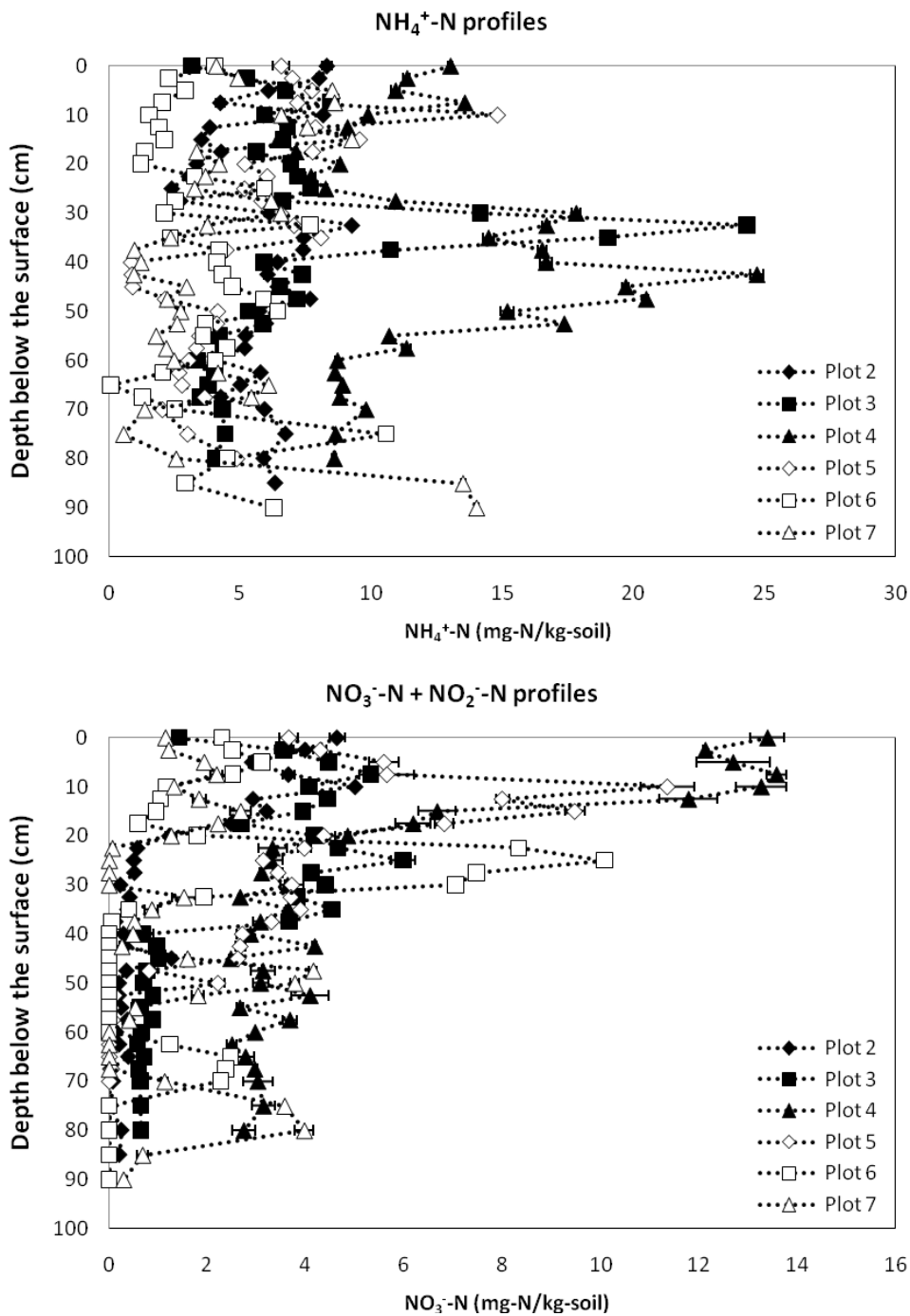


Figure 3-2. Depth profiles of NH₄⁺ (A) and NO₃⁻+NO₂⁻ (B) concentration of each plot. Plot 2 – canopy, plot 3 - canopy, NH₄Cl and KNO₃, plot 4 - canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene. Error bars indicate the range of duplicate measurements.

	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 3-3. Significance of difference in NH₄⁺ and NO₃⁻+NO₂⁻ concentration among plots. Numbers in the box indicate plot with higher concentration. Plot 2 – canopy, plot 3 - canopy, NH₄Cl and KNO₃, plot 4 - canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene. **p* < 0.01; †*p* < 0.05; ‡*p* < 0.10; NS: not significant (*p* > 0.10).

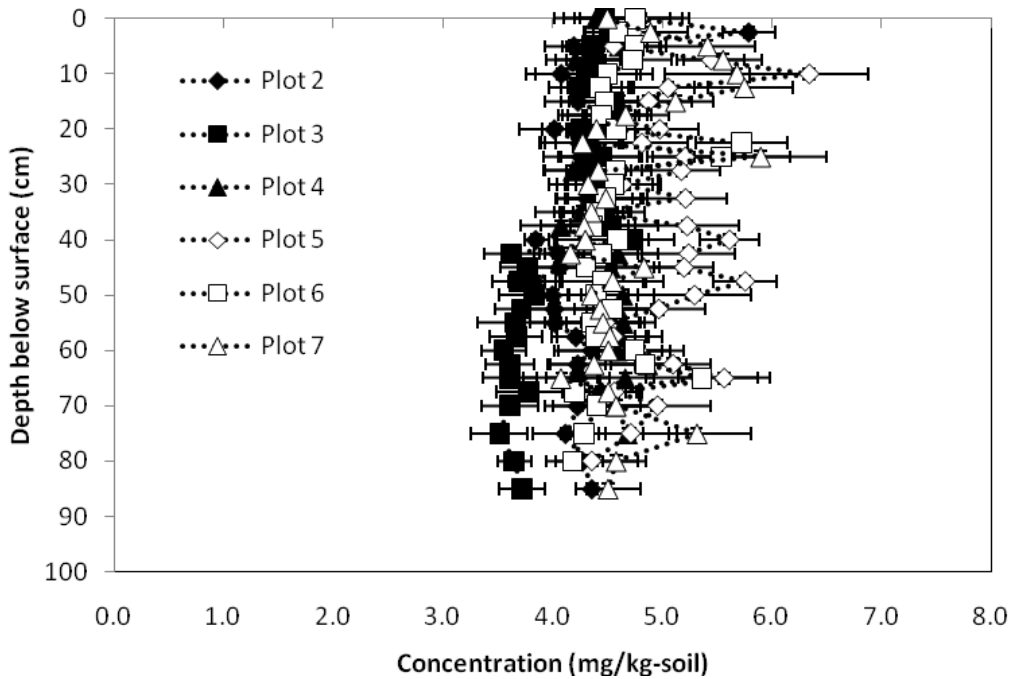
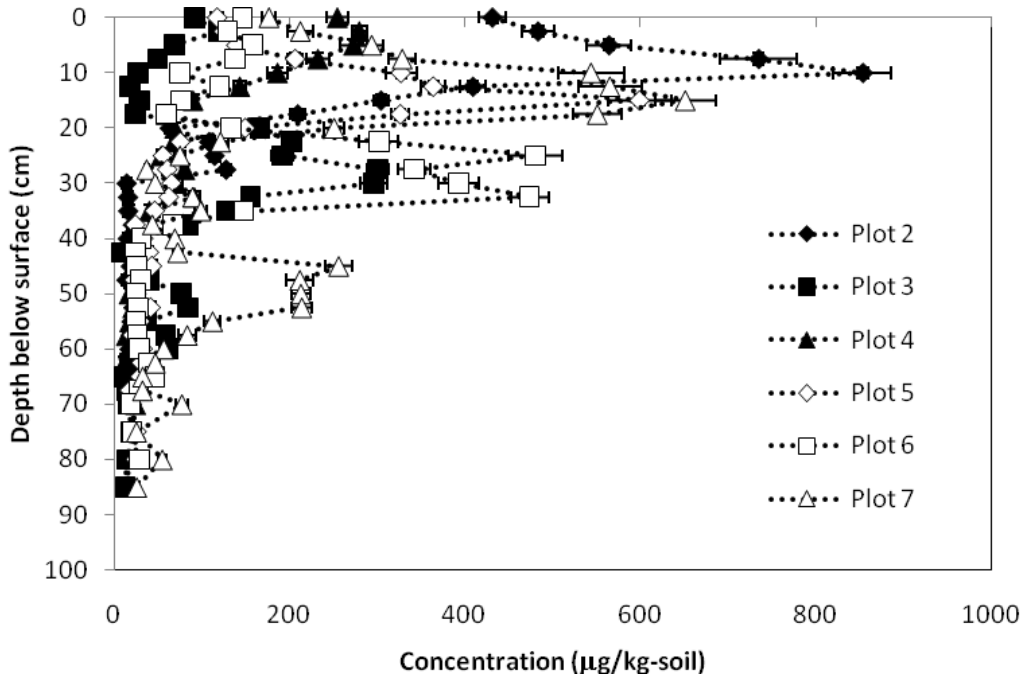


Figure 3-4. Vertical profiles of bioavailable copper (A) and iron (B). Plot 2 – canopy, plot 3 – canopy, NH_4Cl and KNO_3 , plot 4 – canopy, NH_4Cl , KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3 , and plot 7 – NH_4Cl , KNO_3 and phenylacetylene. Error bars indicate the range of duplicate measurements.

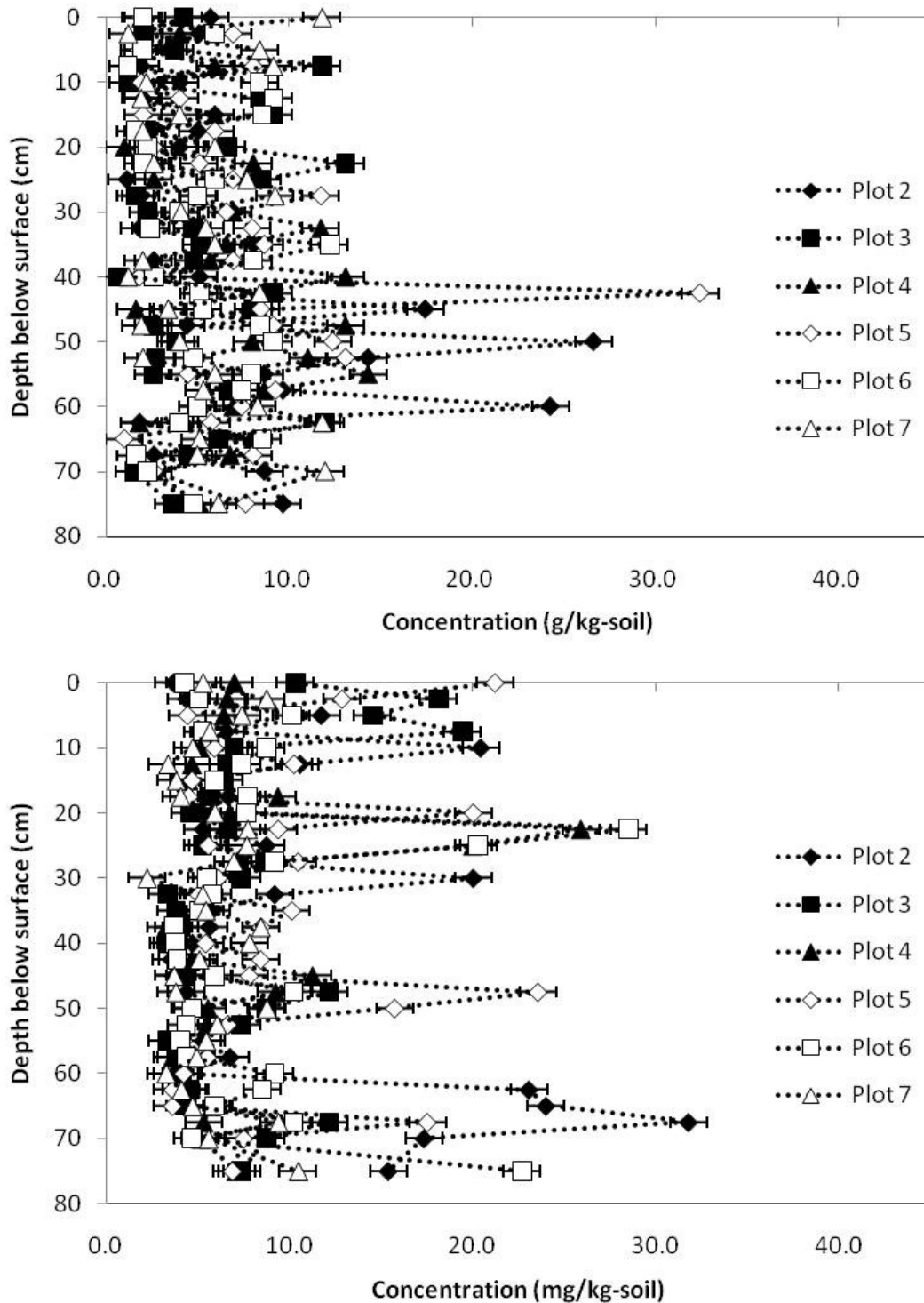


Figure 3-5. Vertical profiles of total copper (A) and iron (B). Plot 2 – canopy, plot 3 - canopy, NH₄Cl and KNO₃, plot 4 - canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene. Error bars indicate the range of duplicate measurements.

Since there are no significant difference in soil pH and total/bioavailable metal content among the plots, it appears that any shifts in microbial activity and/or community structure that occurred (see Chapter 4) are possibly due to differences in nitrogen and/or phenylacetylene.

3.2.2 Monitoring of vertical landfill soil gas profiles

Soil gas samples were collected from a depth of 20 cm to a depth of 100 cm with an interval of 10 cm. N₂O profiles in each plot from November 2007 to July 2009 are shown in Figure 3-6. The profiles showed a significant spatial and seasonal variations with values varying from 0 to 92 ppmv. There were distinct seasonal emission patterns, with higher production during summer and fall and little N₂O detected during the winter and spring. CH₄ also showed strong seasonal pattern, i.e., higher methanotrophic activity in warmer temperatures, as evidenced by higher CH₄ concentration during winter and spring than during summer and fall (Figure 3-7). The seasonal patterns of both N₂O production and CH₄ oxidation suggest that both gases are regulated by microbial activities which are strongly dependent on temperature (Figure 3-8). O₂ profiles showed some variation (Figure 3-9), but there were no specific trends throughout the field test.

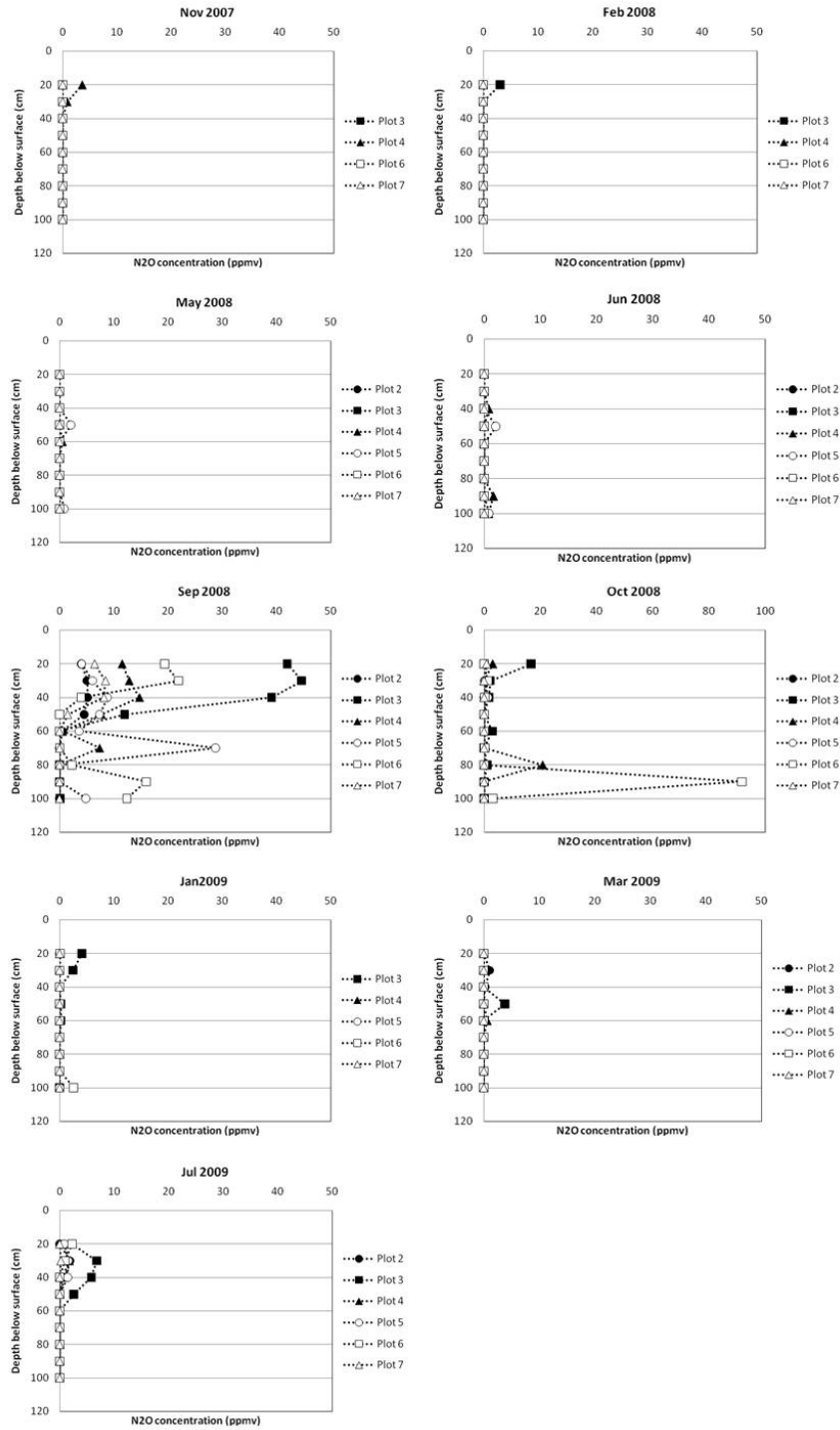


Figure 3-6. Nitrous oxide concentration from each plot from November 2007 to July 2009. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3 , plot 4 - canopy, NH_4Cl , KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3 , and plot 7 – NH_4Cl , KNO_3 and phenylacetylene.

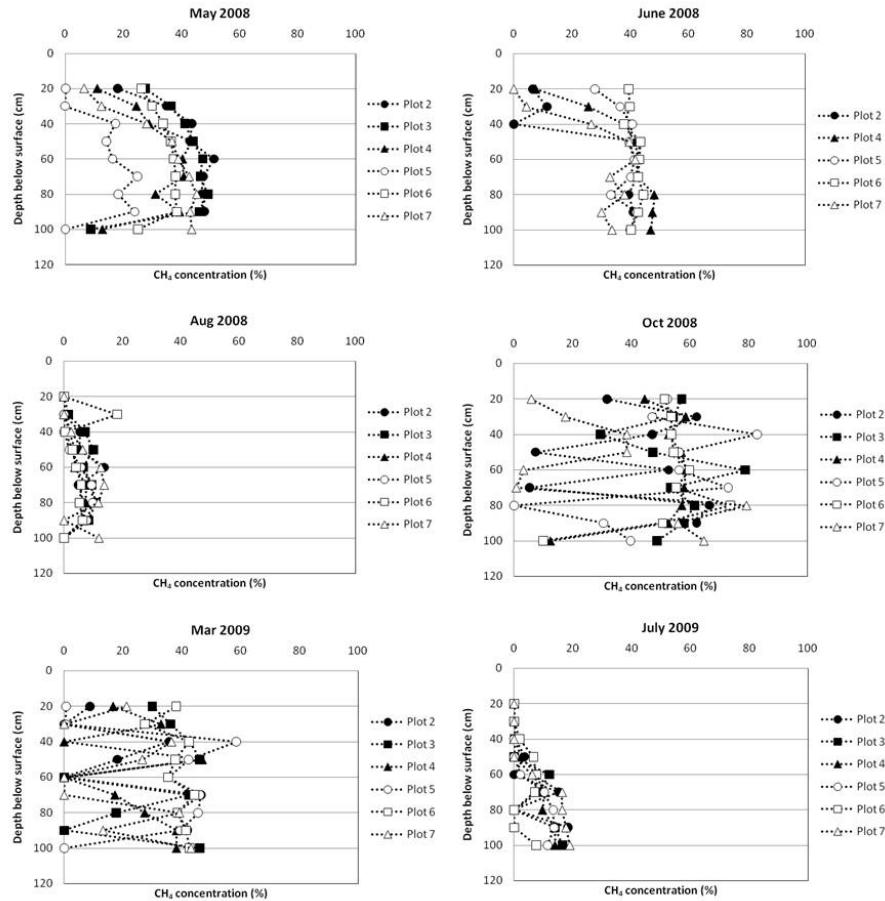


Figure 3-7. Depth profiles of methane concentration of each plot from May 2008 to July 2009. Plot 2 – canopy, plot 3 - canopy, NH_4Cl and KNO_3 , plot 4 - canopy, NH_4Cl , KNO_3 and phenylacetylene, plot 5 – no amendment, plot 6 – NH_4Cl and KNO_3 , and plot 7 – NH_4Cl , KNO_3 and phenylacetylene.

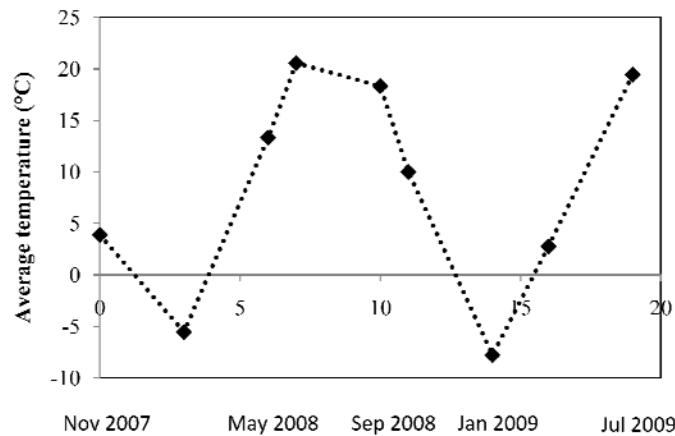


Figure 3-8. Average monthly temperature in Kalamazoo, MI at the time of each amendment addition (Monthly weather summary. National Weather Service. 02 June 2010 <<http://www.nws.noaa.gov/>>).

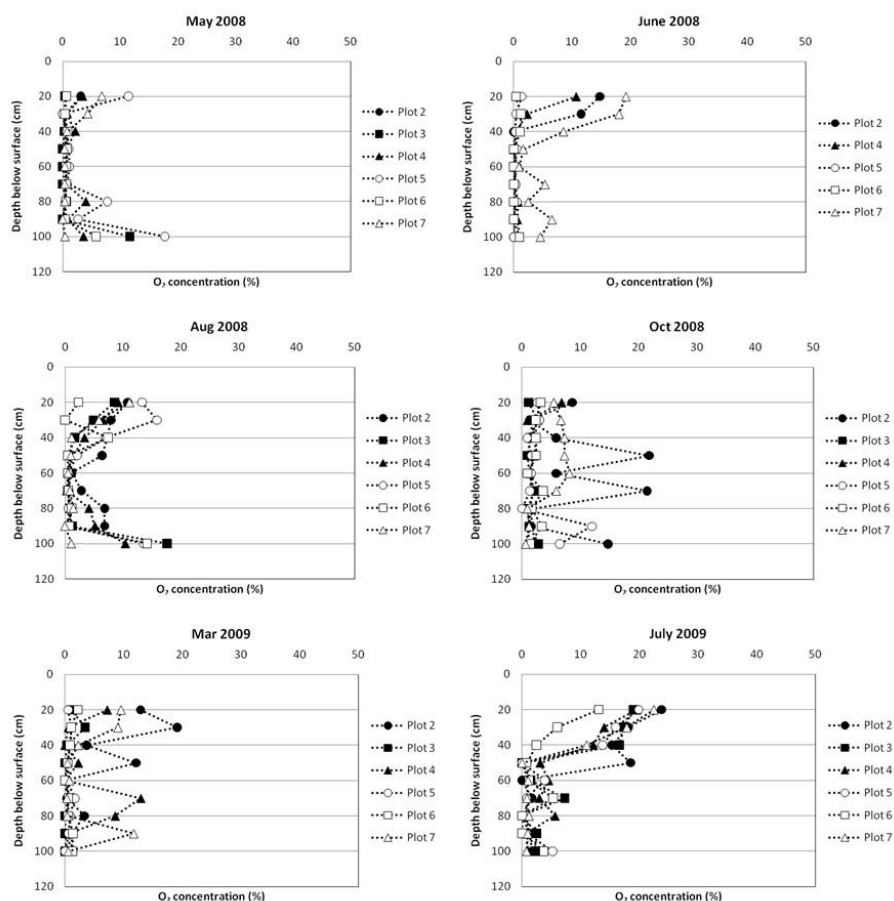


Figure 3-9. Depth profiles of oxygen concentration of each plot from May 2008 to July 2009. Plot 2 – canopy, plot 3 - canopy, NH₄Cl and KNO₃, plot 4 - canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene.

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 N₂O and CH₄ concentrations among the plots to examine if there is any significant effect of the different amendments (Figure 3-10). N₂O concentrations from plots 3 and 6 were higher than those from plots 2 and 5, respectively, suggesting that nitrogen amendments

enhanced N₂O production. N₂O concentration from plots 3 and 6 were also higher than those from plots 4 and 7, respectively, suggesting that phenylacetylene repressed N₂O production. A comparison between plots 3 and 6 suggests that a canopy enhanced N₂O production. Plot 7, which was amended with nitrogen and phenylacetylene, showed significantly lower CH₄ 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 a higher CH₄ concentration than plot 7, suggesting that there might be an inhibitory effect of a canopy on CH₄ oxidation. There was no significant difference in O₂ concentration, indicating that O₂ was dominated by diffusion rather than microbial consumption.

One should be aware that the data collection strategy has certain limitations. One is to do with the representativeness of the sample. The study did not include any replicate plots, and only one core sample was analyzed for each of the six treatments. The

	2	3	4	5	6	7
2		3*	NS	NS	6*	NS
3			3*	3*	3*	3*
4				NS	NS	NS
5					6‡	NS
6						6‡
7	N ₂ O concentration					

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

	2	3	4	5	6	7
2		NS	NS	NS	NS	NS
3			NS	NS	NS	NS
4				NS	NS	NS
5					NS	NS
6						NS
7	O ₂ concentration					

Figure 3-10. 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, NH₄Cl and KNO₃, plot 4 - canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene. **p* < 0.01; †*p* < 0.05; ‡*p* < 0.10; NS: not significant (*p* > 0.10).

collection of a number of replicates is necessary to strengthen any conclusions derived from these data.

Also, the gas measurements showed a high variability of soil gas composition in the different depths within a short range (Figures 3-6 and 3-7). The variability may be due to experimental issues, including: (1) difficulty extracting soil gas from the sampling port due to the limited void space, (2) water being drawn from the sampling point, and (3) a leak in the sampling or purging system (Hinchee and Leeson, 1996). Even though extra care was taken to avoid such problems, such a high variability of soil gas composition suggests that they may have occurred and caused some unexpected variations in gas composition.

However, it should be noted that vertical gas samples were collected for almost two years, and biogeochemical analyses were examined within each core at a depth interval of 2.5 cm, and each core interval was homogenized before performing any chemical and/or molecular biological analyses. For example, we extracted DNA and mRNA from 0.5 g of soil, but the sample was obtained from 50 g of homogenized soil sample. As the statistical analyses showed, there were significant differences among plots ($p \leq 0.01 \sim 0.1$). Also, in field tests, it is not uncommon for a single soil core to be examined for its physicochemical and/or microbial community structures, e.g., Zhou et al. (1997), Henckel et al. (2000, 2001), and Ayres et al. (2009).

With respect to the experimental set-up, a canopy was installed in one half of the plots in an attempt to control soil moisture content whereas it was not installed in the other half. Then, one remaining question is whether the presence of the canopy played a significant role in interacting with the effects of the amendments. For example, the

effects of amendments on NH_4^+ concentration seem to differ substantially depending on whether the canopy was installed or not (Figure 3-4). However, for some other cases, the effects of the canopy seem negligible (e.g., see N_2O or CH_4 concentrations in Figure 3-10). One obvious limitation of the present research is that we did not measure the potential outcomes of the presence of the canopy. Specifically, moisture contents in each plot were not monitored to determine how the canopy affected soil moisture content. Furthermore, the presence of a canopy may have altered the air flow pattern or the amount of sunlight, inducing a temperature change. In future research, one should investigate the potential role of the presence of the canopy further.

3.3 Conclusions

In this chapter, several geochemical parameters and gas concentrations, i.e., pH, inorganic nitrogen, bioavailable/total metal, CH_4 , N_2O , and O_2 concentrations were investigated. Based on the results of inorganic NH_4^+ concentration profile, it appears that (1) the biological activities related to NH_4^+ uptake or oxidation may be inhibited by modified water content created by the presence of a canopy; (2) the amendment of NH_4^+ and NO_3^- enhanced biological activity in the absence of a canopy; and (3) there seemed to be no significant difference in NH_4^+ concentration induced by the addition of phenylacetylene. pH, bioavailable and total copper were also measured but there appeared to be no significant difference among the plots. On the other hand, the analysis of N_2O profiles shows that nitrogen amendments stimulated N_2O production but phenylacetylene amendments repressed the production. The CH_4 concentration analysis suggests that phenylacetylene might help methanotrophs to outcompete other microbial groups for limiting nutrients, while there might be an inhibitory effect of a canopy on

CH₄ oxidation. There was no significant difference in O₂ concentration, suggesting that O₂ was dominated by diffusion rather than microbial consumption.

CHAPTER 4. Effects of amendments on microbial community structure and activity

4.1 Introduction

CH₄ is a primary end-product of anaerobic decomposition, so that it is more likely to find large amounts of CH₄ in deeper parts of the landfill. Since atmospheric O₂ diffuses down into the soil, landfill top covers are complex systems with counter gradient gas profiles of CH₄ and O₂, i.e., low CH₄ to O₂ ratios near the soil surface and higher ratios in deeper regions. Although the two common types of methanotrophs, Type I and Type II, have been found to coexist in many environments, they are known to be sensitive to variations in CH₄ and O₂ concentrations (Bender and Conrad, 1995; Henckel et al., 2000), and it has been suggested that the amount of available CH₄ may influence the distribution of Type I and Type II methanotrophs. Type I methanotrophs appear to outcompete Type II species under low CH₄ and high O₂ conditions, whereas Type II species tend to dominate under the opposite conditions (Graham et al., 1993; Amaral and Knowles, 1995). In nitrogen and/or nutrient-rich environment, Type I methanotrophs are thought to prevail (Graham et al., 1993; Bodelier et al., 2000; Mohanty et al., 2006) and might outcompete Type II methanotrophs, while the inverse has been observed in the nitrogen- and/or nutrient-limiting conditions. The availability of nitrogen affects CH₄ and N₂O fluxes by directly affecting microbial communities and biochemical processes including nitrification and denitrification as well as methanotrophy.

Attempts to stimulate *in situ* CH₄ oxidation in landfill cover soils typically consider amendments to enhance activity of methanotrophs. Amendments commonly include manipulations of soil moisture and/or nitrogen levels (Kightley et al., 1995; Borjesson and Svensson 1997a; Reinhart et al., 2002; Barlaz et al., 2004). Addition of nitrogen is particularly important in landfill cover soils as the C/N ratio is typically high and nitrogen limitation can thus be significant (De Visscher et al., 1999). To circumvent this problem, nitrogen amendments in the form of sewage sludge (Kightley et al., 1995; Borjesson and Svensson, 1997a), leachate (Reinhart et al., 2002; Berge et al., 2005), compost (Barlaz et al., 2004; Halet et al., 2006) and liquid NH₄⁺/NO₃⁻ (Kightley et al., 1995) have been proposed, but such additions can increase N₂O emissions, which is also a potent greenhouse gas (Majumdar, 2003) having a global warming potential ~298 times greater than CO₂ over a 100 year time frame (IPCC, 2007). Any amendment strategy, therefore, should not only attempt to increase methanotrophic activity, but also inhibit the biogenic production of N₂O, particularly by ammonia-oxidizing bacteria (AOB) and/or ammonia-oxidizing archaea (AOA).

In this study, the long-term effects of six amendments identified from the previous study, (1) control, i.e., no amendments; (2) addition of 0.5 M of NH₄Cl + 0.25 M of KNO₃ and; (3) addition of 0.5 M of NH₄Cl + 0.25 M of KNO₃ + 0.01% (w/v) phenylacetylene, each in the presence/absence of a canopy, on the microbial community structure and activity were investigated. NH₄⁺ and NO₃⁻, provided to stimulate methanotrophic activity *in situ*, did indeed enhance CH₄ oxidation, but the nitrogen sources also stimulated microbial N₂O production. However, the addition of phenylacetylene along with nitrogen amendment successfully inhibited N₂O production

while maintaining CH₄ oxidation capacity (Figure 3-10). This raises the questions: (1) which methanotrophic groups are more responsible for the increased CH₄ oxidation? (2) which microbial groups are more responsible for the N₂O production in the landfill soil? To address these questions, several molecular biological analyses, including PCR, RT-PCR, microarray, and clone library analyses, were performed on DNA and mRNA extracted from each soil core taken from the King Highway landfill. A single core was taken from each plot.

4.2 Results and discussions

4.2.1 PCR analyses

DNA and mRNA were extracted from soil samples, 25~27.5 cm of depth, selected based on the landfill gas data and the soil physico-chemical properties to investigate the long-term effects of geochemical parameters on microbial community and their activities. This soil layer was chosen as soils at this depth have a higher potential to exchange gases with the atmosphere, i.e., O₂ and thus be a more favorable habitat for methanotrophs and ammonia-oxidizing microorganisms. Also, the layer was consistent in soil texture since there was sometimes debris such as rocks or concrete observed from the other soil layers. Functional genes that are unique or recognized as key components of CH₄ consumption or N₂O 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 functional markers for studying methanotrophs and AOB/AOA in the environment (Dumont and

Merrell, 2005; 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 different species in environmental samples is currently unknown and can be sample type-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 4-1 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.

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 4-2). Methane oxidizing bacteria, especially those possessing particulate methane monooxygenase, were abundant in every plot as evidenced by amplification of *pmoA*, while those possessing soluble methane monooxygenase were detected more in plots without a canopy as evidenced by amplification of *mmoX*. Interestingly, archaeal ammonia oxidizers were significantly more abundant than their bacterial counterparts in

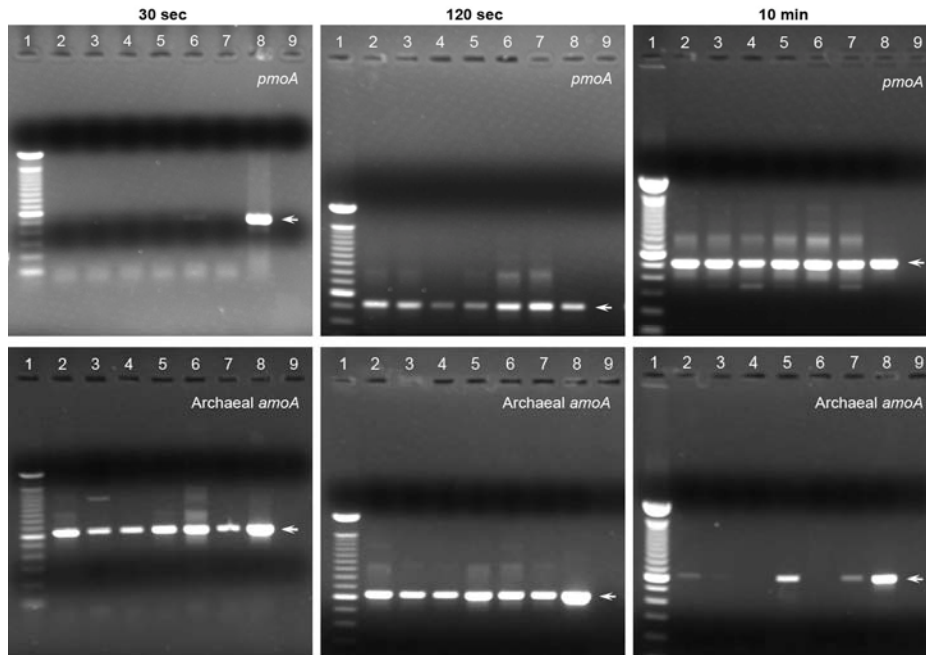


Figure 4-1. PCR amplification of DNA extracted from 25~27.5 cm depth of each soil core using three different bead beating times. Two different primer sets targeting *pmoA* and archaeal *amoA* genes were used. Lane 1: 100 bp DNA ladder; lane 2-7: plot 2-7, respectively; lane 8: positive controls using 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.

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.

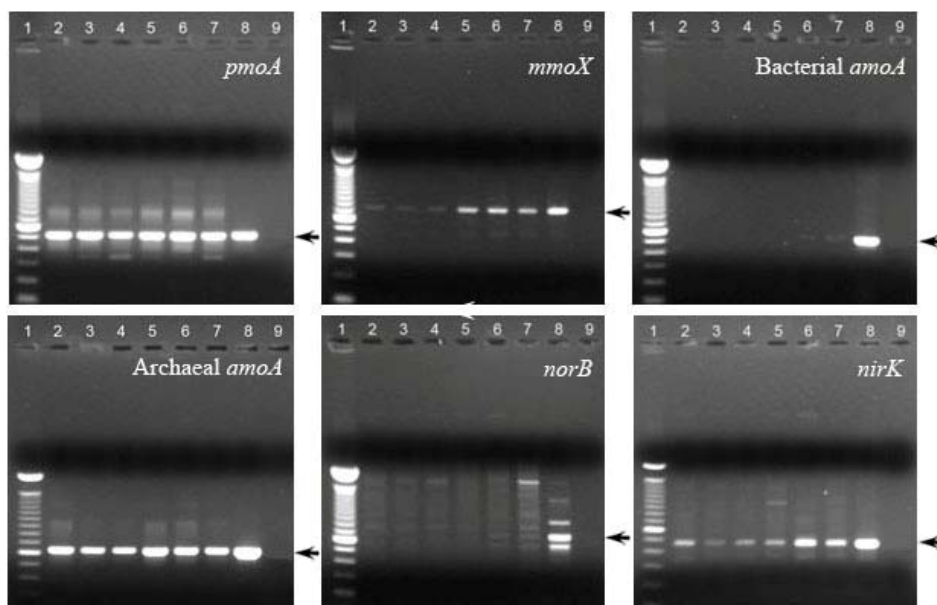


Figure 4-2. PCR amplification of DNA extracted from 25~27.5 cm depth of each soil core targeting specific functional genes. Lane 1: 100 bp DNA ladder; lane 2-7: plot 2-7, respectively; lane 8: positive controls using either chromosomal DNA extracted from *Methylosinus trichosporium* OB3b for *pmoA* and *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 4-3). 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.

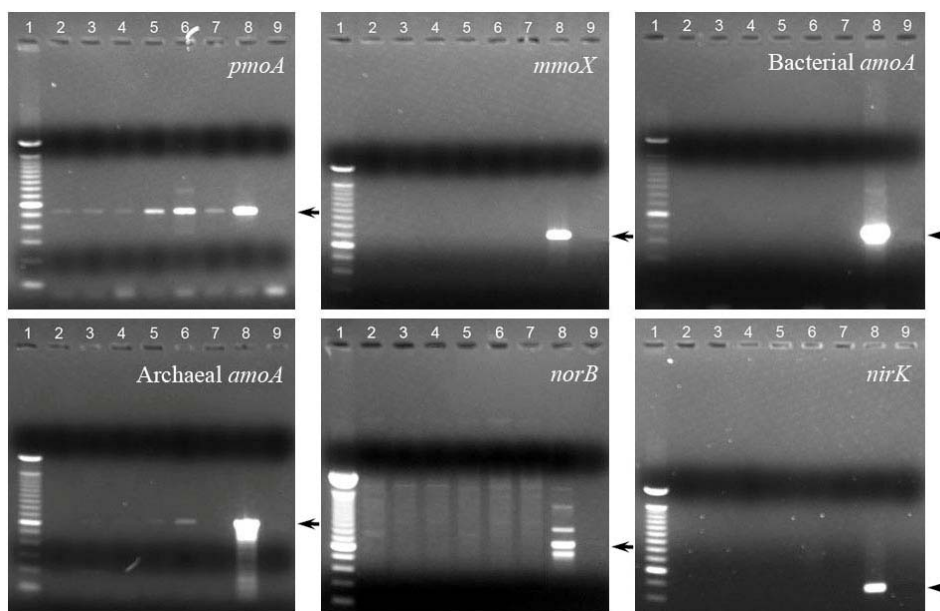


Figure 4-3. PCR amplification of cDNA reverse transcribed from mRNA extracted from 25~27.5 cm depth of each soil core targeting specific functional genes. Lane 1: 100 bp DNA ladder; lane 2-7: plot 2-7, respectively; lane 8: positive controls using either chromosomal DNA extracted from *Methylosinus trichosporium* 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 counterparts, as shown in the PCR analysis on DNA extracted from each soil core (Figure 4-2). The level of transcripts of *amoA* from ammonia oxidizing archaea, however, was much lower than that 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.

However, 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. Also, the primer annealing efficiency might induce a bias in PCR amplification as well.

4.2.2 Effects of amendments on methanotrophic community composition: microarray analyses

pmoA gene PCR amplicons from each of the samples were hybridized against a microarray containing 175 probes encompassing the known breadth of methanotrophic diversity (Table 4-1). As shown in Figure 4-4, 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 amendment was performed. 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 in general, but relatively less than other genera.

Comparisons of plots 2 and 5 with plots 3 and 6, respectively, with the addition of NH₄Cl and KNO₃ regardless of the presence or absence of a canopy, show a decrease in probe intensity compared to that observed in each control plot (plot 2 and plot 5) found for most probes except those targeting *Methylocaldum* and *Methylocystis sp.* Comparing plot 6 with 7, with the addition of phenylacetylene along with NH₄Cl and KNO₃ 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 NH₄Cl and KNO₃ 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 NH₄Cl and KNO₃ 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 plots 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 4-2 shows the indices describing community diversity, richness, evenness and dominance in response to various amendments. Interestingly, comparing plots 2 and

5 with 3 and 6, respectively, the addition of NH_4Cl and KNO_3 reduced methanotrophic diversity index as compared to each control plot both in the presence and absence of a canopy, but had only a small effect on richness index and evenness index. The methanotrophic community also exhibited more dominance with the addition of NH_4Cl and KNO_3 , likely due to the reduced presence of Type I methanotrophs based on the comparison of plots 2 and 5 with plots 3 and 6, respectively. Interestingly, comparing plots 2 and 5 with plots 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 plots 3 and 4 with plots 6 and 7.

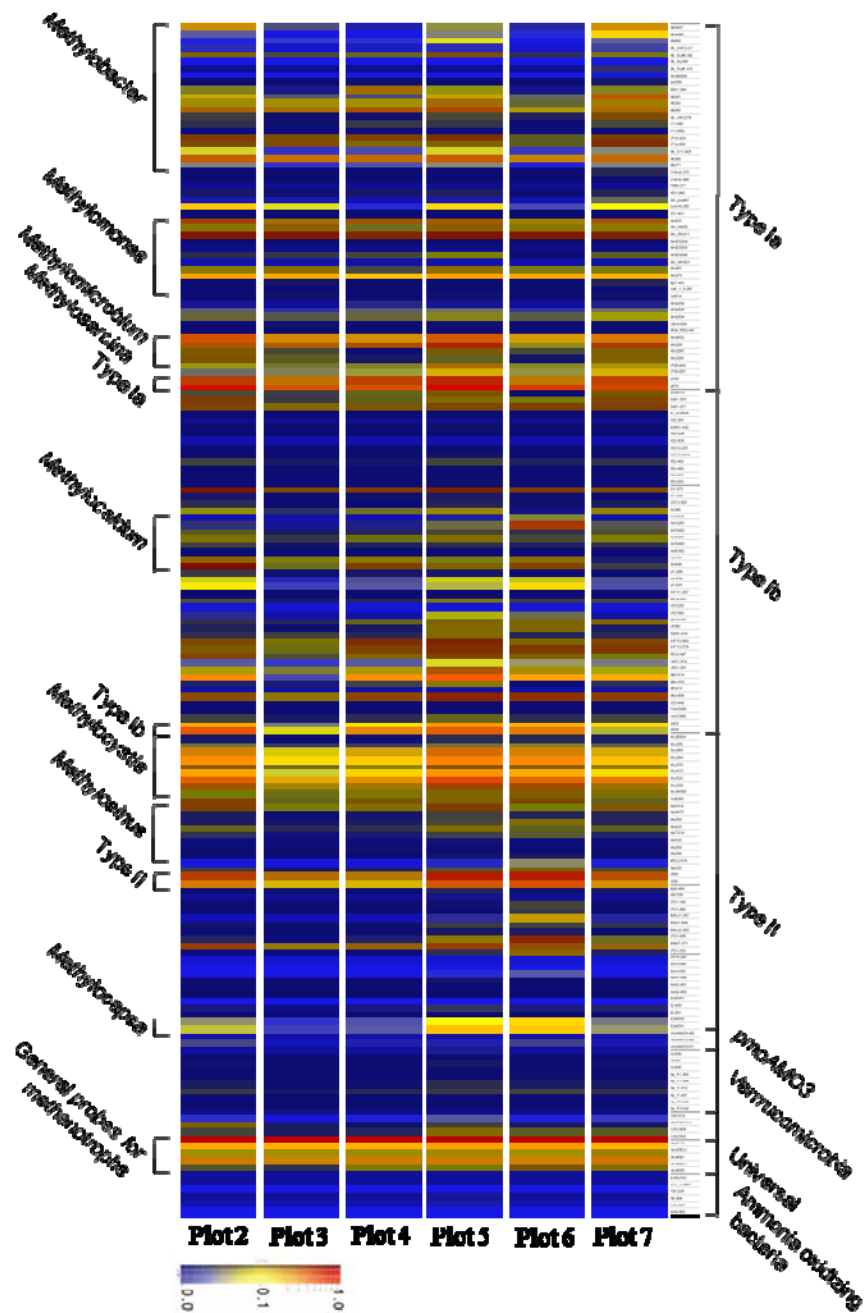


Figure 4-4. Summary of results for the *pmoA*-based methanotrophic community analysis using microarrays. Color coding bar on the right side represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates that 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). Plot 2 – canopy, plot 3 – canopy, NH₄Cl and KNO₃, plot 4 – canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene.

Table 4-1. Probes used for microarray analysis. Column numbers correspond to the order in which the probes are arranged for the microarray analysis as shown in Figure 5-4.

Column number	Probe name	Column number	Probe name	Column number	Probe name	Column number	Probe name
1	MbA557	46	Mmb562	91	JHTY2-578	136	NMsiT-271
2	MbA486	47	Mm229	92	JRC2-447	137	LP21-232
3	Mb460	48	MsQ290	93	LW21-374	138	RA14-299
4	Mb_LW12-211	49	MsQ295	94	LW21-391	139	RA14-594
5	Mb_SL#3-300	50	LP20-644	95	M90-574	140	RA14-591
6	Mb_SL299	51	LP20-607	96	M90-253	141	Wsh1-566
7	Mb_SL#1-418	52	Ia193	97	Mth413	142	Wsh2-491
8	MmbB284	53	Ia575	98	Mha-500	143	Wsh2-450
9	Jpn284	54	Bsed516	99	DS3-446	144	B2rel251
10	BB51-302	55	SWI1-375	100	PmoC640	145	B2-400
11	Mb267	56	SWI1-377	101	PmoC308	146	B2-261
12	Mb292	57	Ne_oce426	102	Ib453	147	B2all343
13	Mb282	58	DS2-287	103	Ib559	148	B2all341
14	Mb_URC278	59	AIMS1-442	104	McyB304	149	pmoAMO3-400
15	511-436	60	DS2-220	105	Mcy255	150	pmoAMO3-486
16	<i>511-436L</i>	61	DS2-626	106	Mcy459	151	pmoAMO3-511
17	LP10-424	62	USCG-225	107	Mcy264	152	Ver330
18	LF1a-456	63	USCG-225b	108	Mcy270	153	Ver307
19	Mb_C11-403	64	JR2-409	109	Mcy413	154	Ver285
20	Mb380	65	JR2-468	110	Mcy522	155	Ma_F1-355
21	Mb271	66	JR3-505	111	Mcy233	156	Ma_F1-594
22	S14m2-270	67	JR3-593	112	McyM309	157	Ma_I1-312
23	S14m2-406	68	501-375	113	Peat264	158	Ma_I1-401
24	PS80-291	69	501-286	114	MsS314	159	Ma_F3-638
25	MS1-440	70	USC3-305	115	MsS475	160	Ma_F3-542
26	Mm_pel467	71	Mc396	116	Msi263	161	ESR-579
27	Kuro18-205	72	MelT272	117	Msi423	162	M84P22-514
28	DS1-401	73	MelG281	118	MsT214	163	TUSC409
29	Mm531	74	MelS402	119	MsT343	164	TUSC502
30	Mm_M430	75	MelS394	120	MM_MsT343	165	mtrof173
31	Mm_RS311	76	MelS400	121	Msi520	166	mtrof362-I
32	Mm_ES294	77	MelE302	122	Msi269	167	mtrof661
33	Mm_ES543	78	Mel404	123	Msi294	168	mtrof662-I
34	Mm_ES546	79	Mel408	124	ARC2-518	169	mtrof656
35	Mm_MV421	80	fw1-286	125	Msi232	170	NmNc533
36	Mm451	81	fw1-639	126	II509	171	Nsm_eut381
37	Mm275	82	fw1-641	127	II630	172	PS5-226
38	Alp7-441	83	JHTY1-267	128	Alp8-468	173	PI6-306
39	peat_1_3-287	84	JRC4-432	129	xb6-539	174	NsNv207
40	Est514	85	OSC220	130	LP21-190	175	NsNv363
41	Mmb259	86	OSC300	131	LP21-260		
42	Mmb303	87	JRC3-535	132	NMcy1-247		
43	Mmb304	88	LK580	133	NMsi1-469		
44	<i>LW14-639</i>	89	RSM1-419	134	NMcy2-262		
45	Mmb_RS2-443	90	JHTY2-562	135	LP21-436		

Table 4-2. Diversity and richness estimates for methanotrophic community analysis. The diversity indices were calculated using probe intensities of microarray. Plot 2-canopy, plot 3-canopy, NH₄Cl and KNO₃, plot 4-canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5-no amendment, plot 6-NH₄Cl and KNO₃, and plot 7-NH₄Cl, KNO₃ 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

4.2.3 Effects of amendments on AOA community composition

The structure of the AOA communities in each soil core was 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 4-3). 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 on plot 7 which was amended with phenylacetylene, NH₄Cl and KNO₃ (Figure 4-5).

Table 4-3 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 Group 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 (Campanella et al., 2003). Of the 13 OTUs from plot 5, where no amendment was added, 5 OTUs fell into Group 1.1a lineage and 8 OTUs fell into Group 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 by plots 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 plots 2 and 5 were more diverse ($H'=1.0\sim 2.0$) than in other plots (plots 3, 4, 6, and 7) receiving nitrogen or nitrogen along with phenylacetylene ($H'=0.5$ or 0). The number of OTUs of Group 1.1b increased when NH_4^+ and NO_3^- were added from 3 to 10 in the presence of a canopy, and from 8 to 22 in the absence of a canopy. Meanwhile, a comparison between plot 2 and plot 3 shows that the number of OTUs of Group 1.1a decreased from 9 to 2 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. A comparison between plot 3 and plot 4, in the presence of a canopy, shows that the number of OTUs of Group 1.1a decreased to 1, and the number of OTUs of Group 1.1b did not change. The Shannon's biodiversity index (H') of Group 1.1a decreased from 0.4 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 22 to 12, 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 2.7 to 2.1, 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).

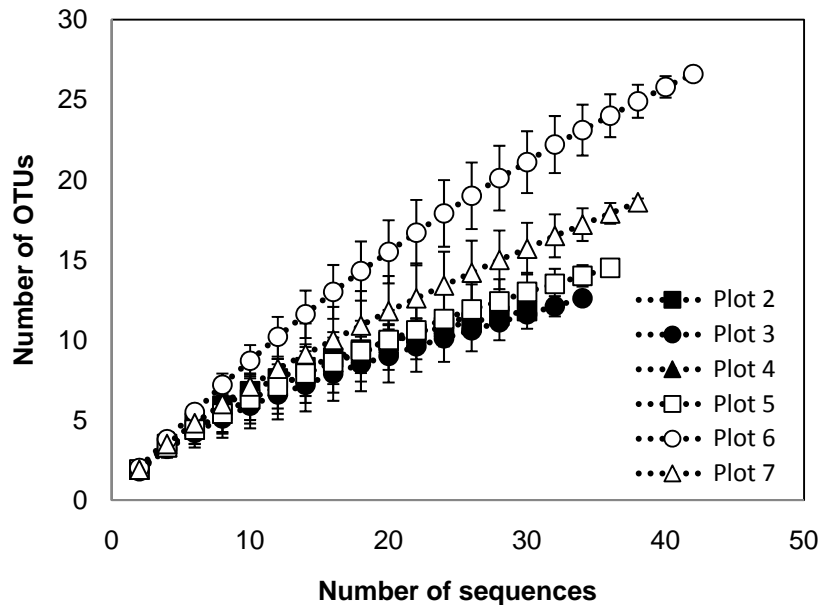


Figure 4-5. Rarefaction analyses of AOA *amoA* genes. OTUs were defined as those with $\geq 97\%$ nucleotide sequence identity. Error bars represent 95% confidence limits. Plot 2 – canopy, plot 3 – canopy, NH_4Cl and KNO_3 , plot 4 – canopy, NH_4Cl , KNO_3 and phenylacetylene, plot 5 – no amendment,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

Table 4-3. 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, NH₄Cl and KNO₃, plot 4 – canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene.

Plot	# of clones	Observed OTUs ^a			Shannon index (<i>H'</i>)		Simpson index of diversity (1/D)		Menhinick index of richness (<i>D_{Mn}</i>)		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

^abased on the similarity of 97% between sequences; N/A: not available.

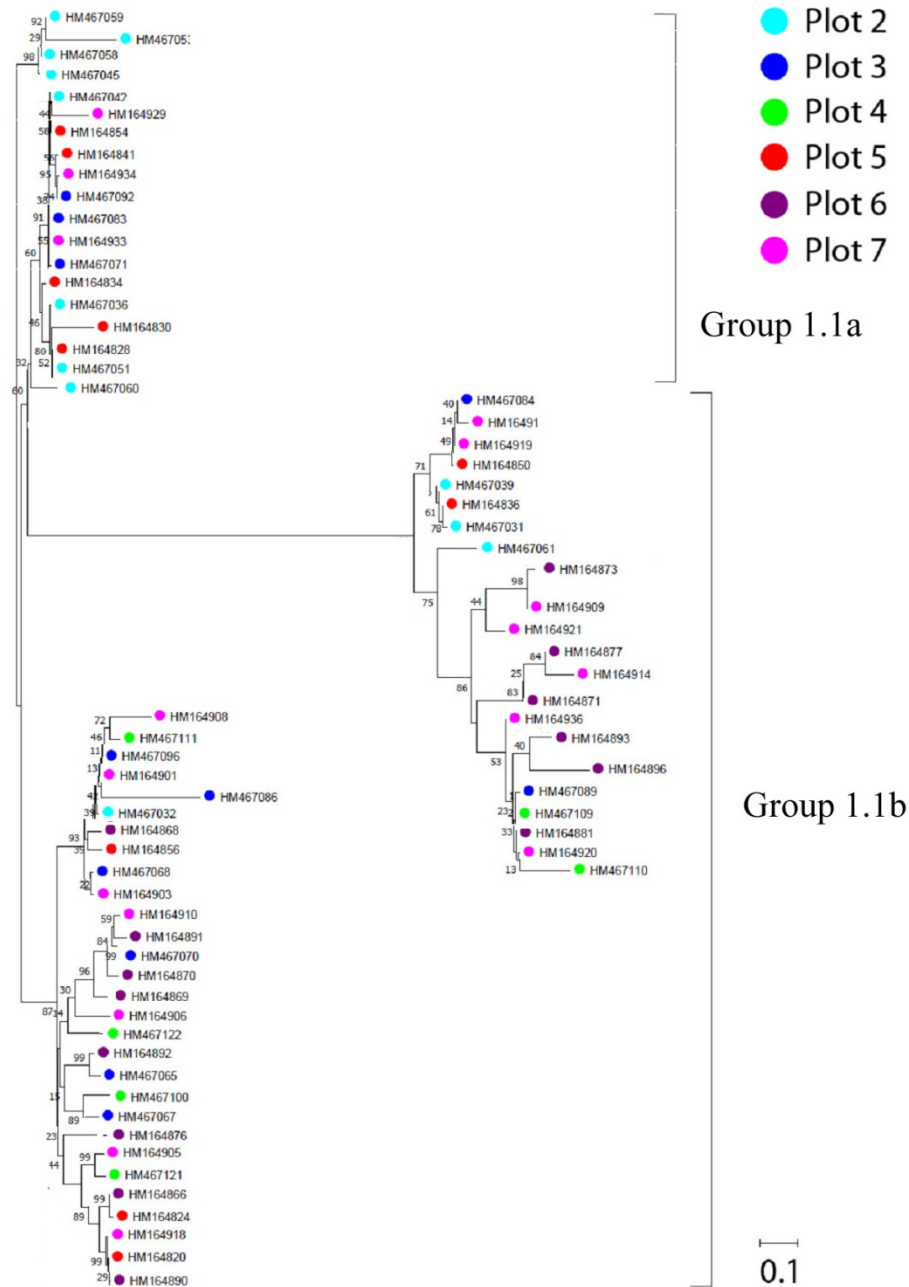


Figure 4-6. Phylogenetic affiliation of archaeal *amoA* sequences obtained from each plot. Redundant sequences were omitted from the tree at level of 95% DNA sequence identity. Sequences are color coded according to plot. Plot 2 – canopy, plot 3 – canopy, NH₄Cl and KNO₃, plot 4 – canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene. Neighbor-Joining method and Jukes-Cantor correction (Jukes and Cantor, 1969) were used, and bootstrap values derived from 100 replicates are shown. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Name corresponds to the unique GeneBank accession number. Scale bar indicate changes per nucleotide position.

Libshuff analysis was employed to assess gross differences in archaeal ammonia oxidizers populations in response to amendments, as represented by the clone libraries (Table 4-4). The analysis showed that there were variations between libraries from each plot suggesting that the amendments may have affected the AOA community structure. More specifically, the effects of amendment of NH_4Cl and KNO_3 on community composition were present regardless of whether there was a canopy or not. Moreover, when phenylacetylene was simultaneously added along with NH_4Cl and KNO_3 , this made a shift in the community composition compared to when NH_4Cl and KNO_3 were added alone. These effects were also observed regardless of the presence of the canopy. However, when we compared the plot where phenylacetylene was simultaneously added along with NH_4Cl and KNO_3 with the control plot, the effects of amendments were different depending on the presence of the canopy. When the canopy was present, the addition of phenylacetylene with NH_4Cl and KNO_3 produced a shift in the community composition compared to the control plot (plot 2 vs. plot 4, $p=0.002$). However, the simultaneous addition of phenylacetylene with NH_4Cl and KNO_3 resulted in the community composition marginally similar to that of control plot in the absence of the canopy (plot 5 vs. plot 7, $p=0.035$). This pattern of data suggests that the AOA community may respond differently to the amendments, depending on the presence of the canopy, particularly when the effects of addition of phenylacetylene with NH_4Cl and KNO_3 are examined. However, it should be noted that, since the number of clones used for the analysis is highly limited, care should be taken when interpreting the analysis result here.

Table 4-4. Pairwise Libshuff comparisons of each archaeal amoA gene sequence libraries.

	<i>p</i> 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. Plot 2 – canopy, plot 3 - canopy, NH₄Cl and KNO₃, plot 4 - canopy, NH₄Cl, KNO₃ and phenylacetylene, plot 5 – no amendment, plot 6 – NH₄Cl and KNO₃, and plot 7 – NH₄Cl, KNO₃ and phenylacetylene.

Here, long-term evaluation of continuous landfill gas monitoring showed that application of NH₄⁺ and NO₃⁻ together with phenylacetylene appeared to effectively enhance CH₄ oxidation and simultaneously repressed N₂O production. Microarray and clone libraries analyses were performed to determine what effects these amendments had on the microbial community, specifically methane and ammonia-oxidizing microorganisms, respectively. Regardless of the amendments, i.e., NH₄⁺ and NO₃⁻, or NH₄⁺, NO₃⁻ 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; Cebon et al., 2007; Lee et al. 2009). With the addition of NH₄⁺ and NO₃⁻, N₂O production was enhanced, but there was no significant change in soil CH₄ concentration from the control plot at a depth between 20 and 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; Cebren 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, the 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^- (Figure 3-10). This may be due to that the modified moisture content induced by the canopy 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 N₂O production in this landfill cover soil. When phenylacetylene was added together with NH₄⁺ and NO₃⁻, N₂O 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 N₂O from NH₄⁺.

The effects of ammonia on the ammonia-oxidizing archaea community are 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 concentration (< 76 µg NH₄⁺-N(g-soil)⁻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 $< 0.03\sim 1 \mu\text{M}$ (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 $7 - 9 \text{ mg/g-soil}^{-1}$ (Nicol et al., 2003) or $0.28 - 3.91 \text{ mg/g-soil}^{-1}$ (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 on DNA samples showed high abundance of archaeal *amoA*, the level of transcripts of *amoA* from AOAs was much lower than those of *pmoA* from methanotrophs. More specifically, archaeal *amoA* DNA was detected from every plot, but mRNA of archaeal *amoA* was detected only from plot 6. 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 a previous study (Lee et al., 2009), a lab-scale microcosm study was carried out using soils obtained from the same landfill with the present study. However, the results of microbial community structure in these microcosms were quite different from those found in the field soil samples. PCR analyses on DNA samples showed 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 was far less diverse than that from the present study. Since the microcosm soil samples were first air-dried and stored at $4 \text{ }^{\circ}\text{C}$ 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. There has been a report that natural populations of ammonia oxidizing bacteria survived in air-dried soils for periods greater than 3 months, especially for the strains capable of producing extracellular polymeric substances as capsular material (Allison and Prosser, 1991). Regarding the methanotrophic community diversity, it can be suggested that there may be different desiccation tolerances among different methanotrophic species as well.

4.3 Conclusions

Given increased concerns over greenhouse gas emissions, the effects of long-term application of NH_4^+ and NO_3^- with and without phenylacetylene on greenhouse gas emissions from landfill were determined. The addition of NH_4^+ and NO_3^- increased N_2O concentration, but had no effect on CH_4 concentrations *in situ* regardless of the presence of a canopy. The simultaneous addition of phenylacetylene reduced N_2O concentration both in the absence and presence of a canopy, but enhanced CH_4 oxidation only in the absence of a canopy. Methanotrophic community diversity decreased in response to the addition of NH_4^+ and NO_3^- but increased with the concurrent addition of phenylacetylene regardless of the presence of a canopy. Furthermore, the presence and activity of AOAs were 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 N_2O 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 N_2O production varies between AOA groups.

CHAPTER 5. Methanotrophic-mediated N_2O production

5.1 Introduction

The production of N_2O in soils can occur during the microbial processes of denitrification and nitrification. The oxidation of NH_4^+ in soils by nitrification is mediated by autotrophic and heterotrophic bacteria as well as presumably autotrophic archaea. Methanotrophic bacteria also co-oxidize NH_4^+ (Yoshinari, 1985; King and Schnell, 1994; Mandernack et al., 2000). This co-oxidation is due to the similarities in size and structure of NH_4^+ and CH_4 and similarities in the physiology and enzymatic oxidation of these substrates by methanotrophs and autotrophic nitrifiers (Bedard and Knowles, 1989; Zahn et al., 1994; Homes et al., 1995; Hanson and Hanson, 1996). The oxidation of NH_4^+ by methanotrophs has been referred to as methanotrophic nitrification and represents a pathway of the nitrogen cycle where methanotrophs may play an important role (Roy and Knowles, 1994; Mandernack et al., 2000). There have been several observations of forest and grassland soils that consume CH_4 and simultaneously produce N_2O (Castro et al., 1994; Neff et al., 1994), and Mandernack and colleagues (2000) have shown that such methanotrophic-mediated N_2O production may be significant. However, the relative importance of methanotrophs compared with autotrophic nitrifiers in nitrification and N_2O production in native soils and sediments is not well understood and requires additional investigation. Therefore, in order to better

understand the environmental and microbial control of N₂O production in soils, we carried out laboratory incubation experiments using eight methanotrophs pure strains.

5.2 Results and discussions

5.2.1 Methanotrophic N₂O production

The first experiment, aimed to investigate the effects of growth condition on N₂O production, was performed using *Methylosinus trichosporium* OB3b. The extent of N₂O production was dependent on growth conditions (Table 5-1), and more specifically, N₂O production was dependent on the concentration of ammonium. N₂O was detected up to 331 ppmv when *Methylosinus trichosporium* OB3b was growing on ammonia mineral salt (AMS) medium in the presence of copper, while no N₂O was observed on AMS with reduced amount of ammonium (0.1X AMS). A small amount of N₂O, up to 27 ppmv, was observed from nitrate mineral salt (NMS) medium grown culture only in the presence of copper. It is also interesting to note that the addition of 1X AMS medium resulted in reduced growth of *M. trichosporium* OB3b, most likely due to oxidation of ammonium by MMO, resulting in inhibition of CH₄ oxidation.

Interestingly, the production of N₂O 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, the intracellular NADH becomes 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 helps to prevent the accumulation of potent precursors for N_2O such as hydroxylamine or nitrite. The absence of copper seemed to inhibit N_2O production from both NMS and AMS, but it will be discussed further in the next chapter. 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.

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 5-2). The amount of N_2O produced varied from 32 to 343 ppmv, which is equivalent to 0.16% to 1.7% conversion rate of $\text{NH}_4^+\text{-N}$ to $\text{NO}_2^-\text{-N}$. 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 that 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, but this should be verified with analyses of

Table 5-1. Nitrous oxide production by *Methylosinus trichosporium* OB3b on various growth conditions. Concentrations of NO₃⁻ and NH₄⁺ in NMS and AMS are 10 mM.

Media	Cu (20 uM)	Formate (20 mM)	Acetylene (1% by vol)	Carbon source	Grow rate (hr ⁻¹)	Max OD ₆₀₀	Max N ₂ O
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.

N.G.: no growth, N.D.: not detected.

Table 5-2. Methanotrophic mediated nitrous oxide production under pMMO-expressing conditions, i.e., AMS with 20 μ M of copper.

Strain	Type	Growth rate (hr ⁻¹)	Max OD ₆₀₀	Max N ₂ O (ppmv)
<i>Methylobacterium album</i> BG8	I	0.054 (0.004)	0.725 (0.01)	ND
<i>Methylococcus capsulatus</i> Bath	I	0.138 (0.007)	0.810 (0.02)	ND
<i>Methylosinus trichosporium</i> OB3b	II	0.088 (0.003)	0.880 (0.04)	343.7 (7.1)
<i>Methylocystis parvus</i> OBBP	II	0.123 (0.004)	0.935 (0.02)	32.3 (2.9)
<i>Methylocystis hirsuta</i> CSC1	II	0.069 (0.001)	0.925 (0.04)	156.5 (2.8)
<i>Methylocystis rosea</i> SV97	II	0.071 (0.002)	0.700 (0.04)	233.1 (9.4)
<i>Methylocystis</i> sp. strain M	II	0.103 (0.008)	0.975 (0.04)	ND
<i>Methylocystis</i> 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.

more methanotrophs, particularly Type I strains. N₂O uptake experiments were performed for each strain to ensure that the results with no N₂O detected are not false negative. At the mid-exponential phase of growth, 100 ppmv of N₂O was injected, but no decrease was observed for over three days.

5.2.2 Effects of copper on N₂O production

Five methanotrophic strains, i.e., *Methylosinus trichosporium* OB3b, *Methylocystis parvus* OBBP, *Methylocystis hirsuta* CSC1, *Methylocystis rosea* SV97, and *Methylocystis* sp. strain SB2 shown to produce N₂O (Table 5-2) were exposed to different concentrations of copper, 0 and 20 μ M, to investigate the effects of copper on the production of N₂O. 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 OD₆₀₀ in the absence of copper (Figure 5-1). Therefore, it was expected that the strains possessing both MMOs would produce more N₂O than the other strains

possessing only pMMO. Interestingly, however, the trend of the amount of N₂O 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 N₂O they produced were not the most. Rather, *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP produced significantly reduced amounts of N₂O, while the other strains produced similar (*Methylocystis rosea* SV97) or more N₂O (*Methylocystis hirsuta* CSC1 and *Methylocystis* sp. strain SB2) (Figure 5-2).

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) N₂O production may not be dependent on the activity of MMOs, i.e., unlike CH₄ oxidation, the rate-limiting step for N₂O production may not be the first reaction, i.e., ammonia oxidation.

5.2.3 Inhibition of methanotrophic N₂O production by phenylacetylene

The effects of phenylacetylene on N₂O production were 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),

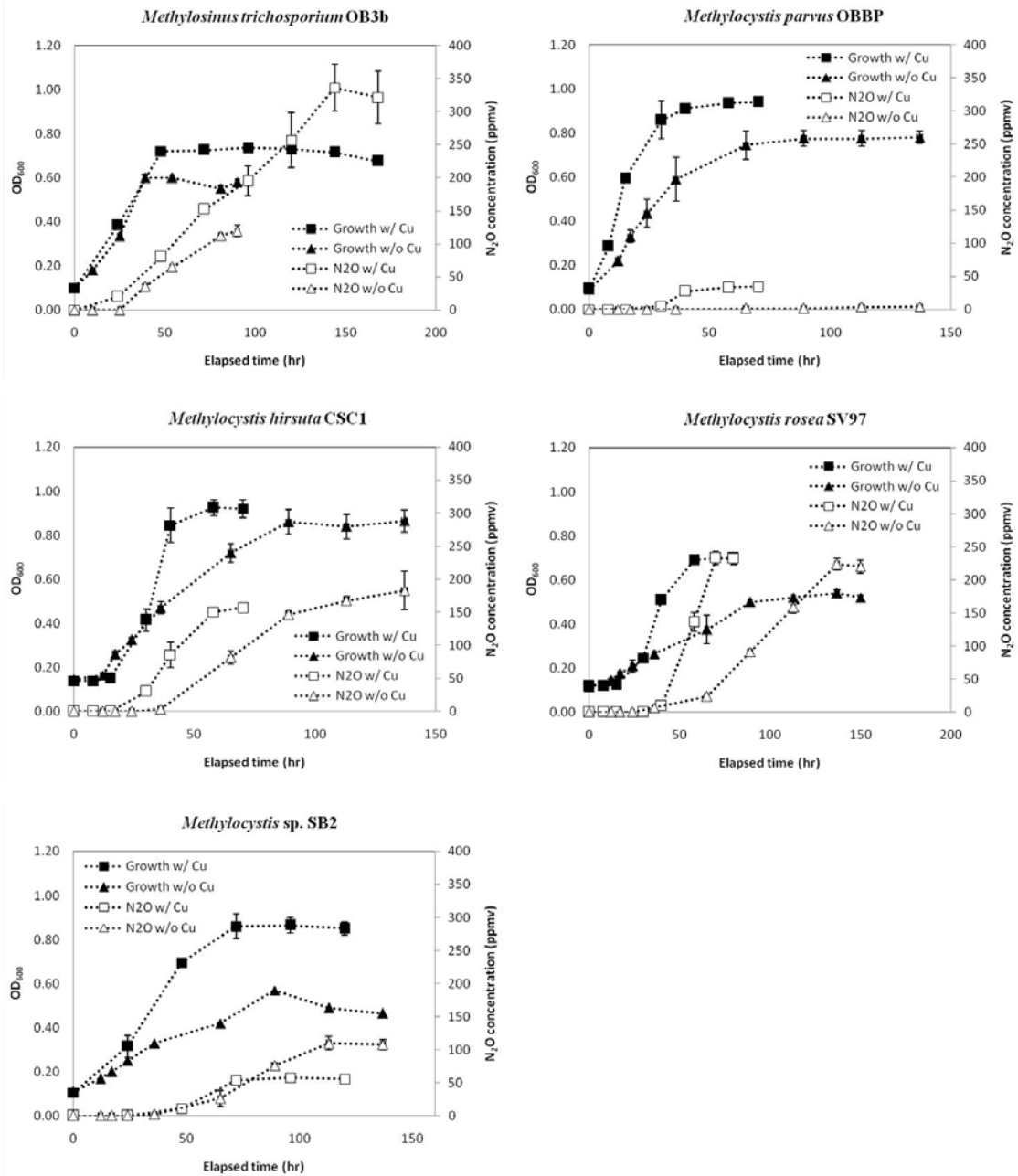


Figure 5-1. Growth and N₂O production of *Methylosinus trichosporium* OB3b^T, *Methylocystis* sp. SB2, *Methylocystis rosea* SV97^T, *Methylocystis parvus* OBBP^T, *Methylocystis hirsuta* CSC1^T when growing on AMS in the presence/absence of 20 μM of copper. Here, only *M. trichosporium* OB3b and *M. hirsuta* CSC 1 have both forms of MMOs.

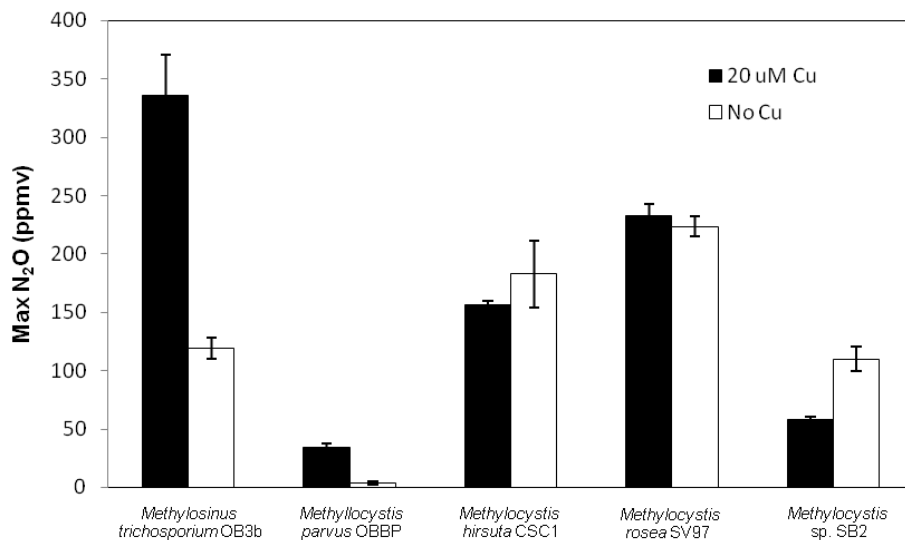


Figure 5-2. Methanotrophic mediated N₂O production in the presence/absence of 20 μM of copper.

phenylacetylene was proposed as a selective inhibitor in this study. To verify the effects of phenylacetylene on methanotrophic N₂O 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 5-3 and Table 5-3 represent a demonstration of differential inhibition of N₂O production by phenylacetylene in *Methylosinus trichosporium* OB3b and *Methylocystis* sp. SB2. Growth rates and N₂O production were 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 N₂O 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

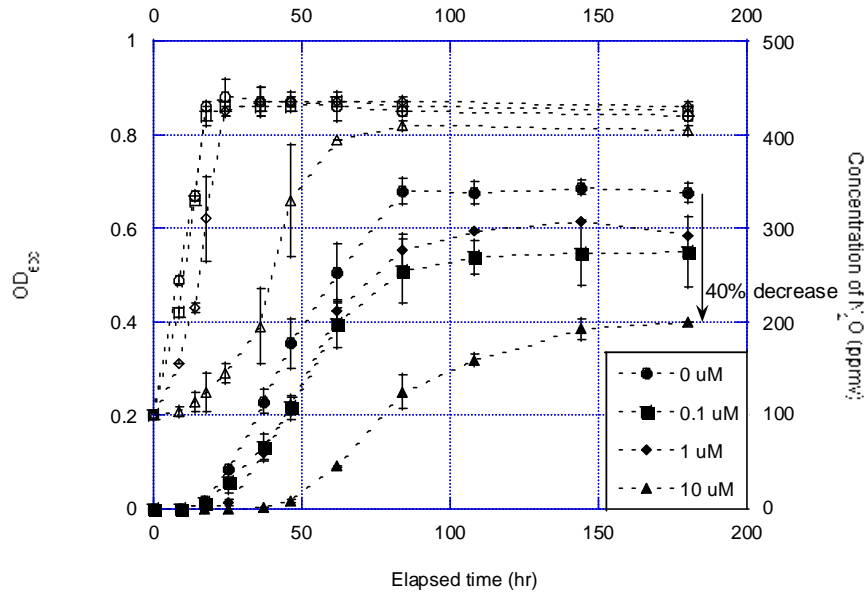
culture density, with the OD values of 0.82~0.87 observed being similar to that observed in the absence of phenylacetylene, 0.88 (± 0.04). For *Methylocystis* sp. strain SB2, N₂O production decreased by 60% when 10 μ M of phenylacetylene was added, but the maximum cell density increased up to 0.7 and the growth rate was reduced only by 20%. This suggests that the reduced N₂O production may be partially due to the reduced cell growth, but the degree of inhibition by phenylacetylene on N₂O production may vary from species to species.

Table 5-3. Effects of phenylacetylene on growth rate and N₂O production of the cells growing on AMS in the presence of 20 μ M of copper.

Strain	C ₈ H ₆ concentration (μ M)	Growth rate (hr ⁻¹)	Max OD ₆₀₀	Max N ₂ O (ppmv)
<i>M. trichosporium</i> 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	-	-
<i>Methylocystis</i> 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	-	-

Values in parentheses indicate standard deviation of duplicate samples

Methylosinus trichosporium OB3b



Methylobcystis sp. SB2

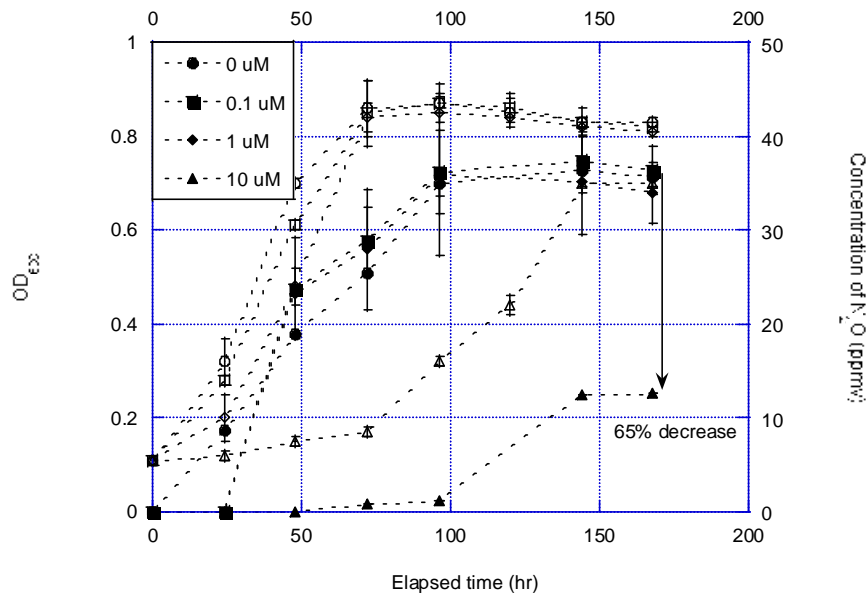


Figure 5-3. Effects of phenylacetylene on N₂O production (open: optical density at 600 nm; closed: N₂O concentration).

5.2.4 Methanotrophic-mediated N₂O production via denitrification

Since N₂O was also produced even in smaller quantity from *Methylosinus trichosporium* OB3b growing on liquid media supplemented with nitrate as a sole nitrogen source (Table 5-1), it was speculated that N₂O might have been produced from denitrification pathway. To validate the hypothesis, *Methylosinus trichosporium* OB3b was incubated with nitrate and various concentrations of nitrite substituted for nitrate, and the initial cell density was set to the optical density that corresponded to the maximum optical density shown from the previous experiment (Table 5-1) to minimize ammonia produced by nitrate reductase for assimilation. A similar amount of N₂O, 51 ppmv was produced from the cell growing on NMS, but it may be due to ammonia as a by-product for 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 5-4. Although growth was not inhibited by the amounts of nitrite less than 1 mM, no significant amount of N₂O was observed, suggesting denitrification pathway can be neglected in methanotrophic-mediated N₂O production.

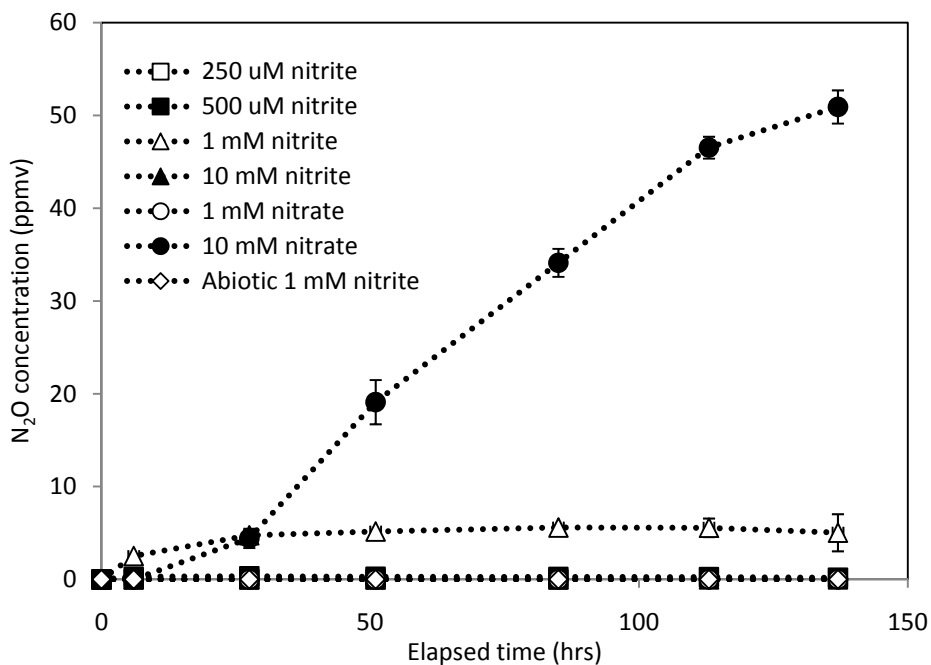
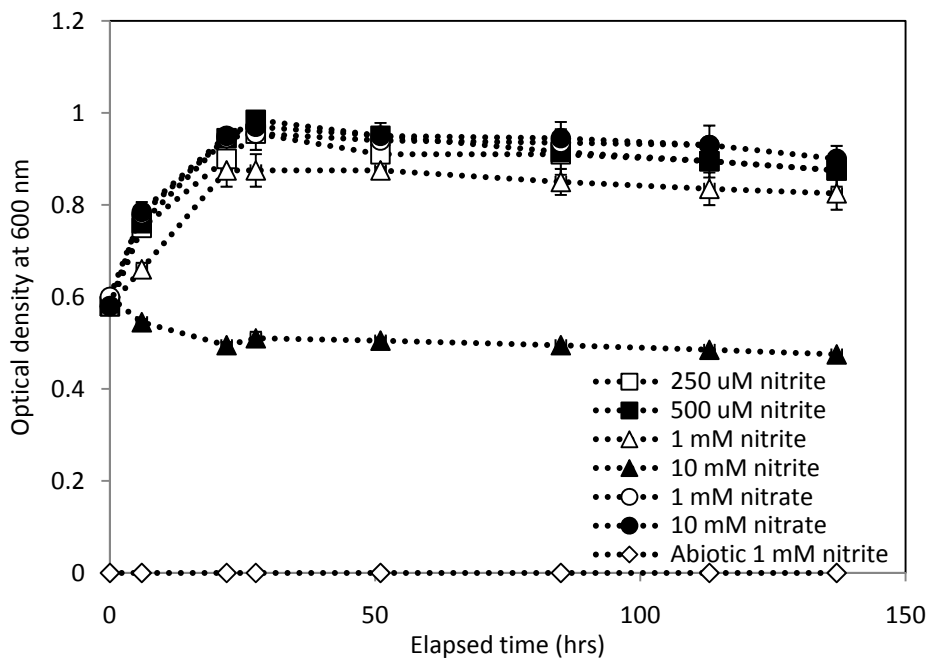


Figure 5-4. N₂O production by *Methylosinus trichosporium* OB3b in mineral salt media supplemented with various concentration of nitrite and nitrate.

5.3 Conclusions

Methanotrophic mediated N₂O production, compared with autotrophic nitrification or denitrification, has not been well characterized and it requires additional investigation. The results in this chapter, however, show that several methanotrophs produce as much N₂O as ammonia oxidizers or denitrifiers. The conversion rate of NH₄⁺-N to NO₂⁻-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 that N₂O production mediated by methanotrophs can be significant in terrestrial ecosystems. Interestingly, five out of six Type II methanotrophic strains were able to produce significant amounts of N₂O when growing on ammonium mineral salt media, while the two tested Type I strains did not produce detectable amounts of N₂O. This result is consistent with the result of the microarray analysis reported in Chapter 4, suggesting that Type II methanotrophs may be more responsible for the N₂O production in terrestrial ecosystems. The effects of copper on N₂O production were different for each strain, i.e., *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP produced significantly reduced amounts of N₂O in the absence of copper, while similar or slightly higher amounts of N₂O were produced by *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 N₂O production may not be dependent on the activity of MMO. The effects of phenylacetylene on methanotrophic-mediated N₂O production were also investigated, but the reduced N₂O production may be partly

due to the reduced cell growth, but the degree of inhibition may vary from species to species. The NH_4^+ concentration used in this study, i.e., 10 mM, is beyond natural concentration ranges in terrestrial ecosystems. However, the results of this study corroborate previous reports of methanotrophic nitrification and N_2O production in aqueous and soil environment, especially in landfill cover soil (Mandernack et al., 2000).

CHAPTER 6. Conclusions and future work

6.1 Conclusions

The overall objective of this study was to investigate proposed strategy to control greenhouse gas emissions from landfill cover soils, more specifically, (1) to understand the long-term effects of geochemical parameters on CH₄ consumption and N₂O production in landfill cover soils and, (2) to examine the role of methanotrophs in producing N₂O. To examine the effects of nitrogen, selective inhibitors and moisture content on CH₄ oxidation and N₂O production *in situ*, 0.5 M of NH₄Cl and 0.25 M of KNO₃, with and without 0.01% (w/v) phenylacetylene and/or a canopy, were applied to test plots at a landfill in Kalamazoo, MI from November 2007 to July 2009. Depth profiles of CH₄, N₂O, and O₂ concentrations were also monitored from 20 cm to 100 cm of depth. Multiple soil cores were also collected at the time of dismantling the field site in July 2009 to determine how these amendments affected the structure and activity of methane and ammonia-oxidizing microbial communities. It was found that NH₄⁺ and NO₃⁻ amendments stimulated N₂O production but did not affect CH₄ oxidation. The simultaneous addition of phenylacetylene stimulated CH₄ oxidation in the absence of a canopy, while reducing N₂O production. In the presence of a canopy, the addition of phenylacetylene had no effect on CH₄ oxidation. Based on NH₄⁺ and NO₃⁻+NO₂⁻ depth profiles of each soil core, the addition of nitrogen source seemed to stimulate overall microbial activity, as evidenced by lower nitrogen contents in the plots with nitrogen amendments than in the control plot. Also, a canopy seemed to inhibit microbial activity,

from the comparisons of nitrogen content of plots with a canopy to those without a canopy. PCR analyses showed that methanotrophs, especially those possessing pMMO, were more abundant than those possessing sMMO, and, interestingly, archaeal ammonia-oxidizers were more abundant than their bacterial counterparts. Microarray analyses showed that the addition of NH_4^+ and NO_3^- caused the 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 NH_4^+ and NO_3^- increased the presence of archaeal ammonia-oxidizers affiliated with Crenarchaeal Group 1.1b regardless of the presence of a canopy, while their presence decreased with the simultaneous addition of phenylacetylene in the absence of a canopy and maintained in the presence of a canopy. Several methanotrophs were investigated to examine the relative importance of methanotrophic mediated N_2O production. Interestingly, several methanotrophs produce as much N_2O as ammonia oxidizers or denitrifiers. More specifically, 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 $\text{NH}_4^+\text{-N}$ to $\text{N}_2\text{O-N}$, while two Type I strains did not produce detectable amount of N_2O . The effects of copper on N_2O production, i.e., some strains produced more N_2O in the absence of copper regardless of the presence of sMMO, suggested that (1) the absence of copper had an inhibitory effect, but in various degrees from species to species; or (2) methanotrophic-mediated N_2O production may not be controlled by the activity of pMMO.

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. Such a possibility is intriguing as pure culture studies indicated that Type II methanotrophs produce more N_2O from ammonia than Type I methanotrophs, but it is currently unknown what the magnitude of N_2O production might be from archaeal ammonia-oxidizers.

6.2 Future work

The results presented here suggest that ammonia oxidizing archaea may be responsible for N_2O production in the landfill cover soil. Such an argument is interesting, particularly as ammonia oxidizing archaea may be more abundant or active in the terrestrial ecosystems (Nicol et al., 2003; Ochseneiter et al., 2003; Nicol et al., 2005; Simon et al., 2005). The simple issue whether they can produce N_2O or not, however, has not been conclusively verified mainly due to the inability to isolate them in pure culture. Phenylacetylene, which has been proven to selectively inhibit the activity of ammonia oxidizing bacteria (Lontoh et al., 2000), seemed to also affect ammonia oxidizing archaea, but this, also, can only be unequivocally affirmed through pure culture studies. While we have generated much information using molecular biological techniques, it should be stressed that cultivation-independent, PCR-based methods also have inherent biases preventing a reliable assessment of the physiology or structure of microbial populations (Head et al., 1998; Forney et al., 2004). Furthermore, much of our basic knowledge of the microbial world has come from investigation of pure cultures, and therefore cultured isolates are still very important in developing our understanding of microbial physiology,

genetics, and ecology (Palleroni, 1997; Zinder and Salyers, 2001). A better understanding of these cells is, therefore, essential for control of greenhouse gas emissions from landfills.

Phylogenetic analyses of AOA partial *amoA* and 16S rRNA gene sequences revealed that there are two distinct lineages that also appear to prefer different niches, i.e., Group 1.1a lineage and Group 1.1b lineage that tend to predominate in low and high nitrogen environments, respectively (Ochsenreiter et al., 2003; Francis et al., 2005; Nicol and Schleper, 2006; Prosser and Nicol, 2008). Such trends are intriguing as the clone library analyses in this research showed that the diversity of Group 1.1b crenarchaeota dramatically increased in response to nitrogen amendments. In order to verify this hypothesis, a simple microcosm study, using sediment or water sample where Group 1.1a AOA predominate, could be performed to examine the effects of ammonium concentration on the community structure of ammonia oxidizing archaea. The phylogenetic composition of the archaea assemblage could be monitored through clone library analyses or new group-specific primers targeting 16S rRNA genes. Alternatively, new group-specific primers targeting ammonia monooxygenase alpha-subunit (*amoA*) genes for Group 1.1a and Group 1.1b crenarchaeota could be designed and applied to monitor their responses on increased nitrogen availability. These effects, however, require more information on AOAs, again requiring more pure culture analyses.

Here, the molecular biological analyses examining the changes in abundance and the transcripts of functional genes allowed an assessment as which microbial groups may be more responsible for greenhouse gas emissions in the landfill cover soils. In addition to this molecular technique based approach, the use of N₂O isotopomers could give an

independent perspective on this matter. The intramolecular distribution of nitrogen isotopes in N₂O, i.e., ¹⁵NNO and N¹⁵NO, has been used extensively for defining the relative importance of microbial sources of N₂O (Yoshida and Toyoda, 2000; Perez et al., 2001; Sutka et al., 2003; 2006; Toyoda et al., 2005; Perez et al., 2006; Well et al., 2006). Previous studies have shown that the site preferences of ~33 ‰ and ~0 ‰ are characteristic of nitrification and denitrification, respectively, to apportion sources of N₂O more in detail (Perez et al., 2001; Stein and Yung, 2003; Sutka et al., 2006). As shown in Chapter 5, *Methylosinus trichosporium* OB3b produced a significant amount of N₂O, up to 51 ppmv, when growing on mineral salt media supplemented with nitrate as a sole nitrogen source, suggesting certain portion of N₂O may be produced via denitrification by methanotrophs. The isotopomer studies, therefore, could provide further information on the mechanism of methanotrophic-mediated N₂O production. This could be achieved through the similar experiments in this study as shown in Chapter 6 using various strains with various nitrogen sources as a sole nitrogen source, i.e., ammonium, nitrate and nitrite.

Finally, one of the conclusions of this work here was that Type II methanotrophs may be more responsible for N₂O production in the terrestrial ecosystem. Unlike ammonia oxidizing archaea, many pure cultures have been isolated from a variety of environments. Therefore, the similar analyses in Chapter 5 should be performed on more extensive range of strains of methanotrophs especially more Type I strains.

Last but not least, once the major contributors on N₂O production are identified through the follow-up works proposed in this chapter, we may be able to come up with a better strategy to mitigate *in situ* GHG emissions from a landfill. For example, since

AOAs might be one of the major contributors of N₂O production in a landfill, a better technology to selectively inhibit the community can be developed through physiology studies using pure cultures. And then, the strategy can be iteratively improved over the course of field scale demonstrations and lab scale experiments. Also, in order to confirm whether the strategy can effectively mitigate GHG from a landfill, it should be tested on more landfills.

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