2023

Metagenomic Insights Into Landfill Intermediate Cover Soil Microbiomes And Their Potential Role In Mitigating Greenhouse Gas Emissions

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METAGENOMIC INSIGHTS INTO LANDFILL INTERMEDIATE COVER SOIL MICROBIOMES AND THEIR POTENTIAL ROLE IN MITIGATING GREENHOUSE GAS EMISSIONS

A Thesis Presented

by

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To

The Faculty of the Graduate College

Of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Civil and Environmental Engineering

August, 2023

Defense Date: June 12, 2023
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ABSTRACT

Landfills are the third largest source of anthropogenic methane emissions in the United States and have also been found to produce nitrous oxide, an even more potent greenhouse gas than methane. Intermediate cover soils (ICS) play a major role in reducing greenhouse gas emissions from landfills because they harbor methanotrophic bacteria that degrade methane. However, the extent of methanotrophs in landfill cover soils and the roles of microbial communities in these complex ecosystems is only beginning to be understood. Additionally, the role of ICS in producing nitrous oxide is poorly understood. While methane consumption is a desirable trait, genes involved in methane oxidation may also promote nitrous oxide production. To better understand the role of ICS microbial communities in nitrogen and carbon cycling, samples were collected from twelve locations and two depths from ICS at an active municipal solid waste landfill. Metagenomic analyses of these samples were performed. Further, composite soil samples were incubated with methane and varying amounts of ammonia to assess the impacts of ammonia on nitrous oxide production. After an initial stabilization period, the incubations showed consumption of approximately 100,000 ppm of methane in the headspace in 9 days. All incubations with methane produced small amounts of nitrous oxide (5 – 8 ppm) even when ammonia was not supplemented. Incubations without methane added, however, produced less nitrous oxide. The methanotrophs *Methylobacter* and unclassified Methylococcaceae were present in the original ICS samples and the incubation samples, and their abundances increased in the incubation with *Methylobacter* being the dominant methanotroph. Other candidate methanotrophs were also enriched, including Verrucomicrobia. Genes encoding particulate methane monooxygenase/ammonia monooxygenase (pMMO/AMO) were much more abundant than genes encoding soluble methane monooxygenase (sMMO) across the landfill ICS, but sMMO genes were enriched during the incubations with methane. Genes associated with nitrous oxide production via ammonia oxidation (the first process in nitrification) and denitrification were also present where only certain genes were enriched during the incubations. Genes encoding hydroxylamine oxidoreductase (the second step in ammonia oxidation) were largely absent. In total, these results suggest that ammonia oxidation via methanotrophs may result in low levels of nitrous oxide production, but ICS microbial communities have the potential to greatly reduce the overall global warming potential of landfill emissions.

ACKNOWLEDGEMENTS

I am extremely grateful for those who have helped and supported me during my graduate studies. I want to thank Dr. Scarborough for his incredible support, guidance, and patience throughout my research. His passion and profound knowledge for what he does encouraged me to pursue my graduate studies, and I cannot thank him enough for his time and effort towards my work. I would also like to thank Dr. Badireddy and Dr. Neher for being a part of my committee and expressing interest in my research. I would like to thank the Vermont Space Grant Consortium provided by NASA for funding my graduate research. Without this funding, I would not have been able to pursue my masters in this research. Also a big thank you to those at the Casella owned Clinton County Landfill in New York, especially Sean Lukas and Amy Davies, for allowing me to take samples from their landfill.

Thank you to those who have supported me along the way and guided me during my research. Thank you to Venus Rohra and Courtney Clement for conducting preliminary analyses of this research. Thank you to Amy DeCola for teaching me how to perform DNA extractions and guiding me through the metagenomic analysis. Thank you to Panagiota Stamatopoulou and Leandro Conrado for showing me how to use the GC-TCD machine and perform other lab tests. Thank you to those in the EMERG Lab and my peers for making my graduate studies more enjoyable. Finally, thank you to my friends and family for believing in me and pushing me to be the best version of myself; I am truly grateful for this incredible experience.

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CHAPTER 1

INTRODUCTION

The production of waste is increasing every day with human activity. In 2018, the United States generated 292.4 million tons of municipal solid waste (MSW) where half of that waste ended up in landfills (EPA, 2023). In the United States, landfills account for 15% of methane emissions (EPA, 2023). Comparatively, the impact of methane on warming the atmosphere is 28 times that of carbon dioxide, and nitrous oxide is 265 times that of carbon dioxide over a 100-year period (EPA, 2023). The microbes present in the organic wastes and the ICS play a role in both methane and nitrous oxide emissions from landfills. However, landfill ICSs are a critical design aspect that can be used to prevent methane from escaping into the atmosphere and contributing to global warming.

The microbial communities present in the ICS are different from those present in the landfill itself. ICS is characterized by a wide range of redox conditions within a small area, and microbial communities in the ICS develop under both anaerobic and aerobic conditions. These differences in the ICS oxygen levels result in a variety of bacteria and archaea that are capable of consuming methane to produce carbon dioxide. Typically, the bottom layer of the ICS consists of bacteria and archaea that can perform anaerobic methane oxidation. Certain groups of bacteria and archaea that are able to perform this have been found in ICSs from previous studies. These groups include anaerobic methanotrophic (ANME) archaea and sulfate reducing bacteria (SRB). One study found an abundance of ANME archaea, SRBs, and NC10 bacteria in the bottom layers of the ICS (Xu & Zhang, 2022). As oxygen levels increase closer to the surface of the ICS, bacteria

that are able to perform aerobic oxidation of methane exist. Certain groups and strains of bacteria and archaea that can perform aerobic methane oxidation have been identified as methanotrophs (members of the α- and γ- Proteobacteria), ammonia-oxidizing bacteria (genera *Nitrosomonas* and *Nitrospira*), and ammonia-oxidizing archaea (Group 1.1a and 1.1b of Crenarchaea). A wide diversity of methanotrophs exists in ICSs around the world, specifically the α- and γ- Proteobacteria. Methanotrophs of the α-Proteobacteria dominate in ICS; the *Methylocystis* species dominates in soils with lower carbon/nitrogen ratios, while the *Methylobacter* species dominates in soils with higher carbon/nitrogen ratios (Semrau, 2011).

Limited research has evaluated these processes occurring in landfills, and it is unknown why nitrous oxide is produced during methane consumption by methanotrophs. The objectives of this research are to quantify methane oxidation and nitrous oxide production from enriched laboratory batch reactors with microbes derived from the studied ICS. Further, the taxonomy and abundance of microorganisms in the laboratory enrichments and ICS samples from various locations and two depths will be analyzed along with the key genes present for methane oxidation and nitrous oxide production. This study aims to identify abundant genes and taxa to assess potential routes of methane oxidation and nitrous oxide production. This knowledge could be beneficial in many aspects related to microbiology and soil management. Once understood, this research could be used to identify engineering controls that can be implemented to reduce greenhouse gas emissions from landfills.

CHAPTER 2

LITERATURE REVIEW

Waste Management and Production of Methane in Landfills

In 2018, the United States landfilled 146 million of the 292.4 million tons of MSW generated. Food waste made up a majority of the landfilled material, accounting for 24%, which was followed by 18% plastics, 12% paper and paperboard, and 21% various materials such as rubber, leather, and textiles (EPA, 2022). Most organic waste in landfills breaks down through a process called anaerobic decomposition (Christy et al., 2014). This process occurs when microbes break down the organic material in the absence of oxygen and transform it into biogas. The biogas produced from this process consists mostly of methane and carbon dioxide.

Anaerobic decomposition (Figure 2.1) consists of four biological and chemical stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Christy et al., 2014). In the first stage of hydrolysis, complex organic matter (i.e., carbohydrates, proteins, nucleic acids, and lipids) are broken down into smaller components by microorganisms producing hydrolases. These microorganisms degrade the complex polymers of organic matter and produce monomers (i.e., sugars, amino acids, nucleotides, and fatty acids). Acidogenesis follows hydrolysis, and it is often the fastest reaction in anaerobic decomposition. Products from hydrolysis are used as substrates for fermentation microorganisms. These microorganisms produce organic acids, short-chain fatty acids, alcohols, hydrogen (H_2) and carbon dioxide (CO_2). This transition from organic matter to organic acids causes the system's pH to drop. However, this acidic environment is suitable

for both acidogenic and acetogenic microorganisms to thrive. During the acidogenic stage, electron sinks such as lactate, propionate, butyrate, ethanol, and higher volatile fatty acids (VFAs) accumulate and are a prerequisite for increases in hydrogen concentration in the medium. Acetogenesis is a stage of anaerobic decomposition performed by anaerobes that produce acetate, CO_2, and H_2 using enzymes and co-factors that are very sensitive to O_2. These anaerobes are slow growing and sensitive to environmental changes and fluctuations of organic loadings. During the final stage of anaerobic decomposition, called methanogenesis, methane is produced by methanogenic archaea in anoxic conditions as a metabolic end-product. Methane production can occur through two pathways. The most common metabolic pathway is hydrogenotrophic methanogenesis, when CO_2 and H_2 are converted to methane. Less common is acetoclastic methanogenesis when acetate is directly converted to methane.

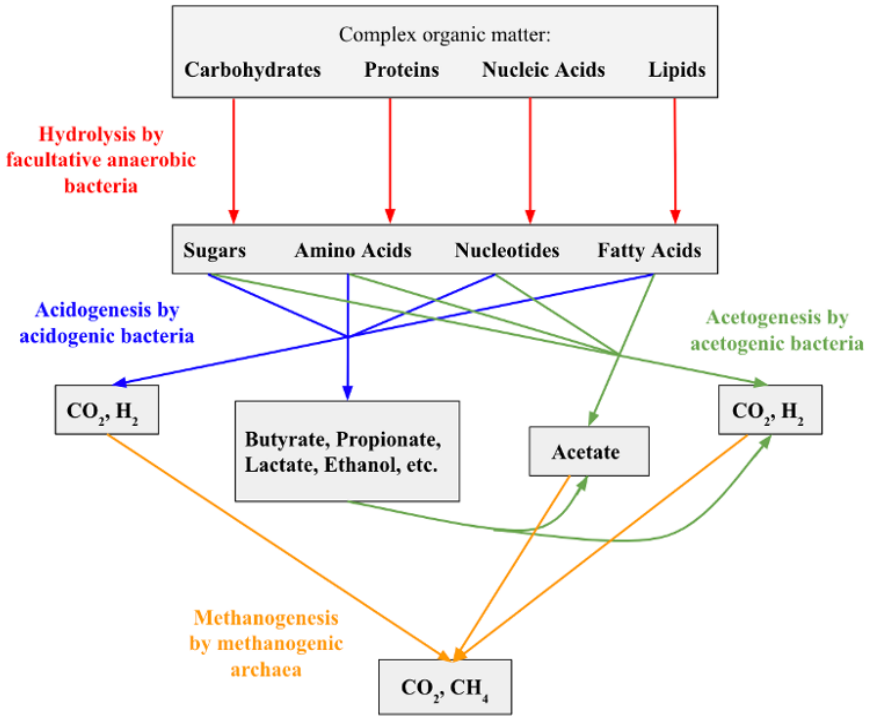

Figure 2.1. *Anaerobic decomposition process diagram.*

Methane Oxidation in Intermediate Cover Soils

Microbial communities present in the ICS differ from those present in the underlying landfill itself. ICS is characterized by a wide range of redox conditions within a small area, and microbial communities in the ICS develop under both anaerobic and aerobic conditions. The anaerobic processes in the ICS occur near the underlying refuse, while the aerobic processes happen on the surface layers of ICS (Xu & Zhang, 2022). These differences in the ICS oxygen levels yield a variety of bacteria and archaea that are capable of consuming methane to produce carbon dioxide and/or nitrous oxide. Typically, the lower layer of the ICS consists of bacteria and archaea that can perform anaerobic methane oxidation. Certain groups of bacteria and archaea have been reported previously for ICSs (Xu and Zhang, 2022). These groups include anaerobic methanotrophic (ANME) archaea and sulfate reducing bacteria (SRB). One study contained an abundance of ANME archaea, SRBs, and NC10 bacteria in the bottom layers of the ICS (Xu & Zhang, 2022). As oxygen levels increase closer to the surface of the ICS, bacteria that can perform aerobic oxidation of methane exist. Certain groups and strains of bacteria and archaea that can perform aerobic methane oxidation have been identified as methanotrophs (members of the α- and γ- Proteobacteria), ammonia-oxidizing bacteria (e.g., *Nitrosomonas, Nitrospira*), and ammonia-oxidizing archaea (Group 1.1a and 1.1b of Crenarchaea). A wide diversity of methanotrophs exists in ICSs around the world, specifically the phyla α- and γ-Proteobacteria. Methanotrophs of the α-Proteobacteria dominate in ICS; the *Methylocystis* genus dominates in soils with relatively low carbon: nitrogen ratios, while the *Methylobacter* genus dominates in soils with relatively high carbon: nitrogen ratios (Semrau, 2011). Optimal carbon: nitrogen ratios for biogas production are in the range of

20-30. Anything above that range would be considered a high ratio whereas measured values below that range would be considered low ratios (Ituen & Etim, 2012).

Knowing the genus of methanotrophs present is important to better understand the type of methanotroph and how it undergoes methane oxidation. To date, three types of methanotrophs have been distinguished based on their metabolic pathways, membrane lipid content, and physical characteristics (Figure 2.2). Type I methanotrophs are γ-Proteobacteria which utilize the ribulose monophosphate (RuMP) pathway for carbon assimilation, as well as having disc shaped intracytoplasmic membrane (ICM) structure comprising of mainly 14-16 carbon phospholipids. Type II methanotrophs, are α-Proteobacteria, use the serine pathway and have ICMs along the periphery of the cells that contain primarily 18-carbon phospholipids (Koo & Rosenzweig, 2021). The third group of methanotrophs, referred to as Type X, are characterized as those in the Verrucomicrobia phylum (Semrau et al., 2010), and exist at higher temperatures than types I and II (Park & Kim, 2019). They are distinguished from Type I methanotrophs but have characteristics of both types including utilization of the RuMP pathway, 16 carbon phospholipid fatty acids, and possessing ribulose-1,5-bisphosphate (Semrau et al., 2010).

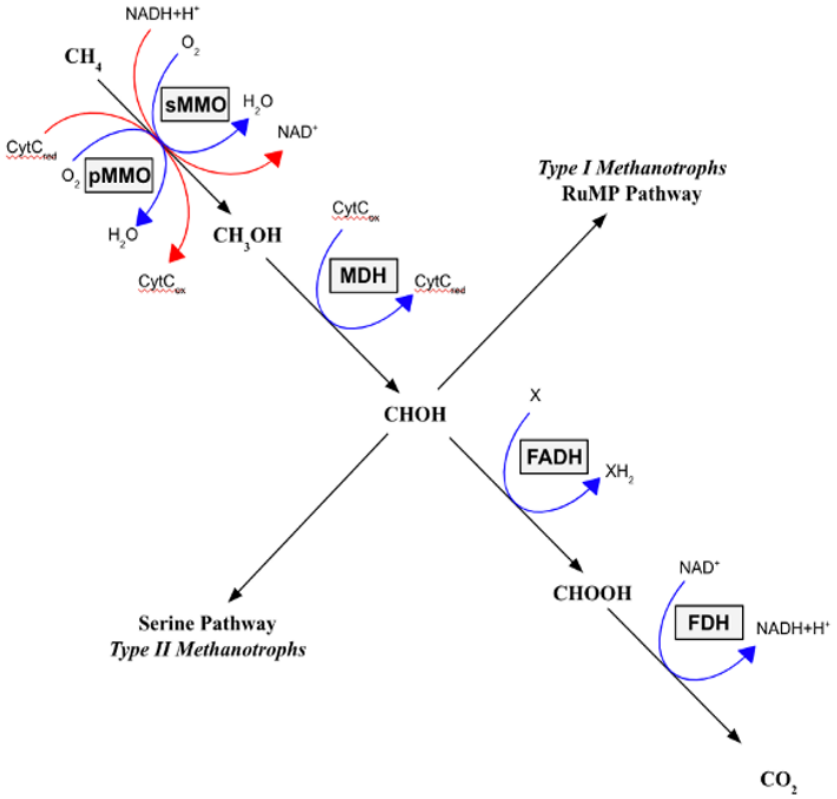

Figure 2.2. *Methane oxidation metabolic pathway displaying enzymes (in boxes), as well as intermediate and by-products. The enzymes shown include particulate methane monooxygenase (pMMO), soluble methane monooxygenase (sMMO), methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FADH), and formate dehydrogenase (FDH). A portion of formaldehyde is directed to biomass precursors via the serine pathway (Type II Methanotrophs) or RuMP pathway (Type I Methanotrophs). Intermediate metabolites include methanol (CH_3OH), formaldehyde (CHOH), and formate (CHOOH). Figure adapted from "The biochemistry of methane oxidation" by A. S. Hakemian and A. C. Rosenzweig, 2007, Review of Biochemistry 76(1) 223-241.*

Contrasting conditions and microbial communities in ICSs suggest microbes produce nitrous oxide from different metabolic pathways. A key factor in the metabolic pathways for methane oxidation are the enzymes present in the methanotrophs. All known aerobic methanotrophs produce the enzyme methane monooxygenase (MMO) that converts methane to methanol during the first step of methane oxidation (Semrau, 2011). There are two forms of the MMO: (1) the particulate methane monooxygenase (pMMO) which is located in the cytoplasmic membrane of most methanotrophs, and (2) the soluble methane monooxygenase (sMMO) which is located within the cytoplasm and found in

some methanotrophs (Choi et al, 2003). Methanotrophs expressing sMMO have faster rates of methane oxidation than those expressing pMMO, especially at high methane concentrations. Thus, methanotrophs expressing pMMO predominate at lower methane concentrations. However, the expression of either pMMO or sMMO depends on the copper concentration present in the medium during the growth of the microbes. Methanotrophs that are cultured in mediums with high copper concentrations tend to express pMMO, while those in low copper concentrations or high iron concentrations express sMMO (Choi et al, 2003).

The rate of methane oxidation also depends on which electron acceptors are present for methane oxidation. Available electron acceptors in ICS vary with respect to the depth and conditions in the soil. Anaerobic methane oxidation occurring in the bottom and middle layers of ICS have different electron acceptors. Sulfate is a dominant electron acceptor in the bottom layer of the ICS, while Fe^{3+} and Mn^{4+} are also an option. This sulfate-anaerobic methane oxidation is catalyzed by SRBs and ANME archaea to produce HCO_3^- and HS^-. Nitrite and nitrate are dominant electron acceptors in the middle depths of typical ICS. Nitrate- and nitrite-anaerobic methane oxidation is catalyzed by ANME-2d archaea and NC10 bacteria, respectively. The top layer of ICS are typically aerobic conditions favoring methane oxidation by methanotrophs using oxygen as an electron acceptor (Xu & Zhang, 2022). Figure 2.3 shows the possible layout of methanotrophs and electron acceptors in a typical ICS.

Methane oxidation is a critical metabolic pathway involved in global carbon cycling. The oxidation of methane as a carbon and energy source by methanotrophic bacteria is crucial for regulating and reducing methane emissions. The metabolic pathways

for methane oxidation have been studied since the 1970s (Murrell, 2008). Various enzymes are involved in methane oxidation that produce by- and intermediate products such as water, methanol (CH_3OH), formaldehyde (CHOH), formate (CHOOH), and carbon dioxide. The pathways available for synthesis of cell materials from formaldehyde are the RuMP pathway catalyzed by Type I methanotrophs and the serine pathway catalyzed by Type II methanotrophs (Fig. 2.2). In this study, I focus on specific genes that encode various enzymes to understand the metabolic pathways available for methane in the studied landfill ICS. The impact of nitrogen as ammonium in the soil will be studied to determine how and by which microbes' nitrous oxide is produced. This study will give more insight into the genes involved in the metabolic pathways of methane oxidation and nitrous oxide production. A better understanding of the microbial community present in landfill ICSs may lead to the possibility of engineering controls to mitigate methane and nitrous oxide emissions.

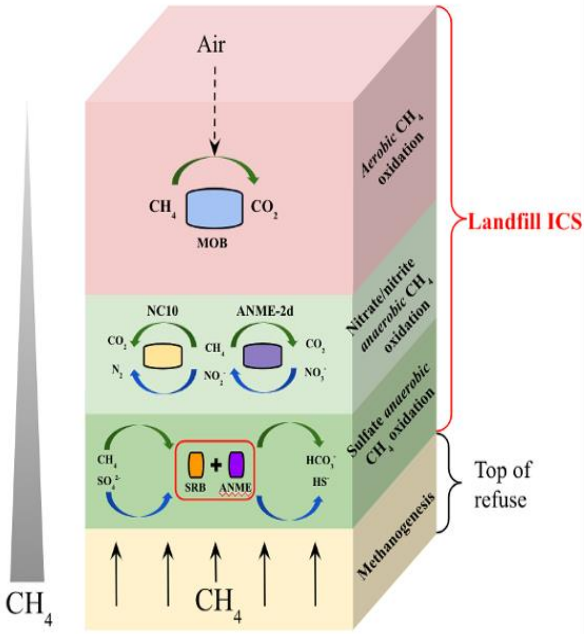

Figure 2.3. *Proposed spatial segregation of microbes and electron acceptors in ICS based on findings from Xu & Zhang, 2022*

Nitrous Oxide Production in Intermediate Cover Soils

In general, soils are the main source of nitrous oxide (N_2O) emissions in the atmosphere where agricultural soil management accounts for 73% of the United States N_2O emissions (EPA, 2023). More specifically, microbial nitrification and denitrification processes are responsible for about 70% of the global N_2O emissions (Butterbach-Bahl et al., 2013). There are also other abiotic processes, which are spontaneous reactions that do not require enzymes, that can lead to N_2O production. The biotic and abiotic processes for nitrous oxide production include autotrophic and heterotrophic nitrification, chemo-denitrification of soil nitrite and abiotic decomposition of ammonium nitrate, nitrifier-denitrification within the same micro-organism, coupled nitrification-denitrification by distinct micro-organisms, denitrification of freely available nitrogen oxides, co-denitrification with NO, and nitrate ammonification (Butterbach-Bahl et al., 2013). This wide range of possibilities leading to nitrous oxide production makes it difficult to determine the exact pathway of nitrous oxide emissions across various settings. However, this information is useful when looking at nitrous oxide production in landfill cover soils where there may be less available nitrogen for the microbes to consume.

There are many factors that can affect the nitrous oxide emissions from landfill ICSs. Methane oxidation and nitrous oxide production are impacted by many factors such as soil moisture content, nitrogen and carbon content in the soil, amount of bioavailable copper, the species of microbes present, and the specific enzymes produced by bacteria and archaea. These have been the primary focus of prior research on methane oxidation and nitrous oxide emissions from landfill ICSs. One study, conducted in 2009, focused on understanding how certain nutrients and other inhibitors impact methane oxidation and

nitrous oxide production while looking at key genes and the role of methanotrophs, nitrifiers, and denitrifiers (Lee et al., 2009).

Moisture content in soils impacts microbial activity. If soils are too wet, methanotrophic activity is reduced due to limited diffusion of methane and oxygen. Methanotrophs need oxygen when consuming methane to produce carbon dioxide and water, so methane oxidation is optimal at a lower moisture content. The rate of methane oxidation is optimum at 5% moisture content. Nitrous oxide production is minimized at the same moisture content. In contrast, maximum nitrous oxide production occurs at a moisture content of 20% (Lee et al., 2009). In my study, I chose 33.3% moisture content on a wet basis to allow for optimal mixing of substrate in the bottles. The substrate was at the consistency of a slurry at this moisture content. However, 33% moisture content did not prohibit methanotrophic activity and allowed microbes to catalyze methane oxidation and nitrous oxide production.

Nitrogen and organic carbon present in soil affects the rate of methane oxidation and nitrous oxide production. Both methane oxidation and nitrous oxide production increase as the amount of ammonium-N increases. In contrast, increases in added organic carbon did not affect methane oxidation, but did greatly increase the nitrous oxide production up to a certain level until it became inhibitory.

Bioavailable copper is a nutrient that is a key factor in regulating the expression of the genes encoding both the pMMO and sMMO in methanotrophs. Bioavailable copper measured in the study by Lee et al. (2009) was 0.2 +/- 0.01 mg/kg of soil which is very close to the copper measured in my study at a value of 0.25 mg/kg of soil. As mentioned previously, sMMO is expressed in conditions of copper deficiency while pMMO is

expressed in higher or varying copper concentrations. This is important because methanotrophs expressing the sMMO have a higher methane oxidation rate than those expressing the pMMO (Semrau, 2011). Adding copper inhibited methane oxidation rates and slightly decreased nitrous oxide production. Lee et al. (2009) also studied methanotrophic species *Methylosinus trichosporium* OB3b in pure culture under both sMMO and pMMO expressing conditions. In axenic culture, *M. trichosporium* OB3b could produce nitrous oxide from ammonia, where the cells expressing pMMO produce ~2.7x more nitrous oxide than those expressing sMMO (Lee et al., 2009).

The combined impacts of multiple factors on nitrous oxide production is important to understand. Lee et al. (2009) compared three different soil microcosms with 20% methane, 10% oxygen, 5% moisture content, and varying addition of ammonium and phenylacetylene (C_8H_6) to understand the differences in methanotrophic community composition. Phenylacetylene was added because it is a known inhibitor of ammonia monooxygenase (AMO), sMMO, and pMMO. When these enzymes were inhibited, all three soil microcosms became dominated by Type II methanotrophs, specifically the genus *Methylocystis*, a common resident in other landfills (Semrau, 2011; Cebron et al., 2007; Stralis-Pavese et al., 2004). The presence of Type I methanotrophs and the production of nitrous oxide increased with the sole addition of ammonium. In contrast, the microcosm containing ammonium and phenylacetylene exhibited an increase in methane oxidation while reducing nitrous oxide production and Type I methanotrophs. In conclusion, nitrous oxide production was a result of Type 1 methanotrophs expressing the pMMO undergoing methanotrophic nitrification (Lee et al., 2009).

Collectively, past studies suggest that nitrous oxide production depends on the soil's characteristics, microbial community composition, and the specific enzymes used during methane oxidation. It is known that the soil moisture content, carbon and nitrogen content in the soil, bioavailable copper, and microbial communities with different metabolic pathways all impact the rate of methane oxidation as well as nitrous oxide production. However, it is still unclear how nitrous oxide is produced by the microbial communities in landfill ICSs. In my study, I examined the impact of nitrogen on methane oxidation and nitrous oxide production during an incubation experiment. I tested the hypothesis that methanotrophs performing methane oxidation are also responsible for producing nitrous oxide. Whole genome shotgun sequencing, functional annotation, and taxonomic classification were used to determine the specific taxa of microbes in the studied ICS. Knowing the exact species is important because it will give insight into the metabolic pathways available for methane oxidation and nitrous oxide production. The DNA analyses will also identify the genes present in the microbes which describes the specific enzymes used during metabolic activity. Knowing the genes that encode for specific enzymes is crucial in understanding the exact metabolic pathways available. The results will increase our understanding of metabolic pathways that lead to the production of nitrous oxide.

CHAPTER 3

JOURNAL ARTICLE

METAGENOMIC INSIGHTS INTO LANDFILL INTERMEDIATE COVER SOIL MICROBIOMES AND THEIR POTENTIAL ROLE IN MITIGATING GREENHOUSE GAS EMISSIONS

TO BE SUBMITTED TO: SCIENCE OF THE TOTAL ENVIRONMENT

Abstract

Landfills are the third largest source of anthropogenic methane emissions in the United States and have also been found to produce nitrous oxide, an even more potent greenhouse gas than methane. Intermediate cover soils (ICS) play a major role in reducing greenhouse gas emissions from landfills because they harbor methanotrophic bacteria that degrade methane. However, the extent of methanotrophs in landfill cover soils and the roles of microbial communities in these complex ecosystems is only beginning to be understood. Additionally, the role of ICS in producing nitrous oxide is poorly understood. While methane consumption is a desirable trait, genes involved in methane oxidation may also promote nitrous oxide production. To better understand the role of ICS microbial communities in nitrogen and carbon cycling, samples were collected from twelve locations and two depths from ICS at an active municipal solid waste landfill. Metagenomic analyses using whole genome shotgun sequencing with the Oxford Nanopore platform was performed for all samples. Further, composite soil samples were incubated with methane and varying amounts of ammonia to assess the impacts of ammonia on nitrous oxide production. After an initial stabilization period, the incubations showed consumption of approximately 100,000 ppm of methane in the headspace in 9 days. All incubations with

methane produced small amounts of nitrous oxide (5 – 8 ppm) even when ammonia was not supplemented. Incubations without methane added, however, produced less nitrous oxide than those incubated with methane. The methanotrophs *Methylobacter* and an unclassified genus within the family Methlyococcaceae were present in the original ICS samples and the incubation samples, and their abundances increased in the incubation with *Methylobacter* being the dominant methanotroph. Other candidate methanotrophs were also enriched, including Verrucomicrobia. Genes encoding particulate methane monooxygenase/ ammonia monooxygenase (pMMO/AMO) were much more abundant than genes encoding soluble methane monooxygenase (sMMO) across the landfill ICS, but sMMO genes were enriched during the incubations with methane. Genes associated with nitrous oxide production via ammonia oxidation (the first process in nitrification) and denitrification were also present where only certain genes were enriched during the incubations. Genes encoding hydroxylamine oxidoreductase (the second step in ammonia oxidation) were largely absent. In total, these results suggest that ammonia oxidation via methanotrophs may result in low levels of nitrous oxide production, but ICS microbial communities have the potential to greatly reduce the overall global warming potential of landfill emissions.

Introduction

Landfills account for 15% of total methane emissions in the United States (EPA, 2023), and landfills may be a large contributor to anthropogenic nitrous oxide production (Rinne et al., 2005). Methane and nitrous oxide are both products of biochemical transformations that can occur within a landfill and in the soils above a landfill. Methane

results from anaerobic decomposition of organic materials in the landfill, and while methane can be captured and used for beneficial purposes, even well-designed landfills emit methane to the atmosphere. Routes of nitrous oxide emissions from landfills are not as well understood, but it likely occurs as a result of nitrogen cycling in soils above the landfill. Carbon and nitrogen cycling in intermediate cover soil (ICS) is of particular interest because these soils serve as a barrier between the landfill and the atmosphere and can potentially reduce GHG emissions. Although most landfills are eventually capped with an impermeable cap, landfill leakage is well-documented, and lessons learned through studying ICS can also be applied to mitigate emissions from capped landfills.

This high potential for ICS to reduce GHG emissions has made it a major focus of recent research, especially with respect to methane oxidation via methanotrophs. Methanotrophs are well-studied for their potential to reduce global methane emissions, and their occurrence in ICS has been established. Most methanotrophs are α- and γ-Proteobacteria, and it was found previously that methanotrophs of the α-Proteobacteria dominate in ICS. The genus *Methylocystis* dominates in soils with low carbon: nitrogen ratios, while *Methylobacter* species dominate in soils with higher carbon: nitrogen ratios (Semrau, 2011). One recent study also found that types of methanotrophs varied across different depths of ICS (Xu & Zhang, 2022). As oxygen levels increase closer to the surface of the ICS, bacteria that are able to perform aerobic oxidation of methane exist. The specific taxa and genomic potential of methanotrophs present in ICS is important because there are multiple routes for methane oxidation with varying levels of specificity and conversion rates.

Methane oxidation is initiated by the conversion of methane to methanol via one of two enzymes: particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO). While both of these enzymes are promiscuous and can oxidize many substrates, pMMO is a homolog of AMO, and they are nearly identical. As such, pMMO is regarded as a multifunctional enzyme that readily oxidizes ammonia to hydroxylamine. Hydroxylamine is a key intermediate of nitrification that can be converted to nitrite, but it can also be converted abiotically (i.e., no enzyme is needed for the transformation) to hydroxylamine. As such, methane oxidation has the potential to promote nitrous oxide production. ICS, however, harbors complex soil microbial ecosystems and nitrous oxide production may result from other nitrogen-cycling pathways, including nitrification and denitrification. The major routes of nitrous oxide production in ICS are not well understood, and there is potential for the same organisms that degrade methane to convert ammonia to nitrous oxide. This trade-off between consuming a potent GHG while producing an even more potent GHG has not been studied in ICS (Yu et al., 2009).

The objectives of this research were to quantify methane consumption and nitrous oxide production from enriched laboratory batch reactors with microbes derived from ICS. Further, the taxonomy and abundance of microorganisms in the laboratory enrichments and ICS samples from 12 locations and two depths were analyzed along with the key genes encoding enzymes present for methane oxidation, methane assimilation into biomass precursors, and nitrous oxide production. Overall, this study demonstrates the potential for microbes in ICS to oxidize methane and the impacts of methane oxidation on nitrous oxide production. Further, this study elucidates the metabolic potential of ICS microbiomes for reducing GHG emissions.

Materials & Methods

Soil Collection and Pre-Treatment

Samples of 2017 and 2018 ICS were collected from the Clinton County Municipal Solid Waste Landfill located in Morrisonville, New York. Samples were taken from 12 different sites on the landfill. At each site, about 50g of soil was collected from two different depths (15 and 30 centimeters) of ICS to determine the spatial segregation of the microbes. A total of 24 soil samples were collected. The location and elevation of each sampling site was also recorded (Table S1).

After collection, each sample was individually sieved using a 2-mm sieve to remove any big rocks or vegetation. 2.5 g of soil at field moisture content from each site was stored in the -80°C freezer for DNA extraction. The remainder of each sample was then air dried for about two days at room temperature. After drying, 200g of each sample was then combined to make a composite of all 24 soil samples for the incubation experiments. Two vials of the composite soil were taken and put in the -80°C freezer for DNA extraction and two aliquots of the composite soil were sent to UVM's Agricultural and Environmental Testing Lab for the analysis of pH, P, K, Ca, Mg, S, micronutrients, reactive Al, base saturation, organic matter, total nitrogen, and total carbon (Tables S2 and S3).

Incubation Experiment

Four treatments (Table 3.1) of ammonium levels were established to determine how the rate of methane oxidation and nitrous oxide production are affected by different ammonium levels in the soil. Each treatment was replicated three times for a total of twelve

125mL glass bottles. Each bottle was sealed with a butyl rubber stopper and was closed for the entirety of the 33-day incubation period.

Table 3.1. *Incubation treatment parameters*

Treatment	Soil Mixture	Gasses in Headspace
Control	30g composite soil + 15mL DI water	90 mL of air
Methane	30g composite soil + 15mL DI water	80 mL air + 10mL of CH_4
Ammonium 5	30g composite soil + 15mL of 5mg/L NH_4Cl solution	80 mL air + 10mL of CH_4
Ammonium 10	30g composite soil + 15mL of 10mg/L NH_4Cl solution	80 mL air + 10mL of CH_4

Measurements of the gasses in the headspace were taken every three days, starting with day 0 on July 15th, 2022, when the bottles were first closed. Methane concentration in the headspace along with carbon dioxide, nitrogen, and oxygen were measured using a Gas-Chromatograph equipped with a Thermal Conductivity Detector (GC-TCD, Shimadzu GC2030, Kyoto, Japan), while the nitrous oxide emissions were measured using a Photoacoustic Infrared Spectroscopy (PAS) gas analyzer calibrated as in Iqbal, Castellano, & Parkin (2013) (Model 1412i, Innova Air Tech Instruments, Ballerup, Denmark). Before the first measurements were taken and after the bottles were closed, 10mL of room air and 10mL of methane were injected into the headspace of the respective bottles. To do this, 25mL of either room air or methane was injected into the bottles using syringe needles. While the needle was still in the bottles, 15mL of gas was removed, leaving 10mL of the injected gas in the bottles. This was only done on day 0 because the GC-TCD required 10mL samples, so reinjection was unnecessary for day 0. Every time after using the GC-

TCD for the rest of the incubation, 10mL of room air or methane were reinjected into the bottles to maintain constant volume. However, day 15 was the last day of reinjecting methane into the headspace for respective bottles; room air was injected for the rest of the incubation to determine the rate and extent of methane oxidation by the microbial communities. Triplicates of nitrous oxide concentrations were measured for each bottle using the PAS machine (, but only single measurements for methane, carbon dioxide, nitrogen, and oxygen were taken using the GC-TCD (manufacturer, city) for each biological replicate. Raw data from the incubation experiments can be found in Tables S5 – S9. The incubation lasted for a total of 33 days.

Ammonia Analysis

Samples for ammonia testing were taken on the last day of the incubation where two vials containing 2.5g of the soil mixture from each bottle were centrifuged for 10 minutes at 10,000 rpm to separate the solids from the liquids. The liquids were transferred to new vials and stored in the -20°C freezer for ammonia testing using the Hach Ammonia Salicylate method (Hach Method 8155). The results can be found in Figures S4 and S5.

Metagenomic Analysis

Samples for DNA extractions were taken on the last day of the incubation where two vials containing 2.5g of the soil mixture from each bottle were centrifuged for 10 minutes at 10,000 rpm to separate the solids from the liquids. As stated in the previous section, the liquids were transferred to new vials and stored in the -20°C freezer for ammonia testing while the solids were stored in the -80°C freezer for DNA extraction. Two vials of soil samples from each site on the landfill at each depth at field moisture content

were stored in -80°C freezer for DNA extraction as well as two vials of the composite soil after air drying. DNA was extracted using commercial DNA extraction kits (Qiagen PowerSoil). DNA quantity and quality was assessed by Qubit and Nanodrop prior to submitting to the Vermont Integrated Genomics Resource for sequencing. Library preparation was performed using rapid PCR barcoding and sequencing was performed with Nanopore using a R 9.4.1 flow cell. Concentration or dilution of the DNA samples was conducted to achieve a concentration close to 20ng/uL measured on a Qubit. The resulting sequencing reads were analyzed using SqueezeMeta to annotate metagenomic reads and to assess abundance and taxonomy of organisms and specific functional genes involved in methane oxidation and nitrogen cycling within each sample (Tamames & Puente-Sanchez, 2019). Nanopore reads were functionally and taxonomically classified using the RefSeq reference database and default SqueezeMeta parameters (O'Leary et al., 2016). Specific genes and pathways of interest were identified based on KEGG and MetaCyc pathways (Kanehisa et al., 2023; Caspi et al., 2014).

Results & Discussion

Methane consumption and nitrous oxide production during incubations with ICS

Treatments with added ammonia or methane produced more nitrous oxide than the unamended controls (- CH4 / 0 NH4) (Figure 3.1). The incubations provided with methane but no ammonia (+ CH4 / 0 NH4), methane and 5 mg/L of ammonia (+ CH4 / 5 NH4), and methane and 10 mg/L of ammonia (+ CH4 / 10 NH4) all had a very similar trend of nitrous oxide production, meaning that higher initial ammonia concentrations did not result in higher nitrous oxide production. However, incubations in which methane was added had a

higher production of nitrous oxide than the incubation without methane added ($p = 4.11x10^{-9}$).

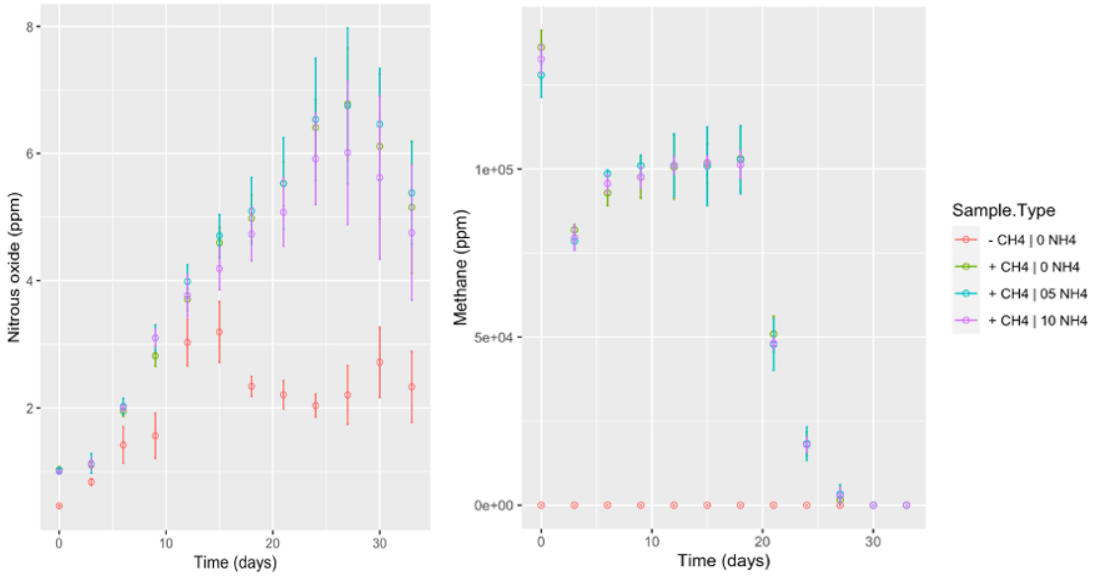

Figure 3.1. *Mean nitrous oxide concentrations (left) and mean methane concentrations (right) over 33-day incubation at room temperature (n = 3). Means and standard error bars are illustrated as open circles and vertical lines, respectively.*

The nitrous oxide production gradually accumulated through time as methane was being consumed by the microbes (Figure 3.1). However, a decrease in nitrous oxide concentrations occurred starting around day 27 which is the same day that almost all the methane was depleted in the bottles. This indicates that nitrous oxide is produced while methane oxidation is occurring, but nitrous oxide is consumed once methane is depleted and provides additional evidence that nitrous oxide production is promoted by methane oxidation. The level of nitrous oxide production, however, is low relative to the amount of methane consumed. A peak of 6 ppm nitrous oxide was observed in the incubations compared to consumption of 100,000 ppm of methane. Comparatively, the global warming potential of 100,000 ppm methane is approximately 1,000-fold higher than 6 ppm of nitrous

oxide. This suggests that microbes in ICS can greatly reduce the global warming potential of gasses emitted from landfills.

Relatively abundant microorganisms in ICS

Given the high level of methane consumption during incubations, metagenomic analyses were performed to further characterize the microbiome in ICS and after incubations. The microbial community present in both the ICS samples and incubations (Figures 3.2 – 3.4) included 24 phyla spanning the bacterial and archaeal kingdoms present at greater than 0.1% relative abundance. Proteobacteria was the most abundant phylum present across all the samples with relative abundances exceeding 25%. This phylum is the largest and most diverse among prokaryotes, encompassing all known methanotrophs, as well as a variety of nitrogen fixing, denitrifying, and ammonia oxidizing bacteria (Gupta, 2000). Proteobacteria have been identified as an abundant phylum in other ICSs (Long et al., 2016). No significant enrichment in proteobacteria, however, was observed after the incubations. Conversely, the Bacteroidetes phylum increased in relative abundance in incubation samples treated with methane (Ma, Mb, Mc, 5a, 5b, 5c, 10a, 10b, 10c). Bacteroidetes are a well-studied human gut microbe and are known for their ability to decompose a wide variety of complex carbohydrates (McKee et al., 2021). Enrichment of Bacteroidetes in soils incubated with methane has been shown by others (Heděnec et al., 2019). This suggests that members of the Bacteroidetes may play a potentially synergistic role in methane oxidation, but this synergy is not yet understood. The phylum Verrucomicrobia, a widespread and diverse phylum containing mostly uncultivated species with unknown phenotypes, was also enriched during incubations with and without added

methane. A previous study looked at one acidophilic methanotroph that belongs to the Verrucomicrobia phylum (Dunfield et al., 2007). Their analysis of the draft genomes of this methanotroph contained genes encoding the pMMO, leading to the possibility that this phylum has the ability to take part in methane oxidation. The phyla Actinobacteria and Planctomycetes showed a decrease in their abundance in the incubation samples compared to the landfill ICS samples. The phylum Nitrospirae had very low abundance in all the samples. This phylum contains nitrite oxidizing organisms and comammox organisms that convert nitrite and ammonia to nitrate, respectively.

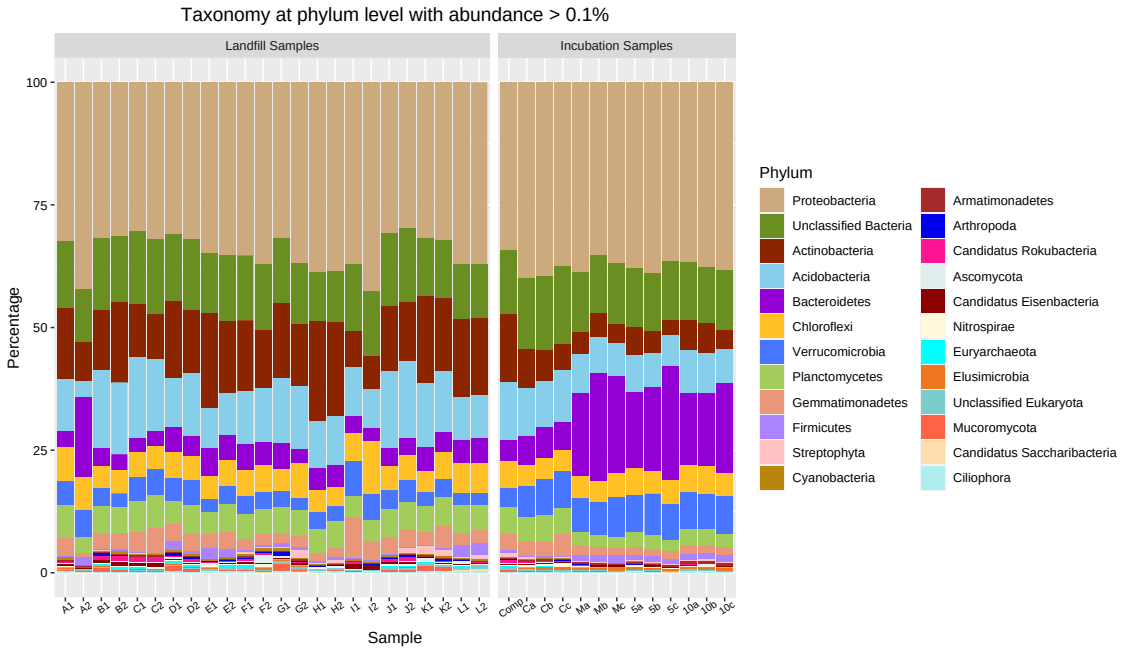

Figure 3.2. *Overall taxonomy at the phylum level with abundance greater than 0.1% in landfill ICS samples (A1 through L2), composite soil (Comp), and incubated samples (Ca/b/c, Ma/b/c, 5a/b/c, and 10a/b/c). (n = 37), e.g., 1 and 2 are samples taken 15cm or 30cm deep, respectively, A – L are locations, C, M, 5 and 10 are no methane or NH_4, methane without NH_4, methane with 5 units NH_4, and methane with 10 units NH_4, respectively.*

The top 20 most abundant families present in the samples were also assessed (Figure 3.3). Similar to the phyla, an unclassified family in Bacteroidetes phylum showed an enrichment with the incubation samples treated with methane. A unclassified family in

the Verrucomicrobia phylum also showed a slight increase in abundance with the incubation samples. The family Methylococcaceae, which contains type I methanotrophs in the γ- proteobacteria class (Nazaries et al., 2013), showed a distinct increase in abundance with the incubation samples treated with methane. This enrichment suggests that this methanotrophic family is responsible for the observed methane oxidation during incubations (Figure 3.1). The unclassified family of α- proteobacteria phylum showed a slight decrease in abundance from the landfill samples to the incubation samples. This family contains type II methanotrophs, suggesting that the methane oxidation is mostly due to type I methanotrophs in the Methylococcaceae family.

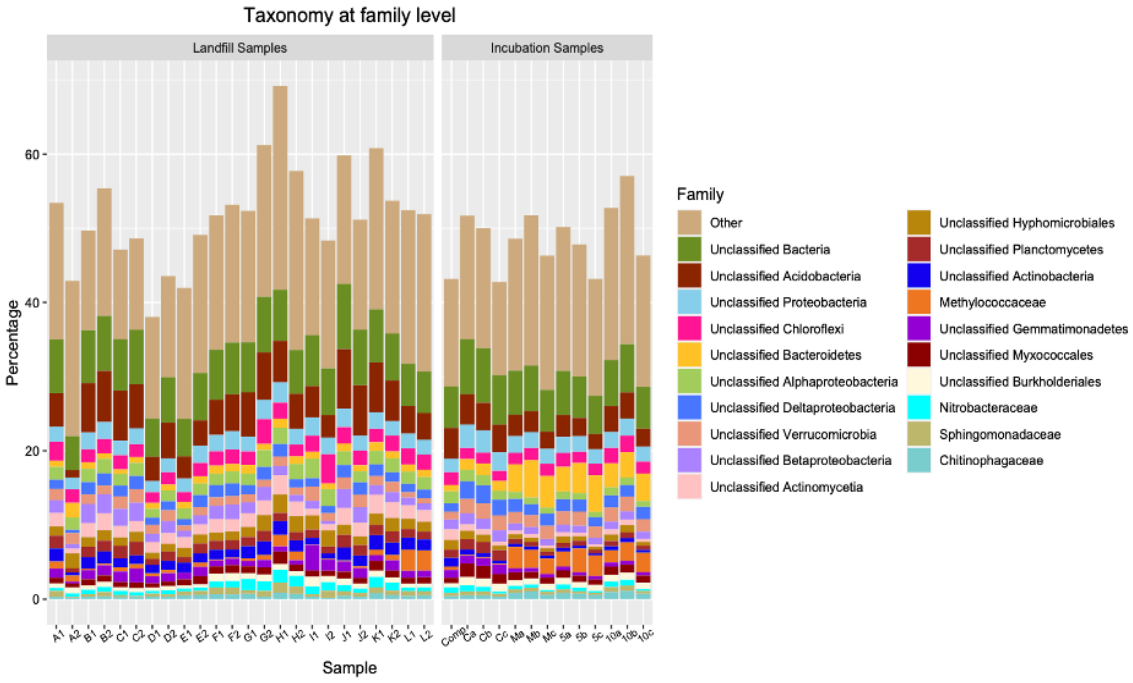

Figure 3.3. *Overall taxonomy at the family level in landfill ICS samples (A1 through L2), composite soil (Comp), and incubated samples (Ca/b/c, Ma/b/c, 5a/b/c, and 10a/b/c) where Unclassified family was ignored and represents the grey shading not listed in the legend.*

Assessment of taxa at the genus level (Figure 3.4) showed that the Methylococcaceae family enriched during the incubations (Type I methanotrophs) contained organisms classified as genera in *Methylobacter* or an unclassified genus in

Methylococcaceae. This finding suggests that the two Methylococcaceae genera are responsible for the methane oxidation occurring in the incubations. In total, the presence of known methanotrophic taxa, specifically type I methanotrophs (*Methylobacter* and Unclassified Methylococcaceae), indicates that these microbes were likely involved in methane consumption observed during the incubations. However, the ambiguity of some taxonomic classifications means that other methanotrophic organisms may be present. Specifically, an unclassified family in each Proteobacteria, α- proteobacteria, Verrucomicrobia, and Hyphomicrobiales may all contribute to methane oxidation, nitrous oxide production, or both. Therefore, further analysis of gene abundance was performed to elucidate potential metabolic pathways within the ICS that may impact carbon and nitrogen cycling.

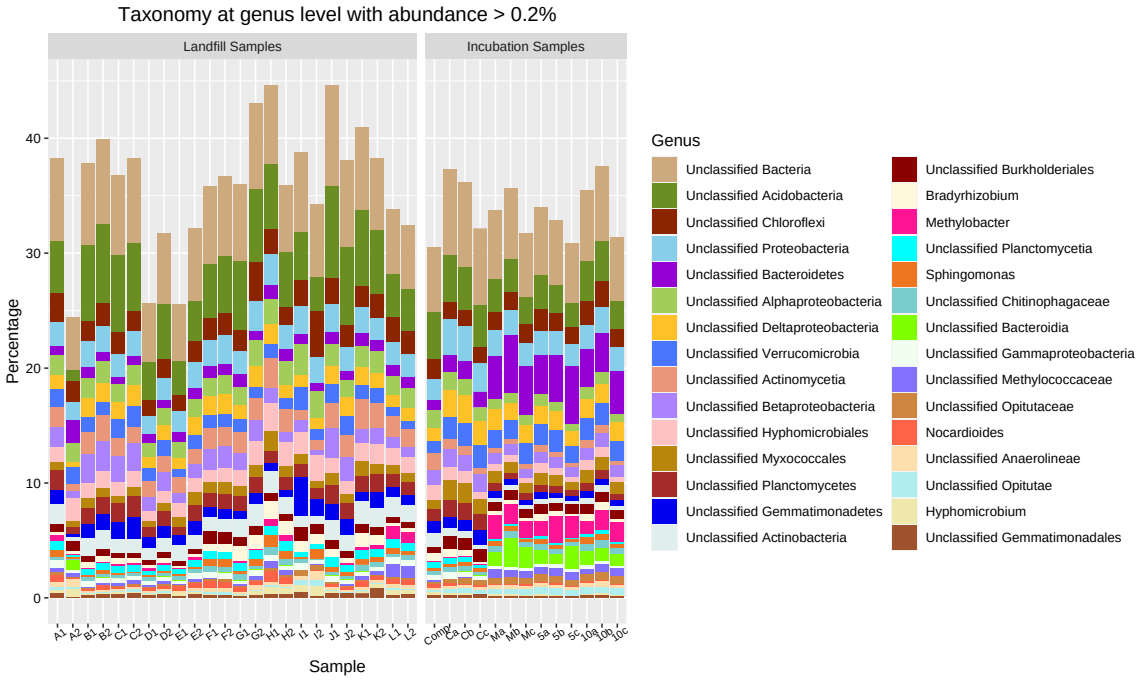

Figure 3.4. *Overall taxonomy at the genus level with abundance greater than 0.2% in landfill ICS samples (A1 through L2), composite soil (Comp), and incubated samples (Ca/b/c, Ma/b/c, 5a/b/c, and 10a/b/c).*

Metagenomics of methane oxidation and assimilation in landfill intermediate cover soils

Methanotrophy relies on the conversion of methane to formaldehyde. In the initial step, methane is oxidized to methanol with a methane monooxygenase. Two well-studied classes of enzymes exist for this conversion: particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO) (Hakemian & Rosenzweig, 2007). pMMO (pmo/amo) is a membrane bound multi-functional enzyme that catalyzes the oxidation of methane to methanol as well as ammonia to hydroxylamine. pMMO contains three subunits encoded by pmoA/amoA, pmoB/amoB, and pmoC/amoC, and pMMO is used by all known methanotrophs (Hanson & Hanson, 1996; Lieberman & Rosenzweig, 2004). sMMO (mmo) is a soluble, cytoplasmic enzyme that is also able to oxidize other substrates, such as ammonia and carbon monoxide, and is associated with Type II methanotrophs but has also been found in Type I methanotrophs (Hanson & Hanson, 1996). The sMMO contains three subunits encoded by mmoX, mmoY, and mmoZ (alpha, beta and gamma subunits, respectively), along with regulatory protein (encoded by mmoB) and a reductase (encoded by mmoC). Another major difference between the two enzymes is that pMMO relies on copper while sMMO relies on iron for the catalytic site, and high copper availability has been shown to promote pMMO activity while low copper availability promotes sMMO (Khider et al., 2021).

There was a much greater abundance of pMMO than sMMO across the samples collected from ICS (Fig. 3.5). All three pMMO subunits were present in the composite samples prior to incubation. pMMO being more abundant than sMMO is expected because most methanotrophs that encode sMMO also encode pMMO (Khider et al., 2021); however, sMMO gene abundance is approximately one order of magnitude less than

pMMO genes in ICS. During incubations, pMMO genes decreased abundance in control experiments in which methane was not added. While remaining less abundant than pMMO genes, sMMO genes increased in abundance during the incubation experiments. The complete lack of mmoD across these samples is noteworthy in that the role of this gene in encoding a regulatory protein associated with sMMO is disputed (Kim et al., 2019; Semrau et al., 2013) and is not required for enzyme function (Merkx & Lippard, 2002). Our results suggest that methanotrophs found in ICS do not contain genes that encode this protein.

After methane oxidation to methanol via pMMO or sMMO, methanol is oxidized to formaldehyde via one of four known enzymes: a cytochrome c -dependent methanol dehydrogenase (EC 1.1.2.7; mdh/mxa), a NADH or NADPH-dependent methanol dehydrogenase (EC 1.1.1.224; mdh), a lanthanide-dependent methanol dehydrogenase (xoxF), or an alcohol: oxygen oxidoreductase (mox). Genes encoding the mdh/mxa complex were most abundant across the ICS and the incubations (Figure 3.5), and these genes increased abundance after incubation. The gene encoding mox was only found in one ICS sample. Neither xoxF or mdh were found in any of the landfill or incubation samples. These results suggest that the mdh/mxa complex is the most widely used by methanotrophs in ICS.

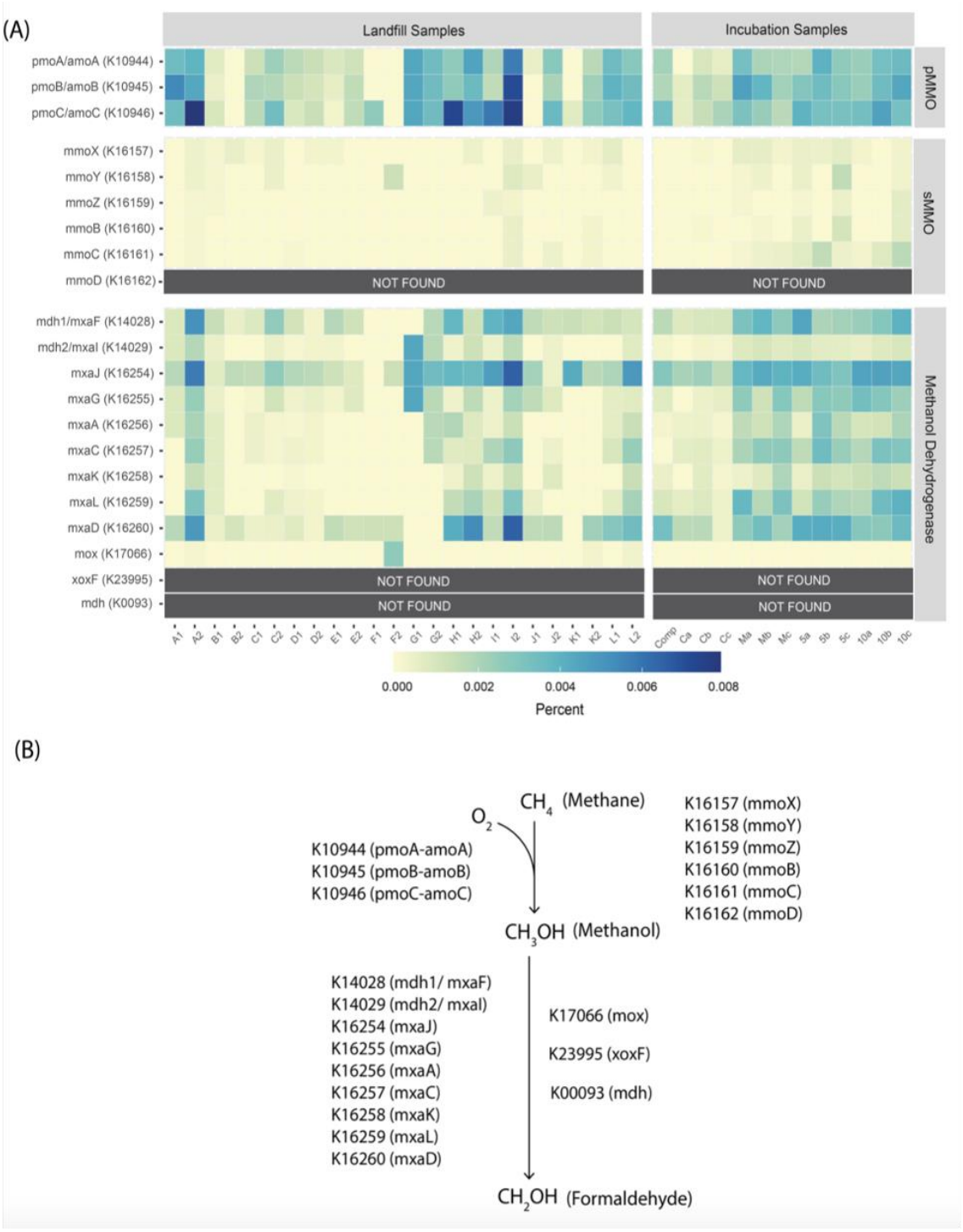

Figure 3.5. *Abundance of genes involved in transformations of methane to formaldehyde in methane oxidation pathway (A), and metabolic pathway of methane to formaldehyde with respective genes (B).*

Formaldehyde is a key intermediate for all known methanotrophic bacteria. It is commonly recognized that Type I methanotrophs convert formaldehyde to biomass precursors with the ribulose monophosphate (RuMP) pathway while Type II methanotrophs incorporate formaldehyde into biomass precursors via the serine pathway. Therefore, we investigated the abundance of genes for both of these pathways in the landfill ICS and incubations (Figure 3.6). The metagenomic results show that genes encoding enzymes involved with the serine pathway (indicative of Type II methanotrophs) were much more abundant than those encoding enzymes for the RuMP pathway. This is likely due to the wide use of genes associated with the RuMP pathway. The initial step of the serine pathway (converting formaldehyde to a 5,10 methylene tetrahydrofolate) is spontaneous (Kallen & Jencks, 1966), thus no gene is associated with this reaction. The second step, which converts glycine to L-serine (encoded by glyA, K00600), is ubiquitous and widely used by bacteria to synthesize the amino acid serine (Florio et al., 2011). The third step involves a serine: glyoxylate aminotransferase (encoded by sgaA, K00830) and this enzyme is present across bacteria and eukaryotes (Zhang et al., 2013; Izumi et al., 1990). The less abundant genes hprA (K00018), mtkA (K14067), mtkB (K08692), and mcl (K08691) may all be indicative of the serine pathway used by methanotrophs (Chistoserdova & Lidstrom, 1994; Chistoserdova & Lidstrom, 1994) and align more closely with the abundance of genes encoding sMMO (Figure 3.6), which is expected to be more common in Type II methanotrophs than Type I methanotrophs.

Overall, the metagenomic results suggest that pMMO is much more abundant than sMMO in landfill ICS and is likely the major route for methane oxidation in the landfill ICS tested. It should be noted that the composite soils had a high concentration of copper

(0.3 ppm, Table S3) which likely plays a role in the high abundance of pMMO since it is a required cofactor. However, when composite ICS samples were incubated with methane, genes encoding sMMO were enriched (Figure 3.5). Results also show that genes encoding the RUMP pathway and serine pathway were present across landfill samples and RUMP pathway genes increased in abundance during incubations with methane while genes associated with the serine pathway decreased. This is unexpected in that sMMO and the serine pathway are expected to show similar patterns as both are commonly associated with Type II methanotrophs. Therefore, these results suggest that sMMO can be enriched independent of serine pathway genes and may be used by organisms using pMMO.

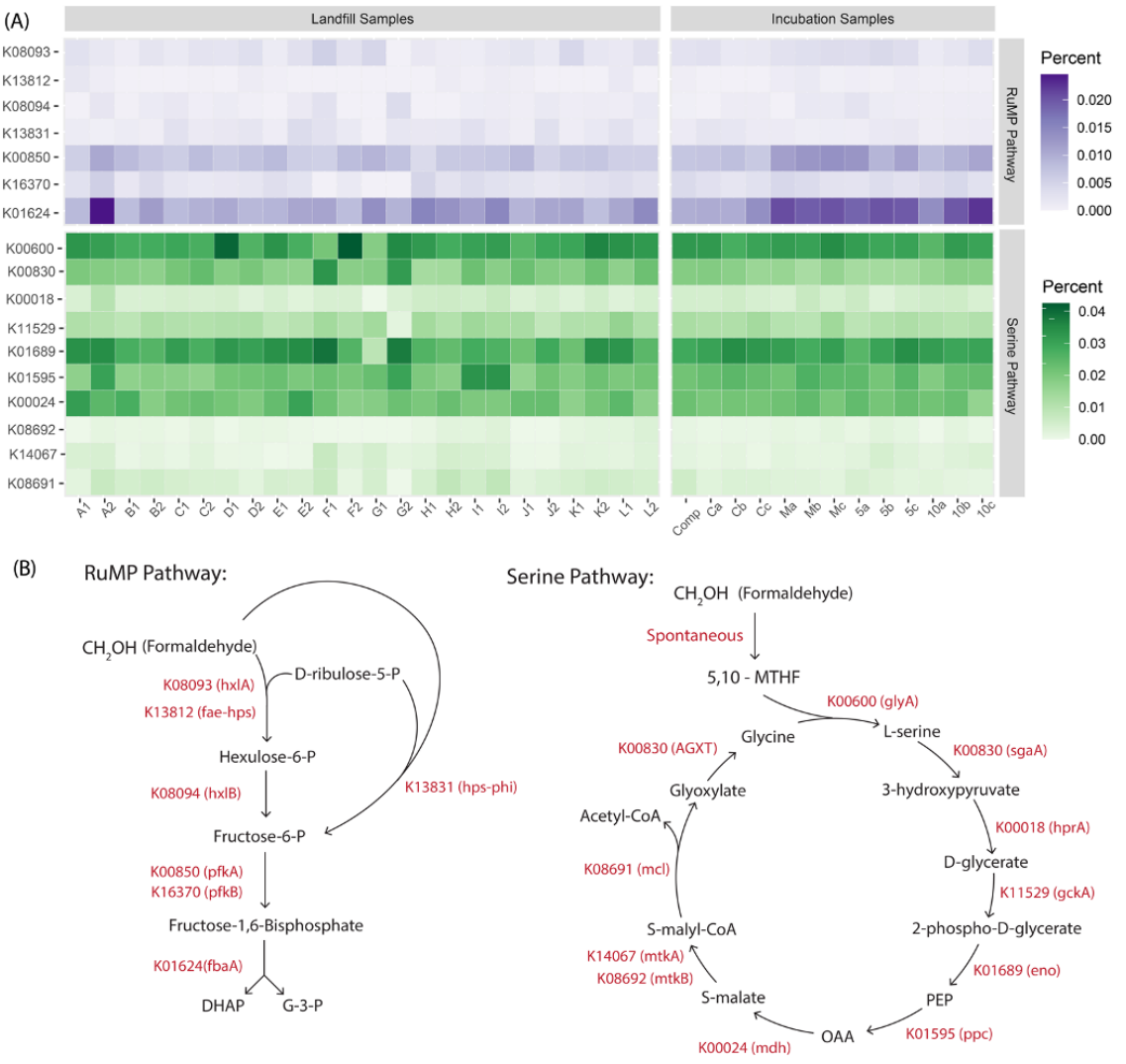

Figure 3.6. *Abundance of genes involved in the Serine and RuMP pathways for methane oxidation (A), RuMP and Serine metabolic pathways with respective genes (B).*

Metagenomics of nitrous oxide production in landfill intermediate cover soils

Factors impacting nitrous oxide production by soils are complex, but both ammonia oxidation and denitrification are expected to contribute to nitrous oxide emissions (Butterbach-Bahl et al., 2013). One proposed source of nitrous oxide is the abiotic conversion of hydroxylamine to nitrous oxide (Butterbach-Bahl et al., 2013). Hydroxylamine is the first intermediate in ammonia oxidation and can result from previously discussed pMMO and sMMO activity. As such, nitrous oxide production may

be a direct result of methanotrophy. Additionally, nitrous oxide is an intermediate of several biotic nitrogen conversion pathways and may be produced by organisms involved in nitrogen cycling in ICS. Furthermore, copper depletion by methanotrophs may increase nitrous oxide production as copper is a required metal in several nitrogen conversion enzymes (Chang et al., 2022). Lastly, nitrous oxide increased in incubations with methane added to ICS. Therefore, to investigate potential routes of nitrous oxide production we assessed the abundance of nitrogen cycling genes across the landfill ICS and during incubations with methane.

While pmoA/amoA, pmoB/amoB, and pmoC/amoC were abundant across landfill samples and after incubations with methane, the gene that encodes hydroxylamine dehydrogenase (hao, K10535) was not found in all samples and was much less abundant than the pMMO genes. Along with the low abundance of taxa known to perform nitrification, this suggests that nitrification is not a major route for N_2O production. However, this does not rule out that pMMO/sMMO may produce hydroxylamine which can be abiotically transformed into nitrous oxide. Due to the lack of hao genes, we also investigated the abundance of tetrathionate reductase genes: ttrA (K08357), ttrB (K08358) and ttrC (K08359). Tetrathionate reductase has been shown to catalyze the conversion of nitrite and hydroxylamine to ammonia. In the reverse, these enzymes could catalyze the conversion of ammonia to hydroxylamine and hydroxylamine to nitrite. We found that these genes were present in ICS samples, though at lower abundance than other nitrogen cycling genes. Further, we did not see any enrichment of these genes during incubations. Genes associated with denitrification were more abundant than nitrification genes, suggesting abundant bacterial populations are capable of respiring nitrate. Nitric oxide

reductase is composed of two subunits, NorB (encoded by norB, K04561) and NorC (encoded by NorC, K02305). NorB was highly enriched during incubations, but norC was much less abundant in landfill ICS and incubated samples. Genes encoding NirK (nirK, K00368), which converts nitrite to nitric oxide were regularly the most abundant denitrification gene in the ICS. Surprisingly, nitrogen fixation genes, specifically those encoding nifDKH showed a high enrichment in incubations with methane, even when ammonia was supplemented. It should be noted that at the time of sample collection for metagenomics, most of the ammonia was depleted in the incubations and final measured ammonia concentrations were similar across all bottles where ammonia was added (Figure S5).

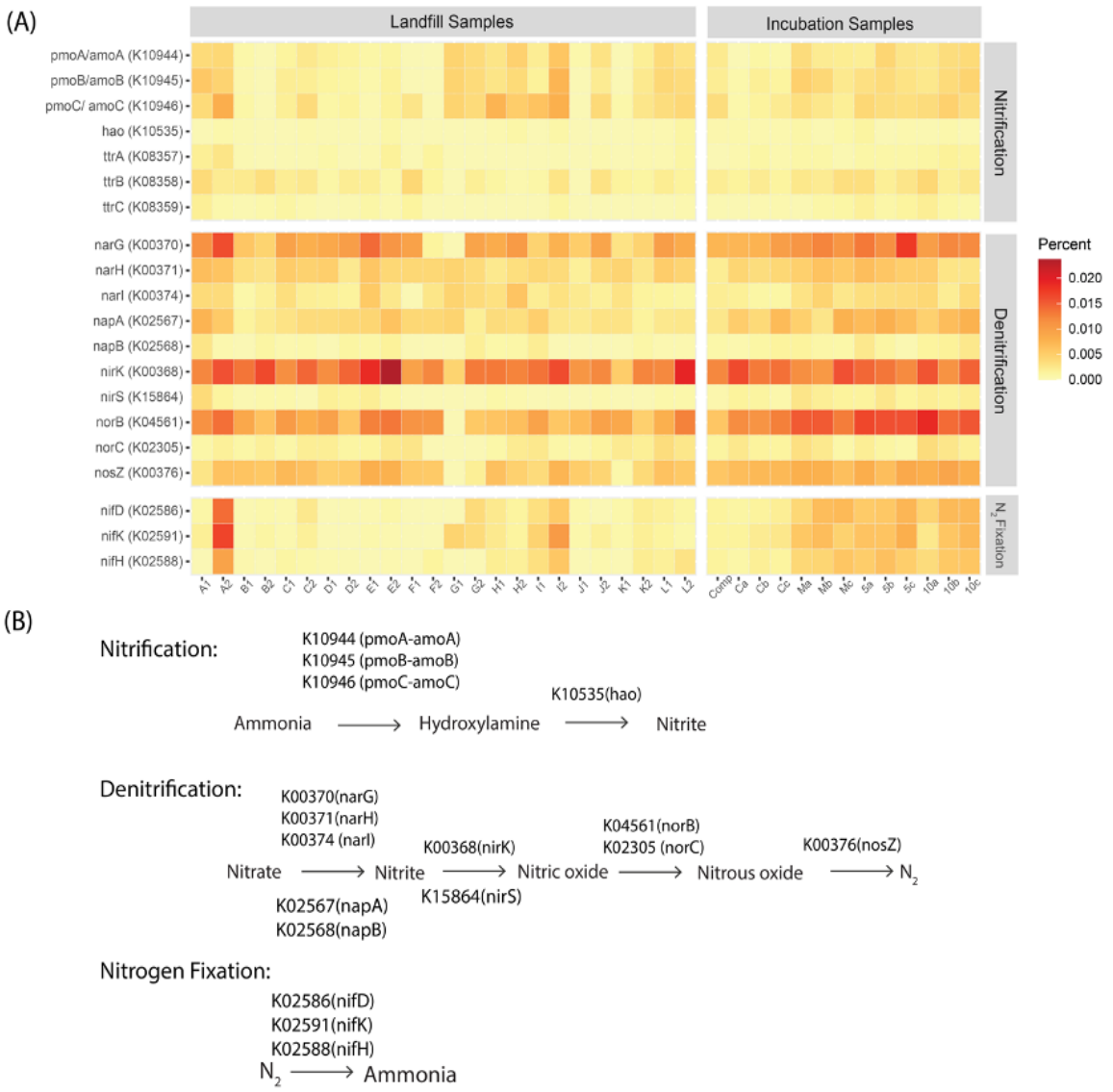

Figure 3.7. *Nitrogen metabolism gene abundance in landfill cover soil samples, composite soil, and incubated samples (A) and nitrogen conversion pathways and associated genes (B).*

Conclusion

Landfills are a major contributor to climate change through greenhouse gas emissions, but they are critical systems for waste disposal and preventing pollution. As the human population continues to grow, the need for sustainable waste management practices is ever increasing. The presence of organic waste in landfills is the main reason why landfills are the third largest source of methane emissions in the United States (EPA, 2023).

However, landfill ICSs can play a crucial role in preventing methane emissions from landfills.

In this study, the microbiomes in landfill ICS were studied to investigate methane oxidation and nitrous oxide production through incubation experiments and metagenomic analyses. Methane oxidation increased nitrous oxide production in 33-day incubation experiments, but ammonia concentration did not significantly impact nitrous oxide production. Type I methanotrophs of the genus *Methylobacter* and *Unclassified Methylococcaceae* were enriched during incubations of ICS with methane. The abundance of key genes involved in methane oxidation and nitrogen cycling were also assessed to determine the available metabolic pathways for methane oxidation, methane assimilation to biomass precursors, and nitrous oxide production. Genes encoding both particulate and soluble methane monooxygenase enzymes (pMMO and sMMO, respectively) were present and pMMO genes were more abundant across ICS and after incubations. However, the sMMO genes increased abundance during incubation whereas the pMMO genes did not.

The RuMP pathway is commonly used by type I methanotrophs, while type II methanotrophs utilize the Serine pathway (Koo & Rosenzweig, 2021). The results showed that genes encoding the RUMP pathway and serine pathway were present across landfill samples and RUMP pathway genes increased in abundance during incubations with methane while genes associated with the serine pathway decreased. Often the sMMO and serine pathway are expected to show similar patterns as both are commonly associated with Type II methanotrophs. These unexpected results suggest that sMMO can be enriched independent of serine pathway genes and may be used by organisms using pMMO. In total,

these results suggest that sMMO may be more abundant in Type 1 methanotrophs than previously thought.

The genes involved in nitrogen cycling were also assessed to identify potential routes for nitrous oxide production. Most genes for denitrification were abundant across ICS but not enriched during incubations. While genes for the initial step in ammonia oxidation to convert ammonia to hydroxylamine (pmoABC/amoABC) were abundant, the genes encoding hydroxylamine oxidoreductase were much less abundant. This suggests that ammonia oxidizing organisms do not play a major role in nitrous oxide production, but nitrous oxide production from denitrification of nitrate present in the ICS used during incubations cannot be ruled out. Further, pMMO produced by methanotrophs may convert ammonia to hydroxylamine which can be abiotically transformed to nitrous oxide.

The results of this study demonstrate that methanotrophs can play a critical role in preventing methane emissions from landfills. While nitrous oxide production by microbes in ICS is a potential concern, the ICS tested here produced very small amounts of nitrous oxide. This may be due to the low organic matter content and high copper content of the particular ICS tested and more analyses are needed to identify ICS conditions that promote methanotrophy while minimizing nitrous oxide production. This research also investigates a critical link between carbon and nitrogen cycling and further understanding the links between methane oxidation and nitrous oxide production is needed and could help reduce global warming. As the planet continues to warm, humans must learn how to harness the natural processes of microbes to reduce methane and nitrous oxide emissions.

Acknowledgements

Peyton Lienhart would like to thank Venus Rohra and Courtney Clement for conducting the preliminary analyses of this research. She also extends her thanks to Amy DeCola for guidance through the metagenomic analysis aspect of this study, as well as those at the Casella owned Clinton County Municipal Solid Waste Landfill in Morrisville, New York for their support in sample collection and analysis. Peyton Lienhart was supported by NASA through the Vermont Space Grant Consortium.

References

1412i. LumaSense Technologies A/S. (n.d.). https://innova.lumasenseinc.com/manuals/1412i/

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., & Zechmeister -Boltenstern, S. (2013). Nitrous oxide emissions from soils: How well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1621), 20130122. https://doi.org/10.1098/rstb.2013.0122

Caspi, R., Altman, T., Billington, R., Dreher, K., Foerster, H., Fulcher, C. A., Holland, T. A., Keseler, I. M., Kothari, A., Kubo, A., Krummenacker, M., Latendresse, M., Mueller, L. A., Ong, Q., Paley, S., Subhraveti, P., Weaver, D. S., Weerasinghe, D., Zhang, P., & Karp, P. D. (2014). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic acids research*, *42*(Database issue), D459–D471. https://doi.org/10.1093/nar/gkt1103

Chistoserdova, L. V., & Lidstrom, M. E. (1994a). Genetics of the serine cycle in Methylobacterium extorquens AM1: Identification of sgaa and MTDA and sequences of sgaa, hpra, and MTDA. *Journal of Bacteriology*, *176*(7), 1957–1968. https://doi.org/10.1128/jb.176.7.1957-1968.1994

Chistoserdova, L. V., & Lidstrom, M. E. (1994b). Genetics of the serine cycle in *Methylobacterium extorquens* AM1: Identification, sequence, and mutation of three new genes involved in C1 assimilation, orf4, mtka, and mtkb. *Journal of Bacteriology*, *176*(23), 7398–7404. https://doi.org/10.1128/jb.176.23.7398 -7404.1994

Chang, J., Peng, P., DiSpirito, A. A., & Semrau, J. D. (2022). Variable inhibition of nitrous oxide reduction in denitrifying bacteria by different forms of methanobactin. *Applied and Environmental Microbiology*, *88*(7). https://doi.org/10.1128/aem.02346-21

Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B., Hou, S., Ly, B., Saw, J. H., Zhou, Z., Ren, Y., Wang, J., Mountain, B. W., Crowe, M. A., Weatherby, T. M., Bodelier, P. L., Liesack, W., Feng, L., Wang, L., & Alam, M. (2007). Methane oxidation by an extremely acidophilic bacterium of the Phylum Verrucomicrobia. *Nature*, *450*(7171),879–882. https://doi.org/10.1038/nature06411

Environmental Protection Agency. (2023b). *Overview of Greenhouse Gases*. EPA. https://www.epa.gov/ghgemissions/overview-greenhouse-gases

Florio, R., di Salvo, M. L., Vivoli, M., & Contestabile, R. (2011). Serine hydroxymethyltransferase: A model enzyme for mechanistic, structural, and evolutionary studies. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1814*(11), 1489–1496. https://doi.org/10.1016/j.bbapap.2010.10.010

Gupta, R. S. (2000). The phylogeny of proteobacteria: Relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiology Reviews*, *24*(4), 367–402. https://doi.org/10.1111/j.1574- 6976.2000.tb00547.x

Hakemian, A. S., & Rosenzweig, A. C. (2007). The biochemistry of methane oxidation. *Annual Review of Biochemistry*, *76*(1), 223–241. https://doi.org/10.1146/annurev.biochem.76.061505.175355

Hanson, R. S., & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiological Reviews*, *60*(2), 439–471. https://doi.org/10.1128/mr.60.2.439-471.1996

Heděnec, P., Angel, R., Lin, Q., Rui, J., & Li, X. (2019). Increased methane concentration alters soil prokaryotic community structure along an artificial pH gradient. *Annals of Microbiology*, *69*(4), 329–339. https://doi.org/10.1007/s13213-018-1421-4

Izumi, Y., Yoshida, T., & Yamada, H. (1990). Purification and characterization of serine-glyoxylate aminotransferase from a serine-producing methylotroph, *Hyphomicrobium methylovorum* GM2. *European Journal of Biochemistry*, *190*(2), 285–290. https://doi.org/10.1111/j.1432-1033.1990.tb15574.x

Kallen, R. G., & Jencks, W. P. (1966). The mechanism of the condensation of formaldehyde with tetrahydrofolic acid. *Journal of Biological Chemistry*, *241*(24), 5851–5863. https://doi.org/10.1016/s0021-9258(18)96350-7

Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M. and Ishiguro-Watanabe, M.; KEGG for taxonomy-based analysis of pathways and genomes, *Nucleic Acids Research*, Volume 51, Issue D1, 6 January 2023, Pages D587-D592, https://doi.org/10.1093/nar/gkac963

Khider, M. L., Brautaset, T., & Irla, M. (2021). Methane monooxygenases: Central Enzymes in methanotrophy with promising biotechnological applications. *World Journal of Microbiology and Biotechnology*, *37*(4). https://doi.org/10.1007/s11274-021-03038-x

Kim, H., An, S., Park, Y. R., Jang, H., Yoo, H., Park, S. H., Lee, S. J., & Cho, U.-S. (2019). MMOD-induced structural changes of hydroxylase in soluble methane monooxygenase. *Science Advances*, *5*(10). https://doi.org/10.1126/sciadv.aax0059

Lieberman, R. L., & Rosenzweig, A. C. (2004). Biological methane oxidation: Regulation, biochemistry, and active site structure of particulate methane monooxygenase. *Critical Reviews in Biochemistry and Molecular Biology*, *39*(3), 147–164. https://doi.org/10.1080/10409230490475507

Long, X.-E., Wang, J., Huang, Y., & Yao, H. (2016). Microbial community structures and metabolic profiles response differently to physiochemical properties between three landfill cover soils. *Environmental Science and Pollution Research*, *23*(15), 15483–15494. https://doi.org/10.1007/s11356-016-6681-6

McKee, L. S., La Rosa, S. L., Westereng, B., Eijsink, V. G., Pope, P. B., & Larsbrink, J. (2021). Polysaccharide degradation by the Bacteroidetes: Mechanisms and nomenclature. *Environmental Microbiology Reports*, *13*(5), 559–581. https://doi.org/10.1111/1758-2229.12980

Merkx, M., & Lippard, S. J. (2002). Why OrfY? Characterization of MMOD, a long overlooked component of the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *Journal of Biological Chemistry*, *277*(8), 5858 –65. https://doi.org/10.1074/jbc.M107712200

Nazaries, L., Murrell, J. C., Millard, P., Baggs, L., & Singh, B. K. (2013). Methane, microbes and models: Fundamental understanding of the soil methane cycle for future predictions. *Environmental Microbiology*, *15*(9), 2395–2417. https://doi.org/10.1111/1462-2920.12149

Nitrogen-ammonia reagent set, salicylate method, 10 ml. Hach. (n.d.). https://www.hach.com/p-nitrogen-ammonia-reagent-set-salicylate-method-10-ml/2668000

O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., Farrell, C. M., … Pruitt, K. D. (2016). Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic acids research*, *44*(D1), D733–D745. https://doi.org/10.1093/nar/gkv1189

Rinne, J., Pihlatie, M., Lohila, A., Thum, T., Aurela, M., Tuovinen, J.-P., Laurila, T., & Vesala, T. (2005). Nitrous oxide emissions from a municipal landfill. *Environmental Science & Technology*, *39*(20), 7790–7793. https://doi.org/10.1021/es048416q

Semrau, J. D. (2011). Current knowledge of microbial community structures in landfills and its cover soils. *Applied Microbiology and Biotechnology*, *89*(4), 961–969. https://doi.org/10.1007/s00253-010-3024-2

Semrau, J. D., Jagadevan, S., DiSpirito, A. A., Khalifa, A., Scanlan, J., Bergman, B. H., Freemeier, B. C., Baral, B. S., Bandow, N. L., Vorobev, A., Haft, D. H., Vuilleumier, S., & Murrell, J. C. (2013). Methanobactin and MMOD work in concert to act as the 'copper-switch' in methanotrophs. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12150

Tamames, J., & Puente-Sánchez, F. (2019). SqueezeMeta, a highly portable, fully automatic metagenomic analysis pipeline. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.03349

Xu, S., & Zhang, H. (2022). First evidence for anaerobic oxidation of methane process in landfill cover soils: Activity and responsible microorganisms. *Science of The Total Environment*, *841*, 156790. https://doi.org/10.1016/j.scitotenv.2022.156790

Yu, T., He, P.-J., Lü, F., & Shao, L.-M. (2009). Mediating N2O emissions from municipal solid waste landfills: Impacts of landfill operating conditions on community structure of ammonia- oxidizing bacteria in cover soils. *Ecological Engineering*, *35*(5), 882–889. https://doi.org/10.1016/j.ecoleng.2008.12.025

Zhang, Q., Lee, J., Pandurangan, S., Clarke, M., Pajak, A., & Marsolais, F. (2013). Characterization of *Arabidopsis* serine:glyoxylate aminotransferase, AGT1, as an asparagine aminotransferase. *Phytochemistry*, *85*, 30–35. https://doi.org/10.1016/j.phytochem.2012.09.017

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

From this research, the studied landfill ICS was found to contain a very diverse microbial community containing methanotrophs that are capable of performing methane oxidation. The incubation experiment also showed low levels of nitrous oxide production during methane consumption by the microbes. The most abundant genera of methanotrophs were the *Methylobacter* and an unclassified genus in Methylococcaceae, both Type I methanotrophs (Nazaries et al., 2013). The abundance of both increased with the addition of methane during the incubation as the primary substrate. The phyla Bacteroidetes, Bacteroidia, and Verrucomicrobia also showed an enrichment with the addition of methane, suggesting that they might play a role in methane oxidation or nitrous oxide production.

At the gene-level, the particulate and soluble methane monooxygenase (pMMO and sMMO, respectively) were found in samples from the landfill ICS and after the incubation with methane. Genes encoding the pMMO enzyme are present in almost all methanotrophs, whereas genes encoding sMMO are less common in methanotrophs (Hanson & Hanson, 1996). Both enzymes are also known to perform ammonia oxidation where hydroxylamine is produced as a byproduct.. The RuMP pathway is often associated with Type I methanotrophs utilizing the pMMO enzyme whereas the Serine pathway is commonly associated with type II methanotrophs, some of which contain sMMO (Koo & Rosenzweig, 2021). The results showed that genes encoding the RuMP pathway and serine pathway

were present across landfill samples, and RuMP pathway genes increased in abundance during incubations with methane while genes associated with the serine pathway decreased. This is interesting since the sMMO enzymes showed an enrichment with the incubation while the genes involved in the Serine pathway did not. These results suggest that sMMO can be enriched independent of serine pathway genes and may be used by organisms using pMMO.

In terms of nitrogen cycling, the genera *Bradyrhizobium, Nocardioides,* and an unclassified genus of Actinobacteria were present in all the samples where their abundances slightly decreased in the incubation samples treated with methane. Contrastingly, the genes involved in nitrogen metabolism were present across all samples and showed an enrichment with the incubation experiment, specifically those treated with methane. The denitrification and nitrogen fixation genes were more abundant than those involved in nitrification, suggesting that the nitrogen gas produced during the incubation was a result of denitrification. However, primary routes for nitrous oxide production are still unknown and its production may have been a result of abiotic processes occurring spontaneously. As an example, nitrous oxide can be a result of the pMMO and/or sMMO enzymes performing ammonia oxidation where hydroxylamine is an intermediate product. Once hydroxylamine is produced, no enzymes are further needed to transform hydroxylamine to produce nitrous oxide (Butterbach-Bahl et al., 2013).

Recommendations for Future Work

Landfill ICSs are a valuable aspect of waste management that help reduce methane emissions from the refuse of landfills. However, additional research is needed to fully

understand the microbial processes occurring in the ICS, specifically methanotrophs and other organisms that are capable of consuming methane and producing nitrous oxide. Although the type of methanotrophs is often associated with specific enzymes (i.e., pMMO and sMMO) and metabolic pathways (i.e., RuMP and Serine), this research showed that there is ambiguity in that some methanotrophs can produce both enzymes and utilize either pathway for methane oxidation and incorporation of methane into cellular biomass.

This study only looked at how the addition of nitrogen as ammonia in the incubation experiment impacted the methane oxidation and nitrous oxide production. There did not seem to be a correlation between the ammonium chloride in the soil and the rate of methane oxidation or nitrous oxide production. Further research should be conducted to see how copper augmentation or depletion impacts methane consumption and nitrous oxide production. Similar incubation experiments could be done with varying copper concentrations in the soil. Additionally, this study could be paired with a meta-transcriptomic analysis to not only determine the genomic potential of the microbiome, but also to assess which genes are actively transcribed and if copper impacts gene expression in landfill ICS.

For the metagenomic analysis, this work used SqueezeMeta to assemble nanopore reads into contigs and to assign taxonomy to the assembled contigs using the NCBI refseq database that contains sequences of more than 160,000 microorganisms. A “gene-centric” approach was used to identify abundant genes within the microbial community. Future work with similar data sets could include a “genome-centric” approach where contigs are binned into metagenome-assemble genomes (MAGs) that represent draft genome sequences of a population. MAGs represent species-level population genomes that can

assess the abundance of specific organisms within the samples. This may lead to a more detailed taxonomy analysis that can help further understand the microorganisms responsible for either methane oxidation or nitrous oxide production.

Furthermore, field-scale studies could further our understanding of GHG emissions from landfills and the use of ICS to combat these emissions. More in-field measurements of nitrous oxide and methane emissions from landfills can further quantify the nitrous oxide and methane emissions from landfills. Engineered strategies to improve methane oxidation in landfills, such as supplementation with copper, could also be tested. This work would require precise measurements of nitrous oxide and methane over larger areas, but methods to perform these analyses, such as using Eddy covariance, are emerging. Therefore, large-scale assessments of ICS that combine analyses of the ICS soil characteristics, vegetation, microbial communities, and GHG emissions should be explored moving forward.

COMPREHENSIVE BIBLIOGRAPHY

1412i. LumaSense Technologies A/S. (n.d.). https://innova.lumasenseinc.com/manuals/1412i/

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., & Zechmeister -Boltenstern, S. (2013). Nitrous oxide emissions from soils: How well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1621), 20130122. https://doi.org/10.1098/rstb.2013.0122

Caspi, R., Altman, T., Billington, R., Dreher, K., Foerster, H., Fulcher, C. A., Holland, T. A., Keseler, I. M., Kothari, A., Kubo, A., Krummenacker, M., Latendresse, M., Mueller, L. A., Ong, Q., Paley, S., Subhraveti, P., Weaver, D. S., Weerasinghe, D., Zhang, P., & Karp, P. D. (2014). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic acids research*, *42*(Database issue), D459–D471. https://doi.org/10.1093/nar/gkt1103

Cebron, A., Bodrossy, L., Chen, Y., Singer, A. C., Thompson, I. P., Prosser, J. I., & Murrell, J. C. (2007). Identity of active methanotrophs in landfill cover soil as revealed by DNA-stable isotope probing. *FEMS Microbiology Ecology*, *62*(1), 12 –23. https://doi.org/10.1111/j.1574- 6941.2007.00368.x

Chang, J., Peng, P., DiSpirito, A. A., & Semrau, J. D. (2022). Variable inhibition of nitrous oxide reduction in denitrifying bacteria by different forms of methanobactin. *Applied and Environmental Microbiology*, *88*(7). https://doi.org/10.1128/aem.02346-21

Chistoserdova, L. V., & Lidstrom, M. E. (1994a). Genetics of the serine cycle in *Methylobacterium extorquens* AM1: Identification of sgaa and MTDA and sequences of sgaa, hpra, and MTDA. *Journal of Bacteriology*, *176*(7), 1957–1968. https://doi.org/10.1128/jb.176.7.1957-1968.1994

Chistoserdova, L. V., & Lidstrom, M. E. (1994b). Genetics of the serine cycle in *Methylobacterium extorquens* AM1: Identification, sequence, and mutation of three new genes involved in C1 assimilation, orf4, mtka, and mtkb. *Journal of Bacteriology*, *176*(23), 7398–7404. https://doi.org/10.1128/jb.176.23.7398 -7404.1994

Choi, D.-W., Kunz, R. C., Boyd, E. S., Semrau, J. D., Antholine, W. E., Han, J.-I., Zahn, J. A., Boyd, J. M., de la Mora, A. M., & DiSpirito, A. A. (2003). The membrane -associated methane monooxygenase (pmmo) and pMMO-NADH:quinone oxidoreductase complex from *Methylococcus capsulatus* bath. *Journal of*

Bacteriology, *185*(19), 5755–5764. https://doi.org/10.1128/jb.185.19.5755-5764.2003

Christy, P., Gopinath, L. R., & Divya, D. (2014). A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renewable and Sustainable Energy Reviews*, *34*, 167–173. https://doi.org/10.1016/j.rser.2014.03.010

Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B., Hou, S., Ly, B., Saw, J. H., Zhou, Z., Ren, Y., Wang, J., Mountain, B. W., Crowe, M. A., Weatherby, T. M., Bodelier, P. L., Liesack, W., Feng, L., Wang, L., & Alam, M. (2007). Methane oxidation by an extremely acidophilic bacterium of the Phylum Verrucomicrobia. *Nature*, *450*(7171), 879–882. https://doi.org/10.1038/nature06411

Environmental Protection Agency. (2022a). *Importance of Methane*. EPA. https://www.epa.gov/gmi/importance-methane

Environmental Protection Agency. (2022b). *National Overview: Facts and Figures on Materials, Wastes and Recycling*. EPA. https://www.epa.gov/facts-and-figures-about-materials-waste-and-recycling/national-overview-facts-and-figures-materials

Environmental Protection Agency. (2023a). *Municipal Solid Waste Landfills*. EPA. https://www.epa.gov/landfills/municipal-solid-waste-landfills

Environmental Protection Agency. (2023b). *Overview of Greenhouse Gases*. EPA. https://www.epa.gov/ghgemissions/overview-greenhouse-gases

Florio, R., di Salvo, M. L., Vivoli, M., & Contestabile, R. (2011). Serine hydroxymethyltransferase: A model enzyme for mechanistic, structural, and evolutionary studies. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1814*(11), 1489–1496. https://doi.org/10.1016/j.bbapap.2010.10.010

Gupta, R. S. (2000). The phylogeny of proteobacteria: Relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiology Reviews*, *24*(4), 367–402. https://doi.org/10.1111/j.1574- 6976.2000.tb00547.x

Hakemian, A. S., & Rosenzweig, A. C. (2007). The biochemistry of methane oxidation. *Annual Review of Biochemistry*, *76*(1), 223–241. https://doi.org/10.1146/annurev.biochem.76.061505.175355

Hanson, R. S., & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiological Reviews*, *60*(2), 439–471. https://doi.org/10.1128/mr.60.2.439-471.1996

Heděnec, P., Angel, R., Lin, Q., Rui, J., & Li, X. (2019). Increased methane concentration alters soil prokaryotic community structure along an artificial pH

gradient. *Annals of Microbiology*, *69*(4), 329–339. https://doi.org/10.1007/s13213-018-1421-4

Ituen, E., & Etim, G. (2012). Carbon-nitrogen ratio as a basis for the selection of substrates for optimum biogas production. Advances in Science and Technology, 6(1), 33-38.

Izumi, Y., Yoshida, T., & Yamada, H. (1990). Purification and characterization of serine-glyoxylate aminotransferase from a serine-producing methylotroph, *Hyphomicrobium methylovorum* GM2. *European Journal of Biochemistry*, *190*(2), 285–290. https://doi.org/10.1111/j.1432-1033.1990.tb15574.x

Kallen, R. G., & Jencks, W. P. (1966). The mechanism of the condensation of formaldehyde with tetrahydrofolic acid. *Journal of Biological Chemistry*, *241*(24), 5851–5863. https://doi.org/10.1016/s0021-9258(18)96350-7

Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M. and Ishiguro-Watanabe, M.; KEGG for taxonomy-based analysis of pathways and genomes, *Nucleic Acids Research*, Volume 51, Issue D1, 6 January 2023, Pages D587-D592, https://doi.org/10.1093/nar/gkac963

Khider, M. L., Brautaset, T., & Irla, M. (2021). Methane monooxygenases: Central Enzymes in methanotrophy with promising biotechnological applications. *World Journal of Microbiology and Biotechnology*, *37*(4). https://doi.org/10.1007/s11274-021-03038-x

Kim, H., An, S., Park, Y. R., Jang, H., Yoo, H., Park, S. H., Lee, S. J., & Cho, U.-S. (2019). MMOD-induced structural changes of hydroxylase in soluble methane monooxygenase. *Science Advances*, *5*(10). https://doi.org/10.1126/sciadv.aax0059

Koo, C. W., & Rosenzweig, A. C. (2021a). Biochemistry of aerobic biological methane oxidation. *Chemical Society Reviews*, *50*(5), 3424–3436. https://doi.org/10.1039/d0cs01291b

Hanson, R. S., & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiological Reviews*, *60*(2), 439–471. https://doi.org/10.1128/mr.60.2.439-471.1996

Lee, S.-W., Im, J., DiSpirito, A. A., Bodrossy, L., Barcelona, M. J., & Semrau, J. D. (2009). Effect of nutrient and selective inhibitor amendments on methane oxidation, nitrous oxide production, and key gene presence and expression in landfill cover soils: Characterization of the role of methanotrophs, nitrifiers, and denitrifiers. *Applied Microbiology and Biotechnology*, *85*(2), 389–403. https://doi.org/10.1007/s00253-009-2238-7

Lieberman, R. L., & Rosenzweig, A. C. (2004). Biological methane oxidation: Regulation, biochemistry, and active site structure of particulate methane monooxygenase. *Critical Reviews in Biochemistry and Molecular Biology*, *39*(3), 147–164. https://doi.org/10.1080/10409230490475507

Long, X.-E., Wang, J., Huang, Y., & Yao, H. (2016). Microbial community structures and metabolic profiles response differently to physiochemical properties between three landfill cover soils. *Environmental Science and Pollution Research*, *23*(15), 15483–15494. https://doi.org/10.1007/s11356-016-6681-6

McKee, L. S., La Rosa, S. L., Westereng, B., Eijsink, V. G., Pope, P. B., & Larsbrink, J. (2021). Polysaccharide degradation by the Bacteroidetes: Mechanisms and nomenclature. *Environmental Microbiology Reports*, *13*(5), 559–581. https://doi.org/10.1111/1758-2229.12980

Merkx, M., & Lippard, S. J. (2002). Why OrfY? Characterization of MMOD, a long overlooked component of the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *Journal of Biological Chemistry*, *277*(8), 5858 –65. https://doi.org/10.1074/jbc.M107712200

Murrell, C. (2008). Professor Sir Howard Dalton FRS (8th February 1944–12th January 2008).

Nazaries, L., Murrell, J. C., Millard, P., Baggs, L., & Singh, B. K. (2013). Methane, microbes and models: Fundamental understanding of the soil methane cycle for future predictions. *Environmental Microbiology*, *15*(9), 2395–2417. https://doi.org/10.1111/1462-2920.12149

Nitrogen-ammonia reagent set, salicylate method, 10 ml. Hach. (n.d.). https://www.hach.com/p-nitrogen-ammonia-reagent-set-salicylate-method-10-ml/2668000

O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., Farrell, C. M., … Pruitt, K. D. (2016). Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic acids research*, *44*(D1), D733–D745. https://doi.org/10.1093/nar/gkv1189

Park, S., & Kim, C. (2019). Application and development of methanotrophs in environmental engineering. *Journal of Material Cycles and Waste Management*, *21*(3), 415–422. https://doi.org/10.1007/s10163-018-00826-w

Rinne, J., Pihlatie, M., Lohila, A., Thum, T., Aurela, M., Tuovinen, J.-P., Laurila, T., & Vesala, T. (2005). Nitrous oxide emissions from a municipal landfill. *Environmental Science & Technology*, *39*(20), 7790–7793. https://doi.org/10.1021/es048416q

Semrau, J. D. (2011). Current knowledge of microbial community structures in landfills and its cover soils. *Applied Microbiology and Biotechnology*, *89*(4), 961–969. https://doi.org/10.1007/s00253-010-3024-2

Semrau, J. D., DiSpirito, A. A., & Yoon, S. (2010). Methanotrophs and copper. *FEMS Microbiology Reviews*, *34*(4), 496–531. https://doi.org/10.1111/j.1574-6976.2010.00212.x

Semrau, J. D., Jagadevan, S., DiSpirito, A. A., Khalifa, A., Scanlan, J., Bergman, B. H., Freemeier, B. C., Baral, B. S., Bandow, N. L., Vorobev, A., Haft, D. H., Vuilleumier, S., & Murrell, J. C. (2013). Methanobactin and MMOD work in concert to act as the 'copper-switch' in methanotrophs. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12150

Stralis-Pavese, N., Sessitsch, A., Weilharter, A., Reichenauer, T., Riesing, J., Csontos, J., Murrell, J. C., & Bodrossy, L. (2004). Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. *Environmental Microbiology*, *6*(4), 347–363. https://doi.org/10.1111/j.1462-2920.2004.00582.x

Tamames, J., & Puente-Sánchez, F. (2019). SqueezeMeta, a highly portable, fully automatic metagenomic analysis pipeline. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.03349

The UniProt Consortium, UniProt: the Universal Protein Knowledgebase in 2023, *Nucleic Acids Research*, Volume 51, Issue D1, 6 January 2023, Pages D523–D531, https://doi.org/10.1093/nar/gkac1052

Xu, S., & Zhang, H. (2022). First evidence for anaerobic oxidation of methane process in landfill cover soils: Activity and responsible microorganisms. *Science of The Total Environment*, *841*, 156790. https://doi.org/10.1016/j.scitotenv.2022.156790

Yu, T., He, P.-J., Lü, F., & Shao, L.-M. (2009). Mediating N2O emissions from municipal solid waste landfills: Impacts of landfill operating conditions on community structure of ammonia- oxidizing bacteria in cover soils. *Ecological Engineering*, *35*(5), 882–889. https://doi.org/10.1016/j.ecoleng.2008.12.025

Zhang, Q., Lee, J., Pandurangan, S., Clarke, M., Pajak, A., & Marsolais, F. (2013). Characterization of *Arabidopsis* serine:glyoxylate aminotransferase, AGT1, as an asparagine aminotransferase. *Phytochemistry*, *85*, 30–35. https://doi.org/10.1016/j.phytochem.2012.09.017

APPENDIX

Table S1: Samples with their elevation and coordinates

Site Name	Elevation (ft)	Coordinates
A	701	44.6964583N, -73.59665167E
B	700	44.696443N, -73.596785E
C	704	44.6964767N, -73.5969283E
D	709	44.69655167N, -73.5968967E
E	711	44.696573N, -73.5966367E
F	708	44.69713167N, -73.5966683E
G	712	44.697385N, -73.596793E
H	711	44.697413N, -73.5963567E
I	691	44.69734167N, -73.596205E
J	676	44.697223N, -73.595995E
K	696	44.6970567N, -73.59631167E
L	705	44.69684167N, -73.597175E

Table S2: Soil nutrient and pH analysis

Analysis	Value Found	Optimal Range (for most crops)
Soil pH (2:1, water)	7.5	6-7
Modified Morgan extractable, ppm		
Phosphorus (P)	12.9 (high)	4-7
Potassium (K)	28 (low)	100-130
Calcium (Ca)	3005	>1000*
Magnesium (Mg)	136 (high)	50-100
Soil Organic Matter (%)	0.3	*

CEC, meq/100g	16.2	*

* Ca content, organic matter %, and CEC are dependent on soil texture. They tend to be high in soils with a lot of clay and low in soils with a lot of sand.

Table S3: Micronutrient analysis

Analysis	Value Found	Typical Ranges in VT (ppm)**
Iron (Fe)	15.8	2.4-10.6
Manganese (Mn)	15.9	2.1-9.3
Boron (B)	0.3	0.10-0.60
Copper (Cu)	0.3	0.16-0.30
Sulfur (S)	12.0	5-17
Zinc (Zn)	1.1	0.4-3.2
Sodium (Na)	11.0	6-21
Aluminum (Al)	46	8-107

** Ranges shown represent 90% of > 7000 recent soil test results. Micronutrient deficiencies are rare in VT when soil pH is in the optimal range. Al and Na are not nutrients but are shown because at high levels they can cause plant toxicity.

Table S4: Total nitrogen and carbon

Description	Value	Unit of Measure
Total Nitrogen	<0.01	%
Total Carbon	0.41	%

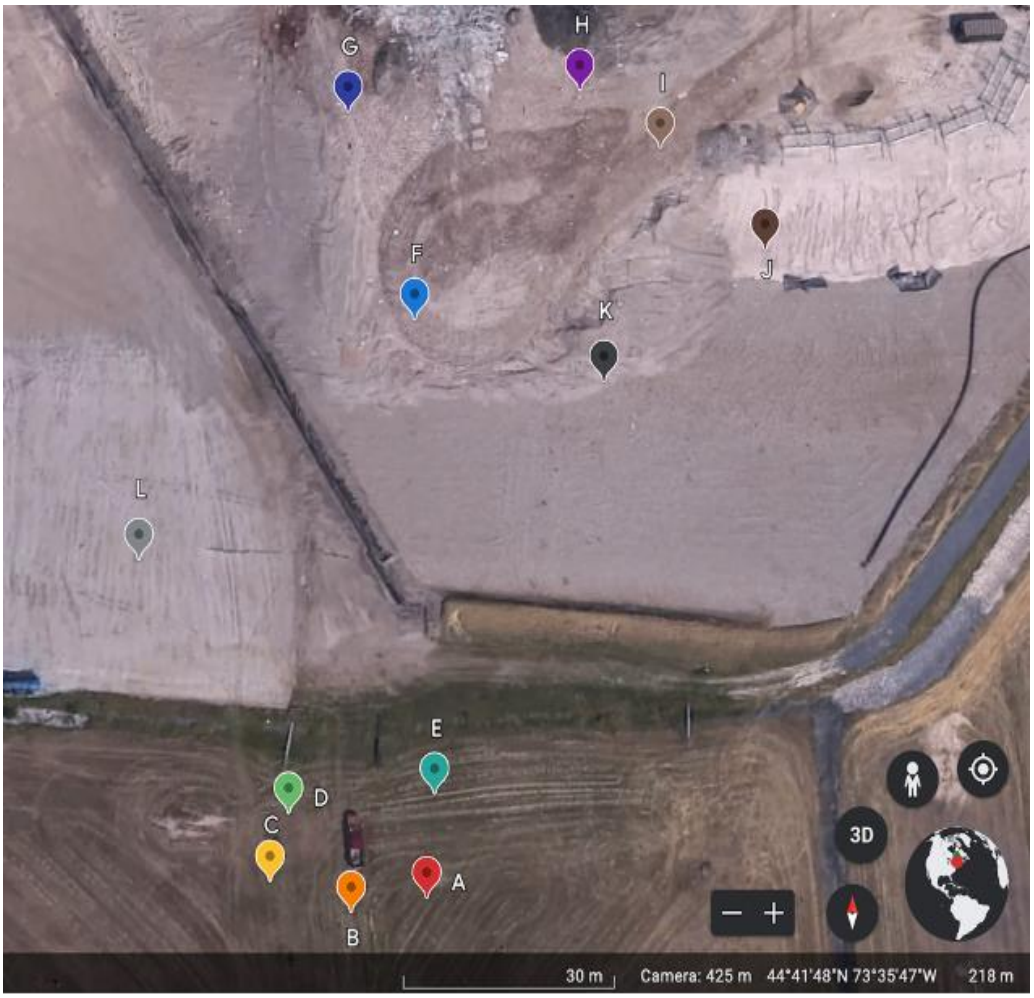

Figure S1: Map of sample locations with labels

Table S5: Methane concentration in ppm through time (days) measured using the GC-TCD

Day	C1	C2	C3	CH4-1	CH4-2	CH4-3	10NH4-1	10NH4-2	10NH4-3	5NH4-1	5NH4-2	5NH4-3
0	0	0	0	1.42E+05	1.35E+05	1.32E+05	1.36E+05	1.35E+05	1.28E+05	1.33E+05	1.21E+05	1.30E+05
3	0	0	0	8.02E+04	8.22E+04	8.34E+04	8.38E+04	7.70E+04	7.79E+04	7.88E+04	7.63E+04	8.06E+04
6	0	0	0	8.88E+04	9.64E+04	9.35E+04	9.85E+04	9.29E+04	9.56E+04	9.95E+04	9.90E+04	9.74E+04
9	0	0	0	9.05E+04	9.96E+04	1.03E+05	1.01E+05	9.66E+04	9.50E+04	9.75E+04	1.03E+05	1.02E+05
12	0	0	0	8.95E+04	1.06E+05	1.06E+05	1.04E+05	9.92E+04	1.00E+05	9.04E+04	1.06E+05	1.07E+05
15	0	0	0	9.48E+04	1.05E+05	1.05E+05	1.04E+05	1.01E+05	1.01E+05	8.74E+04	1.07E+05	1.09E+05
18	0	0	0	9.23E+04	1.06E+05	1.11E+05	1.06E+05	1.00E+05	9.79E+04	9.16E+04	1.06E+05	1.11E+05
21	0	0	0	4.54E+04	5.15E+04	5.59E+04	4.93E+04	4.89E+04	4.64E+04	3.93E+04	4.99E+04	5.41E+04
24	0	0	0	1.55E+04	1.70E+04	2.22E+04	1.55E+04	1.96E+04	1.89E+04	1.33E+04	1.85E+04	2.31E+04
27	0	0	0	1.38E+03	9.80E+02	2.46E+03	0	3.96E+03	4.35E+03	8.60E+02	2.75E+03	6.32E+03
30	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0

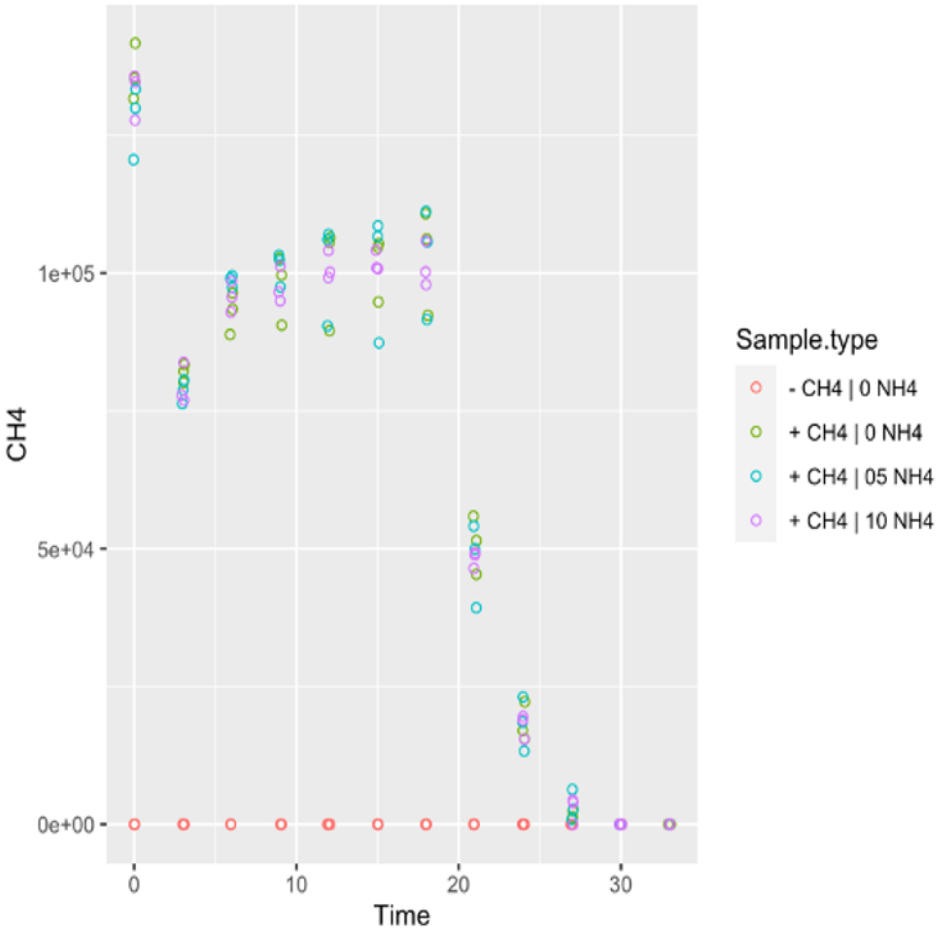

Figure S2: Methane concentration (ppm) of each treatment with their biological replicates over time in days during the 33-day incubation.

Table S6: Nitrogen concentration in ppm through time (days) measured using the GC-TCD

Day	C1	C2	C3	CH4-1	CH4-2	CH4-3	10NH4-1	10NH4-2	10NH4-3	5NH4-1	5NH4-2	5NH4-3
0	8.61E+05	8.70E+05	8.70E+05	7.55E+05	7.59E+05	7.61E+05	7.62E+05	7.62E+05	7.64E+05	7.62E+05	7.72E+05	7.64E+05
3	8.68E+05	8.66E+05	8.66E+05	8.02E+05	8.00E+05	7.98E+05	7.96E+05	8.04E+05	8.00E+05	8.00E+05	7.99E+05	7.97E+05
6	8.63E+05	8.63E+05	8.65E+05	7.93E+05	7.87E+05	7.91E+05	7.89E+05	7.90E+05	7.91E+05	7.86E+05	7.87E+05	7.89E+05
9	8.67E+05	8.66E+05	8.58E+05	7.96E+05	7.89E+05	7.91E+05	7.89E+05	7.94E+05	7.94E+05	7.96E+05	7.87E+05	7.88E+05
12	8.70E+05	8.69E+05	8.69E+05	8.02E+05	7.86E+05	7.95E+05	7.96E+05	7.99E+05	7.96E+05	8.01E+05	7.88E+05	7.86E+05
15	8.73E+05	8.68E+05	8.65E+05	8.01E+05	7.91E+05	7.96E+05	7.95E+05	7.94E+05	7.94E+05	8.07E+05	7.88E+05	7.82E+05
18	8.57E+05	8.59E+05	8.56E+05	7.88E+05	7.83E+05	7.83E+05	7.88E+05	7.85E+05	7.97E+05	8.01E+05	7.83E+05	7.81E+05
21	8.65E+05	8.53E+05	8.55E+05	8.33E+05	8.35E+05	8.16E+05	8.31E+05	8.25E+05	8.21E+05	8.39E+05	8.22E+05	8.20E+05
24	8.50E+05	8.47E+05	8.45E+05	8.52E+05	8.51E+05	8.47E+05	8.59E+05	8.49E+05	8.44E+05	8.52E+05	8.55E+05	8.41E+05
27	8.54E+05	8.48E+05	8.56E+05	8.61E+05	8.62E+05	8.65E+05	8.70E+05	8.69E+05	8.60E+05	8.63E+05	8.64E+05	8.59E+05
30	8.59E+05	8.59E+05	8.57E+05	8.65E+05	8.64E+05	8.68E+05	8.64E+05	8.71E+05	8.66E+05	8.62E+05	8.70E+05	8.70E+05
33	8.53E+05	8.51E+05	8.51E+05	8.53E+05	8.51E+05	8.60E+05	8.58E+05	8.56E+05	8.55E+05	8.28E+05	8.56E+05	8.61E+05

Table S7: Oxygen/Argon concentration (ppm) through time in days measured using the GC-TCD

Day	C1	C2	C3	CH4-1	CH4-2	CH4-3	10NH4-1	10NH4-2	10NH4-3	5NH4-1	5NH4-2	5NH4-3
0	2.50E+05	2.45E+05	2.45E+05	2.13E+05	2.15E+05	2.17E+05	2.14E+05	2.14E+05	2.18E+05	2.14E+05	2.17E+05	2.15E+05
3	2.41E+05	2.41E+05	2.42E+05	2.22E+05	2.21E+05	2.21E+05	2.22E+05	2.22E+05	2.25E+05	2.24E+05	2.26E+05	2.21E+05
6	2.40E+05	2.38E+05	2.37E+05	2.14E+05	2.11E+05	2.12E+05	2.08E+05	2.12E+05	2.09E+05	2.10E+05	2.07E+05	2.08E+05
9	2.35E+05	2.37E+05	2.42E+05	2.09E+05	2.07E+05	2.02E+05	2.01E+05	2.04E+05	2.01E+05	2.01E+05	2.00E+05	2.00E+05
12	2.35E+05	2.37E+05	2.38E+05	2.06E+05	2.04E+05	1.96E+05	1.94E+05	1.97E+05	1.99E+05	2.06E+05	1.98E+05	2.01E+05
15	2.34E+05	2.36E+05	2.40E+05	1.98E+05	1.96E+05	1.92E+05	1.93E+05	1.99E+05	1.98E+05	2.02E+05	1.99E+05	2.02E+05
18	2.42E+05	2.39E+05	2.39E+05	1.99E+05	1.91E+05	1.86E+05	1.83E+05	1.94E+05	1.89E+05	1.94E+05	1.90E+05	1.88E+05
21	2.38E+05	2.44E+05	2.39E+05	2.02E+05	1.96E+05	2.01E+05	1.94E+05	2.03E+05	2.09E+05	2.05E+05	2.04E+05	2.03E+05
24	2.40E+05	2.41E+05	2.41E+05	2.07E+05	2.02E+05	2.00E+05	1.90E+05	2.03E+05	2.11E+05	2.10E+05	1.96E+05	2.05E+05
27	2.37E+05	2.41E+05	2.37E+05	2.15E+05	2.09E+05	2.01E+05	1.97E+05	2.01E+05	2.09E+05	2.16E+05	2.06E+05	2.05E+05
30	2.38E+05	2.36E+05	2.43E+05	2.22E+05	2.22E+05	2.12E+05	2.13E+05	2.13E+05	2.16E+05	2.26E+05	2.12E+05	2.11E+05
33	2.40E+05	2.38E+05	2.43E+05	2.30E+05	2.31E+05	2.17E+05	2.18E+05	2.27E+05	2.26E+05	2.51E+05	2.22E+05	2.18E+05

Table S8: Carbon dioxide concentration (ppm) through time in days measured using the GC-TCD

Day	C1	C2	C3	CH4-1	CH4-2	CH4-3	10NH4-1	10NH4-2	10NH4-3	5NH4-1	5NH4-2	5NH4-3
0	0	7.30E+02	6.60E+02	5.00E+02	6.00E+02	5.50E+02	7.60E+02	6.70E+02	7.30E+02	6.80E+02	6.90E+02	6.40E+02
3	1.40E+03	1.86E+03	1.49E+03	1.44E+03	1.71E+03	1.68E+03	1.97E+03	1.70E+03	1.81E+03	1.90E+03	2.18E+03	2.07E+03
6	3.20E+03	4.00E+03	4.05E+03	4.15E+03	4.77E+03	4.69E+03	5.31E+03	4.81E+03	5.15E+03	4.87E+03	5.74E+03	5.62E+03
9	6.83E+03	6.41E+03	5.28E+03	7.51E+03	8.53E+03	8.89E+03	1.04E+04	9.27E+03	9.45E+03	9.27E+03	1.02E+04	1.04E+04
12	8.19E+03	8.80E+03	7.21E+03	9.99E+03	1.09E+04	1.18E+04	1.34E+04	1.22E+04	1.23E+04	1.05E+04	1.25E+04	1.26E+04
15	9.26E+03	1.00E+04	7.76E+03	1.35E+04	1.42E+04	1.58E+04	1.68E+04	1.46E+04	1.51E+04	1.28E+04	1.49E+04	1.46E+04
18	7.60E+03	8.49E+03	8.58E+03	1.63E+04	1.74E+04	1.93E+04	2.10E+04	1.73E+04	1.71E+04	1.57E+04	1.81E+04	1.84E+04
21	7.26E+03	7.99E+03	8.63E+03	1.96E+04	2.13E+04	2.21E+04	2.48E+04	2.01E+04	1.93E+04	1.73E+04	2.15E+04	2.02E+04
24	6.72E+03	7.67E+03	6.52E+03	2.13E+04	2.38E+04	2.54E+04	2.90E+04	2.31E+04	2.16E+04	1.91E+04	2.49E+04	2.33E+04
27	5.88E+03	9.04E+03	6.69E+03	2.08E+04	2.47E+04	2.82E+04	3.01E+04	2.35E+04	2.29E+04	1.75E+04	2.59E+04	2.52E+04
30	7.08E+03	8.70E+03	5.89E+03	1.78E+04	1.78E+04	2.50E+04	2.54E+04	2.05E+04	2.15E+04	1.45E+04	2.27E+04	2.38E+04
33	6.82E+03	8.60E+03	5.32E+03	1.55E+04	1.56E+04	2.27E+04	2.29E+04	1.74E+04	1.85E+04	1.21E+04	1.95E+04	2.12E+04

Table S9: Nitrous oxide concentration (ppm) through time in days measured using the PAS

Day	C1	C2	C3	CH4-1	CH4-2	CH4-3	10NH4-1	10NH4-2	10NH4-3	5NH4-1	5NH4-2	5NH4-3
0	0.459	0.448	0.453	1.053	1.027	1.004	1.049	1.023	1.005	0.996	0.998	0.996
	0.453	0.474	0.453	1.034	1.038	1.013	1.025	1.010	1.007	1.002	0.983	1.026
	0.430	0.483	0.478	1.027	1.027	1.012	1.035	1.010	1.005	0.965	0.992	1.013
3	0.919	0.831	0.812	1.080	1.155	1.100	1.320	1.002	1.105	1.219	1.047	1.142
	0.883	0.814	0.786	1.072	1.139	1.077	1.283	0.991	1.044	1.177	1.024	1.115
	0.875	0.801	0.778	1.086	1.127	1.081	1.286	1.021	1.065	1.192	1.026	1.099
6	1.210	1.330	1.762	1.887	2.057	2.008	2.205	1.933	2.035	1.994	2.063	2.008
	1.178	1.326	1.735	1.848	2.002	1.941	2.136	1.907	2.008	1.943	2.042	1.991
	1.176	1.297	1.701	1.843	2.027	1.936	2.133	1.877	1.974	1.951	2.048	1.972
9	1.208	1.813	1.743	2.686	2.927	2.989	3.379	3.036	3.075	2.983	3.211	3.222
	1.132	1.813	1.709	2.614	2.882	2.912	3.332	2.947	2.995	2.912	3.152	3.166
	1.118	1.799	1.697	2.585	2.866	2.891	3.283	2.902	2.941	2.933	3.173	3.133
12	3.422	3.275	2.666	3.579	3.832	3.921	4.367	3.905	3.883	3.426	4.032	4.032
	3.261	3.173	2.588	3.498	3.742	3.829	4.265	3.779	3.844	3.356	3.911	3.901
	3.188	3.119	2.561	3.463	3.709	3.800	4.229	3.769	3.840	3.371	3.924	3.924

15	3.657	3.521	2.688	4.517	4.602	5.003	5.181	4.573	4.620	3.837	4.418	4.448
	3.467	3.394	2.636	4.365	4.455	4.832	5.077	4.459	4.513	3.804	4.351	4.389
	3.407	3.372	2.601	4.341	4.465	4.771	5.014	4.427	4.499	3.775	4.325	4.352
18	2.201	2.379	2.551	4.761	5.002	5.462	5.753	4.891	4.862	4.324	4.976	5.089
	2.163	2.292	2.471	4.625	4.851	5.352	5.676	4.799	4.722	4.208	4.875	5.000
	2.188	2.331	2.466	4.612	4.852	5.331	5.664	4.813	4.693	4.222	4.866	5.031
21	2.017	2.213	2.447	5.287	5.583	5.999	6.438	5.345	5.067	4.526	5.533	5.327
	1.986	2.193	2.428	5.181	5.425	5.858	6.294	5.225	4.941	4.484	5.541	5.196
	1.979	2.148	2.439	5.144	5.441	5.816	6.298	5.204	4.952	4.442	5.486	5.170
24	2.021	2.253	1.900	6.068	6.563	6.940	7.727	6.341	5.838	5.192	6.604	6.210
	1.972	2.251	1.862	5.904	6.383	6.790	7.527	6.191	5.709	5.095	6.484	6.055
	1.994	2.193	1.877	5.869	6.400	6.755	7.576	6.188	5.722	5.075	6.456	6.079
27	1.898	2.815	2.035	6.006	6.937	7.786	8.328	6.136	6.133	4.813	6.900	6.569
	1.868	2.690	1.974	5.854	6.745	7.620	8.088	5.998	6.009	4.676	6.781	6.502
	1.865	2.690	1.997	5.794	6.723	7.560	8.087	5.985	5.987	4.673	6.742	6.479
30	2.958	3.288	2.160	5.586	5.521	7.578	7.642	5.970	6.177	4.207	6.324	6.577
	2.831	3.176	2.086	5.427	5.378	7.371	7.404	5.801	5.976	4.125	6.230	6.425

	2.753	3.106	2.088	5.458	5.355	7.334	7.350	5.828	6.002	4.095	6.159	6.435
33	2.467	2.879	1.764	4.721	4.556	6.464	6.395	4.822	5.157	3.602	5.203	5.625
	2.385	2.832	1.726	4.570	4.488	6.341	6.264	4.752	5.050	3.524	5.106	5.531
	2.369	2.815	1.725	4.515	4.470	6.261	6.221	4.739	5.018	3.539	5.115	5.530

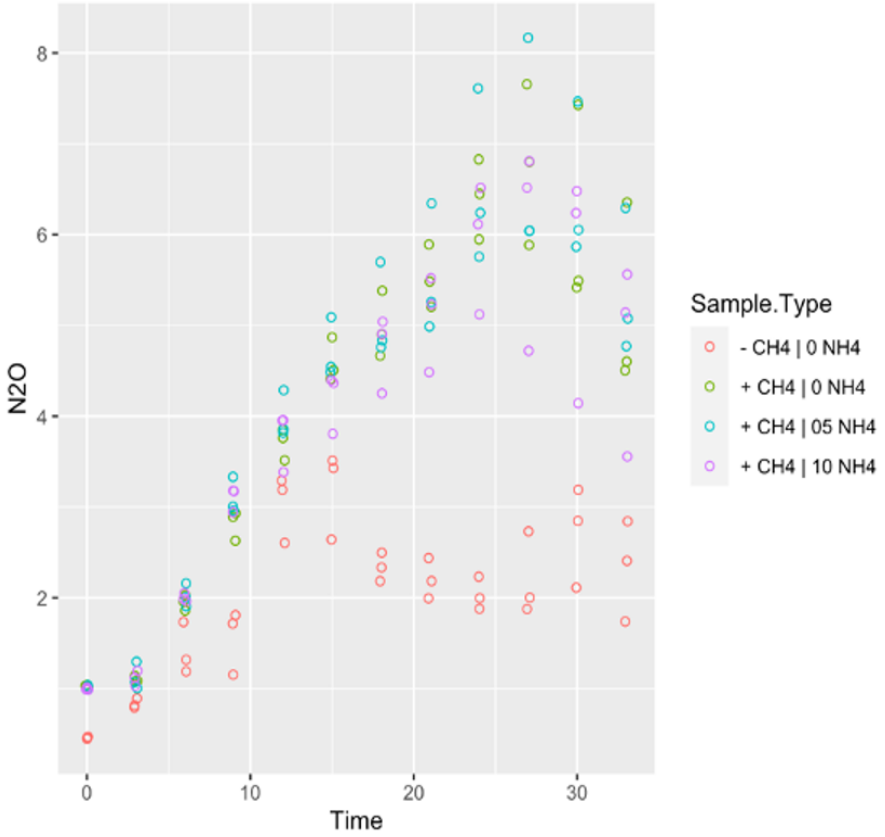

Figure S3: Nitrous oxide concentration (ppm) of each treatment with their biological replicates over time in days during the 33-day incubation.

Table S10: Mean nitrous oxide concentrations through time (n = 3)

Day	C1	C2	C3	CH4-1	CH4-2	CH4-3	10NH4-1	10NH4-2	10NH4-3	5NH4-1	5NH4-2	5NH4-3
0	0.447	0.468	0.462	1.038	1.031	1.010	1.037	1.014	1.006	0.987	0.991	1.012
3	0.892	0.815	0.792	1.079	1.140	1.086	1.296	1.005	1.071	1.196	1.032	1.119
6	1.188	1.318	1.733	1.859	2.029	1.961	2.158	1.906	2.006	1.963	2.051	1.991
9	1.153	1.808	1.716	2.629	2.892	2.931	3.331	2.962	3.004	2.942	3.179	3.174
12	3.290	3.189	2.605	3.514	3.761	3.850	4.287	3.818	3.855	3.384	3.956	3.952
15	3.510	3.429	2.642	4.408	4.507	4.869	5.091	4.486	4.544	3.805	4.365	4.397
18	2.184	2.334	2.496	4.666	4.902	5.382	5.697	4.835	4.759	4.251	4.906	5.040
21	1.994	2.185	2.438	5.204	5.483	5.891	6.343	5.258	4.987	4.484	5.520	5.231
24	1.996	2.232	1.880	5.947	6.449	6.828	7.610	6.240	5.756	5.121	6.515	6.115
27	1.877	2.732	2.002	5.885	6.802	7.655	8.167	6.039	6.043	4.720	6.808	6.516
30	2.847	3.190	2.111	5.490	5.418	7.428	7.466	5.866	6.052	4.142	6.238	6.479
33	2.407	2.842	1.738	4.602	4.505	6.355	6.293	4.771	5.075	3.555	5.141	5.562

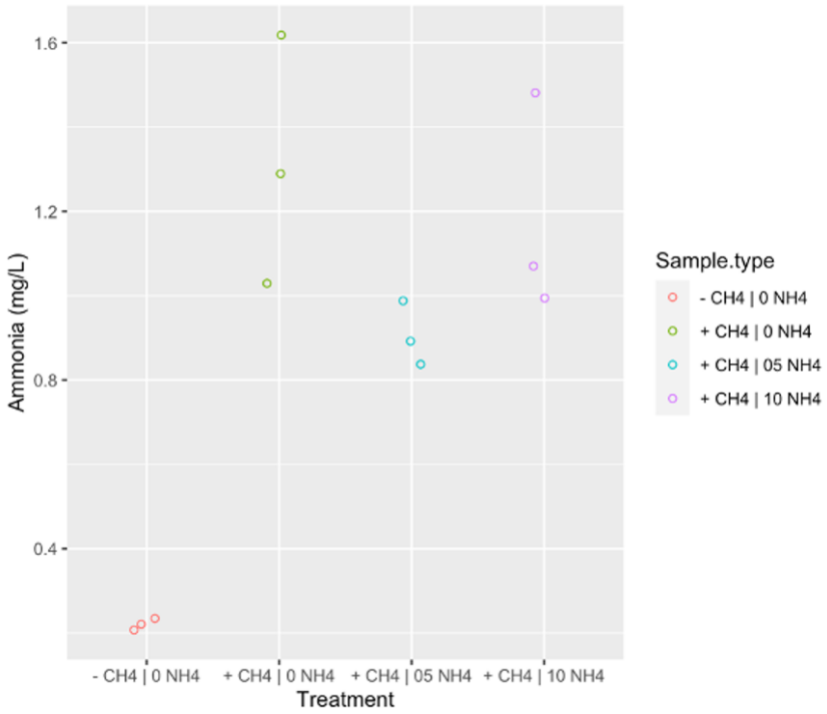

Figure S4: Ammonia concentrations (mg/L) for each bottle at the end of the 33-day incubation

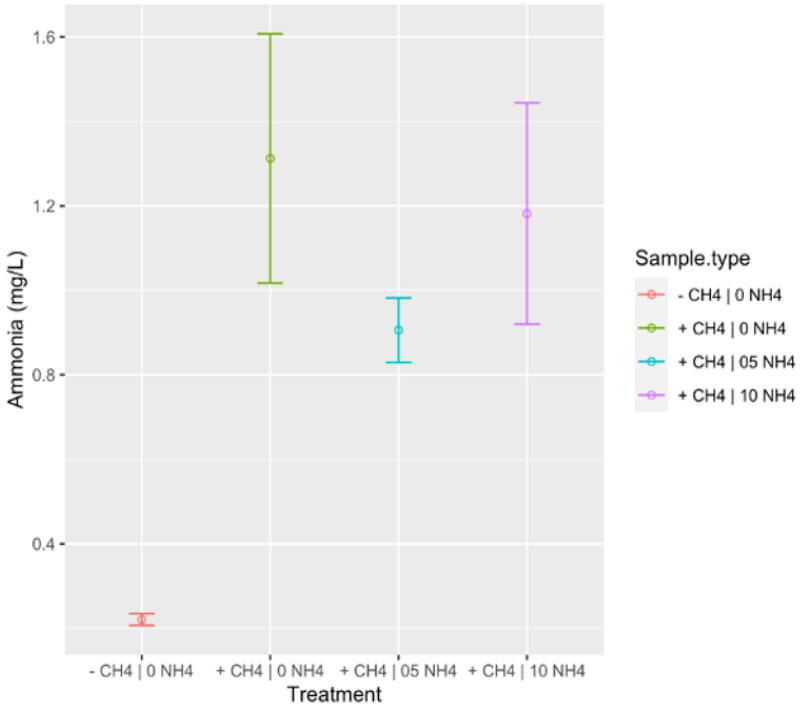

Figure S5. Mean ammonia concentrations (mg/L) of each treatment at the end of the 33-day incubation (n = 3).

Table S11: One way ANOVA test for nitrous oxide production vs. ammonium added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	40.0	20.010	5.444	0.00528
Residuals	141	518.2	3.675		

Table S12: One-way ANOVA test for nitrous oxide production vs. methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Methane added	1	121.0	121.00	39.3	4.11e-09
Residuals	142	437.2	3.08		

Table S13: Two-way ANOVA test for nitrous oxide production vs. ammonium added and methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	40.0	20.01	6.444	0.0021
Methane added	1	83.5	83.51	26.895	7.38e-07
Residuals	140	434.7	3.11		

Table S14: One-way ANOVA test for day 0 nitrous oxide production vs. ammonium added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	0.2117	0.10584	1.968	0.195
Residuals	9	437.2	0.05377		

Table S15: One-way ANOVA test for day 0 nitrous oxide production vs. methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Methane added	1	0.6927	0.6927	2371	3.22e-13
Residuals	10	0.0029	0.0003		

Table S16: One-way ANOVA test for day 27 nitrous oxide production vs. ammonium added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	11.53	5.765	1.331	0.312
Residuals	9	38.99	4.332		

Table S17: One-way ANOVA test for day 27 nitrous oxide production vs. methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Methane added	1	41.83	41.83	48.13	4.01e-05
Residuals	10	8.69	0.87		

Table S18: One-way ANOVA test for methane oxidation vs. ammonium added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	3.750e+10	1.875e+10	8.532	0.000318
Residuals	141	3.099e+11	2.198e+09		

Table S19: One-way ANOVA test for methane oxidation vs. methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Methane added	1	1.144e+11	1.144e+11	69.77	5.45e-14
Residuals	142	2.329e+11	1.640e+09		

Table S20: Two-way ANOVA test for methane concentration vs. ammonium added and methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	3.750e+10	1.875e+10	11.27	2.89e-05
Methane added	1	7.695e+10	7.695e+10	46.25	2.78e-10
Residuals	140	2.329e+11	1.664e+09		

Table S21: One-way ANOVA test for day 0 methane concentration vs. ammonium added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	1.164e+10	5.822e+09	1.871	0.209
Residuals	9	2.801e+10	3.112e+09		

Table S22: One-way ANOVA test for day 0 methane oxidation vs. methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Methane added	1	3.937e+10	3.937e+10	1405	4.35e-12
Residuals	10	2.802e+08	2.802e+07		

Table S23: One-way ANOVA test for day 27 methane concentration vs. ammonium added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Ammonium added	2	15445433	7722717	2.172	0.17
Residuals	9	32005933	3556215		

Table S24: One-way ANOVA test for day 27 methane concentration vs. methane added with 5% significance level

	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Methane added	1	14771211	14771211	4.52	0.0594
Residuals	10	32680156	3268016		