DEVELOPMENT OF A METHANOTROPHIC ALTERNATIVE DAILY COVER TO REDUCE EARLY LANDFILL METHANE EMISSIONS

by

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A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biology

Charlotte

2009

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ABSTRACT

BRYN LAUREN ADAMS. Development of a methanotrophic alternative daily cover to reduce early landfill methane emissions. (Under the direction of DR. HELENE HILGER)

Final covers, especially when supplemented with gas collection, are highly engineered systems to prevent landfill methane release into the atmosphere. However, some methane production begins even before open cells are covered and often well before final capping, representing an unaddressed source of methane release. A number of biotic cover designs, such as biofilters, biocovers, and bio-"windows", have been proposed as supplements to gas collection or as top covers on older landfills lacking gas collection systems. These systems employ media that promote the growth of bacteria which are able to oxidize methane to carbon dioxide and water. The purpose of this investigation is to explore the potential use of the methane oxidation capacity of methanotrophs embedded in a "biotarp" to mitigate methane release from open, active landfill cells. If successful, the biotarp could serve as an alternative daily cover during routine landfill operation.

A mixed methanotroph cell population was enriched and isolated from landfill cover soil. Three cell immobilization techniques were evaluated, including cell entrapment in alginate beads and in liquid-core gel capsules. Adsorption to a synthetic geotextile was found to be most feasible and yield the best methane oxidation rates (2.0 g CH_4/day). Evaluation of nine geotextiles produced two that would likely be suitable biotarp components. Pilot tarp prototypes were tested in continuous flow systems simulating landfill gas conditions. Multilayered biotarp prototypes consisting of alternating layers of the two geotextiles were found to remove 16% of the methane

flowing through the biotarp. The addition of landfill cover soil, compost, or shale as amendments to the biotarp increased the methane removal to over 30%. With successful methane removal in a laboratory bioreactor system, prototypes were evaluated at a local landfill using flux chambers installed atop a landfill section with an intermediate cover layer. The 4-layered biotarp and amended biotarp configurations were all found to decrease landfill methane flux; however negative controls were also observed to reduce methane flux equally well. Spatial and chronological variations in methane flux were also noted.

ACKNOWLEDGEMENTS

Foremost, I would like to express my utmost gratitude to Dr. Helene Hilger, advisor extraordinaire. Your role has extended far beyond that of advisor, the least of which include advocate, grammar instructor, role model, motivator, and friend. Thank you for being an exceptional example of a mentor, scientist, and a successful woman; and relentlessly challenging me, whilst always mindful of my best interests. An equally heartfelt gratitude is also extended to my dissertation committee members for their support and guidance throughout this entire endeavor.

Many individuals have directly contributed to this research and their involvement has been essential. I thank Dr. Jean Bogner for her valuable guidance concerning many aspects of this project, particularly the field work. I also thank David Jones and Chris Timpson for providing materials, Gary Abernathy for constructing the continuous flow chambers, Dr. Levente Bodrossy for conducting the diagnostic microarrays, Laura Shewmon for embedding and slicing geotextile samples, Jill Hudak and Dr. Didier Dreau for microscopy assistance, and Richard Jones for assistance with bioreactor optimization and much more. I also appreciate the assistance of many former and current students: Lindsey Talmadge, Julianne Todd, Kristen Sangliani, Bryant Scarlett, Craig Midget, Gabbi Martinson, Swetha Manian, Mary Fabian, Darla DeStephanis, Jean Hamm, and especially Fabien Besnard, who worked side-by-side with me throughout this entire project.

And finally, I owe my deepest gratitude to my family and dear friends. Thank you all for your support and encouragement over the years, and for always, at least, pretending you understood what my research was all about. I love you all.

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CHAPTER 1: INTRODUCTION AND RESEARCH OBJECTIVES

Landfills are one of the largest anthropogenic sources of methane in the U.S. and throughout the world. Upon landfill closure, final caps are highly engineered to prevent fugitive methane emissions into the atmosphere. However, during the time an open cell is being filled, no measures are taken to mitigate the early methane emissions. Methane production begins soon after waste placement and is likely routinely released and emitted through the 15 cm layer of soil placed over the waste at night. In a 2001 investigation of a French landfill site, Bogner found methane fluxes as high as 100-200 g/m^2 day over open cells (unpublished).

One category of methane mitigation technology that may be adaptable for open cell emissions mitigation is biotic cover designs. These include biofilters (120), biocovers (154), and biowindows (279), all of which are being tested for the removal of low-level emissions from closed landfill sites. These systems employ media that promote the growth of methanotrophic bacteria, a robust group of bacteria that use methane as their sole carbon and energy source. This energy is harvested from the oxidation of methane to carbon dioxide and water (133). They are abundant in ecosystems where methane is present, such as peat soils (281), arctic wetlands (334), freshwater lake sediment (74), rice paddies (72), and landfill cover soil (60, 166, 300, 308, 347). In addition to the mitigation of methane, these organisms are also capable of co-metabolizing some non-methane hydrocarbons (17, 205, 282).

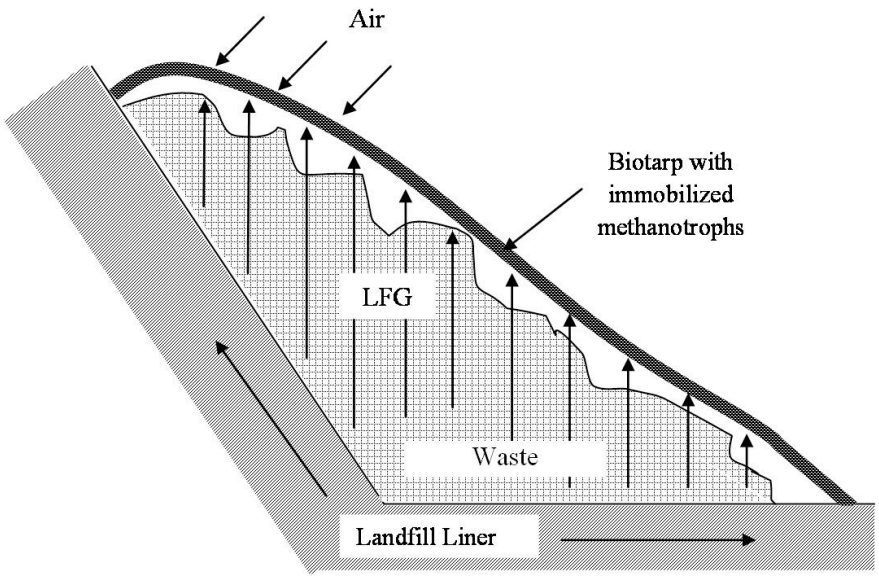

Figure 1. Conceptualized diagram of a methanotroph embedded biotarp to serve as an alternative daily cover and mitigate early methane emissions from landfilled waste.

U.S. federal law requires that daily cover be placed over open landfill cells at the end of each working day, and approved alternative daily cover (ADC) materials may be used in place the 15 cm of soil. The objective of this project was to investigate the feasibility of creating a methanotroph-embedded ADC, or " biotarp", to reduce early methane emissions from landfilled waste in open cells or under temporary cover (Fig. 1). As conceptualized, the biotarp would be portable, amenable to reactivation, and cost effective due to its ability conserve landfill air space. Specifically, the biotarp would be placed atop the refuse in open landfill cells at the conclusion of each working day and stored off the landfill during operation hours. While in place, early methane emissions

from the refuse would be oxidized by methanotrophs immobilized within the biotarp. As methane is produced by the waste, it is oxidized by embedded methanotrophs as it passes through the tarp.

In order to develop and determine the feasibility of a methanotroph embedded biotarp, the following specific research objectives were investigated:

i) Isolation of a mixed methanotroph population from landfill cover soil
ii) Identification of a feasible immobilization technique that enhanced methane oxidation
iii) Determination of immobilized methanotroph responses to temperature variation, methane starvation, and washing
iv) Evaluation and selection of biotarp prototype components
v) Construction and evaluation of biotarp prototypes using continuous flow chambers
vi) Visualization and verification of immobilized methanotrophs in prototypes
vii) Evaluation of biotarp prototypes under field conditions

CHAPTER 2: LITERATURE REVIEW

Methane and Global Climate Change

Methane is the most abundant organic gas in the atmosphere (67, 77) and is the second largest contributor to greenhouse gases after carbon dioxide (160). Although the concentration of methane is significantly lower than carbon dioxide, it has a much higher energy reemission. Solar radiation is absorbed by the Earth and a portion emitted into space in the form of infrared radiation. Methane and other greenhouse gases trap this radiation within the atmosphere. At higher green house gas concentrations, more energy is absorbed and radiated back to the Earth's surface. Molecule for molecule, methane absorbs infrared radiation about 21 times more efficiently than carbon dioxide (199).

As a result of methane's increased radiation adsorption capacity, a decrease in methane emissions has been estimated to be 20-60 times more efficient than an equivalent reduction in carbon dioxide for mitigating global climate change (146, 325). Atmospheric methane has a lifetime of approximately 12 years, with 90% being oxidized by hydroxyl radicals in the troposphere (331). Studies of air samples trapped in polar ice provide a 420,000 year record of atmospheric methane and carbon dioxide levels (260). These data indicate that current methane levels are unprecedented and that increases in methane correspond to increases in global temperatures. In the last 300 years, the concentration of atmospheric methane has increased from 0.75 to 1.7 parts per million by volume (ppmv). This concentration continues to increase by 0.8-1.0% per year, and it is

estimated to reach 2.1 to 4.0 ppmv by the year 2050 (267). Lelieved *et al.* (199) predicted that increases in atmospheric methane concentrations will decrease hydroxyl radical concentrations and thus, increase the methane lifetime up to 20% by the year 2050.

Using methane levels during the 1970's, Donner and Ramanathan (99) calculated the global surface temperature will increase by 1.3 K due to the effects of methane alone. The Intergovernmental Panel on Climate Change (IPCC) has indicated that anthropogenic increases in greenhouse gases contribute, in part, to global climate change. Furthermore, current climate models show that by 2100, the average temperature will increase by 1.4–5.8°C, with the greatest increases occurring at higher latitudes and over land (160). Global average annual rainfall is also predicted to increase and flooding could become more severe, although many mid and lower latitude land regions will become drier.

Methane Sources

Methane emissions, both natural and anthropogenic in origin, have been estimated to be over 500 teragrams (Tg)/year. Sixty percent of methane emissions have been attributed to human activities, such as agriculture, fossil fuel use and waste disposal, while the main natural sources of methane are wetlands, termites, oceans, and methane hydrates.

Wetlands. Wetlands, defined as environments with standing water for all or part of the year, produce 27% of the total methane emissions (approx. 145 Tg/year) (160). The wetlands provide an anaerobic, organic carbon rich environment, optimal conditions for the establishment of a large population of methanogenic bacteria. These bacteria then produce methane from acetate and hydrogen (258). A number of environmental

parameters influence the amount of methane released from wetlands, including nutrient availability, vegetation cover, water table depth, and soil temperature (232, 327).

Termites. Although the amount of methane released by a single insect is small, the large number of termites, along with their wide geographical distribution, makes them a significant source of methane (167). Termite methane production accounts for approximately 4% (20 Tg/year) of the total methane emissions. Methanogenic bacteria located in the hindgut region of the termite gut (132) convert carbon dioxide and hydrogen into methane (251). Some studies suggest that diet may influence the amount of methane produced, with soil-fed termites emitting more methane than wood-fed termites (26, 46).

Oceans. Methane is slightly supersaturated in seawater, with the highest concentrations in near-surface water (67). This source is responsible for only 2% (10 Tg/year) of methane emissions (160). The exact sources of oceanic methane are not clear, although anaerobic niches in fish intestines, fecal pellets and decaying plankton are likely sources (254). Cold gas seeps and hydrothermal vents are also sources of methane, although the extent to which they contribute is not currently clear.

Hydrates. Methane hydrates are the frozen form of natural gas. They usually form in deep sea sediments, which are high in organic content. Methane and other low molecular weight gases are trapped in a water lattice as a clathrate (291). Changes in the surrounding temperature, pressure, or salinity cause the release of methane from the hydrates (315). It has been estimated that 5 Tg/year are released from methane hydrates, contributing to approximately 1% of total methane production.

Fossil fuels. Fossil fuels, an anthropogenic methane source, account for 18% of the total methane emissions, releasing 95 Tg methane into the atmosphere each year. The primary source is natural gas leaks, which occur during processing, transportation and distribution (21). The exact leakage rate remains unclear, as they vary between countries (197) and leakage may be lower in developed countries because transportation is more tightly controlled (181). Methane is also emitted from coal mines, where it is released from layers of coal during the mining process (21).

Domestic ruminates. Methane emissions from domestic ruminants, including cattle, sheep, buffalo, and goats, are associated with methane production in the gastrointestinal tract. Many dietary factors influence methane production, including feed processing, as well as starch and lipid content (28). These emissions account for 18% (93 Tg/year) of annual methane emissions (160) and results from the incomplete digestion of low quality feed.

Waste decomposition. Approximately 59 Tg (9%) of methane is emitted yearly due to the decay of biogenic waste in anaerobic environments, mainly landfills and wastepools (288). Wastewater has been identified as a source of methane, resulting from the anaerobic digestion of organic materials. It has been estimated that approximately 13.6m^3 methane is produced per 1000 people/day (312). Methane production in landfills will be discussed in detail in following sections.

Rice cultivation. Rice paddies are one of the most important sources of anthropogenic methane, producing 11% of the total methane released annually. They are flooded most of the year for rice cultivation and methane is produced in a manner similar to that in natural wetlands (18). Three distinct pathways of methane release in rice

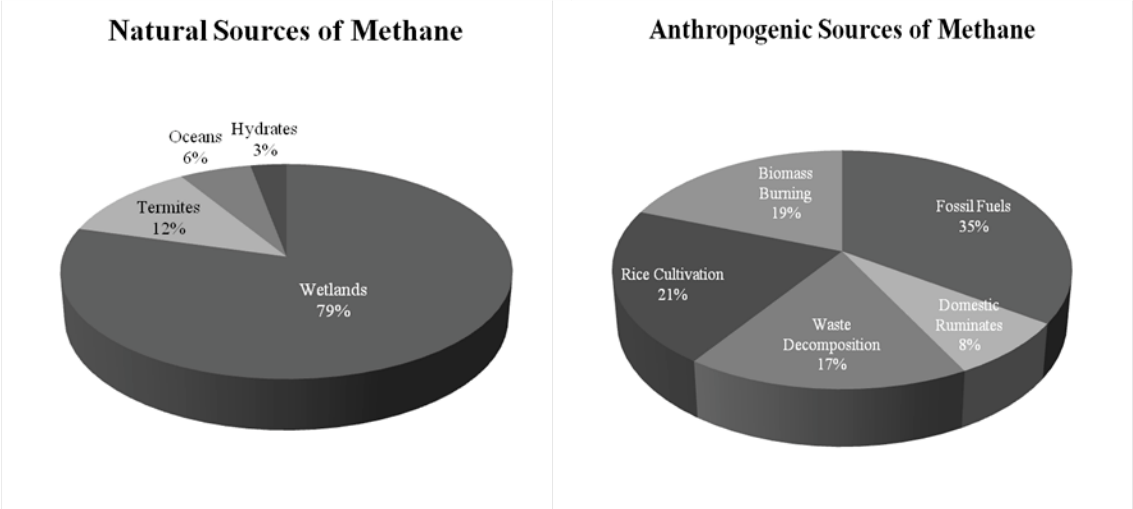

Figure 2. Natural and anthropogenic sources of methane

paddies have been identified; methane dissolved gas bubbles, dissolved methane diffusing into the vapor phase, and plant mediated transport (282).

Biomass burning. Biomass burning represents the smallest percentage of anthropogenic methane emissions, 10% (52 Tg/year). However, this is an important source of other pollutants in addition to methane. If combustion is incomplete, large quantities of methane and other higher-order hydrocarbons are released (200).

Methane Sinks

Although there are numerous sources of methane, there are only three types of methane sinks, or means of methane removal. The major methane sink, a reaction between methane and hydroxyl radicals in the troposphere, consumes 90% of the atmospheric methane released. The minor methane sinks are removal by dry soil oxidation (approximately 5%) and transport to the stratosphere, where methane is consumed by reactions with chloride ions (124, 289).

Reactions with hydroxyl radicals. Hydroxyl radicals are produced in the atmosphere when ozone reacts with water vapor in the presence of ultraviolet light. In the troposphere, the hydroxyl radicals react with methane to form water and carbon dioxide (67). Other greenhouse gases, including carbon monoxide, may also react with the hydroxyl radicals. Therefore, when the concentration of these other gases increase and theycompete for the hydroxyl radicals, the lifetime of methane in the atmosphere also increases (209). When NO_x is plentiful in the atmosphere, it can catalyze the breakdown of formaldehyde, an intermediate of methane oxidation, ozone and carbon dioxide (268).

Oxidation in soil. Methane oxidation in soil is thought to be due largely to the metabolism of methane by microorganisms, primarily a group of methane-oxidizing bacteria called methanotrophs (166, 337), although nitrifiers, some yeasts, and even some anaerobes likely undergo reverse methanogenesis (66, 266, 336, 350, 351). Among methanotrophs, there are two distinct groups, each distinguished by their methane affinity. Although much is known about the low affinity methanotrophs that can initiate metabolism only at high methane concentrations (> 40 ppm), the bacterial population responsible for oxidation at low methane levels (<12 ppm) is less well characterized (22). Methane oxidation by methanotrophs will be discussed in further detail in following sections.

Other methane sinks. In the stratosphere, methane reacts with chloride atoms to form hydrochloric acid. It is estimated that less than one Tg/year is removed in this manner (78).

Landfill and Methane Production

Landfills are among the largest anthropogenic sources of methane, and were reported to account for approximately 37% of the annual methane emissions in the United States for 2001 (160). For this reason, solid waste management remains a critical element of climate change mitigation. Approximately 245.7 million tons of municipal solid waste (MSW) was generated in the US in 2005, with 54% of the waste being buried in landfills. Although the number of landfills in the US has decreased significantly over the last eighteen years, the size of individual landfills has increased (324). Landfills will likely remain the primary means of waste disposal because landfilling is the least expensive waste management alternative in the U.S.

Structure and Design

An engineered landfill is a controlled method of solid or hazardous waste disposal that is designed to prevent pollution or degradation of the surrounding environment. Subtitle D of the Resource Conservation and Recovery Act (RCRA), as well as state regulations, seeks to ensure that buried waste is sequestered from the environment. A landfill site is typically excavated and a liner system installed along the bottom and lateral sides. This liner system includes a 0.67-1.0 m compacted clay layer overlain with a 1.5 mm thick geosynthetic (impermeable synthetic noncellulose) liner material. It serves to prevent leachate (liquid produced from the degradation of waste) from contaminating groundwater. A leachate collection system is also installed within the liner system and directs the leachate to low points at the bottom landfill. The collected leachate is removed either by gravity flow or pumping, and is then treated, recirculated, or transported off site for disposal.

The basic unit of a landfill is a cell, which includes daily deposits of compacted waste and daily layers of cover material. A cell is typically 3 m in height, although heights of 10 m have been employed. Cells typically have a rectangular area and steeply sloping sides. Waste is deposited into a cell each day and compacted to 710-950 kg/m^3. At the end of each work day, the waste is covered by soil, which excludes disease vectors, rodents, and some rainwater, and minimizes odor and windborne litter. A given cell is filled to a designated height, after which a new cell is begun. After adjacent cells in a sector are filled to the same height, they are collectively referred to as a lift (Fig. 3). An intermediate cover is then installed. This cover is typically a 15 cm layer of soil or combination of soil and compost that provides a more permanent barrier to odor and stormwater. New cells are then established over the intermediate cover until the landfill section has reached a pre-determined height. A final cover is then placed on the landfill to minimize infiltration of rain water and dispersion of waste. This cover also aides in the long-term maintenance of the landfill. The exact composition of the final cover is set forth by local governments. It will typically consist of a gas control layer that routes gas to flares or a gas collection system, a filter and drainage layer, and a layer of seeded topsoil for erosion control.

Methane Generation in Landfills

The biological decomposition or organic matter in the buried waste and the concomitant generation of gaseous by product is mediated by microorganisms. Overall, these processes pass through a number of defined phases, although the rate can vary from region to region in a given landfill due to the heterogeneous nature of the deposited waste. The end-product of these processes is methanogenesis. The first phase is aerobic,

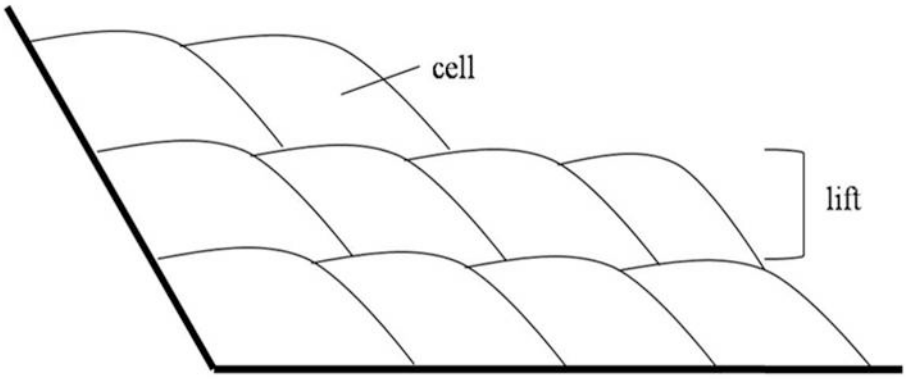

Figure 3. Diagram of a typical landfill.

with both O_2 and NO_3 used as electron acceptors for the metabolism of readily biodegradable soluble sugars (3). After oxygen and nitrogen are depleted, an anaerobic phase begins (4), and carboxylic acids, ammonia, CO_2 and H_2 accumulate, as well as acetate and alcohols, due to the hydrolysis of polymers such as carbohydrates, fats, and proteins by fermentative microorganisms. As these new by-products accumulate, the anaerobic, methanogen population expands. Methanogen catabolism follows one of two paths, with cells deriving energy by either producing methane from hydrogen and carbon dioxide or converting acetic acid to methane and carbon dioxide. When these reactions are fully developed, the degradation is considered to be in accelerated methane production, designated as the third phase (5). The two paths for methane production are shown below (225):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$

Accelerated methane production is followed by a decelerated phase, where the rate of methane production decreases as substrates become depleted (235).

Landfill Alternative Daily Covers

Title 40, Part 258 of the Code of Federal Regulations, Solid Waste Disposal Facility Criteria requires landfill owners or operators to cover compacted waste with 15 cm of earthen material at the end of each operating day; more frequent coverage is required if there are problems with disease vectors, fires, odors, wind-blown litter or scavengers. This type of daily landfill cover consumes valuable landfill space and reduces the landfill operating life. In cases where soil is not available on site, it must be purchased and transported to the site, which significantly increases operating costs. As a result, several types of alternative daily covers (ADC) have been developed and can be categorized as blankets, sprays, and slurries of waste materials.

Blanket ADCs are large tarps that cover the working surface of a landfill. This type of ADC is out in place at the end of each operating day, and although many are placed by landfill staff, some are applied with dedicated motorized roller machines. Reusable tarps made of various types of polypropylene or polyethylene geomembranes are taken up each morning, while non-reusable blanket ADCs are composed of thin polyethylene, polypropylene, or polyvinyl chloride. Some non-reusable blankets will thermally degrade in 4-6 weeks; however, others must be perforated to allow them to be left in place without acting as an impermeable layer.

Spray ADC may be applied as either a slurry or a foam. Slurries are solids, such as newspaper, mixed paper, wood fiber, cement kiln dust or fly ash, mixed with water and sprayed over the working landfill surface. The slurry is applied in a thin layer and is

designed to harden over the waste after 20 minutes to 2 hours. Foams are composed of synthetic materials, such as resins or soaps, and are mixed with water prior to application as a thin layer with a specialized foam sprayer. However, unlike a slurry ADC, the foam does not harden. Compared to daily cover soil, both blanket and spray ADCs take up negligible landfill volume.

Waste ADCs may employ yard waste, municipal or industrial sludge, auto shredder waste, shredded tires, cement kiln dust, and impacted soil. Although the waste material consumes fill capacity that is approximately equal to that of traditional daily soil cover, it does generate some tipping fee revenue (137).

Biotic Methane Mitigation Systems

Final capping systems for modern landfills are highly engineered to efficiently prevent methane release into the atmosphere (312). At large landfills, they typically include some type of active or passive gas collection system. However, once methane generation has entered the decelerated phase of production, the costs of operating a collection system may not be warranted, and a variety of engineered bio-based covers are being pilot tested to control methane emitted during the low production period.

Bio-based systems exploit the fact that some microorganisms can oxidize methane as it permeates upward into the aerobic regions located at the top of the waste (79, 207, 214, 337). Such systems seek to provide ideal conditions for methane oxidizer growth and enhance the microbial population interface between the waste and the atmosphere. These systems may also be used alone during new landfill start up or as a supplement to gas collection in order to capture fugitive methane emissions. They are also suitable at small landfills where gas collection is not technically or economically feasible (153).

Three basic types of biogas collection systems have been designed and pilot tested: biocovers, biofilters, and biowindows (153).

Biocovers

One of the original biocovers investigated was a compost biocover that was employed to reduce methane emissions from a closed landfill site in Austria (158). Several types of compost and configurations were assessed including (a) well composted municipal solid waste underlain with coarse gravel; (b) composted sewage sludge layered directly over the waste; (c) sewage sludge compost underlain by compacted loam; and (d) sewage sludge compost underlain with coarse gravel. Methane was found to be emitted from control plots and plots not underlain with gravel. However, no methane was detected from plots where either sewage sludge compost or municipal solid waste compost was underlain with gravel. The authors concluded that the gravel layer was important for gas distribution and porosity, while the compost provided the proper water-holding capacity and good thermal insulation properties. Subsequent laboratory investigations found that a mature and porous compost enhanced methane uptake over that achieved in conventional landfill cover soil (154). The addition of a substrate that increased porosity also proved to be important in the optimization of methane consumption in simulated biocover samples.

In the US, pilot tests using compost biocovers have demonstrated potential for mitigating methane emissions in a variety of landfill settings (19). Methane uptake in areas topped with a biocover was almost double that in control sites with intermediate cover. A biocover configured for a closed landfill by Stern *et al.* (297) used crushed glass for a gas distribution layer covered by pre-composted yard or garden waste over

intermediate cover. Their biocover cells reduced methane emissions 10-fold relative to non-treated control cells. The authors concluded that the increased methane oxidation by the biocover was a result of longer gas retention times due to higher contact time in the cover. Greater insulation against moisture loss likely resulted in less desiccation in the thicker compost cover material and, thus, a reduced methane flux was observed.

Biofilters

Biofilters are also designed to host a methane oxidizer population, as well as other microbes that can remove odor and no-methane organic compounds (NMOC's); however, they are confined to smaller areas and require an active or passive system to feed the landfill gases into the filter. Oxygen is obtained from the air diffusing downward into the material; therefore, a particular biofiltration material must have high gas permeability, large surface area, and proper environmental conditions to promote methanotrophic growth and methane oxidation. (153). Various types of media have been investigated under laboratory conditions, including assorted composts (95, 112, 299, 304, 344), wood chips, bark mulch, peat, or glass (292), bottom ash (221), porous clay pellets (120), sand and soils (256, 262), and mixtures of organic and inert materials (100, 228).

Laboratory columns simulating biofilters showed successful methane removal (136, 256, 292, 305, 344). Although the biofilter composition (soil, glass tubing, wood fiber, and compost) and methane inflow rates varied among experiments, the studies noted that methane removal was dependent on the initial methane concentration. Field studies of biofilters have also shown success in methane reduction. A field scale compost biofilter was able to remove 10-20 g $CH_4/m^3 \cdot$hour with methane loading rate of 9-112.5 $g/m^3 \cdot$hour (303), while a biofilter constructed with pelleted inorganic porous clay topped

with 10 cm of grassed removed a maximum 80 g CH_4/m^3·hour, almost 100% of methane input (121). In a biofilter containing compost underlain with bark or wood chips, 90% methane removal was observed with a methane loading rate of 1.1-2.5 g CH_4/m^3·hour (299). Although various biofilter configurations showed successful methane removal in the field, it was found that high landfill gas input can limit performance if the methane flux from below outpaces the rate at which oxygen can diffuse downward (121). Temperature (120, 304), moisture holding capacity (262) and exopolymeric substance (EPS) formation (305) are also important influences in biofilter functioning. It has been suggested that nutrient imbalance (316) or other stressors (118) can promote EPS formation, which can be problematic in bio-based systems as it tends to clog the system and slow the rate of methane oxidation (145).

Biowindows

Biowindows are similar to biocovers, but they cover only small regions of a landfill rather than the entire surface. They are used where a full biocover is not warranted (e.g. to address isolated "hot spots", where cracks in the landfill surface develop), and no gas collection system is present to feed a biofilter. Biowindows are integrated into a conventional landfill cover in discrete sections, offering a "path of least resistance" for leaking gas (153). A field-scale project is currently underway at the Fakse landfill in Denmark in which sections of the final cover has been replaced with high methane oxidation materials. 10 x 10 m biowindows were constructed by placing 1 m of composted garden waste over 10-15 cm gravel layer (115). The maximum methane oxidation capacity of the compost was measured to be approximately 150 g CH_4/day using laboratory batch and column studies (116).

Immobilization

Immobilization techniques have been widely explored over the last 30 years and have been applied to all types of cells, organelles, as well as enzymes, proteins, and other subcellular structures (61, 175, 342). An immobilized cell is defined as a cell, or remnant thereof, that by natural or artificial means is prevented from moving independently of its neighbors to all parts of the aqueous phase of the system under study (310). Numerous investigations have demonstrated the advantages associated with the use of immobilized cells. Pashova *et al.* (257) found pectinolytic enzyme activity levels were greatly increased in immobilized cells of *Aspergillus niger* compared to free cells. Others found that when *Pseudomonas* sp. and *Xanthomonas maltophilia* were immobilized, the degradation rate for acrylamide increased (243). Further examples of immobilized cells and organelles has been discussed by Mattaisson (220).

Six distinct types of general immobilization methods have been defined: covalent coupling, affinity immobilization, adsorption, confinement in a liquid-liquid emulsion, capture behind a semi-permeable membrane, and entrapment (212). Covalent coupling is a common technique used to immobilize enzymes to a solid support by permanently covalently bonding them together (310). This method is not used often with viable cells, as the covalently immobilized cells are unable to divide (212). Affinity immobilization takes advantage of the fact that some cells have unique surface characteristics that allow them to selectively bind to a substrate (310). *Staphylococcus aureus* cells were immobilized to plastic coated with fibronectin and collagen due to this organism's natural affinity for these substrates (223). An *Escherichia coli* strain expressing a cellulose binding domain was successfully immobilized to cellulose supports (332). The

confinement of cells, organelles, or molecules within a liquid-liquid emulsion is an efficient means to forgo problems associated with the use of a solid support. In this technique, the biocatalyst of interest is entrapped within one liquid phase that forms small droplets when introduced into a second liquid phase. The cells must be stable in the two liquid phase systems selected and the required nutrients must be portioned along with the cell. Although such systems can prove suitable for small-scale laboratory work, they are considered impractical for industrial scale applications because of the high material costs (310). Capture behind a semi-permeable membrane involves the use of hollow fibers. In this process, macromolecules are retained within the system, but free diffusion of some low molecular weight solutes is permissible. Multiple individual fibers are bundled together to allow bulk flow in and out of the system. The space between the fibers can be packed with cells in such a manner that nutrients are continuously delivered to the cells and waste removed. Although this system can be very useful for maintaining cells, fiber pores can easily become blocked, and it is difficult to remove adherent cells from the apparatus (310). Adsorption and entrapment will be discussed in detail below.

Adsorption

Adsorption involves nonspecific interactions between cells and the surface support material. Bhamidimarri (25) describes three types of forces involved in microbial adsorption: short range forces, interfacial reactions, and long range forces. Short range forces are thought to be the most important (259) and include dipole-dipole interactions and hydrogen bonding. Interfacial reactions are those involved in the conditioning of the surface by microbial production of EPS. Long-range forces consist of Van der Waals forces and electrostatic interactions. The electrostatic forces result from the charges

associated with the cell and the surface of the support. Mozes *et al.* (236) presented evidence that adsorption of microorganisms to a support was the result of electrostatic interactions. Adsorption has been optimized by altering the electrostatic charges of cells and a support surface to increase the immobilization yield (231). The role of Van der Waals forces in adsorption has been shown empirically by Klotz *et al.* (184). They demonstrated the adherence of *Candida albicans* and other *Candida* spp. to inert plastic surfaces was a result of Van der Waals attractive forces. In addition to cells being attached to a surface by adsorption, a portion of the cell population may remain physically trapped within the support, but dispersed in the liquid phase and not physically attached (183).

In addition to physio-chemical attachments, cellular structures can contribute to adsorption. Three types of cell- mediated attachments to surfaces have been identified: extracellular adhesions, holdfasts, and lipopolysaccharide attachment (25). Furthermore, flagella are thought to aid in chemotactic responses and hold cells in close proximity to the surface.

It is likely that some combination of cellular, physical and chemical factors mediate the passive attachments of cells to surfaces, depending on the particular microbe and surface involved. What is clear is that cell adsorption is a fairly common phenomenon, and it has even been shown to increase the activity of cells. Slabova and Nikitin (290) employed methanol-degrading bacterial strains immobilized via adsorption to polypropylene or polyvinyl formal foam. Foam granules were incubated in a turbid bacterial culture (OD 2-2.5) for 1-1.5 hours, washed, and placed in fresh nutrient medium for 1 day. The granules were then removed, washed and placed in fresh medium, with

this process repeated seven times to obtain granules with well-absorbed cells. The three strains examined were able to maximally utilize the substrates when adsorbed to both types of supports. A recombinant *Escherichia coli* strain, which produced human epidermal growth factor (hEGF), was adsorbed onto porous polyurethane foam particles in order to enhance plasmid stabilization (62). The cells were added to a flask or bubble column containing the foam particles and allowed to adhere in culture. These immobilized cells resulted in enhanced hEGF secretion rates.

The use of adsorbed cells has been shown to have many practical applications. *Pseudomonas putida* cells were immobilized by adsorption onto magnetite in order to treat Cu^{2+}-containing municipal wastewater (333). A strain of blue green microalgae was immobilized on a loofa sponge in a continuous flow fixed bed column reactor to efficiently remove heavy metal ions from aqueous solution (278). Tse and Yu (319) adsorbed a *Pseudomonas* strain capable of degrading synthetic dyes to porous glass beads and solid polyvinyl alcohol particles to increase degradation efficiency from an initial rate of less than 10% to 80%. Cells adsorption is also used to mount samples for atomic force microscopy (AFM), such that three-dimensional images of cells under physiological conditions can be obtained (106).

Adsorption has several advantages over other immobilization techniques. It is the most gentle immobilization method because it is passive, and only the natural properties of the cells and support surface are involved (310). Furthermore, it typically requires no changes in cultivation conditions (183). Many investigations of adsorbed cells demonstrate increased cellular activity (217, 242); however this is not always the case

(56, 298, 341). It has been suggested that the increase in activity is a result of increase nutrient concentration and not the physical attachment (362).

Despite the advantages, there are several potential problems with using adsorption to immobilize cells. When the adsorption is mainly physio-chemical and nonspecific, cells may desorb from the surface as readily as they attach (310). Furthermore, changes in ionic strength (219, 342) or pH (342) can lead to cell desorption, as can gas or liquid shearing forces (310). For these reasons, cell immobilization by adsorption is not ideal for harsh or highly variable environments.

The type of support selected for adsorption is also critical. The support must be nontoxic and have a high surface area accessible to the cell (188). Atkinson *et al.* (14) expanded the description of a desirable support material to include the ability to withstand heat sterilization, to resist microbial degradation, have cost appropriate to the application, and possess the ability to be reused. A variety of organic and inorganic supports have been explored, including polyurethane foam (216), wood shavings (127), stainless steel wire meshes (126), natural cellulose sponge (278), ceramics (217), brick (253), porous glass (242), and alumina (191).

In addition to retention, many applications require that the cells be able to grow and replicate. Microorganisms attached to a surface by physio-chemical interactions often lead to the formation of biofilm (250). Biofilms are actually quite common in nature, with attached microorganisms vastly out-numbering planktonic organisms in natural environments (104).

The formation of a biofilm enhances attachment of cells to the support and increases their resistance to environmental stresses. In a review exploring the incentives

for bacterial biofilm production, Jefferson (161) suggests that biofilms play a protective role by allowing the cells within it to withstand shear forces, nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms. However, there can also be some limitations for cells deep in a biofilm if substrate cannot easily diffuse through the matrix layers (237). A portion of cells within a biofilm can become nutrient and oxygen deprived, leading to lowered cellular activity. The trade-off between some of these benefits and drawbacks may offer an explanation in the inconsistency in reported cell performance among various investigations.

Entrapment

Cell entrapment is the most frequently used immobilization technique, wherein cells are contained in an artificial three-dimensional gel matrix. Unlike adsorption, there are many different methods to entrap cells, and they are typically independent of the natural properties of the cells themselves (310). There are also many different materials that can be utilized to entrap cells, and material selection depends on cell type and application properties. A few of the most common materials include alginate, polyvinyl alcohol, and proteins.

Polyvinyl alcohol (PVA) is a hydrophilic polymer in which hydrogen bonding occurs between neighboring hydroxyl groups of the polymer chain to form a non-covalent network (349). At temperatures below 0°C, this bonding is enhanced and is considerably stronger (208). PVA is very stable and resists biodegradation, making it ideal for nonsterile conditions. The gel strength can be modified by the degree of deacetylation, polymer chain length, concentration, and thaw time (349). In 1998, Jekel *et al.* (162) introduced a new method that allowed gelation at room-temperature, which

avoided much of the cell loss that occurred during the freezing process. Application of PVA-entrapped include ethanol production (270), wastewater nitrification (151), enzyme production (218), nucleoside synthesis (317, 318) and gasoline desulferization (201).

Proteins have many properties that make them excellent candidates for use in entrapment techniques. The type of film formed depends on their composition (proportion of hydrophobic and hydrophilic residues) and the degree of unfolding they undergo, with the film forming as the unfolded protein separates from the solvent phase. Most protein films are moisture sensitive, but provide an excellent barrier to nonpolar substances, such as oxygen and fats (329). Good film performance correlates with good surface active properties, film forming and mechanical properties, high gas barrier properties, and a high resistance to organic solvents and fats (88). Other beneficial properties are that it be biodegradable, and easily modifiable. Each protein film type may have unique properties that make it suitable for a particular application. Both animal and plant proteins are available, and include collagen, gelatin, keratin, wheat gluten, soy and pea proteins (329).

The methods by which cells can be entrapped are numerous. One such method is spray drying, where a cell suspension is atomized using compressed air or nitrogen. The product is collected in a desiccation chamber and dried under a current of hot air (329). Entrapment by extrusion disperses cells within a molten mass, which is then cooled and solidified (314). During the coacervation method, the protein is precipitated onto the cell as a coating (329). Recent applications include the use of whey protein to immobilize probiotics (272), the use of a starch-milk-gluten matrix to co-immobilize lactic acid

producing bacteria (261), and the conversion of sucrose via intracellular invertase by cells immobilized in gelatin (307).

One common immobilization technique is to entrap cells in alginate beads or sheets (229). Alginates are natural marine polymers that have been used in various applications for emulsification, thickening, film formation and gelation (252). They are composed of copolymers of D-mannuronic (M) and L-glucoronic (G) acid joined in a blockwise fashion by a glycosidic bond, allowing for three possible configurations: M-blocks, MG-blocks, and G-blocks.

Bead preparation involves two main steps; first the formation of an alginate bead with an internal cell-containing core, and then gelation of the alginic acid by multivalent cations (310). Cells are added to a solution of alginate and added dropwise into a bath of dilute aqueous $CaCl_2$. The Ca^{2+} ions react with the alginate molecules, causing them to cross-link (229). This alginate gels and traps the cells inside a solid-gel bead. The bead size is an important element in a successful entrapment procedure. Beads should be large enough to contain the cells and be handled with ease. The exact size depends on the type of nozzle used, the viscosity of the alginate solution, and the fall distance to the $CaCl_2$ bath. The gelling solution can also affect bead size, with a low G-content alginate being more susceptible to volumetric changes (229).

Some applications of alginate entrapment include the use of immobilized organisms to deliver probiotic organisms beads (193), to remove contaminates in wastewater treatment processes (309), and degrade soil contaminates (202) among numerous other uses. One of the most notable uses of alginate beads is in the

development of tissue replacement and artificial organs, such as the immobilization of islet of Langerhans as an artificial pancreas for the treatment of Type I diabetes (361).

Alginate gel entrapment has many advantages, mainly that it tends to be very mild on cells and maintains high viability (310). It is easily performed, and the alginate itself is inert and nontoxic (174, 175, 264, 269). However, the ionotrophic nature of the alginate makes it highly susceptible to chelating agents, such as phosphate, lactate, and citrate (229). Also, cells that are located at the bead surface are likely to proliferate more rapidly, leading to mass transfer resistance and bead leakage at the surface (286, 287, 311). The alginate bead has a gel polymeric matrix pore size of approximately 10nm (182), which reduces the space in which cells can proliferate and prevents high cell densities from being reached. Furthermore, as the cell density increases within the bead, the strength of the matrices decreases (249).

Entrapment of cells using liquid core alginate gel (also referred to as hydrogel membrane) capsules is similar to the formation of alginate beads, in that similar components are used. However, gel capsule formation is accomplished by a reversal of solutions – cells mixed in a dilute $CaCl_2$ solution are added dropwise to an alginate solution. Calcium ions diffuse from the center of the droplet and bind alginate chains at the surface, such that an alginate membrane will form around a soft gel core of $CaCl_2$/cell mixture. The gel core will not solidify, as it does in the alginate bead (295). This technique has several advantages over the use of alginate beads, primarily that the cells never directly contact the alginate solution. Additionally, capsule size, membrane thickness, and pore-size can be altered during gel capsule production. Capsule size can range from 100 μm to several mm in diameter and is determined by the microdroplet

generator aperture size. The membrane thickness is determined by the droplet incubation time in the alginate solution; decreasing this time produces a thinner membrane, while increasing this time thickens the membrane. The addition of various molecular weights and the concentration of non-gelling polymer, such as dextran, to the $CaCl_2$ solution can be used to create specific pore sizes within the capsule. After capsule formation, these non-gelling polymers will diffuse out (247).

Methanotrophic Bacteria

Methanotrophic bacteria are aerobic, Gram negative microbes that can utilize methane as their sole carbon and energy source. Methanotrophs are able to enzymatically oxidize methane (from both atmospheric and high methane sources) by combining it with oxygen to form carbon dioxide and water. As a result of their methane oxidation activity, they play a role in the global methane cycle, serving as the largest biological methane sink (133, 179).

Taxonomy

At present, there are 13 recognized methanotroph genera (29, 44, 93, 94, 133, 143, 321, 348), which were originally classified as Type I, II, or X based on morphology and physiology. Whittenbury *et al.* (339) isolated over 100 methane utilizing organisms and grouped them according to a Type I or Type II membrane structure. Type X was later added to accommodate the characterization of *Methylococcus capsulatus* and other similar organisms (133). 16S ribosomal DNA sequence analysis confirmed the presence of three distinct groups, with Type I and X methanotrophs forming distinct clusters in the gamma subdivision of the *Proteobacteria* and Type II clustering in the alpha subdivision (45, 47). However, methanotroph taxonomy was later revised, and Type X organisms

Table 1. Methanotroph Classifications

Characteristics	Type I	Type II
Proteobacterial subdivision	gamma	alpha
Cell morphology	short rods, usually occur singly; some cocci or elipsoids	crescent-shaped rods, rods, pear-shaped cells, sometime occurs in rosettes
Membrane arrangement		
bundles of vesicular disks	yes	no
paired membranes aligned to periphery of cells	no	yes
Nitrogen fixation	no	yes
Resting stages		
Exospores	no	some strains
Cysts	some strains	some strains
RuMP pathway present	yes	no
Serine pathway present	no	yes
Major phospholipd fatty acids	14.0, 16:1ω7c, 16:1ω5t	18:1ω8c

Adapted from Hanson and Hanson (133)

were grouped with Type I as Family *Methylococcacea* (45, 47). A summary of characteristics distinguishing the two types is presented in Table 1.

Methane Oxidation

The first step in the metabolic pathway of methane oxidation is initiated by the enzyme methane monooxygenase (MMO) (10, 11, 82-84). This enzyme catalyzes the insertion of oxygen into the methane molecule to form methanol and water. Two isoforms of this enzyme have been identified in methanotroph: soluble MMO (sMMO) and membrane-bound or particulate MMO (pMMO). All but one methanotroph genera express the pMMO, and only a small group is capable of expressing both isoforms (101).

sMMO is composed of three components: a hydroxylase, where the active site is located, a reductase that transports electrons from NADH to the active site, and a regulatory protein (230). As the name implies, this enzyme remains soluble after high speed centrifugation (82, 206) and is highly conserved among species that express it

(238, 239). sMMO has a wide range of substrate specificity, oxidizing various alkanes, alkenes, and aromatics compounds (71). pMMO, though much more prevalent in methanotrophs, is less well characterized due to being an integral membrane protein. This isoform has three subunits, α, β, and γ (203, 359) and evidence suggests that the active site contains copper ions (57, 203).

After methane is oxidized to methanol, it is then further oxidized to formaldehyde by periplasmic methanol dehydrogenase (MDH) (10, 86, 352). The formaldehyde is then assimilated into the cell by either the serine or ribulose monophosphate (RuMP) pathway, or oxidized to formate by the formaldehyde dehydrogenase (FalDH) (11, 133). The serine pathway is utilized by Type II methanotrophs, whereas Type I methanotrophs utilize the RuMP pathway. Both pathways allow formaldehyde to be converted to intermediates, which are then used for the biosynthesis of cellular products (9, 96, 263). Two moles of formaldehyde and 1 mole of carbon dioxide are used to form a three-carbon intermediate in the serine pathway. In the RuMP pathway, 3 moles of formaldehyde are used (9, 11, 90, 194, 263). Formate, which results from the oxidation of formaldehyde, is further oxidized to carbon dioxide by a NAD-dependent formate dehydrogenase (9, 96).

Factors Influencing Methane Oxidation

Methanotrophs are ubiquitous and have been found in swamps, rivers, rice paddies, oceans, ponds, soil from meadows, deciduous forests, streams, sewage sludge, as well as deep sea mussels (73, 134, 135, 142, 147, 155, 284, 285, 301, 339). A number of factors influence the type of methanotroph present in a given environment, as well as the population density of each type. One such factor is methane concentration. High concentration methane environments, such as landfills, as well as low methane

concentration environments (atmospheric concentrations) are dominated by different methanotrophic species (102, 129, 130). With the exception of one strain, all pure culture isolates are low affinity methane oxidizers that require rather high methane concentrations to initiate metabolism (186). Thus, little is known about the physiology of the high affinity methanotrophs.

Several key environmental parameters that affect methanotroph methane oxidation rates are temperature, the presence of ammonia and nitrate compounds, and moisture content. Landfill soil incubated under batch conditions shows a clear oxidation response to temperature, with lower methane uptake rates at lower temperatures (below 20°C) and an optimal temperature at 35-37°C (89, 330). Similar temperature optima were found with biofilter material (120). Einola et al (105) found methane oxidation occurred in landfill cover soil samples incubated over the range of 1-19°C. Some evidence suggests that temperature may also affect the population structure of methanotrophic communities. Borjesson *et al.* (40) observed that landfill soil samples incubated at temperatures between 3°C and 10°C consisted of only Type I methanotrophs, whereas samples incubated at 20°C consisted of both types of methanotrophic bacteria. Furthermore, microarray analysis of biofilter samples showed that incubations at different temperatures led to distinct changes in methanotrophic community composition (119).

Methane oxidation can be inhibited by ammonia, which acts as both a competitive inhibitor and leads to the production of hydroxylamine (27, 48, 51, 76, 178, 248). The lack of specificity of sMMO and pMMO leads to its reaction with ammonia, as well as methane. These enzymes oxidized ammonia to nitrate; however methanotrophs can derive no energy from the reaction (213). Furthermore, the oxidation of ammonia

produces hydroxylamine as a by-product, a compound that can also inhibit MMO activity (156).

Soil moisture content is possibly the most important factor affecting methane oxidation. Most investigations have found that approximately 15% moisture is optimal for methane oxidizers in soil (33, 43). In contrast compost, which is much more porous and water absorbent, supports optimum methane oxidation in the range of 45-110% moisture(234). The lower porosity of soil and sand (relative to compost) requires that soil moisture levels be sufficiently low enough to allow gas permeation (176). If a soil pore is completely filled with water, the transport of oxygen to the soil bacteria is slowed markedly. The diffusion coefficient of methane is 10^4-fold lower in water than in air. Castro *et al.* (53) observed the negative effect of high moisture content in forest soil. Methane consumption declined as moisture increased from 60 to 100% water-filled soil pore space (190). Lower than optimal moisture levels are also problematic (34); Whalen *et al.* (337) demonstrated that deviations from the optimal soil moisture content resulted in decreased methane oxidation rates in landfill cover soil. At the optimal moisture content of 11%, approximately 35% of the methane was oxidized in 12h; however as the moisture content increased to 46% or decreased to 5%, the methane oxidation rate was essentially unchanged.

Molecular Detection Techniques

The use of traditional cultivation techniques to isolate and characterize methane oxidizers in environmental samples has proved difficult due to their slow growth rate of methanotrophs and their susceptibility to competition from bacterial and fungal heterotrophic contaminates. During a survey of methanotrophic isolates, Whittenbury *et*

al. (339) noted the frequency of heterotroph contamination, and this has been echoed in subsequent methanotrophic isolation attempts by other investigators (15, 91, 120, 186, 246, 308, 335). For these reasons, molecular techniques have been routinely used to assess methanotrophic populations.

Polymerase chain reaction (PCR) has been utilized in several different capacities to detect methanotrophic bacteria. It has been used to verify indirect enrichment and isolation techniques, where methanotrophs are not surveyed directly from their natural environment. Svenning *et al.* (308) enriched soil samples by incubating them with methane and isolated putative methanotrophic organisms using a membrane diffusion process that yielded colonies on the surface of a polycarbonate membrane. PCR of conserved 16S rRNA sequences from the resulting colonies was then used to confirm that isolates were, in fact, methanotrophs. PCR has also been used in conjunction with other molecular techniques, such as restriction fragment length polymorphisms (RFLP) or rRNA sequencing to identify methanotrophs (226, 245). Additionally, real-time PCR has been used to detect and quantify methanotrophic DNA in environmental samples. Kolb *et al.* (187) employed real-time PCR targeting the *pmoA* gene to document the abundance of various methanotroph groups in DNA extracts from rice paddy soil.

Denaturing gradient gel electrophoresis (DGGE) has been frequently used to provide a direct visualization of dominant methanotrophic populations, and it is often a precursory step in phylogenetic studies. Identical length PCR fragments are separated based on sequence variation on a denaturing gradient polyacrylamide gel. Differences in DNA sequences lead to variations in mobility within the gel, and distinct banding patterns therefore emerge (241). Henckle *et al.* (138) first utilized DGGE to monitor a

rice field soil methanotroph population using amplified sequences for genes encoding the subunit of the pMMO enzyme. Although the use of 16S rRNA primers yields more unique sequences than from pMMO genes, the rRNA based method was slower to develop due to the need for nested PCR during the amplification step to increase specificity to methanotrophs (320, 347). Bodelier *et al.* (28) devised an improved DGGE strategy using combinations of universal and specific primers to avoid nested PCR and improved the specificity of the technique, allowing more phylogenetic data to be gathered on environmental samples with a high abundance of methanotrophic bacteria.

Fluorescent *in situ* hybridization (FISH) utilizes labeled probes to stain and enumerate cells without their prior isolation or purification from a variety of natural environments (240). Due to the diversity of methanotrophs, multiple rRNA probes are necessary to detect all genera. Gulledge *et al.* (128) constructed a suite of 16S rRNA probes that could distinguish between Type I and Type II species with a high degree of specificity. Of 87 methanotrophic sequences surveyed, this probe suite provided 97% coverage. Eller *et al.* (107) also utilized methanotroph type-specific probes and general eubacterial probes to differentiate microbes isolated from rice soil and root samples. Dedysh *et al.* (92) was the first to report the use of FISH to detect and enumerate methanotrophs in indigenous environments. Using the Eller probes, as well as novel probes, the authors were able to visualize and quantify bacterial cells on a peat matrix. The use of FISH has recently been combined with fluorescence activated cell sorting (FACS) to enumerate and sort methanotrophic cells enriched from complex environments (170). Cells derived from lake sediment were first hybridized with methanotrophic Type I and II specific probes. FACS was then used to count and separate the cells, such that a

subsequent diagnostic RT-PCR could be performed to survey the methanotrophic species present in the sediment sample.

An analysis of phospholipid fatty acids (PLFA) can also be used to detect and characterize methanotrophic populations. This technique is based on the unusual fatty acids (16:1ω8 and 18:1ω8 derivatives) found in methanotroph cell membranes. The methanotrophic biomass and population characterization of peat samples were successfully performed using PLFA analysis, and the cell number estimates derived from the analysis corresponded well with enumeration studies using fluorescently- tagged antibodies (306). PLFA analysis has also been used to identify unique methanotrophic species (122). Analysis of extracts from a biofilter charged with landfill gas containing significant amounts of trace organics revealed a highly specific population of Type II methanotrophs.

With the emergence of microarray technology, and particularly, the development of methanotroph -specific diagnostic microarrays, this technique is becoming more common for studying methanotrophs. A DNA-based microarray was developed by Bodrossy *et al.* (32) and has been used to identify methanotrophic genera in simulated landfill cover (300) and actual landfill cover soil (54), as well as a method to monitor shifts in population composition due to temperature changes (119) or nutrient supplementation (55). The development of mRNA-based DNA microarrays has allowed for the analysis of the composition and function of methanotrophic communities (31). The expression of the two types of MMO were monitored in various acidic soil samples and differences in the community structure among the samples examined using mRNA-based microarrays (63).

CHAPTER 3: ENRICHMENT AND SELECTION OF A MIXED METHANOTROPHIC POPULATION

Introduction

The existence of methanotrophs was proposed by Söhngen to explain the lower atmospheric methane concentrations as compared the amount produced in nature. He suggested that these differences in methane levels were due to microbial methane oxidation in soil. In 1906, he isolated the first methanotroph, *Bacillus methanicus*, from aquatic plants (293). Few investigations into the detection, isolation and characterization of methanotrophs were conducted until the 1960's, at which time Fosters and Davis began to isolate methane-utilizing bacteria (114). In discussing methane-oxidizing bacteria, Howard Dalton described the turning point in methanotrophic microbiology to be work conducted by Whittenbury and colleagues (81). In their studies, over 100 aerobic, methane-oxidizing bacteria were isolated, and from this, a classification system based mainly on morphology and carbon assimilation was devised (338, 339). Since this time, many investigations have detected or isolated methanotrophs from diverse environments, including sanitary landfills.

Methanotroph detection and characterization in landfill cover soil has proved to be somewhat easier than their isolation. The development of a variety of reliable and specific molecular techniques has enabled detection and identification of methanotrophs, in a culture independent manner. For example, Wise *et al.* (347) employed primer sets

specific for Type I and Type II 16S ribosomal DNA sequences to amplify DNA extracted from landfill soil. Clone libraries were developed and sequenced to elucidate phylogenetic relationships between the isolates and known methanotrophs. The authors also utilized denaturing gradient gel electrophoresis (DGGE) analysis to establish a methanotrophic community profile based on differences in the variable region 3 of 16S ribosomal DNA. Another molecular detection technique - catalyzed reporter deposition (CARD)-FISH has been used to enumerate methanotrophs in cover soil samples. Horseradish peroxidase-bound probes were used to detect methanotrophic cells in cover soil, and the signal was further amplified by fluorescently-labeled tyramide (168). The results suggested that 10^8-10^9 methanotroph cells/g dry soil were present in landfill soil samples - counts several orders of magnitude higher than observed by traditional most probable number (MPN) techniques. The use of stable isotope probing (SIP) containing labeled methane has also been valuable in the detection of methanotrophs, as they utilize methane as their sole carbon source and will therefore integrate carbon isotopes into DNA, as well as other cellular molecules containing carbon. Peat landfill cover soil samples were incubated in a ^{13}C-methane atmosphere. Active methanotrophic DNA fractions were detected by the presence of ^{13}C-DNA. These sequences were then identified using clone libraries, microarray analysis, and DGGE to develop a methanotrophic community profile in acidic peat cover soil (54).

Early reports of isolation attempts of methanotrophs from landfill soil document the challenges of this process, perhaps as a result of large heterotrophic populations in environmental samples. Whalen *et al.* (337) attempted to isolate methanotrophs from landfill cover soil by incubating a soil slurry in a 1:1 methane-in-air headspace for one

month before streaking this enriched sample onto solid media. This process ultimately yielded only a single isolate, which was not classified. Jones and Nedwell (166) also struggled to isolate methanotrophic cells from landfill soil samples. They initially used standard bacteriological agar to solidify a mineral salts medium; however, high levels of contaminating organisms were found to "apparently [grow] on organic impurities in the agar." Dalton (81) suggested that a heterotroph population were surviving on excreted methanotroph products, as opposed to organic impurities in the agar. Jones and Nedwell investigated other solidifying agents and ultimately utilized silicon dioxide, which reduced the growth of contaminating organisms and permitted methanotroph enumeration.

Wise *et al.* (347) successfully isolated several methanotrophs from landfill cover soil using an extraction-dilution technique. Soil samples were serially diluted every 8 days and the nutrient and pH levels were adjusted to promote methanotrophic growth. They found that high nutrient levels favored the growth of Type I methanotrophs, whereas low nutrient levels promoted Type II growth. The use of sample dilution for methanotroph isolation has also been employed by other investigators. Svenning *et al.* (308), used a modified dilution technique that utilized a membrane surface on which cells could form colonies. Specifically, a landfill soil slurry was diluted and spread onto a membrane floating atop a sterile soil slurry. This method permitted direct enumeration without prior enrichment. Interestingly, only an agricultural soil slurry was found to be a successful growth medium and poor results were obtained using the soil from which the samples originated. Also, a 47d incubation period was required for colony development.

Of the landfill cover methanotroph isolation methods published thus far, some have been more successful than others. However, all are time intensive and hindered by frequent heterotroph contamination, and no selective methanotroph medium has yet been developed to aid in the isolation of methanotrophs from environmental samples.

The purpose of this investigation was to explore a variety of isolation techniques in order to obtain a mixed population of methanotrophic bacteria from landfill cover soil. A diagnostic microarray analysis was used to confirm the presence of methanotrophs and identify genera within the population. Additionally, heterotrophs present in the methanotroph population were also isolated and identified.

Materials and Methods

Site and Sampling

Fresh landfill cover soil core samples (24.5 cm x 4 cm) were collected from Renaissance Park (Charlotte, NC), where recreational fields have been constructed atop a closed landfill. This site has a history of methane production, and soil regions with high methane emissions had previously been identified (144).

Soil Enrichment

Large stones and debris were manually removed and the core sample soil was mixed. The mix was divided into duplicate 50 g subsamples and placed in 1000 mL gas-tight jars with a threaded cap. The caps were fitted with a Swage-lok compression fitting and sealed with a silicone septum. A gas-tight syringe was used to prepare a 9% or 45% methane-in-air headspace (347). This headspace concentration was monitored by gas chromatography and maintained for 12 days at room temperature, with the headspace replenished as needed.

Isolation of Methanotrophs by Enriched Soil Dilution in NMS

Whittenbury's Nitrate Mineral Salts (NMS) (339), the standard methanotrophic growth medium, was prepared at three different concentrations: $^{1}/_{100}$ strength, $^{1}/_{10}$ strength, and full strength. A 0.3 g sample from the 9% enriched soil sample was added to 10 mL of each NMS dilution in customized 100 mL gas-tight bottles (Pyrex). The bottles were sealed with metal port fittings made for use with chemostat systems (Bellco, Vineland, NJ). The port opening was capped with a white plastic septum (Kontes Glass Company, Vineland, NJ) in which a silicone septum had been secured using silicone caulking. A silicone gasket (45 mm) was placed between the metal fitting and the threaded bottle cap (45 mm with 33.3 mm hole) (Fig. 4). A gas-tight syringe was used to create a 20% methane-in-air headspace, and the headspace was monitored by gas chromatography for 21 days at room temperature.

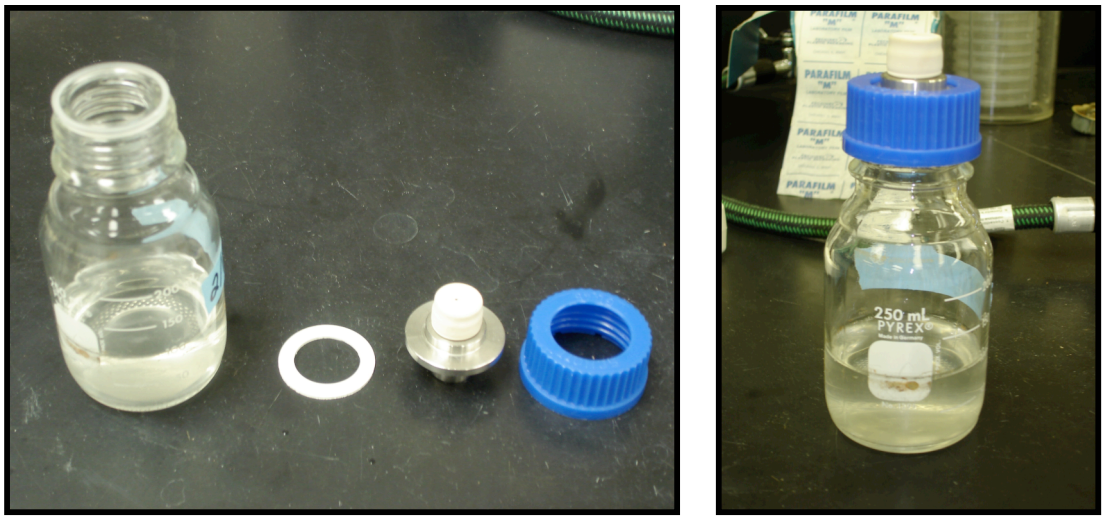

Figure 4. Gas-tight bottle sealed with metal port fittings and capped with a white plastic septum containing a silicone septum.

Isolation of Methanotrophs by Adsorption from Enriched Soil

The moisture content of 20 g subsamples of the landfill cover soil incubated under 9% headspace methane was adjusted to 15% (w/w) and placed in 100 mL gas-tight bottles. Five materials were tested for their ability to adsorb and host methanotrophic cells present in the soil. The supports tested were natural sponge (Florida Sponge, Pinellas Park, FL), a 0.95 cm x 2 cm x 4 cm sample of a highly wettable polypropylene (PP) nonwoven geotextile (Ten Cate Nicolon, Pendergast, GA), a small sub-section of injection molded polypropylene plastic tower packing material (AceChemPack Tower Packing Co, Hangzhou,China), a 90 mm diameter circle of polycarbonate membrane with a 0.22 μm pore size (GE Osmonics, Minnetonka, MN), and glass beads with a 200-300 mm diameter (Polysciences, Warrington, PA) (Fig. 5). The enriched soil was incubated with the supports for 20 day at room temperature under a 20% methane-in-air headspace. Methane uptake was monitored by gas chromatography and methane replenished as needed.

After the 20 day incubation, supports from bottles with the highest methane oxidation capacity were removed to fresh 100 mL gas-tight jars containing 10 mL of NMS. The samples were shaken at room temperature for 21 days under a 20% methane-in-air headspace, replacing the headspace as needed. The spent media was collected, pooled, and diluted 1:1 in fresh NMS to create liquid cultures containing a mix of soil methanotrophs released from the supports. The fresh liquid cultures were shaken at room temperature in 100 mL gas-tight bottles in a 10% methane-in-air headspace. Methane uptake was monitored by gas chromatography and the resulting mixed methanotroph

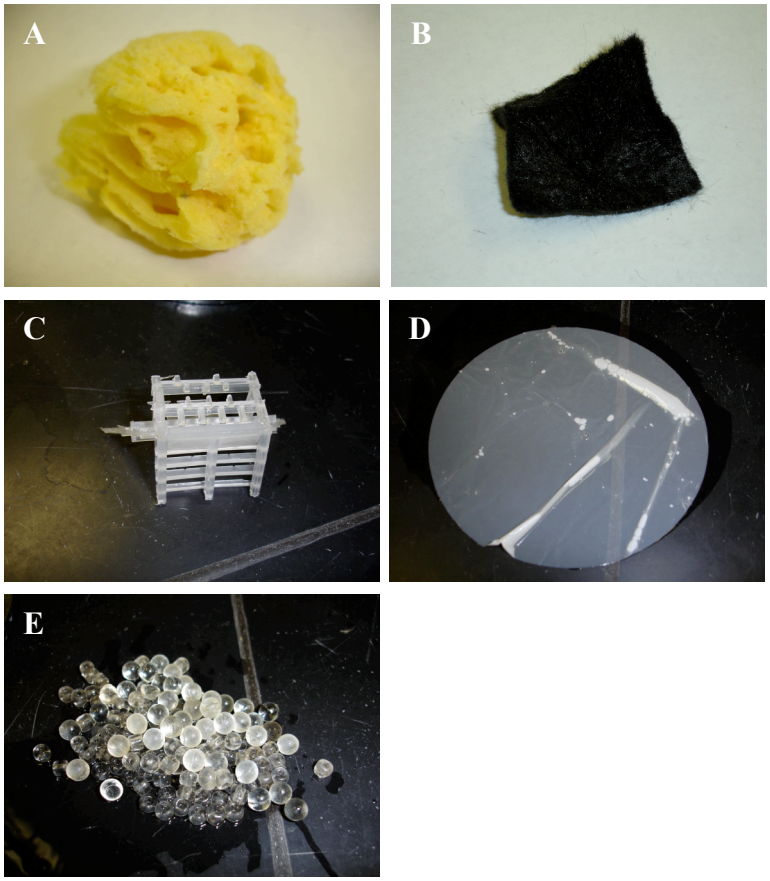

Figure 5. Five supports incubated in enriched landfill soil for isolating methanotrophs by adsorption. **A** natural sponge, **B** highly wettable PP nonwoven geotextile, **C** subsection of injected molded polypropylene plastic tower packing material, **D** polycarbonate membrane, **E** glass beads

stock was maintained by fresh inoculations into NMS as the methane headspace was depleted. This cell population was used in subsequent laboratory investigations, including the population characterization described in the following sections.

DNA Isolation and Methanotrophic Diagnostic Microarray Analysis

DNA was extracted from an overnight mixed methanotroph liquid culture using a DNeasy Tissue Kit (Qiagen, Inc.), according to manufacturer instructions. DNA microarray analysis was conducted as previously described (300) by Dr. Levente

Bodrossy at the Austrian Research Centers in Seibersdorf, Austria. Briefly, the *pmoA*/*amoA* genes were amplified from the samples to obtain RNA transcripts. The purified RNA was fragmented and tested for hybridization with a variety of molecular probes. These probes were derived from sequences specific to various types of methanotrophs with diverse origins. Hybridized slides were scanned, and the results were normalized to a positive control (300).

Isolation of Non-methanotrophic Organisms

In order to detect the presence of non-methanotrophs in the mixed culture, streak plates were prepared on Luria-Bertani (LB) agar and incubated at room temperature overnight. Colonies were isolated and restreaked on NMS agar plates. The plates were incubated in a 1:1 methane-in-air headspace for 3 weeks in an anaerobic chamber fitted with a gas-tight quick release valve. Gram staining was also performed using a four-step Gram stain kit according to manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ). The identity of isolates was determined by using 16S rRNA amplification by SeqWright, Inc. (Houston, TX) and comparing the isolate sequences to known microbial sequences using MicroSeq® ID Software.

Gas Chromatography

A gas chromatograph (Shimadzu GC-14A) equipped with a CTR1 column (Alltech, Deerfield, IL) and a thermal conductivity detector was used to simultaneously measure the methane, oxygen, carbon dioxide, and nitrogen concentrations in injected gas samples. The helium carrier gas was set at a flow rate of 60 cm^3/minute, and the detector temperature was set to 75ºC. The injector and oven temperature were both maintained at 60ºC. Standard curves were generated using ultra-high purity methane and carbon

dioxide (National Welders, Augusta, GA), and oxygen and nitrogen were obtained from atmospheric air sampling each time the GC was employed.

Results and Discussion

Various optimal methane headspace concentrations have been offered in the literature for optimal methanotroph enrichment from environmental samples (50, 169, 196). Therefore, a low and high initial enrichment methane headspace concentration was tested. Throughout the 21 day soil enrichment, both headspace concentrations showed an overall decrease in methane and oxygen concentrations with a concomitant increase in the carbon dioxide concentration. These changes are indicative of methane oxidation and suggest that an active methanotroph population was present. Methane oxidation rates in soil enriched in a 9% methane-in-air headspace were statistically higher, with a $p < 0.05$ (Fig. 6). These soil samples consumed an average of 47 g CH_4/day, while soil incubated in 45% methane headspace had an average methane uptake rate of only 5 g CH_4/day. Despite having a lower initial methane concentration, the 9% methane headspace samples contained more oxygen, which is also needed for methane consumption to occur. These data suggest that methanotrophs in the high methane headspace incubation quickly depleted the oxygen levels, yielding a low daily methane consumption. Joergensen and Degn (165) measured an oxygen to methane ratio of 1.7 for Type I methanotroph, *Methylosinus trichosporium*, and a ratio of 1.5 for a Type II methanotrophic strain. Similarly, environmental samples containing methanotrophs (wetland, agricultural, and forest soil, as well as lake sediment) were found to have an oxygen to methane ratio of 1:1.57-1:1.97. Methanotroph growth was observed when the methane oxidation reaction was optimally reached (6). Czepiel *et al*. (79) showed that methane oxidation rates were

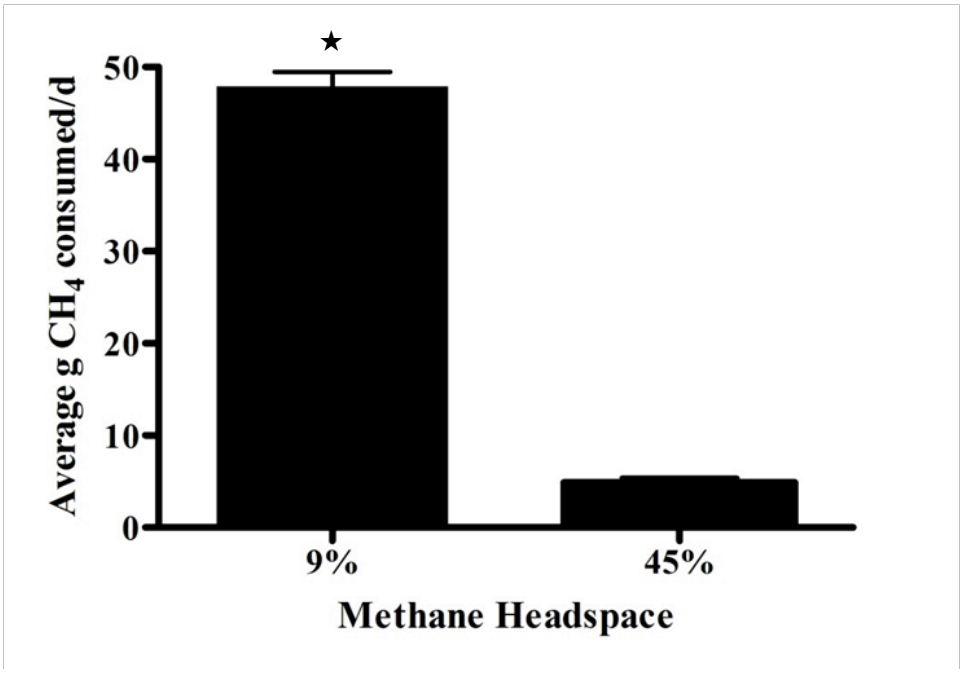

Figure 6**.** Average daily methane uptake of landfill cover soil enriched in a 9% or 45% methane-in-air headspace. Error bas represent the standard deviation of two replicate samples. ★ indicates a statistically significant difference ($p<0.05$) compared to soil enriched in a 45% methane headspace.

independent oxygen concentration at compositions greater than 3%. Based on the observed experimental data and reported oxygen levels, a methane headspace concentration of 20% methane was employed, which prevented oxygen concentrations from falling below 3% during subsequent enrichment attempts.

Wise *et al.* (347) found that the medium nutrient concentration was an important factor in the ability to isolate methanotrophs. Therefore, three concentrations of NMS were investigated as diluents for methanotroph isolation from enriched landfill cover soil. Overall, there were no differences in methane uptake between the three concentrations (Fig. 7). Initially, all cultures showed reasonable methane uptake rates, which declined to no detectable methane uptake after 21 day (data not shown). Although such soil dilution

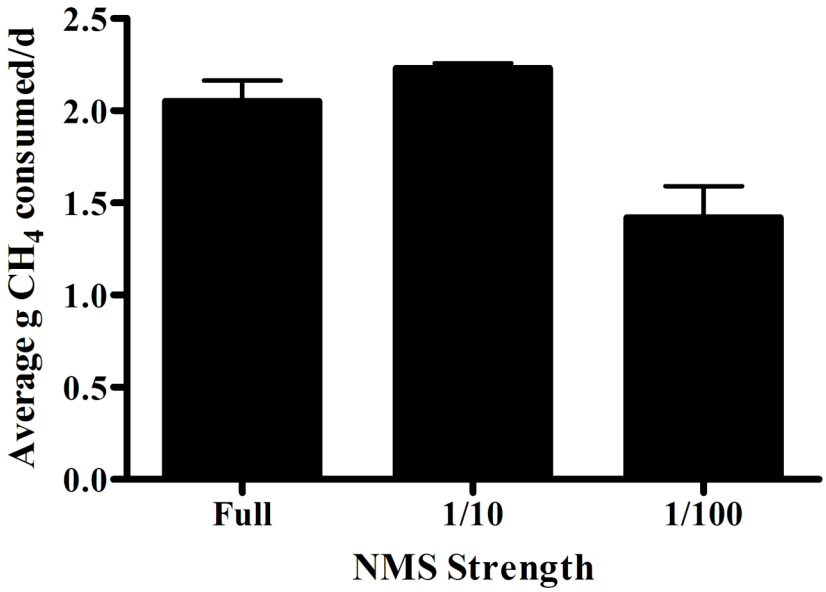

Figure 7. Isolation of methanotrophs by incubation of enriched landfill cover soil in various strengths of NMS. Error bas represent the standard deviation of two replicate samples.

techniques have been employed in previous studies to isolate methanotrophs (108, 339, 347), sustained methane uptake was not successful in this investigation. The lack of prolonged methane consumption may have been due to the growth of heterotrophic organisms utilizing organic substrates in the soil, as samples were observed to become turbid in the absence of detectable methane oxidation.

A second alternative method of isolating methanotrophs was devised whereby supports were placed directly into the soil. This method offered an attachment surface for growth, in addition to soil particles. After incubation, the supports were removed to liquid NMS media. The supports tested included materials with characteristics likely to support

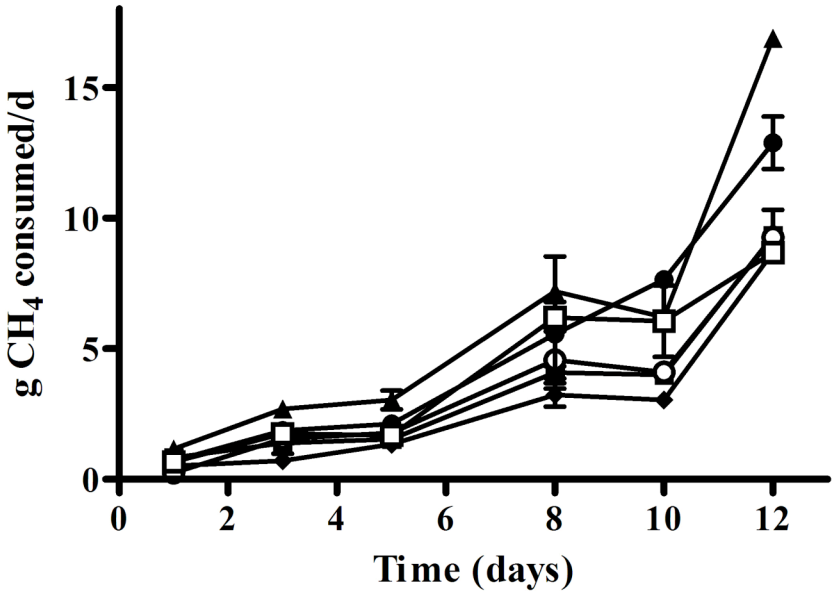

Figure 8**.** Isolation of methanotrophs from enriched landfill cover soil by adsorption onto natural sponge (▲), synthetic geotextile (●), glass beads (♦), plastic filter packing (□), polycarbonate membrane (○), and negative control (■). Error bars represent the standard deviation of two replicate samples.

cell attachment and biofilm growth, including high moisture holding capacity and a large surface area available for colonization.

Methane oxidation was observed to increase in all samples, including soil only controls. After 20 days incubation, only the sponge and synthetic geotextile samples had methane uptake rates significantly different from the controls (Fig. 8). The geotextile and natural sponge consumed 17 g CH_4/day and 13 g CH_4/day, respectively, compared to the 9 g CH_4/day consumed by the soil only control and other supports. The sponge and geotextile both had high moisture holding capacity and high surface area, which are

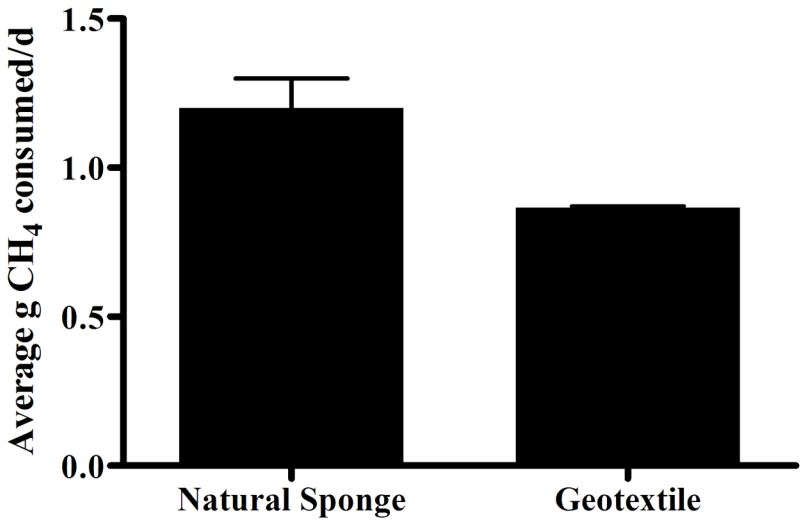

Figure 9**.** Average daily methane uptake by cells adsorbed to supports placed in NMS. Error bars represent the standard deviation of two replicates.

predicted to be important for successful colonization. It is likely that the high moisture holding capacity allowed cells to be *adsorbed* as moisture was *absorbed*.

Sustained methane uptake was observed after transferring the sponge and geotextile to fresh NMS (Fig. 9). An average methane uptake of 1.1 g CH_4/day was observed in samples containing the natural sponge and 0.85 g CH_4/day was observed in samples containing the geotextile. There was no statistically significant difference found between the methane uptake by samples containing the natural sponge and geotextile. Furthermore, this rate of methane uptake was sustained over the 21 days of enrichment for both supports (data not shown). Negative controls, containing NMS alone, showed negligible methane uptake.

There was also evidence that continued enrichment of the supports in liquid media (with aeration) allowed microbes to move from the supports into the solution. The NMS

was observed to increase in turbidity, and the pooling of spent media from these samples yielded a liquid culture capable of consuming methane and producing carbon dioxide. As cultures were further enriched by dilution in fresh NMS over several weeks, a rapid and high methane oxidation rate of nearly 100% methane oxidation in 24 hours was obtained.

In order to confirm that the mixed methanotroph culture derived in this way did, in fact, contain methanotrophic cells, DNA was extracted from the enriched sample and a diagnostic microarray performed. The resulting analysis confirmed the presence of methanotrophs belonging to the *Methylobacter, Methylosinus,* and *Methylocystis* genera. *Methylobacter* species are Type I methanotrophs, while *Methylosinus* and *Methylocystis* species are Type II methanotrophs. Hybridization with probe Peat264, designed against *pmoA* sequences derived from a peat soil sample (265), was also observed (Appendix A). The genera found in the enrichment culture are among those that grow optimally under mesophilic conditions (31). Various studies of methanotroph populations in environmental samples have also found that only a few genera dominate (16, 28, 31, 92, 187). The species found here are consistent with those commonly found together (347), although they represent two very different optimal growth conditions. Type I methanotrophs dominate in low methane, high oxygen conditions, while the opposite is true for Type II methanotrophs (6, 139). Additionally, the DNA sequence, from which Peat264 probe was derived, was found to be closely related to *Methylocystis parvus* (265). This is consistent with the positive *Methylocystis* probe results in the microarray assay.

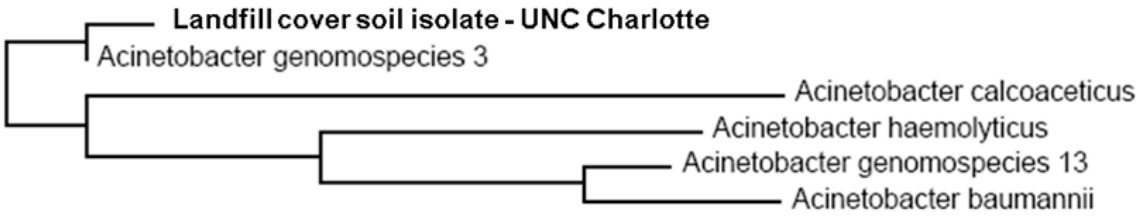

Figure 10**.** Phylogenetic relationship of the landfill cover soil isolate to *Acinetobacter* species based on 16S rRNA sequencing.

In addition to multiple methanotrophic genera, one non-methanotrophic microorganism was isolated from the mixed methanotroph culture. Streak plates on nutrient rich agar revealed one smooth, off-white colony on LB agar. When colonies were streaked on NMS agar plates and incubated under a 50% methane-in-air headspace, no growth of this isolate was evident. This indicates that the isolate was not a facultative methanotroph. Gram staining showed that the isolate was a Gram negative coccobacillus, and 16S rRNA sequencing indicated it was a member of the *Acinetobacter* genus, having 99.81% sequence homology to *Acinetobacter* genomospecies 3 (Fig. 10). Others similarly report non-methanotrophic species in environmental methanotrophic samples. For example, Dunfield *et al.* (103) isolated four distinct non-methanotrophs from a four year old methanotroph culture. The non-methanotroph found in our studies, *Acinetobacter*, is ubiquitous in soil and water (140), and therefore its occurrence is not considered notable. Although no organic carbon source was provided in the culture, *Acinetobacter* likely utilized metabolic by-products produced by the methanotrophs. No

attempts were made to screen for additional non-methanotrophs using other growth conditions; therefore ,other organisms may be present in the mixed methanotroph culture.

Due to their slow growth and unique nutritional requirements, the isolation of methanotrophs from environmental sources is difficult. Only a small number of studies have reported attempts to isolate methanotrophs from landfill cover soil, an environmental source in which they are abundant (308, 337, 347). In this study, cell adsorption to a porous support was shown to successfully aid in the enrichment of a robust, mixed methanotroph population from landfill cover soil. By using a diagnostic microarray, exhaustive culturing of individual methanotrophic isolates was not required to broadly characterize the population. The use of nutrient rich agar was an efficient, (although not absolute) technique to quickly detect non-methanotrophs. Both culture-dependent and culture-independent methods could be used to monitor the heterogeneity of the mixed methanotrophic culture. To our knowledge, this is the first time such an adsorption technique has been used for a methanotrophic population enrichment and isolation from landfill cover soil samples. When coupled with microarray analysis, it proved to be an effective method to develop liquid cultures of methanotrophs.

CHAPTER 4: IMMOBILIZATION OF A MIXED METHANOTROPHIC POPULATION BY ADSORPTION AND ENTRAPMENT

Introduction

Attachment of cells or cell components to a surface has been exploited for a variety of purposes, including water and air treatment (164, 177, 212, 294, 309); enzyme production (8, 172, 189); and biosensors (24, 198, 326). As described in Chapter 2, methods of immobilization fall into six general categories: covalent coupling, affinity immobilization, adsorption, confinement in a liquid-liquid emulsion, capture behind a semi-permeable membrane, and entrapment (212). In this investigation, the immobilization of methanotrophic bacteria was examined with the aim of creating a methanotroph embedded tarp matrix to mitigate methane emissions from landfills.

Two immobilization schemes were investigated because, of the six types, these were the mildest and most likely to be feasible for use in a biotarp. The first, adsorption, is the simplest and most gentle immobilization technique, relying on natural bacterial attachment properties, such as biofilm formation (25). The ability of methanotrophs to form a biofilm, or extracellular polymeric substance (EPS), is well documented (65, 93, 145, 149, 163, 211, 339, 353). Adsorption has been employed to coat porous glass beads with a *Methylosinus* sp. cell paste in order to enhance propylene conversion to propylene oxide (148). Methanotrophic cells have also been adsorbed to pretreated activated carbon supports to increase the production of methanol from methane (358). Xin *et al.* (354)

utilized methanotrophic bacteria adsorbed to diatomite particles in a fluidized bed system to produce epoxypropane from a mixture of methane, propene, and oxygen gases.

The second immobilization scheme evaluated here was cell entrapment, where bacteria are integrated into a liquid matrix that gels or solidifies. *Methylosinus trichosporium* cells immobilized in DEAE cellulose were used in the biosynthesis of methanol from biogas in batch and continuous cell reactor systems (227). Uchiyama *et al.* (323) used a variety of immobilization techniques, including entrapment in Ca-alginate, κ-carrageenan, and agarose beads, to enhance trichloroethylene (TCE) degradation by methanotrophic cells. These immobilized cells yielded comparable or higher activity than planktonic cells.

To our knowledge, no immobilization technique has ever been utilized to enhance methane oxidation by methanotrophic cells. Three immobilization procedures were selected for evaluation: adsorption, entrapment in alginate beads and entrapment in a liquid core alginate gel. The methane oxidation performance of cells immobilized by each procedure was assessed, and the feasibility of each product for biotarp use was taken into account in the assessment.

Materials and Methods

Cell Culture and Conditions

A mixed methanotrophic cell population, enriched and isolated from landfill cover soil as previously described, was grown in Whittenbury's NMS (339). Cells were incubated under a 10% methane-in-air headspace in 100 mL gas tight bottles at room temperature with constant shaking.

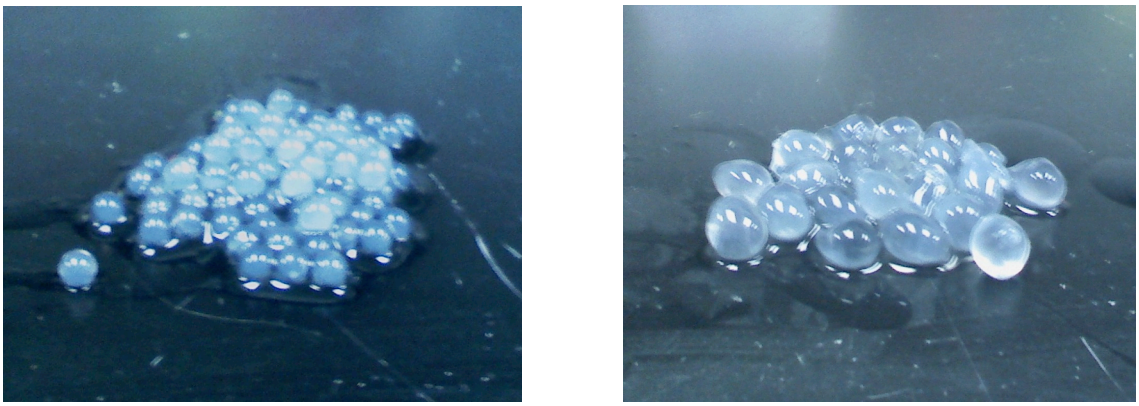

Figure 11. Immobilization of a mixed methanotroph population by entrapment in alginate beads (left) or liquid-core gel capsule beads (right).

Synthesis of Alginate Beads

Alginate beads were prepared using a modification of the method described by Knaebel *et al.* (185) (Fig. 11). A 50 mM HEPES solution was prepared and pre-heated to 80°C and divided into 30 mL, 25 mL, and 20 mL aliquots. To each, sodium alginate (Sigma Aldrich, St. Louis, MO) was added under continuous stirring and heat, such that the final concentration after the addition of cells was 6% (w/v). The alginate solutions and a 500 mL 0.1 mM $CaCl_2$ bath solution were then sterilized by autoclaving, and allowed to cool to room temperature overnight.

Two different cell concentrations were tested by the addition of 5 mL or 10 mL of an overnight methanotroph population to the appropriate solution to bring the final volume to 30 mL and mixed by gentle stirring. This yielded a 33% cell suspension bead solution containing approximately $2.5x10^7$ colony forming units (cfu)/ mL, a 17% cell

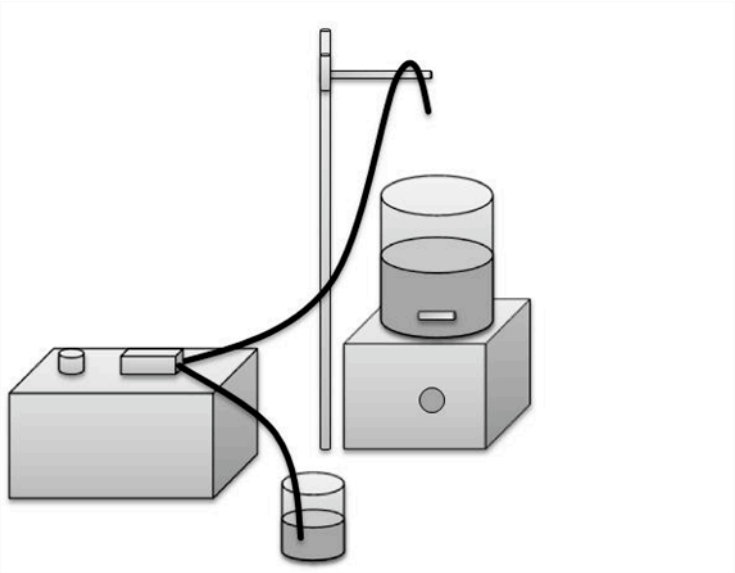

Figure 12**.** Apparatus for synthesis of alginate beads. The alginate/cell mixture was pumped and added dropwise to a $CaCl_2$ bath.

suspension bead solution containing approximately $5.0x10^8$ cfu/mL and a bead solution containing no methanotrophic cells.

Beads were synthesized by feeding droplets of the solution into a continuously stirred 0.1mM $CaCl_2$ bath (Fig. 12). Droplets were created using a low-flow peristaltic pump that fed the alginate solution through 2 mm diameter silicone tubing with a 1mm diameter connector fitted at the end. The droplets fell from a height of approximately 17 cm above the $CaCl_2$ bath. Beads were formed at a rate of 1 bead/5 seconds, and they were stirred in for an additional 30 minutes before removal by straining through a sterile mesh. The beads from each cell concentration were divided into duplicate 100 mL gas-tight bottles with a 10% methane-in-air headspace. Beads were incubated at room temperature for 3 day, with the headspace methane concentration monitored each day by gas chromatography.

Synthesis of Liquid-Core Gel Capsules

Synthesis of liquid-core gel capsules was based on a method described by Koyama and Seki (192) (Fig.11) . A sterile solution containing 2% (w/v) $CaCl_2$ and 20% (w/v) PEG 8000 was prepared. Either 5 mL or 10 mL aliquots of an overnight mixed methanotroph population were added to this solution, with a final volume of 30 mL. This yielded a 33% cell suspension bead solution containing approximately $5.3x10^7$ cfu/mL and a 17% cell suspension bead solution containing approximately $1.1x10^8$ cfu/mL. A 30 mL solution containing no cells was also prepared as a negative control. A 1.92 % (w/v) alginate solution was prepared by slowly adding the sodium alginate to a 0.1% (w/v) Tween 60 solution that was pre-warmed to approximately 70°C. The solution was incubated overnight in a 70°C water bath to completely dissolve the alginate before autoclave sterilization.

Beads were synthesized using a peristaltic pump as described previously, but here $CaCl_2$ droplets were dispensed into an alginate bath. Beads were formed at a rate of 1 bead/45 seconds, and after 10-15 beads were formed, they were removed with sterile forceps and placed in a sterile 2% (w/v) $CaCl_2$ gelation solution (pH 6.0) for 10 minutes. This process was repeated until 30 mL of gel capsule beads were synthesized. After formation, each batch was divided between two sterile gas-tight bottles and incubated under a 10% methane-in-air headspace at room temperature. The methane concentration in each bottle was monitored by gas chromatography for 3 days.

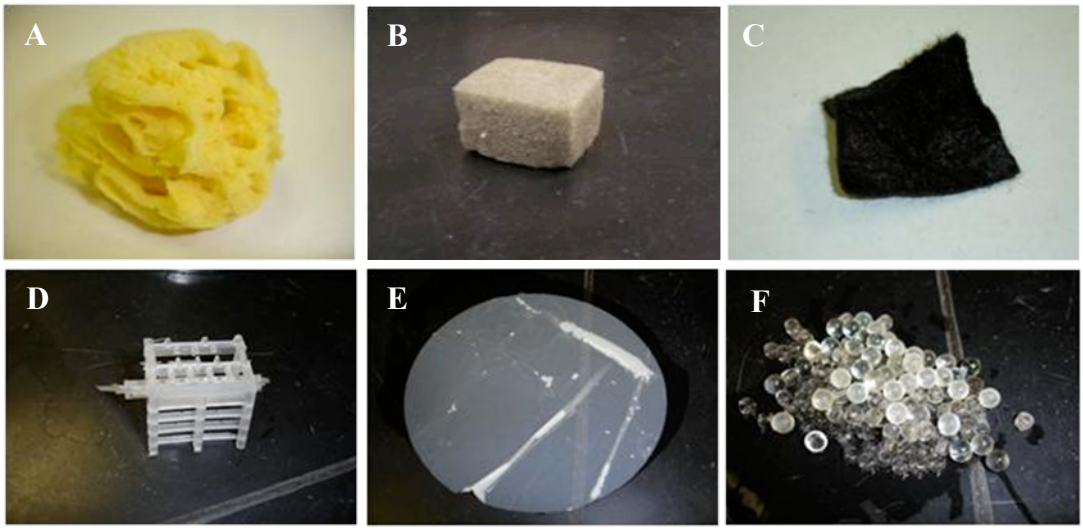

Figure 13. Immobilization of a mixed methanotroph population by adsorption to various support materials, **A** natural sponge, **B** foam padding, **C** nonwoven geotextiles, **D** plastic trickling filter medium, **E** polycarbonate membrane filter, and **F** glass beads.

Adsorption of Cells to Various Materials

Six different support types were tested for their ability to maintain a robust population of methanotrophs: natural sponge (Florida Sponge, Pinellas Park, FL); a 0.95 cm x 2 cm x 4 cm sample of a highly wettable PP nonwoven geotextile (Ten Cate Nicolon, Pendergast, GA); a 2.5 x 3 x 4 cm piece of synthetic foam padding with a 1.2 lb/ft^3 density (Foamorder, San Francisco, CA); a small sub-section of injection molded polypropylene plastic tower packing material (AceChemPack Tower Packing Co, Hangzhou,China); a 90 mm diameter circle of polycarbonate membrane with a 0.22 µm pore size (GE Osmonics, Minnetonka, MN); and glass beads with a 200-300 mm diameter (Polysciences, Warrington, PA) (Fig. 13). The supports were selected because they possessed one or more of the following properties: (i) high surface area; (ii) good

water holding capacity; and/or (iii) a known propensity for methanotroph or bacterial biofilm attachment. An overnight mixed methanotrophs population was diluted 1:10 in fresh NMS and 5 mL aliquots were added to each gas-tight bottle containing a sterile support material. Positive controls consisted of 5 mL portions of culture without the addition of a support. All samples were incubated under a 10% methane-in-air headspace concentration and incubated at room temperature. After 24 hours, the methane headspace concentration was analyzed by gas chromatography.

Accumulation of Biomass on Supports

All supports were sterilized, dried for 6 hours in a pre-warmed 105°C oven, and cooled in a desiccator before pre-weighing. Overnight mixed methanotrophs were diluted 1:10 in fresh NMS, and 10 mL were placed in a gas-tight bottle with each support type in triplicate. The headspace gases were initially adjusted to 10% methane-in-air, and readjusted to this concentration every 2-3 days for 15 days during incubation at room temperature. After this incubation period, supports were then placed in a pre-warmed, 105°C oven to dry for 6 hours, cooled in a desiccator, and re-weighed. The biomass accumulation on each support was calculated as the increased weight of the supports after incubation.

Statistical Analysis

Data were compared using a One-way ANOVA with a Tukey's multiple comparison test. Statistical analysis was performed with Prism GraphPad software (GraphPad Software Inc., San Diego, CA).

Gas Chromatography

A gas chromatograph (Shimadzu GC-14A) equipped with a CTR1 column (Alltech, Deerfield, IL) and a thermal conductivity detector was used to simultaneously measure the methane, oxygen, carbon dioxide, and nitrogen concentrations in injected gas samples. The helium carrier gas was set at a flow rate of 60 cm^3/min, and the detector temperature was set to 75ºC. The injector and oven temperature were both maintained at 60ºC. Standard curves were generated using ultra-high purity methane and carbon dioxide (National Welders, Augusta, GA), and oxygen and nitrogen were obtained from atmospheric air sampling each time the GC was employed.

Results and Discussion

Alginate beads were successfully synthesized, with a 4 mm diameter and a solid inner core. Methane oxidation by alginate beads containing both cell concentrations was initially very low, but increased over several days. After three days, the $5.0x10^8$ cfu/mL beads consumed an average of 0.72 g CH_4/day and the $2.5x10^7$ cfu/mL beads consumed an average of 0.3 g CH_4/day. The cell free control beads had an average methane oxidation rate of 0.15 g CH_4/day (Fig. 14). There was a statistically significant increase in methane uptake in the $5.0x10^8$ cfu/mL beads, as compared to the control and the $2.5x10^7$ cfu/mL beads. However, there was no significant difference between the control and $2.5x10^7$ cfu/mL beads. Methane removal in bottles with control beads is likely due to methane dissolution into the carry-over liquid surrounding the beads, since no carbon dioxide production was observed here. Carbon dioxide production with concomitant methane and oxygen consumption was observed in beads containing methanotrophic cells.

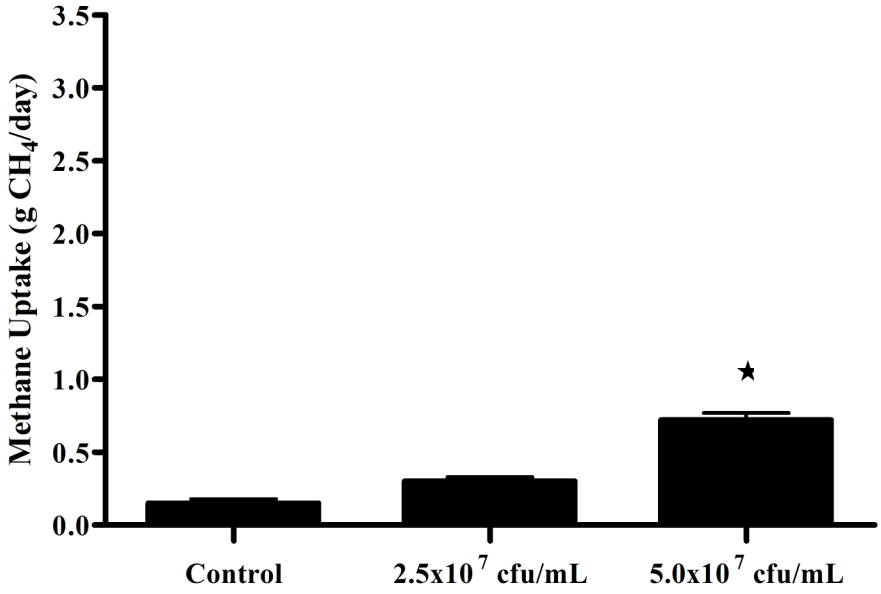

Figure 14**.** Methane oxidation by alginate beads synthesized with various amounts of a mixed methanotroph population. ★ indicates a statistically significant difference from negative controls (no cells) ($p<0.01$). Error bars represent the standard deviation of two replicates.

Although the results clearly indicate that embedded methanotrophic bacteria can successfully oxidize methane, and that the methane uptake rate is proportional to the number of cells embedded, it should be noted that there is a limit to the number of cells that can be embedded per bead. Beads composed of a 50% methanotroph culture were attempted but were unsuccessful. Once dissolved in the HEPES, the bead solution was too viscous for bead formation.

The lack of significant methane oxidation during the first two days of bead incubation suggests that an acclimatization period was necessary. This may be due to a delay in the transfer of methane molecules into the beads or an adjustment of the methanotrophic cells to growth conditions and methane oxidation within the alginate

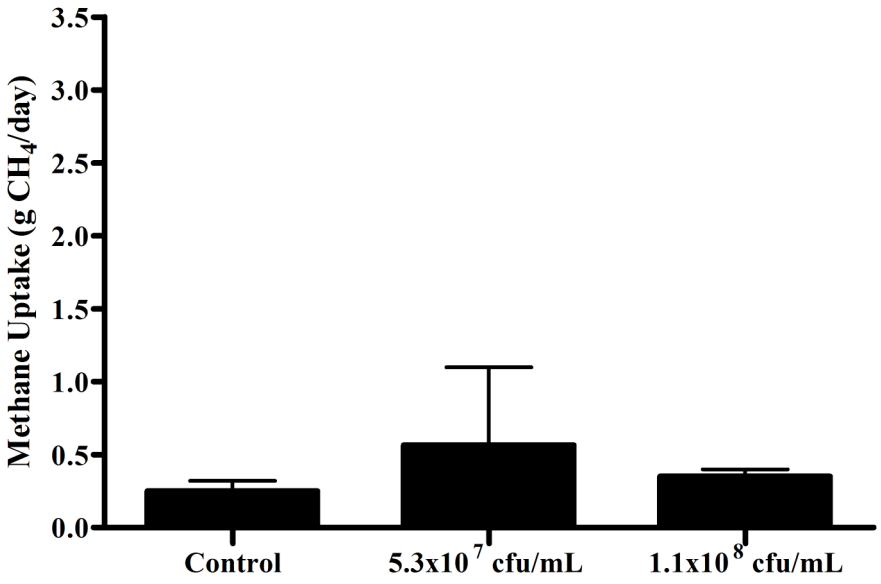

Figure 15**.** Methane oxidation by liquid-core gel capsules synthesized with various amounts of a mixed methanotroph population and negative control capsules containing no cells. Error bars represent the standard deviation of two replicates.

beads. There was also likely some cell replication during this period, although it was not possible to obtain a final cell count. A more serious problem with the beads was their rapid desiccation rate in open air. They also failed to rehydrate when soaked in water or HEPES. This propensity to desiccate could not be overcome and was the impetus to pursue liquid-core gel encapsulation.

During the synthesis of liquid-core gel capsule beads, gelation and proper bead formation was found to be highly influenced by the $CaCl_2$ concentration and the shear forces of the stirring alginate solution. At lower $CaCl_2$ concentrations, gelation occurred too slowly and spherical beads did not form. The stirring rated needed to be slow enough to prevent the formation of comet-shaped bodies, but fast enough to prevent beads from

aggregating and fusing. Further, bead detention time in the alginate solution affected the thickness of the capsule. Multiple trials led to a detention time of 40 minutes, and formation of 10-15 beads at a time allowed batches of liquid-core gel capsules to be formed with relatively similar capsule thickness.

Unfortunately, the successfully prepared methanotrophic liquid-core gel capsules showed no statistically significant increased methane oxidation beyond that of controls (Fig. 15) or that of cells embedded in solid core alginate beads. Addition of NMS to the incubation bottles did not stimulate activity. One explanation may be that the higher levels of calcium ion exposure introduced by the 2% $CaCl_2$ solution were inhibitory. A typical methanotroph culture medium contains only 0.02% $CaCl_2$ (339). No reports on calcium homeostasis or calcium toxicity in methanotrophs have been published, but Rosch found that increased calcium levels were toxic to *Streptococcus pneumoniae* cells and a calcium efflux pump was required to survive under such conditions (275). Also, the alginate gel capsule may have been too thick to allow for sufficient gas exchange or nutrient transfer. Bead synthesis systems are commercially available and can easily allow the various parameters to be manipulated in order to optimize capsule formation. Perhaps successful methane oxidation by cells entrapped in a gel capsule could be achieved with further capsule optimization. However, like the alginate beads, the liquid core gel capsules quickly dessicated in air and were not considered suitable immobilization methods for biotarp development.

When the mixed methanotroph culture was applied to various support materials and monitored for activity, the natural sponge support showed the greatest methane uptake rate (2.9 g CH_4/day), which was 7.5-fold higher than the positive control

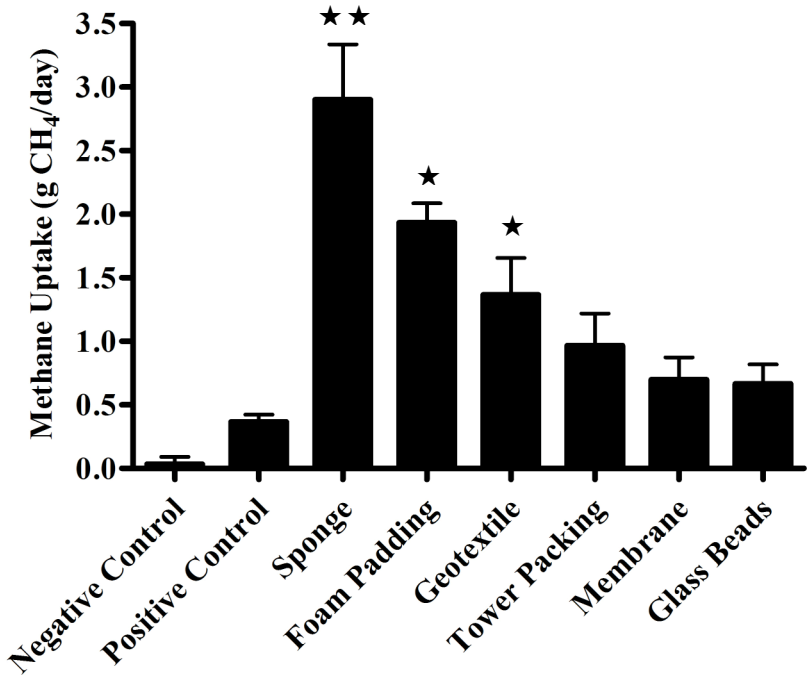

Fig. 16**.** Methane uptake by a mixed methanotroph culture adsorbed to various supports. Negative controls contained sterile NMS (no cells) and positive controls contained planktonic methanotrophs in NMS (no supports) Error bars represent the standard deviation of three replicate samples. ★ indicates a $p<0.05$ and ★★ indicates a $p<0.001$ for means compared to all other conditions.

(planktonic methanotrophs) and significantly different ($p<0.001$) from all other supports tested (Fig. 16). The geotextile and synthetic foam padding also supported high methane oxidation (1.4 and 2.0 g CH_4/day, respectively) at rates that were significantly higher than the positive control ($p<0.001$), but significantly lower than that of methanotrophs incubated onto the sponge. Differences in methane uptake activity between the other support materials examined and the control were not statistically significant.

The relationship between absorbency and methane oxidation was not immediately assumed because the material properties contributing to methanotrophic cell attachment

were unknown. The materials selected had a variety of physical properties and were utilized in other cell immobilization configurations. By surveying methane uptake of methanotrophs attached to the materials, characteristics which contributed to increased methane oxidation could be identified. Findings from these experiments suggest that methanotrophs applied to and incubated with the natural sponge, synthetic foam padding, and a geotextile consumed methane more efficiently than planktonic cells. Furthermore, the supports with the highest methane uptake were found to absorb all liquid culture applied. In samples with the least absorbent supports (plastic filter packing, polycarbonate membrane, and glass beads), the majority of the liquid culture remained suspended in the culture bottle, resembling the positive control. This is consistent with these supports yielding a methane oxidation rate similar to that of the positive controls.

Clearly, the greater methane uptake by the more absorbent materials suggests that the higher methane oxidation was likely due to more cells having attached to the more absorbent material. Therefore, the amount of attached biomass on each material surface was measured. Geotextile samples were found to have a higher average biomass accumulation than the sponge (87 mg vs. 57 mg); however, the sponge replicates showed high variability, and overall, there was no significant difference between the biomass accumulated on any of the various supports (Fig. 17). These data suggest that the methane oxidation levels observed was not merely a function of the number of attached cells on a particular surface. Furthermore, this indicates that differences in previously observed methane oxidation rates between materials were not due to differences in cell numbers. The enhanced performance by methanotrophs incubated with the natural sponge, geotextile or foam padding is likely due to properties of these materials

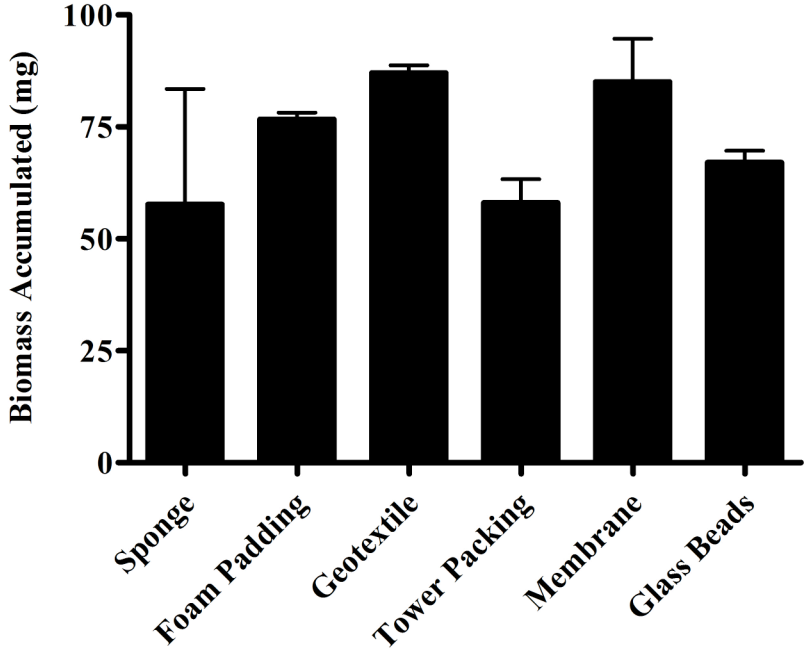

Figure 17**.** Biomass accumulation on various supports after incubation in a mixed methanotroph population for 15 days. Error bars represent the standard deviation of three replicate samples.

themselves. Perhaps the higher water holding capacity of these materials increased the concentration of dissolved methane and thus, higher oxidation rates were possible.

As screening experiments were concluded, several of the support matrices (plastic filter packing, glass beads, and polycarbonate membrane) were eliminated from further consideration due to their low methane uptake performance, handling difficulties, and low water holding capacities. The sponge and foam showed good potential for supporting methane oxidizing organisms, but it was felt that gas permeability through them would be limited, especially at high water content. Additionally, the natural sponge was found to be subject to degradation over time. The synthetic geotextile was selected for further study

because of its low propensity for biological degradation, good performance in the methane uptake capacity comparisons, and its ability to hold water but still maintain good gas permeability. It was also much thinner than both the natural sponge and foam padding, making it more suitable for handling under field conditions.

Three different types of cell immobilization were investigated as possible means to immobilize methanotrophic bacteria into a biotarp. The use of cells entrapped in alginate beads or liquid-core gel capsules were eliminated due to their high propensity for desiccation and the inability to re-hydrate them. Adsorption was found to be a more successful immobilization technique, particularly adsorbing cells to supports which had a high moisture holding capacity. Of the six supports examined, the synthetic geotextile was chosen for further study. The increased metabolic activity of immobilized cells compared to planktonic is consistent with many other reports; however this investigation was the first to show that methane oxidation can be increased by adsorbing methanotrophic cells to a support.

CHAPTER 5: EFFECTS OF TEMPERATURE, STARVATION, AND WASHING ON METHANE OXIDATION BY METHANOTROPHIC CELLS IMMOBILIZED ON A SYNTHETIC GEOTEXTILE

Introduction

The effects of temperature fluctuations and carbon starvation on immobilized methanotrophs are of interest when biological-based methane mitigation systems are designed for landfills. In this study, the feasibility of a methanotroph-embedded geomembrane biotarp is assessed in terms of whether or not methane oxidation can be sustained when temperature and methane availability fluctuate. In general, laboratory studies on methanotroph cell responses to various types of stress are limited, and this is particularly true with respect to carbon starvation. Furthermore, no studies to date have examined the effects of temperature and methane starvation on immobilized methanotroph methane oxidation.

Seasonal differences in ambient and landfill soil methane oxidation rates are linked, in part, to temperature (41, 42, 59). Temperature effects were clearly demonstrated in laboratory landfill cover soil columns, with methane oxidation rates increasing as a function of temperature (33, 40, 89, 337). More recently, temperature changes have been shown to influence the methanotroph population composition (40, 119, 233). King and Adamsen (180) examined the methane oxidation of *Methylomonas rubra* in liquid culture at various temperatures from 5°C to 45°C at a low (100 ppm) or high (10,000 ppm) methane headspace concentration. Methane oxidation was found not

only to vary with temperature, but the amount of variation was a function of the methane concentration. Uchiyama *et al.* (322) investigated the molecular response of *Methylocystis* sp to temperature stress by exposing cells to cold and heat. They found four polypeptides were *up-regulated* and 12 were *induced* by cold stress. Another 5 polypeptides were induced by heat stress as well. These proteins and the exact cellular mechanisms for these responses were not determined.

Carbon starvation effects on methanotrophs are poorly characterized. In forest soil columns, Schnell and King (280) found that methane oxidation rates during methane deprivation decreased exponentially over 38 days. Furthermore, after 16 days of methane re-introduction, oxidation activity could not be restored. Kightly *et al.* (176) found that even after 8 days without methane, landfill soil columns packed with one meter of sandy soil resumed methane uptake within 48 hours of methane re-introduction. The recovered oxidation rate was the same as the steady-state rate reached prior to starvation. Based upon the contradicting findings of these studies, the response of methanotrophs to methane starvation is not clear and may depend on the soil type, the soil's previous history to methane exposure, the methanotroph population characteristics, and the methane concentration. Roslev and King observed that 70 days methane starved liquid cultures of *Methylosinus trichosporium* OB3b showed a nearly 100% decrease in methane oxidation capacity and a 28% cell protein loss. Furthermore, methane addition for 48 hours did not stimulate oxidation. After six weeks incubation on nitrate mineral medium under a 30% methane-in-air headspace, only 4% of the initial cells remained culturable. Their results suggested that methanotroph type and growth phase influenced the methane starvation response (276, 277).

The present study aims to evaluate the effects of temperature and methane fluctuations on methanotrophs immobilized in a potential biotarp the methane uptake of component. As the biotarp will be used on an active landfill cell that could be open for a year's duration, methane oxidation over a typical annual temperature range for a temperate climate will be monitored. Since the biotarp will only be employed to cover an open landfill cell during evenings and weekends, it will experience cyclical changes in methane availability. When it is not in service, it will not be exposed to methane. The response of immobilized cells to methane starvation is therefore, an important indicator of potential biotarp success under field conditions.

Materials and Methods

Cell Culture and Conditions

A mixed methanotrophic cell population, enriched and isolated from landfill cover soil as previously described, was grown in Whittenbury's NMS (339) under a 10% methane-in-air headspace in 100mL gas tight bottles at room temperature with constant shaking.

Effects of Temperature on Methane Oxidation

An overnight mixed methanotroph cell population was diluted 1:10 into fresh NMS and 5 mL aliquots were placed into gas tight bottles containing a 38 x 63.5 mm piece of 20 oz/yd^2 (osy) wettable PP geotextile. Previous studies showed that the cells will adsorb to the geotextile matrix create a methanotroph-embedded biotarp. A 10% methane-in-air headspace was prepared and replicate samples were placed at 5, 15, 25 or 35°C for 24 hours. Sterile NMS incubated at room temperature served as a negative. The

initial and finial methane headspace concentrations were determined by gas chromatography.

Effects of Long-term Methane Starvation on Renewed Methane Oxidation

An overnight mixed methanotroph population was diluted 1:10 in fresh NMS and 5 mL aliquots added to gas tight bottles containing a 38 x 63.5 mm piece of 20 osy wettable PP geotextile. A negative control was prepared with sterile NMS. All bottles were prepared with a 10% methane-in-air headspace and the initial methane headspace concentration was measured using gas chromatography. Samples were incubated at room temperature for 24 hours, after which the methane concentration was measured and used to calculate the initial methane oxidation rate.

Samples were starved by opening the bottles and allowing atmospheric air to enter and replace the headspace gases. After recapping, gas chromatography was used to confirm that no methane was present, the samples were incubated at room temperature and a 10% methane-in-air headspace was reintroduced after 2, 5, 7, or 9 days. The methane headspace concentration was measured by gas chromatography after a 24 hour incubation and the final methane uptake rate calculated.

Effect of Intermittent Methane Starvation on Methane Uptake

An overnight mixed methanotroph population was diluted 1:10 in fresh NMS and a 5 mL aliquot added to gas-tight bottles containing a 38 x 63.5 mm piece of 20 osy wettable PP geotextile. A negative control was prepared with sterile NMS. A 10% methane-in-air headspace was prepared in each bottle, and the initial methane headspace concentration was determined by gas chromatography. All samples were incubated with methane for 18 hours, and then the headspace was sampled to determine the final

methane concentration and the methane oxidation rate. All sample headspace volumes were refreshed, and positive control samples were prepared with a 10% methane-in-air headspace. Starved sample headspace gases consisted of atmospheric air only. After 12 hours, methane was reintroduced into starved samples and all samples were further incubated for an additional 12 hours, followed by the calculation of methane uptake. After this initial 24-hour period, all headspace gases were refreshed and the 12-hour starvation cycle was repeated for 5 times over 5 days.

Cell Stability Assay

An overnight mixed methanotroph population was diluted 1:10 in fresh NMS to a final volume of 35 mL in 250 mL gas-tight bottles in triplicate. Seven pieces of 38 x 63.5 mm 20 osy wettable PP geotextile were placed in each bottle and a 10% methane-in-air headspace was prepared. The geotextiles were incubated for 15 days, with the methane headspace refreshed every 2-3 days. After incubation, three geotextile sections were placed directly into sterile gas-tight bottles with a 10% methane-in-air headspace as positive controls. The remaining 18 geotextile sections were removed to 50 mL conical tube containing 40 mL sterile DI water. The sections were shaken at 450 rpm, and three samples removed to gas-tight bottles, with a 10% methane-in-air headspace, each hour over 5 hours. The initial and finial methane headspace concentrations at 24 and 48 hours were determined by gas chromatography.

Statistical Analysis

Data were compared using a One-way ANOVA with a Tukey's multiple comparison test and a Two-Way ANOVA. Statistical analysis was performed with Prism GraphPad software (GraphPad Software Inc., San Diego, CA).

Gas Chromatography

A gas chromatograph (Shimadzu GC-14A) equipped with a CTR1 column (Alltech, Deerfield, IL) and a thermal conductivity detector was used to simultaneously measure the methane, oxygen, carbon dioxide, and nitrogen concentrations in injected gas samples. The helium carrier gas was set at a flow rate of 60 cm^3/min, and the detector temperature was set to 75ºC. The injector and oven temperature were both maintained at 60ºC. Standard curves were generated using ultra-high purity methane and carbon dioxide (National Welders, Augusta, GA), and oxygen and nitrogen were obtained from atmospheric air sampling each time the gas chromatograph was employed.

Results and Discussion

Methane uptake was highly influenced by temperature (Fig 18). Samples held at 5°C performed similarly to negative controls, with 0.1 g CH_4/day removed. Samples incubated at 15°C had slightly higher methane oxidation rates; however this increase was not statistically significant. Samples incubated at 25°C and 35 °C had average methane uptake rates of 2.2 g CH_4/day and 3.3 g CH_4/day, respectively. These rates were significantly higher ($p<0.001$) than those at the lower temperatures. Additionally, the 35°C oxidation rates were significantly higher ($p<0.01$) than the 25°C rates.

Increased methane oxidation with increasing temperature is consistent with landfill cover field observations (41, 59), laboratory landfill soil investigations (33, 40, 89, 337), and laboratory investigations involving pure methanotroph cultures (180). Maximum methane oxidation for the immobilized mixed methanotroph population in this investigation occurred at 35°C, however methane oxidation may be maintain at temperatures higher than 35°C (330). Such temperatures were not examined, as they are

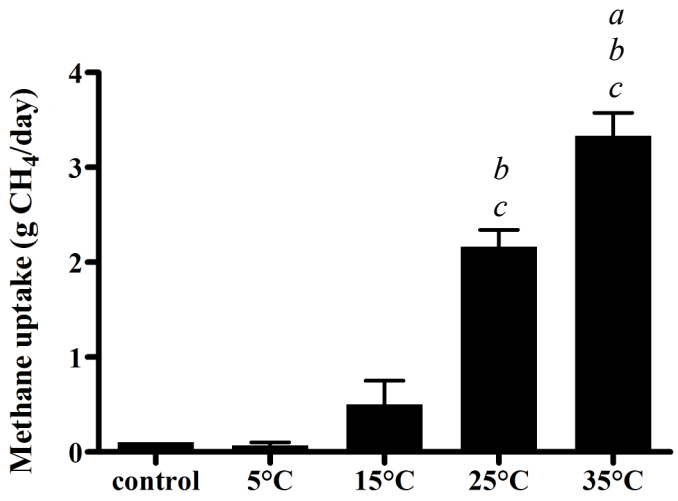

Figure18. Methane uptake by cells adsorbed to a geotextile at various temperatures. Error bars represent the standard deviation of three replicate samples. Control samples contained planktonic cells held at room temperature. *a* indicates a $p<0.01$ for means compared to 25°C. *b* indicates a $p<0.001$ for means compared to 15°C. *c* indicates a $p<0.001$ for means compared to 5°C.

unlikely to be encountered under field conditions. These data also indicate that, neglecting other factors, a methanotroph immobilized biotarp will function optimally at higher temperatures and may not provide much mitigation at lower temperatures. The methanotroph population employed was enriched and maintained at room temperature, and the optimal growth conditions were likely the moderate temperatures of 25 and 35°C. However, there is evidence that mixed methanotroph populations can shift to meet altered growth conditions. Gebert *et al.* (120) enriched biofilter media samples containing a mixed methanotroph population was incubated at 28°C and found to have a methane oxidation temperature optimum of 38°C. However, when the media samples were enriched at 10°C, the optimal temperature for methane oxidation was 22°C. Examination of the methanotroph in each samples revealed that the dominant methanotrophic species

had shifted. This was later supported by diagnostic microarray analysis, which confirmed the population shift was due to temperature (119). The mixed methanotroph population utilized in this investigation was maintained at 25°C, favoring mesophilic methanotrophs. It was incubated at low temperatures for only 24 hour. Incubation of these samples at lower temperature for longer time periods might allow for a population shift to occur, as observed by Gebert *et al.* (119), so that more robust and psychrophilic or psychrotolerant methanotrophs could mediate methane oxidation in the low temperature range.

Methanotrophs absorbed to a geotextile had an initial average methane uptake rate of 1.9 g CH_4/day. Renewed methane uptake was observed for all starvation durations (Fig. 19). After two days, a mean renewed methane uptake of 3.4 g CH_4/day was observed, which is almost a 2-fold increase over the baseline uptake rate. However, after 5, 7, and 9 days in the absence of methane, uptake rates declined 2.3, 1.7, and 12.5 fold, respectively. Negative controls with cell-media showed no methane uptake.

The increased methane uptake rate observed in the two day starved samples indicated that cell growth took place during the starvation period. As methane is soluble in both distilled water and seawater (356), it is also likely soluble in NMS. It is possible that the methanotrophs utilized methane that dissolved during the initial incubation period. It is also possible that cell growth did not occur, but that the stress of starvation induced a physiological response that increased the subsequent methane uptake rate. These data indicate that immobilized cells can tolerate a two day period of methane starvation and that short periods of starvation may enhance the oxidation rate.

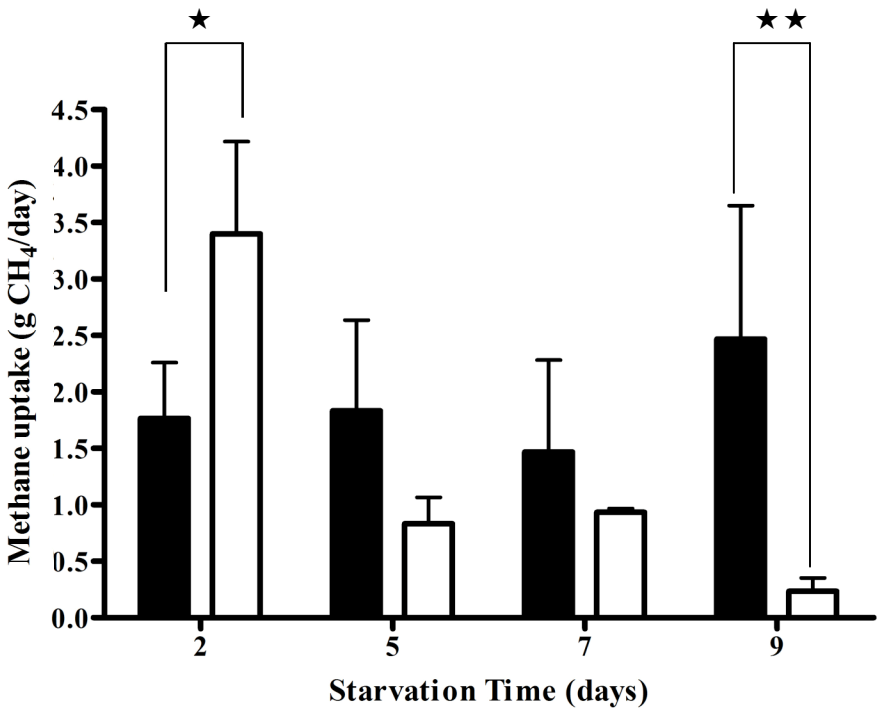

Figure 19**.** Initial (■) and final (□) methane uptake rates by a mixed methanotroph population adsorbed to a geotextile and methane starved for various amounts time. Error bars represent the standard deviation of three replicate samples. ★ indicates a $p<0.05$ and ★★ indicates a $p<0.01$ as compared to the means specified.

Although methane uptake rates were much lower in samples exposed to a 5 day starvation period, detection of some methane oxidation indicated that a portion of the cells survived starvation and were metabolically active when methane was added to the headspace. Other cells in these populations may have been dying or entering a dormancy state (338). *Methylosinus trichosporium* forms exospores when methane starved (271), while other methanotrophs, such as *Methylobacter, Methylococcus, and Methylomonas*, form cysts (338). If these survival structures were present in the longer starved samples, the 24-hour period after methane re-introduction may not have been sufficient to allow for germination. Whittenbury and Dalton (338) noted that older exospores (7 days to 18

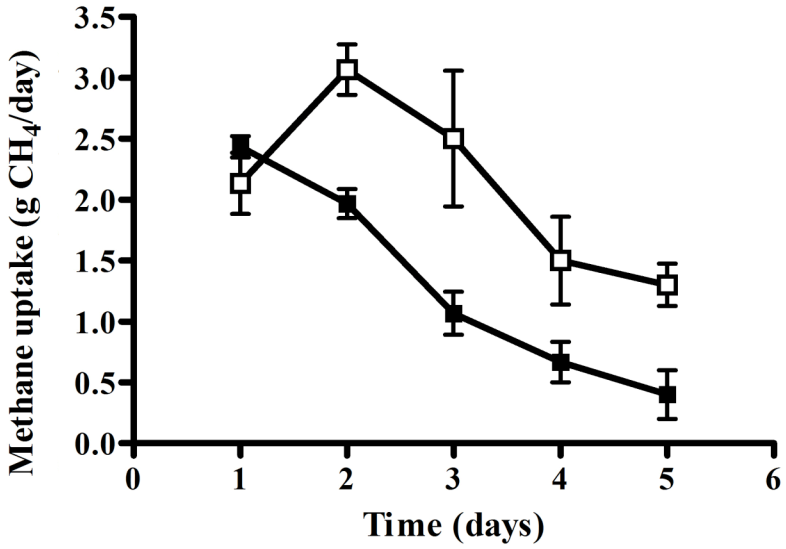

Figure 20**.** Methane uptake by geotextile adsorbed methanotrophs under a constant methane atmosphere (□) or cycle of 12 hours methane, then 12 hours air (■). Error bars represent the standard deviation of three replicate samples.

months) required 7-15 days to germinate. Longer recovery incubations with methane may be required for methane oxidation to return to its initial oxidation rate. This evidence also suggests that a methanotroph embedded biotarp could be stored off the landfill surface for short periods of time, without causing a loss of methane uptake potential.

Conceptually, a biotarp would be methane starved approximately every 12 hours while a landfill cell is being filled; therefore, the effects of methane starvation cycles was investigated. After the first 12-hour starvation cycle, there was no difference in the methane uptake by starved and control cells (Fig. 20). However, after the second cycle of starvation, methane oxidation levels began to decline, and methane uptake fell to only 0.5 g CH_4/day after the fifth cycle. Control samples that received methane every 24 hours with no starvation period, showed an initial methane uptake increase, but it was not

sustained. By day three, uptake in controls began to decline as well. This was unexpected, as methane was plentiful in these samples, and a steady state rate was anticipated by day 3 or 4. The results suggest that multiple 12 hours cycles of on-off methane cycling had a more significant effect on renewed methane uptake than a single 24 hours interruption of continuous methane provision.

On the other hand, the decline in methane uptake rates by control samples also indicated that methane starvation was not the only factor influencing oxidation rates. Although the methane headspace was refreshed every 24 hours in the controls, the inorganic nutrients were not. The depletion of the inorganic salts in the NMS medium may have caused the decline in methane uptake by controls and amplified the effects of starvation in the methane cycled samples. Subsequent experiments were conducted in which additional NMS was added to the samples (data not shown). However, no increase in methane uptake was observed.

In addition to temperature and starvation stress, biotarp methanotrophs will also be subject to the effects of precipitation in the field. For this reason, the firmness of cell attachment to the geotextile was determined by monitoring methane uptake after washing. Methane uptake by unwashed samples was 2.3 g CH_4 after 24 hours, and generally decreased over the 5 hours of washing (Fig. 21). Oxidation rates declined by approximately 74% in washed samples, however there was no further decline in methane oxidation after the first hour. These data suggest that there is a significant cell loss initially, as indicated by the lower methane oxidation rate. However, a population of cells remains attached in the biotarp and methane oxidation continues through at least 5 hours of washing. Methane uptake increased further after 48 hours post- washing in all samples

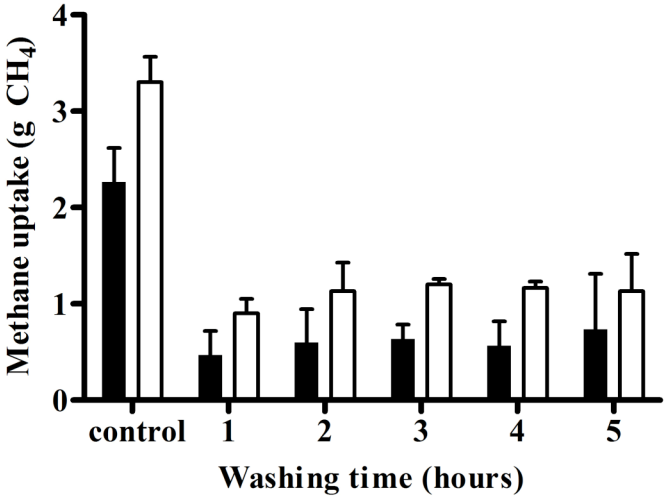

Figure 21. Methane uptake by a mixed methanotroph population adsorbed to geotextile sections at 24 hours (■) and 48 hours (□) after washing in dI water for various lengths of time. Controls were unwashed geotextiles sections. The error bars represent the standard deviation of three replicate samples.

(with a daily methane uptake similar to the 24 hour post-washing rate), indicating continued activity of the remaining cells. These data suggest the biotarp may be capable of repopulation following cell during a precipitation event.

The methanotroph biotarp will ultimately be employed atop open landfill cells, where it will need to perform over a range of seasonal temperatures. Its daily removal from the landfill cells during operational hours requires that it be able to resume methane uptake after intermittent starvation. These results suggest that changes in temperature and methane availability may be significant challenges for immobilized methanotroph cells. Although these findings suggest that methane oxidation rates will be poor at lower temperatures, it may be possible for the population to shift and acclimate to field conditions. Evidence also indicates that the daily removal of the methanotroph biotarp

will likely result in the inability of the biotarp to maintain its methane oxidation capacity. Furthermore, nutrient addition may be required in some form to sustain the biotarp under field conditions. Precipitation during field use may also contribute to lower methane oxidation, likely resulting from cell loss from the biotarp. As part of a functional biotarp, these factors will impact its effectiveness during field use.

CHAPTER 6: EVALUATION OF CANDIDIATE GEOTEXTILE TO ASSESS THEIR SUITABILITY FOR A METHANOTROPH EMBEDDED BIOTARP PROTOTYPE

Introduction

Previous experiments compared a variety of materials for use in a biotarp, and synthetic nonwoven geotextile proved to be among the best for supporting methanotroph growth and robust methane oxidation. The materials examined were selected based on reports of their successful use in other cell immobilization applications. Water holding capacity (WHC) proved to be the most significant factor affecting methane oxidation capacity, and the geotextile proved to be the most feasible for field use among the materials possessing this characteristic.

Geotextiles, also referred to as geosynthetics, are a family of geomaterials used in a wide variety of civil engineering applications. These are permeable, synthetic textiles that are used as a separator, filter, reinforcement, protection, or as a liquid barrier, and most American geotextiles are manufactured from polyester or polypropylene fibers. Two types of geotextiles are commercially available: woven and nonwoven. Woven geotextiles are made of fibers that are interlaced together to form a fabric, whereas nonwoven geotextile fibers are randomly distributed into layers to form a felt-like web. Depending on the specific properties and configuration, geotextiles are used in roads and pavements, subsurface drainage, erosion and sediment control, reinforced soil systems, and seepage control systems (123). Geotextiles are also used in landfill designs as part of a liner system.

A variety of commercial and custom-manufactured geotextiles were provided by project collaborator TenCate Geosynthetics, and each was evaluated as a potential biotarp components. The geotextiles were compared for their relative WHC and ability to support high methane oxidation activity. The results from these evaluations were used to select geotextile materials for biotarp prototype design.

Materials and Methods

Cell Culture and Conditions

A mixed methanotrophic cell population, enriched and isolated from landfill cover soil as previously described, was grown in Whittenbury's NMS (339) under a 10% methane-in-air headspace in 100 mL gas tight bottles at room temperature with constant shaking.

Geotextiles

Nine geotextiles fabrics (TenCate Geosynthetics, Pendergrass, GA) were tested for their ability to support methanotrophs and methane oxidation. The samples differed in thickness, fiber density, water affinity, and chemical composition (Table 2).

Geotextile Water Holding Capacity

Each type of geotextile was cut into 7.5 cm squares and the thickness and dry weight of each was measured. The swatches were then soaked in deionized (DI) water for 10 min and the saturated weight measured. Each swatch was then squeezed by hand until no further water could be removed and the final weight obtained. Geotextiles were assigned a letter designation to allow materials to be easily distinguished in this investigation.

Table 2. Geotextile comparison for potential biotarp components

	Geotextile	Thickness (cm)	Color	Characteristics
A	20 osy wettable PP	0.81 ± 0.04	White	Common geotextile
B	160N	0.30 ± 0.06	Black	Common geotextile
C	20 osy wettable PP 3 denier	0.97 ± 0.01	White	Version of 20 osy wettable PP with a lighter thread
D	6 osy wettable PP 3 denier	0.46 ± 0.04	White	Version of 160N with a lighter thread
E	FR 60	0.36 ± 0.05	White	Treated with polyphosphate-based additive to release inorganic phosphate when wetted
F	160N + FR 60	0.61 ± 0.05	White and Black	Composite of 160N and FR 60
G	30 osy PP	1.27 ± 0.01	White	Thicker version of the 20 osy wettable PP
H	S1600	0.50 ± 0.01	Grey	Needle-punched, nonwoven PP fibers
I	IR 26	0.70 ± 0.01	Black	One side heat fused during fabrication

Methane Oxidation Capacity

Each type of geotextile was cut into 7.5 cm squares and washed thoroughly in DI water. Washing consisted of three sequences of soaking in DI water for 10 minutes followed by rinsing in running DI water. The wash process was important because some of the materials tested produced soap-like foam when wetted. Preliminary trials showed that this wash procedure was adequate for removing all traces of foam and debris from

the fabrication process. After washing, the swatches were sterilized. The geotextile pieces were inoculated with 10mL of an overnight mixed methanotroph population and incubated in gas tight bottles with a 10% methane-in-air headspace at room temperature. The methane headspace concentration in each bottle was measured by gas chromatography at the start of a trial and measured again after a 24 hour incubation.

Phosphate Release by Geotextiles with a Phosphate Additive

Candidate geotextile material E (FR60) with phosphate incorporated and FR120, which is composed of two thicknesses of FR60 fused together were evaluated for their phosphate release rate. If the phosphate leached slowly, it could act as a slow-release nutrient for associated methanotrophs. This feature could be an asset for biotarp methanotrophs as previous experiments indicated nutrient depletion may contribute to poorly sustained methane oxidation, particularly when methane starved. It was thought that the phosphate, if released slowly and continually, might enhance methanotroph performance. However, if the phosphate leached rapidly and at high concentrations, it could challenge the osmotic stability of the microbes. Therefore, the phosphate release rates of these geotextiles were assessed.

Newly cut, square (4x4cm) sections of geotextiles FR60 and FR120 (with FR120 being twice as thick as FR60) were placed in a flask containing 100mL DI water and shaken at 400 rpm. After 5, 10, 20, and 30 minutes, 5 mL water samples were removed and diluted 1:10 in fresh DI water. The phosphate concentration was measured using the PhosVer® 3 method (Hach Co., Loveland, CO) for reactive phosphorous (orthophosphate) and a HachDR2500 colorimeter. The geotextile swatches were then transferred to 100 mL fresh DI water to determine if additional phosphate release would

occur. After shaking for 5 minutes, 5 mL of liquid were removed and the phosphate concentrations of the undiluted samples were measured.

Statistical Analysis

Data were compared using a One-way ANOVA with a Tukey's multiple comparison test. Statistical analysis was performed with Prism GraphPad software (GraphPad Software Inc., San Diego, CA).

Gas Chromatography

A gas chromatograph (Shimadzu GC-14A) equipped with a CTR1 column (Alltech, Deerfield, IL) and a thermal conductivity detector was used to simultaneously measure the methane, oxygen, carbon dioxide, and nitrogen concentrations in injected gas samples. The helium carrier gas flow rate was 60 cm^3/min, and the detector temperature was set to 75ºC. The injector and oven temperature were both maintained at 60ºC. Standard curves were generated using ultra-high purity methane and carbon dioxide (National Welders, Augusta, GA), and oxygen and nitrogen were obtained from atmospheric air sampling each time the GC was employed.

Results and Discussion

The WHC of the nine geotextiles varied widely (Table 3). The relative performance of the swatches was compared by expressing the highest WHC, that geotextile I, at 100% and expressing all others as percentages of that maximum. Geotextiles A, H and I had the best water holding capacity of the nine materials tested after draining, with A and H holding 99.3% and 92.5% as much as I, respectively. None of the other drained samples retained more than 70% of the water retained by I. Material H had the maximum water holding capacity of the group when wrung dry, with I and A

Table 3. Water holding capacity of geotextiles tested drained and wrung dry

	Thickness (m) and volume (m^3) of a 1.0 m^2 swatch	Dry Density g/cm^3	Water Retained Drained g/cm^3	Water Retained Wrung Dry g/cm^3	Relative Water Holding Capacity Drained % of max	Relative Water Holding Capacity Wrung Dry % of max
A	0.0081	0.12	0.803	0.378	99.3	82.8
B	0.0028	0.104	0.237	0.176	29.3	38.7
C	0.0097	0.079	0.3	0.068	37.1	15
D	0.0041	0.052	0.571	0.211	70.7	46.4
E	0.0041	0.055	0.456	0.139	56.4	30.5
F	0.0064	0.083	0.529	0.203	65.5	44.6
G	0.0127	0.075	0.522	0.26	64.6	56.9
H	0.005	0.144	0.748	0.456	92.5	100
I	0.007	0.165	0.808	0.403	100	88.5

retaining 88.5% and 82.8% as much water as material H, respectively. Based on their water holding performance and thinness relative to the other materials, geotextiles H (S1600) and I (IR 26) were judged to be excellent candidates for further study.

Of the nine geotextiles, G, H, and I (30 osy PP, S1600 and IR26) supported more than 0.7 g CH_4 uptake/day (Fig.22), which was significantly higher ($p<0.05$) than the rates of most other materials. Of these three high performers, the uptake rate of H (S1600), one of the thinner materials tested, was also significantly higher than that of the other two.

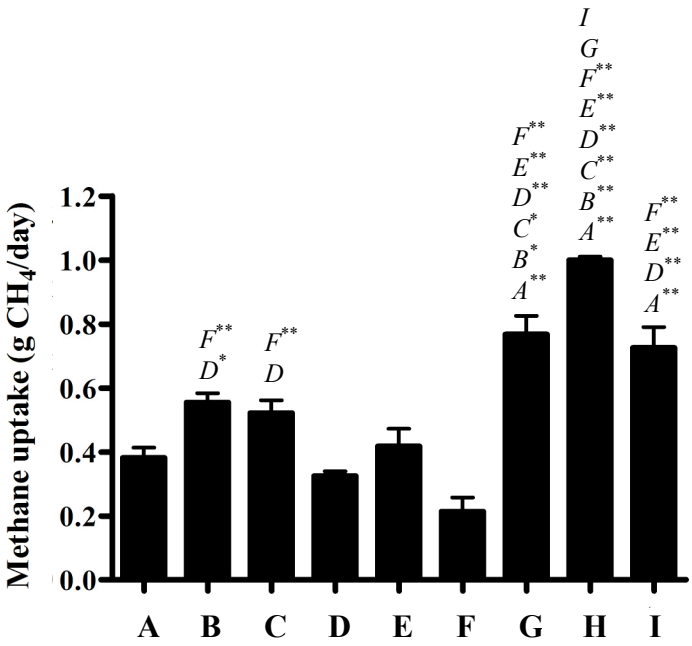

Figure 22. Comparison of methane uptake by methanotrophic cells immobilized in various geotextiles materials, as described in Table 2. A $p< 0.05$ compared to a specific geotextile is indicated by the letter designation. * indicates $p<0.01$, and ** indicates $p<0.001$. Error bars represent the standard deviation of three replicate samples.

It is important to note that for these experiments, each material was allowed to adsorb a cell suspension and then drain. Therefore, the number of methanotroph cells held in each material depended on its WHC. Assuming the WHC in Table 3 apply and the culture contained approximately 10^8 cells/mL, the cells present in each test swatch were calculated and used to normalize the methane uptake according to the number of cells present (Table 4). Based on the normalized results, material B had the highest uptake rate per 10^8 cells. This rate was more than two-fold higher than material H, the next highest performer and the performance of material I was about half that of H.

Table 4. Batch methane oxidation rates for samples normalized for cells adsorbed

	Volume/ swatch (cm^3)	Culture Retained (est.'d mL)	Methane Consumed in 24h ($mL/10^8$ cells)
A	0.0081	5.69	67.1
B	0.0028	0.73	760
C	0.0097	2.8	186
D	0.0041	1.95	167
E	0.0041	1.6	261
F	0.0064	2.99	71.6
G	0.0127	5.77	133
H	0.005	3.4	294
I	0.007	5.19	140

When the WHC and methane oxidation activity are considered together (Table 5), it is clear that sample B with the highest methane oxidation activity had the lowest WHC of all candidates (37.1%). It may be that the poorer water adsorption capacity of material B facilitated better gas penetration to the cells, allowing more oxygen and methane to reach the cells. However, the next highest methane oxidation activity was evident in material H, which had the third highest WHC (92.5%).

When a field setting was considered, water holding capacity was judged to be a critical factor for good performance under varying weather conditions, and subsequent continuous flow tests were performed with materials H and I. Additionally, materials H and I were existing commercial Ten Cate products, available in 20 ft wide rolls. Although the size of geotextile pieces needed for testing samples in the continuous flow chambers was quite small (17.8 x 30.5 cm), the ability to create large test samples for field trial was

Table 5. Relative water holding capacities and weight of geotextile materials

	Thickness (m) and volume (m^3) of a 1.0 m^2 swatch	Relative Water Holding Capacity Drained (% of max)	Methane Consumed in 24h (mL/10^8 cells)
A	0.0081	99.3	67.1
B	0.0028	29.3	760
C	0.0097	37.1	186
D	0.0041	70.7	167
E	0.0041	56.4	261
F	0.0064	65.5	71.6
G	0.0127	64.6	133
H	0.005	92.5	294
I	0.007	100.0	140

important. Also, geotextile I, (IR 26) had a fused side, which was considered a potentially valuable characteristic. If the fused surface was placed face-up, the gas might compress beneath it, retarding the flow rate through it and resulting in longer gas retention time within tarp and with the methanotrophs.

Results from tests of the phosphate treated tarps indicated that both types tested released most of the leachable phosphate they contained within five minutes of being in excess DI water (Fig. 23). The thinner material (FR60) released less phosphate (average 3.87 mg PO_4-P/L) than the thicker FR120 geotextile (average 20.52 mg PO_4-P/L). After the first five minute trial was completed, geotextiles were removed to fresh DI water and shaken an additional five minutes, but no further phosphate release was detected (data not shown). The methanotroph growth media, Whittenbury's NMS, has a phosphate concentration 0.42 mg phosphate/L (339). Cells adsorbed to these phosphate-enhanced

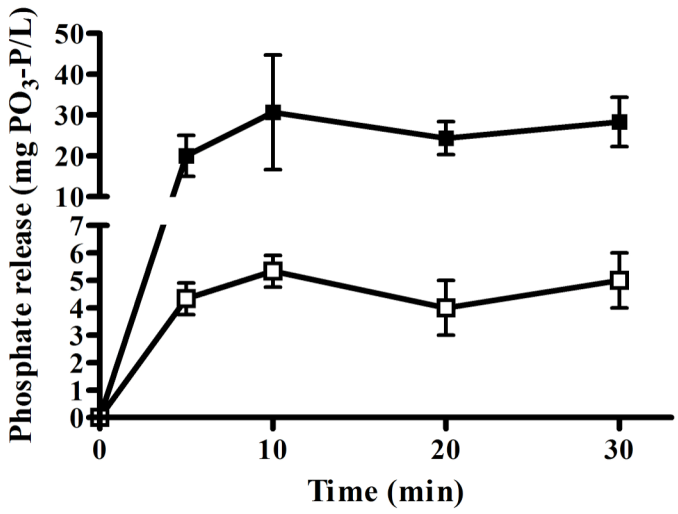

Figure 23. Phosphate release by FR60 (□) and FR120 (■). Error bars represent the standard error of the mean of three replicate samples.

geotextiles would be exposed to phosphate concentrations far higher than those found in routine culture, where they could suffer from damaging osmotic shock. Therefore, the FR60 and FR120 materials were eliminated as suitable candidates for a biotarp prototype.

CHAPTER 7: USE OF CONTINUOUS FLOW CHAMBERS IN THE EVALUATION OF POTENTIAL BIOTARP PROTOTYPES

Introduction

Previous experiments showed that immobilization of a mixed methanotroph population in a synthetic geotextiles produced an increased methane oxidation rate and represents a practical material for use as a biotarp on open landfill cells. Geotextile materials S1600 and IR26 were selected for further trials based on their methane oxidation capacities and water holding capacities relative to other candidate geotextiles. The focus of subsequent laboratory trials was to test the candidate materials under continuous flow rather than batch conditions.

In batch tests, methane is sealed in a gas-tight bottle so that the methanotrophic cells are surrounded by a given supply of methane for the entire incubation period, which is typically 24 hours. This configuration is suitable for some initial relative comparisons of material performance, but it is not fully representative of landfill conditions, where exposure of methanotrophs to a particular mass of methane is more fleeting. While it is true that methanotrophs in landfill cover soil may be exposed to a continuous supply of methane daily that methane is part of a biogas mix (about 50% methane and 50% carbon dioxide) that is part of a moving stream of emitted gas. A typical emission rate from an open landfill cell might range from 100-200 g CH_4/m^2 day (Bogner, unpublished), as such landfill methanotrophs have a limited time with a given group of methane molecules

as the gases move through the soil and into the atmosphere. The natural landfill methane environment presents a unique set of challenges for biotarp methanotrophs.

In order to simulate conditions of constant gas flow, continuous flow chambers were designed and constructed. Briefly, a synthetic landfill gas mix entered a chamber and was routed beneath a biotarp prototype affixed in the chamber. The gas flowed upward through the tarp at a constant rate to simulate the flow of biogas from the landfill into the biotarp. Compressed air was introduced near the top of the chamber above the prototype, to simulate atmospheric air in field conditions. The continuous flow chamber design allowed all gas flows to be controlled and monitored. By combining flow and concentration measurements, biotarp methane removal efficiency was calculated.

Preliminary experiments were conducted using single and double geotextile (S1600 and IR26) layer tarps. These experiments revealed that, not only was methane oxidation in these prototypes very low, but that air currents within the building were interfering with accurate gas flow measurements. To prevent current interferences, acrylic shields were placed around the continuous flow chambers. Smoke tests were also used to visualize the path of gas flow paths and confirm that there was no short circuiting or leaks (23). Based on these tests, a final chamber was configured, and it is that final design that is presented in the following sections.

Materials and Methods

Continuous Flow Chamber Design

Continuous flow chambers were fabricated from 25.4 cm diameter cylindrical acrylic plastic cut to 45.7 cm lengths. The cylinders were oriented horizontally so that each chamber was 25.4 cm high and 45.7 cm long. Each cylinder was sealed closed at

one end and equipped with a gas-tight removable lid at the other end. Holes were drilled for two 3.175 mm brass bulkhead tube fittings (Swagelok, Solon, OH) to pass through the sealed wall. The fittings were spaced 7.6 cm apart horizontally from the center. A 3.175 mm brass union tube fitting (Swagelok, Solon, OH) was installed at the center of the lid (Fig.24).

The lower bulkhead fittings on the closed end of the chamber were used to accommodate a 20.3 cm length of 3.175 mm diameter stainless steel tubing. The tubing was perforated with holes to deliver a synthetic landfill gas mix to the chamber. A bed of gravel was spread beneath the bottom pipe to enhance gas mixing. A 38.1 cm length of 3.175 mm diameter perforated stainless steel tubing was used to form a U-shaped air inlet, which was fitted in the upper bulkhead fitting on the closed end of the chamber.

A circle of furnace filter fabric was cut from a 2.5 cm thick rectangular commercial furnace filter (E-Z flow II, Flanders PrecisionAire, St-Petersburg, FL). The circle was sized to snugly cover the cross-sectional area of the cylindrical, near the open end of the chamber to ensure that gases from the chamber were well mixed before entering the septum-covered outlet of the chamber. The removable exit lid was fitted with a butyl rubber gasket to ensure a gas-tight seal when the chamber was closed. Six bolts equipped with wing nuts were screwed in the body of the chamber. The lid was secured gas-tight by tightening the wing nuts, which engaged the gasket.

Inside Frame

A 9.525 mm thick acrylic base section was permanently fixed inside the chamber. It was 30.5 cm long and 17.8 cm wide with a 17.8 cm x 11.3 cm opening in the center.

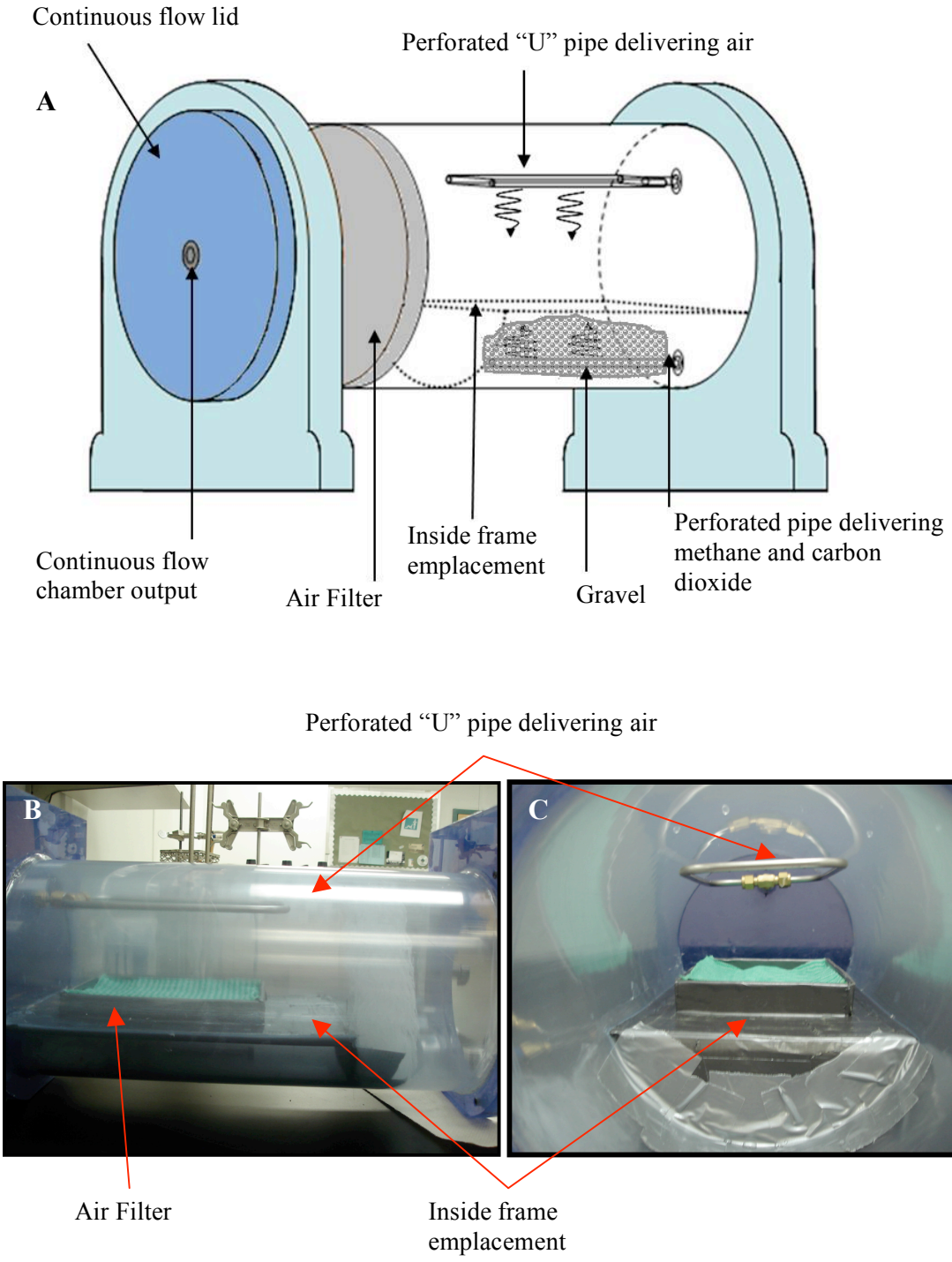

Figure 24. Laboratory continuous flow chamber bioreactor. **A)** Schematic drawing. **B)** Side view photo of chamber. **C)** Inner view photo of chamber.

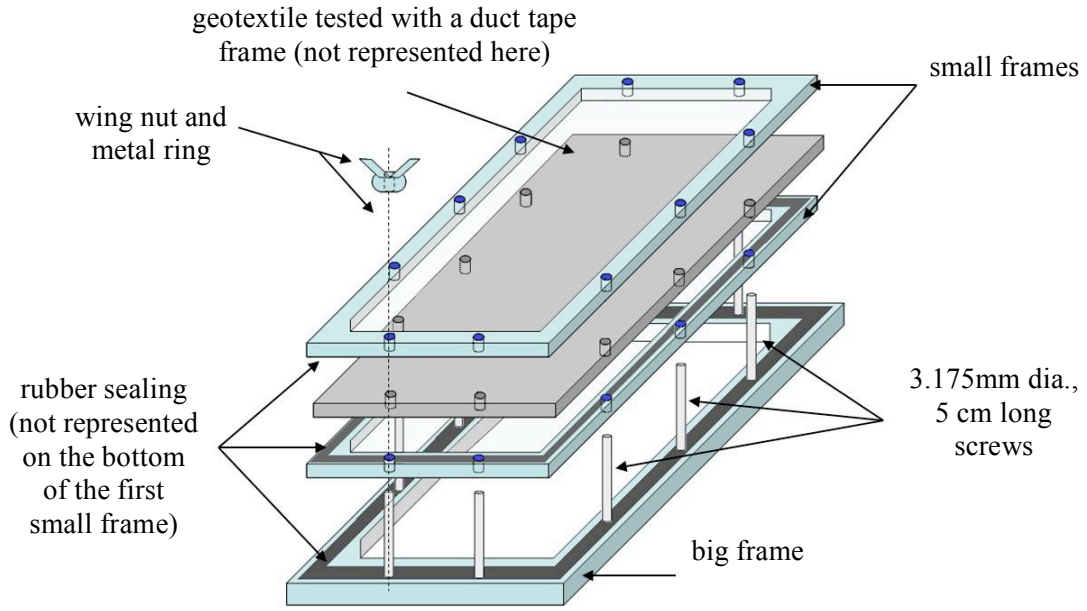

Figure 25. Biotarp in smaller removable frame secured in permanent frame

Ten, 5 cm long screws (3.175 mm diameter) were distributed evenly around the inside frame. The screws were equipped with metal washers and wing nuts.

A second internal unit consisted of two 3.175 cm thick Plexiglas plates (25.4 cm x 16.5 cm). They were used to sandwich the test prototype, and they had a 17.8 cm by 11.3 cm opening that matched the opening of the larger frame. A line of holes was drilled on opposite sides of each plate. The frames were stacked vertically with a biotarp sample in place, and the bolts and wing nuts were used to secure the tarp. The secured prototype was then placed in the chamber and sealed to the base support piece with silicone (Silicone II, GE) at the time of testing (Fig.25).

Inflow Gases

The inflow gases consisted of synthetic landfill gas entering the chamber through the lower inlet and air entering though the upper inlet. The synthetic landfill gas was a

1:1 mixture of ultra high purity methane and dry bone carbon dioxide (National/Specialty Gases, Durham, NC). The gases were combined in a tubular mixing chamber before entering the bioreactor. The mixing chamber was a 45.7 cm length of 5.4 cm diameter white PVC pipe filled with glass wool to enhance mixing. The gas delivery system was plumbed with stainless steel tubing and Swagelok fittings. Each gas was metered through flow controllers (0-5 mL/min range, VCD 1000, Porter Instrument Inc.) into the mixing chamber.

The mixture exited through a single outlet at the other end of the mixing chamber and passed (via a bulkhead fitting) to the stainless steel sparging tube inside and near the bottom of the continuous flow chamber. An additional fitting plugged with a silicone septum (Sheet Mat 250c, Alltech, Deerfield, IL) was placed at the end of the mixing chamber to monitor mixing efficiency. The flows were calibrated and monitored with a mass flow meter (ADM 2000, Humonics J&W Scientific, Folson CA), and pretests were conducted to ensure that the flow entering and exiting the mixing chamber were equal. A 1 mL/min inlet flow rate of synthetic landfill gas yielded a flux rate of 20-25 g/ m^2 day through a tarp secured in the bioreactor. In order to attain this rate, a rate of 0.5 mL/min each of methane and carbon dioxide was required. Medical grade air (Linde Gas, Independence, OH) simulating the atmosphere was metered through a flow controller (range 0-25 mL/min) to the upper stainless steel perforated tubing at a rate of 5 mL/min.

Cell Culture and Conditions

A mixed methanotrophic cell population, enriched and isolated from landfill cover soil as previously described, was grown in Whittenbury's NMS (339) under a 10% methane-in-air headspace in 2 L gas tight flasks. Each flask was capped with a butyl

rubber stopper fitted with a silicone septum filled Swagelok cap. The methanotroph population was maintained at room temperature with constant stirring.

Biotarp Prototype Configurations

A multilayer tarp was prepared by alternating layers of 16.5 cm x 25.4 cm sections of washed and sterilized IR26 and S1600. The IR26 piece were placed fused side up in the second and fourth (top) layers to yield a configuration with a fused top surface. A one liter overnight mixed methanotroph population was washed and resuspended in fresh NMS. The swatches were soaked in the resuspended methanotroph population for at least 10 min, removed, and allowed to drain until no further liquid dripped from them. The edges of a test sample were covered with duct tape to prevent gas short circuiting and then "sandwiched" between the two small Plexiglas frames and anchored to the larger frame inside the bioreactor. All biotarp samples were tested against negative control samples that were similarly prepared without the addition of methanotrophic cells to the NMS solution.

A second prototype was assembled that consisted of the methanotroph-embedded 4-layered biotarp and a ⅓ cm thick layer of intermediate cover soil (Allied Waste landfill, Cabarrus County, NC) between the second and third layers. Before addition to the tarp, approximately 400 g of the soil was enriched by incubation in a gas-tight jar under a 50% methane-in-air headspace for 2 days. Negative controls consisted of a NMS soaked 4-layered biotarp.

A compost addition to the 4-layered tarps as described above was examined by adding a ⅓ cm layer of finished compost (Compost Central Municipal Yard Waste Composting Facility, Charlotte, NC) between the third and fourth layer. Like the soil, the

compost was first pre-incubated in a gas-tight jar with a 50% methane-in-air headspace for 2 days. The negative control tarp was a multilayer tarp prepared with sterile NMS only.

Shale was also examined as an additional biotarp additive. The shale Fines (Carolina Stalite) were sieved to produce a 2.00-4.76 mm particle size fraction, washed with DI water and autoclaved. It was then pre-incubated in either excess solution of an overnight mixed methanotroph population or in sterile NMS for about 30 min. The biotarp was amended with the methanotroph-soaked shale, while the negative control tarps were amended with NMS-soaked shale. Like the soil and compost-amended tarps, a ⅓ cm layer of shale was placed in the middle of the four geotextile layers.

Continuous Flow Chamber Sampling and Data Analysis

Three continuous flow chambers were operated simultaneously, two containing biotarp prototypes and the other containing a corresponding negative control. To calculate methane oxidation in each chamber, gas flows and concentrations into and out of each chamber were monitored and used in a mass balance analysis. For gas concentrations, results from duplicate samples were averaged. Each sample was collected in a 50 µl gas-tight syringe (Hamilton syringe, Reno, Nevada). Inlet gas was sampled from the PVC tube mixing chamber, and outlet gas was sampled at the continuous flow chamber exit. Inlet and outlet flows were measured using a volumetric flow meter ADM 2000 (Humonics J&W Scientific, Folson CA). During flow measurement, the flow meter was connected to a computer equipped with companion software, and flow measurements were recorded at 2 sec intervals for 7 min and averaged. The percentage of methane removed by the biotarp was calculated according to the following equation:

$$\% \text{ removal} = \frac{Q_{in}\, C_{in} - Q_{out}\, C_{out}}{Q_{in}\, C_{in}} \times 100$$

Where Q_{in} = flow in (mL gas/min)

C_{in} = methane concentration in inflow (mL CH_4/100 mL gas)

Q_{out} = exit flow (mL gas/min)

C_{out} = methane concentration in exit flow (mL CH_4/100 mL gas)

Results and Discussion

The overall average methane uptake rate of two independent evaluations of a four-layered biotarp was 16%, with a maximum removal of 23% attained during one trial. Methane uptake remained constant for the first 4 days, after which it decreased regularly each day until reaching 3% uptake on day 9 (Fig. 21A). This four-layered biotarp configuration yield higher methane uptake rates than single or double layer designs examined in preliminary trials, and methane uptake was sustained for a much longer time in the multi-layered tarps (23). It is not clear whether the improved performance was due to the higher number of methanotrophs present in the four-layered biotarps or to the increased retention time the greater thickness offered, though both likely contributed. It would then follow that a six- or eight-layered biotarp would increase methane uptake further. Although this is likely, these types of prototypes were not constructed or tested because they would be too bulky for storage and handling under field conditions. Since increasing the number of geotextile layers was not an option, biotarp amendments were investigated to increase gas detention, and as a result, methane uptake.

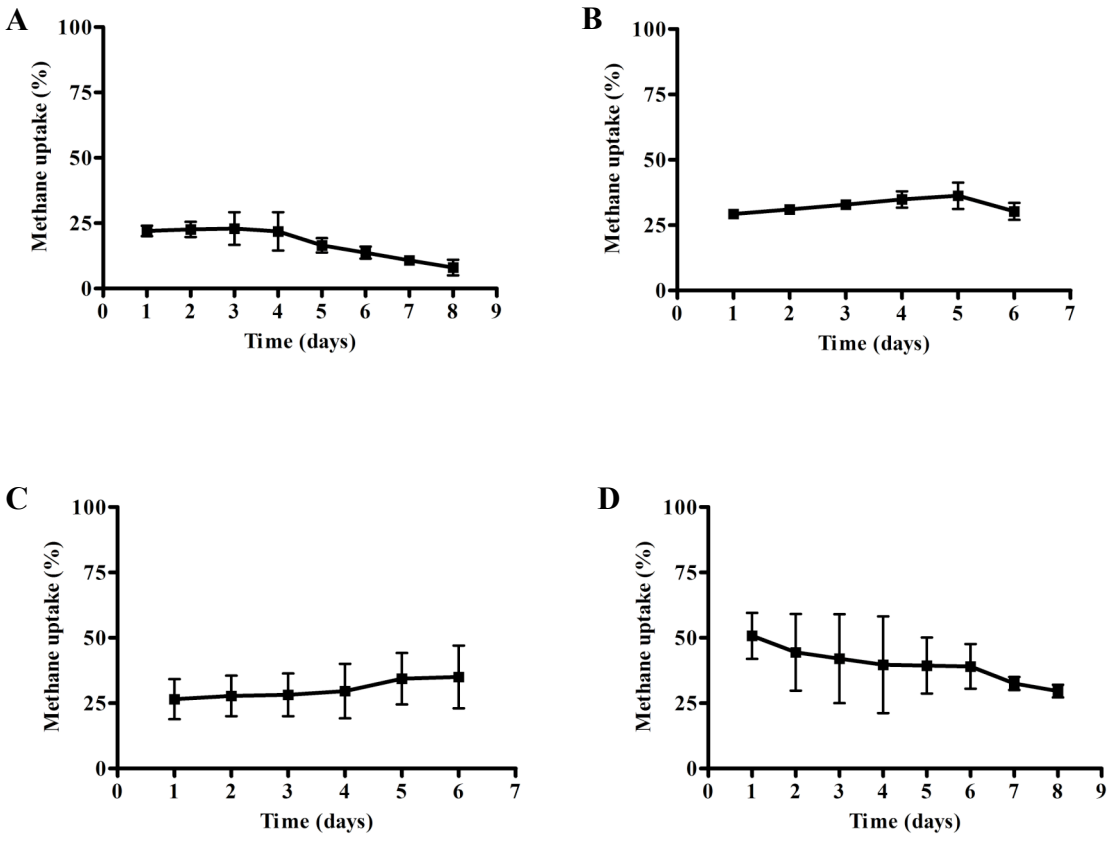

Figure 20. Methane uptake by multilayered biotarp prototype. Error bars represent the standard deviation of duplicate samples. **A** four layered biotarp prototype with no amendments. **B** four layered biotarp prototype with a landfill soil amendment. **C** four layered biotarp prototype with a compost amendment. **D** four layered biotarp prototype with a shale amendment.

The addition of landfill soil to the biotarp proved beneficial, increasing the average methane uptake rate of three independent trials to 26% removal, a rate over 1.5-fold higher than the 4-layered prototype (Fig. 21B). There was also considerable variation between replicates, ranging from 21% to 31%, particularly early in the time course. However, unlike the unamended 4-layered biotarp, performance was sustained, with little overall change during the 9 days of monitoring.

The compost additive yielded similar results with the overall average methane uptake rate of two independent compost-amended multilayered biotarps calculated to be 27% (Fig. 21C). The variability between replicates was very high, ranging from 20% to 35% removal, but like the soil, this average was higher than the unamended four-layered biotarp. There was no difference in the between compost and soil amended biotarps.

The addition of shale to the multilayer biotarp produced an overall average methane removal for three independent evaluations of 32%, which was found to be 2-fold higher than the unamended four-layered, soil amended ($p<0.001$), and compost amended ($p<0.01$) prototypes. Although methane removal began at 50%, it was observed to decline to 28% by day 8 (Fig. 21D). As observed in other prototype trials, there was large variability between the biotarp replicates, and values ranged from 59% to 21%. During each continuous flow chamber trial, condensation was evident on the walls of biotarp chambers, but not on the walls of the controls. This is noteworthy because water is a product and indicator of methane oxidation. The removal of methane and oxygen by the biotarps with an accompanying production of carbon dioxide and condensed water were not observed in any negative control chambers. Therefore, methane reduction was not likely the result of its adsorption or dissolution, but rather the result of biotic activity by the methanotroph immobilized geotextiles.

Table 6. Mass of Methane Consumed by Biotarp Prototypes

	Amendment			
	None	Landfill soil	Compost	Shale
Mean	163.4	305.9	285.2	374.9
SD	5.9	2.8	3.6	6.6
Maximum	217	342.8	330.5	479.7
Minimum	75.5	276.7	251.2	279.5

It is well known that soil microbes can require trace micronutrients from the soil or by-products from other microbes to flourish. Since the methanotroph population employed in this investigation was enriched from landfill soil, the soil addition may have provided nutrients or other factors that enhanced methane oxidation. The soil itself also contained methanotrophs, and enrichment likely further increased the number of methanotrophs present in the biotarp. Thus, the landfill soil amended biotarp may have contained a larger number of methanotrophic organisms than the multilayered biotarp alone, which was a confounding factor in the comparison of the two types of tarps. Additionally, the large variation observed between replicates may have resulted from variations in the soil. Fresh soil samples were collected for each trial, and therefore the number and composition of microbes was likely different. Even slight differences in the soil added to each replicate may have contributed to the variability. Nevertheless, the soil addition did add a valuable attribute to the biotarp prototype, namely increased performance duration and stability.

Compost has been shown to be a good host matrix for methanotrophs (153) and has been used in various types of experimental biotic landfill covers to successfully reduce methane emissions (1, 19, 158, 234, 297, 305, 360). This success is likely due in

part to its excellent water holding capacity, which is a property previously identified as important for biotarp performance. It is not surprising that the addition of compost to the biotarp led to increased methane uptake over the unamended multilayered biotarp. Like the addition of intermediate landfill cover soil to the biotarp, methanotrophs were also likely present in the compost samples, particularly after enrichment. However, it is not clear, whether the increased methane removal was due to the introduction of additional methanotrophs or to particular compost properties (i.e. moisture, nutrients, or gas retention) that stimulated the existing methanotrophs. The large variation between prototype evaluations further confounded the methane removal trend. As in the case of soil addition, compost was collected fresh each time the prototype was assembled, so that variability in the physical, chemical, and microbial composition of the samples may account for some of the variation observed.

Shale is a very light, small rock frequently used to reduce the density of concrete. It is extremely porous, and it is similar to the expanded clay particles that have been used as effective methanotroph supports in methane biofilters (120). Its high porosity and surface area may have allowed better gas penetration, greater water holding capacity in pores, or more sites for cell attachment. Additionally, the shale may have functioned as an additional gas distribution layer, a factor important in enhancing methane oxidation (154). Again, as previously noted with other additives, the pre-incubation of shale in methanotroph-rich NMS may have added additional methanotrophs to the biotarp relative to those in the unamended trials. There was high variability within replicates. Although the measurements relied on instrumentation that was subject to air pressure fluctuations,

which could have contributed to the high variability, efforts were made to control this interference by the time these trials were conducted.

Batch conditions are useful in rapid methane removal evaluations by methanotrophs in various configurations and conditions. However, the use of continuous flow chambers provides a more realistic methane environment, and is very different from the batch methane environment. The primary difference is the number of contacts a single methane molecule has with a given methanotrophic cell. In batch, methanotrophic cells are surrounded by methane molecules, and a given methane molecule will eventually contact a methanotrophic cell and diffuse into it for oxidation. This is a result of the gas being confined to the culture bottle. Under continuous flow conditions, a methane molecule has a finite time interval in which it is available for diffusion into a given biotarp methanotrophic cell. If the molecule does not enter a methanotroph for oxidation, the cell will have another opportunity to capture another methane molecule, as the methane supply is continuous. However, the methane molecule escapes from the biotarp to be emitted into the atmosphere and is not available again for oxidation. For this reason, performance in batch cannot be used to predict performance under field conditions.

The evaluation of a multilayered biotarp under continuous flow indicated that gas detention time within the biotarp was critical for methane removal. The addition of additives, including landfill soil, compost, and a methanotroph/shale mix led to an increase in the methane removal over the unamended biotarp. The addition of amendments may have contributed to higher methanotrophic cell numbers in the biotarp, provided additional nutrients, or increased the gas detention and distribution. The data also suggest that the densification and addition of more methanotrophic cells to the

biotarp may increase methane removal, as well. The combination of increased gas detention and cell numbers within the biotarp seem to be central to improving biotarp performance.

CHAPTER 8: DEVELOPMENT AND USE OF A BIOFILM STAINING TECHNIQUE TO VISUALIZE METHANOTROPH ATTACHMENT

Introduction

Biofilms consist of a community of bacterial cells enclosed in a self-produced polymeric matrix and are typically adherent to inert and living surfaces (75). They are ubiquitous in nature, with surface attached bacterial cells out numbering planktonic cells (104). Biofilm formation has been characterized into two general stages, the first being primary bacterial adhesion. Initially, cells must come into contact with the surface through Brownian motion, sedimentation (255), or active transport mediate by flagella and chemotaxis (87). The cells then attach reversibly due to the surface physico-chemical properties (104), such as electrostatic and hydrophobic interactions, steric hindrance, van der Waal's forces, temperature and hydrodynamic forces (7, 52). The second phase of bacteria surface attachment is the locking or anchoring phase. During this time, adhesion is strengthened by producing exopolysaccharides that complex with the surface irreversibly (7). Biofilm formation has several advantages for the cells, helping them concentrate nutrients, promoting genetic exchange (98), and protecting them from hostile environmental conditions and external predation (104).

Due to the environmental, industrial, and medical importance of biofilms, numerous techniques have been developed to visualize them (49, 110, 141, 152, 244, 355). One technique utilizes lectins, which are plant, animal or microbial proteins that bind to specific carbohydrate residues that compose polysaccharides and biofilms (173).

Concanavalin A is a plant derived lectin (125) that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues (131, 215) of bacterial polysaccharides (204). Concanavalin A has been used to stain biofilms on various surfaces (58, 171, 210) and originating from various microbes (70, 195, 340). Furthermore, lectins have been previously utilized in this laboratory to successfully visualize the methanotroph colonization of filter membrane segments incubated in soil column reactors (109).

In previous work, it was assumed that incubation of geotextile sections in a mixed methanotroph population led to the cell adsorption and methane oxidation activity was due to these adsorbed cells. Methanotrophic biofilm production is well documented (12, 13, 30, 38, 65, 111, 145, 211, 273, 343), and it was thought that their attachment to the geotextile fibers was mediated by biofilm production. However, there was no direct evidence of this. In order to visualize how the methanotrophs were associated with the geotextile, a technique was developed in which embedded biotarp prototype samples were sliced and stained to identify polysaccharides and methanotrophic cells. The validity of each staining protocol was first established, and then the two techniques were combined to provide a method for the simultaneous visualization of cell population and extracellular polysaccharides (EPS) architecture within the geotextile.

Materials and Methods

Cell Culture and Conditions

A pure culture of Type I methanotroph, LW13 (16), Type II *Methylocystis parvus* OBBP (339), and a mixed methanotrophic cell population, were grown in Whittenbury's NMS (339). Cells were incubated under a 10% methane-in-air headspace in 100 mL gas tight bottles at room temperature with constant shaking.

Preparation of Control Geotextile Samples

Geotextile material 160N was washed, sterilized, and cut into 4x4cm squares prior to placement in 100 mL gas-tight bottles with 5 mL of an overnight methanotroph culture and the headspace was adjusted to 10% methane-in-air. The cells were incubated with the geotextile sections for 7 days, with the methane headspace refreshed every two days. Negative controls were also prepared from the washed and sterilized geotextile, however no cells were applied.

Preparation of Biotarp Samples

After a complete trial in the bioreactor, a multi-layered biotarp prototype containing shale was sectioned into 4x4 cm squares. The shale pieces were removed to improve embedding and slicing. Two types of negative controls were prepared; one from a prototype sample that was not exposed to cells and another that was formalin fixed just after soaking in a cell preparation.

Cell Fixation, Embedding, and Slicing

Each geotextile section was fixed in 50 mL of a 5% formalin solution for 5 min at the North Carolina State School of Veterinary Medicine Histology Laboratory, the geotextile samples were further cut to fit 12 x 16 x 5 mm plastic molds (ES Sciences, East Granby, CT). Samples were dehydrated in a series of increasing ethanol concentrations (70%, 80%, 95% and 100%) for one hour each under gentle vacuum. Samples were then transferred to Technovit 7100 (Heraeus Kulze, Wehrheim) infiltrate with a 30-60 min vacuum treatment during infiltration, followed by infiltration overnight at room temperature. After 24 hours, the infiltrate was changed again, and samples

Table 7. Oligonucleotide probes targeting methanotrophic bacteria

Probe	Probe sequence (5' ⇨3')	Fluorescent tag	Probe Target
Am445	CTTATCCAGGTACCGTCATTATCGTCCC	FLUOS	_-Methanotrophs
Gm633	AGTTACCCAGTATCAAATGC	CY-3	*Methylobacter* and *Methylomicrobium*
Gm705	CTGGTGTTCCTTCAGATC	CY-3	ϒ-Methanotrophs except *Methylocaldum*

remained in infiltrate until embedded. Samples were embedded in Technovit 7100 glycol methacrylate resin (Heraeus Kulze, Wehrheim) according to manufacturer instructions. Hardened blocks were placed in a 65°C oven for one hour and stored in a desiccator box prior to microtoming. A glass knife was used to cut 2.5 micron sections, which then placed on charged slides.

Fluorescent In Situ Hybridization (FISH)

A hybridization buffer was prepared from 720 µL 5 M NaCl, 80µL Tris-HCl, 4 µL 10% SDS, and 800 µL deionized formamide and the volume brought to 4 mL with RNase free water. A Kim-wipe dampened with hybridization buffer was used to create a moist chamber in a Petri dish, and 200 µL of hybridization buffer was applied to each geotextile sections. The chamber was placed in a pre-heated 46°C incubator for 30 min. Oligonucleotide probes (128) are described in Table 6 (MWG Biotech, High Point, NC) and were applied to each section at a concentration of 0.01 µg/µL. They were mixed well with the hybridization buffer in the dark and further incubated in a 47°C pre-warmed oven hybridization chamber for 90 min. A wash buffer was prepared by the addition of

2000 µL 1 M Tris-HCl, 4300 µL 5 M NaCl, 1000 µL 0.5M EDTA, 100 µL 10% SDS, and brought to 100 mL with RNase free water. The wash buffer was prewarmed to 51°C in a water bath before use. Slides were rinsed well with the wash buffer and then flooded before being incubated for 10 min at 51°C. Slides were rinsed with DI water and dried overnight at room temperature.

Biofilm Staining

A 1 mg/mL stock solution of Concanavalin A conjugated to Alexa Fluor 488 (green fluorescence) or Alexa Fluor 594 (red fluorescence) (Invitrogen, Eugene, OR) was prepared in a 0.1 M sodium bicarbonate (pH 8.3) solution. Sections were stained by diluting 10 µL of the Concanavalin stock in 90µL sodium bicarbonate solution and incubated in the dark at room temperature for 30 min. Slides were rinsed with DI water and dried overnight. Type I RNA probes were utilized in combination with Alexa Fluor 488 tagged-Concanavalin and Type II probes were used with Alexa Fluor 594 tagged-Concanavalin.

Microscopy

Slides were examined on an inverted fluorescent microscope (Olympus 1X71) with the appropriate filters and images captured using a digital camera (Olympus DP70) mounted atop the microscope.

Results and Discussion

Geotextile Batch Incubations. For EPS detection, geotextile samples were embedded in acrylic resin, sliced, and stained with a Concanavalin A, where fluorescence indicated the presence of EPS. Positive controls, prepared from methanotroph incubated samples, were observed to stain positive for EPS when both fluorochromes were utilized

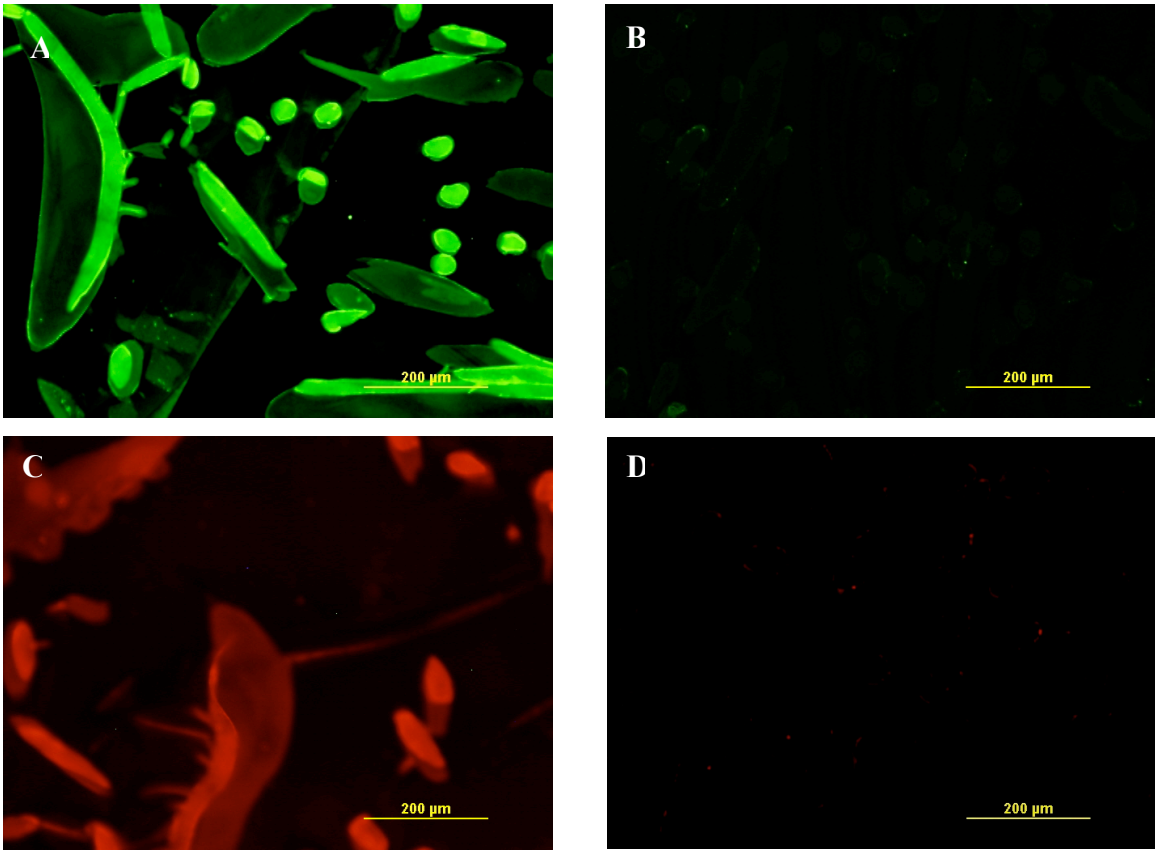

Figure 27. Concanavalin A staining for methanotroph EPS. **A)** Geotextile sample incubated for one week with methanotrophs and stained with Concanavalin A-Alexa Fluor 488. **B)** Negative control geotextile (no cells) stained with Concanavalin A-Alexa Fluor 488. **C)** Geotextile sample incubated for one week with methanotrophs and stained with Concanavalin A-Alexa Fluor 594. **D)** Negative control geotextile (no cells) stained with Concanavalin A-Alexa Fluor 594. All sections were viewed a 100X magnification.

(Fig. 27A and C). The fiber structure could clearly be distinguished from the background, indicating that EPS coated the fibers and resulting in either green or red fluorescence. As the culture contained almost exclusively methanotrophs, the EPS observed is most likely of methanotroph origin. Negative control geotextile samples showed virtually no fluorescence (Fig. 27B and D). This lack of staining indicated that the stain was specific for EPS and did not bind to the geotextile or embedding material.

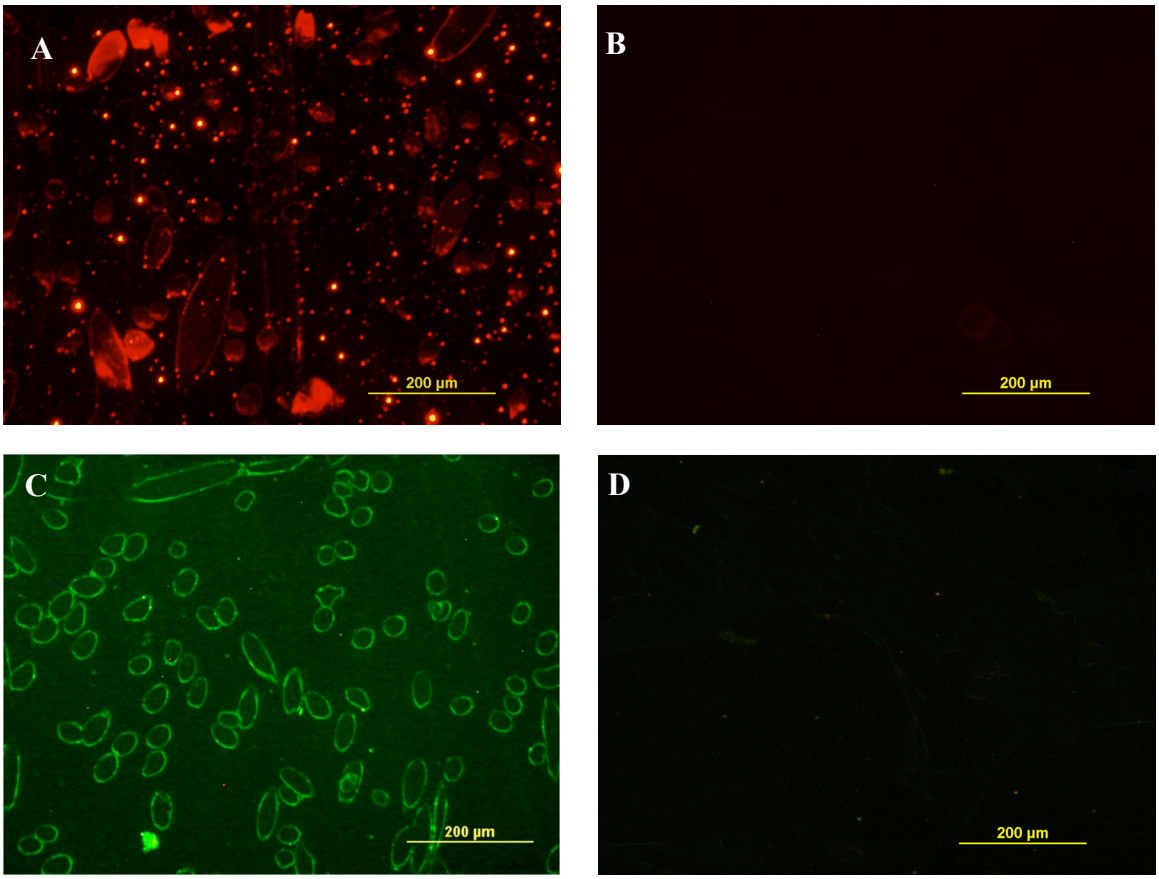

Figure 28. Fluorescent *In Situ* Hybridization controls on synthetic geotextile sections. **A)** Type I positive control methanotroph LW13 hybridized with CY-3 (red) tagged RNA probes. **B)** Type I negative control (no cells) hybridized with CY-3(red) tagged RNA probes. **C)** Type II positive control methanotroph *Methylocystis parvus* OBBP hybridized with FLUOR (green) tagged RNA probes. **D)** Type II negative control (no cells) hybridized with FLUOR (green) tagged RNA

Methanotroph EPS is synthesize as part of a capsule (339, 353) and biofilm (150, 163), and as much as 62% of the cell biomass has been measured to be polysaccharide (150). Therefore, it is not surprising that significant amounts of EPS were present on methanotroph incubated geotextile fibers. Concanavalin A is specific for glucose and mannose residues (131, 215), which have been detected in methanotroph EPS. However, other monosaccharide residues are present in methanotroph EPS as well (64, 150, 353). It

is also important to note that Concanavalin A is not specific for methanotroph produced EPS, and will also bind to polysaccharides originating from other microbial species.

When the batch incubated geotextile samples were subjected to FISH using probes specific for Type I and II methanotrophs, significant amounts of red fluorescence was observed as indicated by either red or green fluorescence (Fig. 28 A and C). Negative control geotextile samples, which contained no cells, lacked any detectable fluorescence from the methanotroph RNA probes (Fig. 28 B and D). Individual geotextile fibers could be distinguished in both methanotroph incubated samples, suggesting the bacterial cells are associated with the fiber surface. Furthermore, the presence of EPS around the fibers (Fig. 27 A and C) suggests that it is mediating cell attachment, as would be expected. The microscopy also indicates that attachment was higher on *M.* parvus OBBP geotextile sections, as almost all cells appeared to be associated with the geotextile fibers. On the other hand, there was significant amount of Type I probe hybridization independent of the geotextile fibers. Such difference may be the result of differences in the propensity of attachment or EPS production between the two strains. As sections were viewed at 100X magnification, the fluorescent points are not single cells, but rather cell aggregates. It is not certain if the unattached cells were an artifact of the embedding and slicing, or were never attached to the fibers at all. It is possible that not all cells became associated with the geotextile fibers, but some remain suspended in the NMS liquid trapped between fibers.

Biotarp Incubations in Continuous Flow Chambers. The combined EPS and cell staining technique was applied to subsamples of shale amended biotarp prototypes that either received no incubation; that were incubated without methanotrophs; and that were

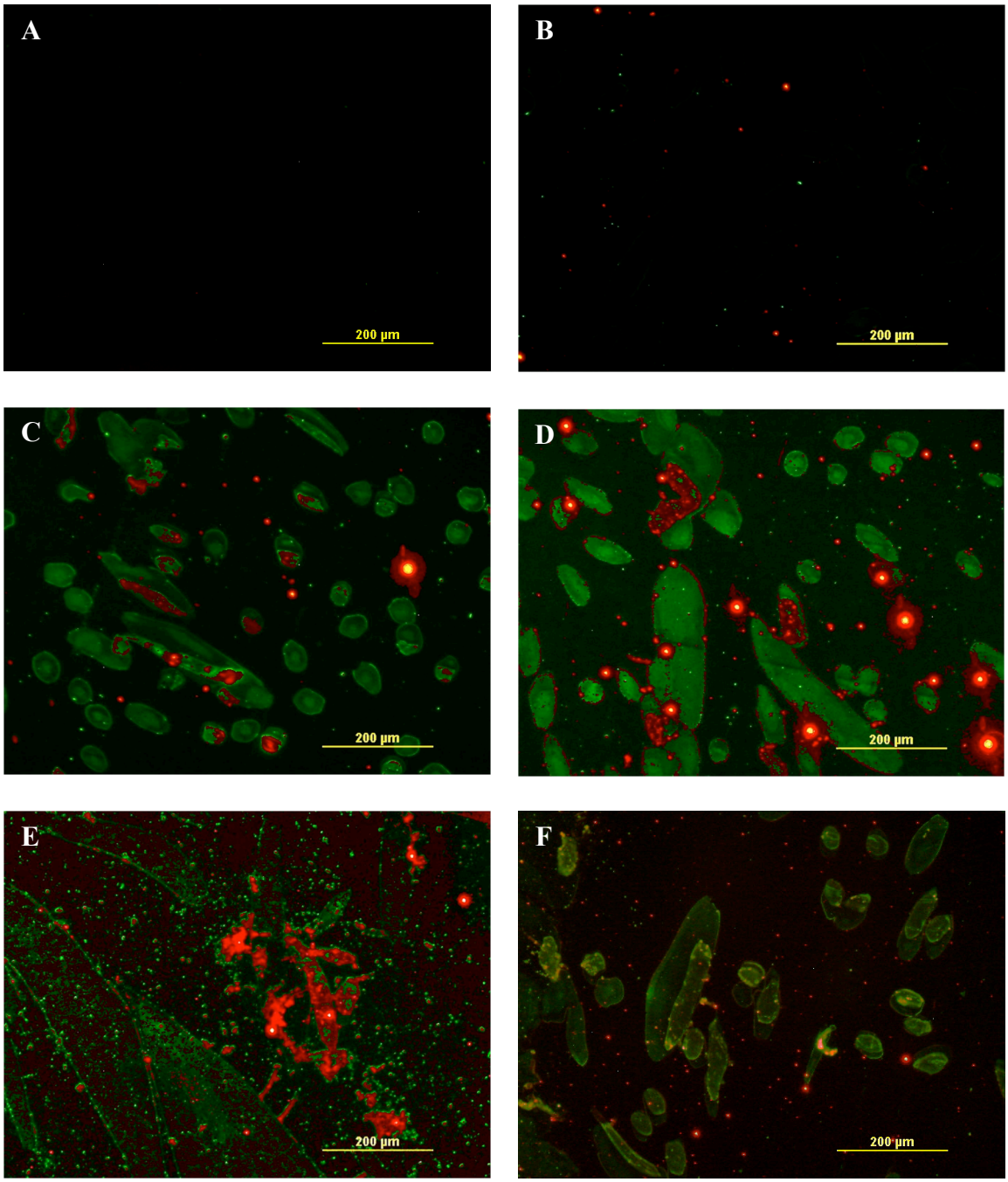

Figure 29. Shale amended biotarp prototype sections stained for Type I methanotrophs (red) using FISH and EPS (green) using Concanavalin A. **A)** Negative control biotarp section (no cells). **B)** Initial biotarp sample (fixed immediately after cell application). **C-F)** Biotarp layers from bottom to top, after 9 days incubation in a laboratory continuous flow chamber. All sections viewed at 100X magnification.

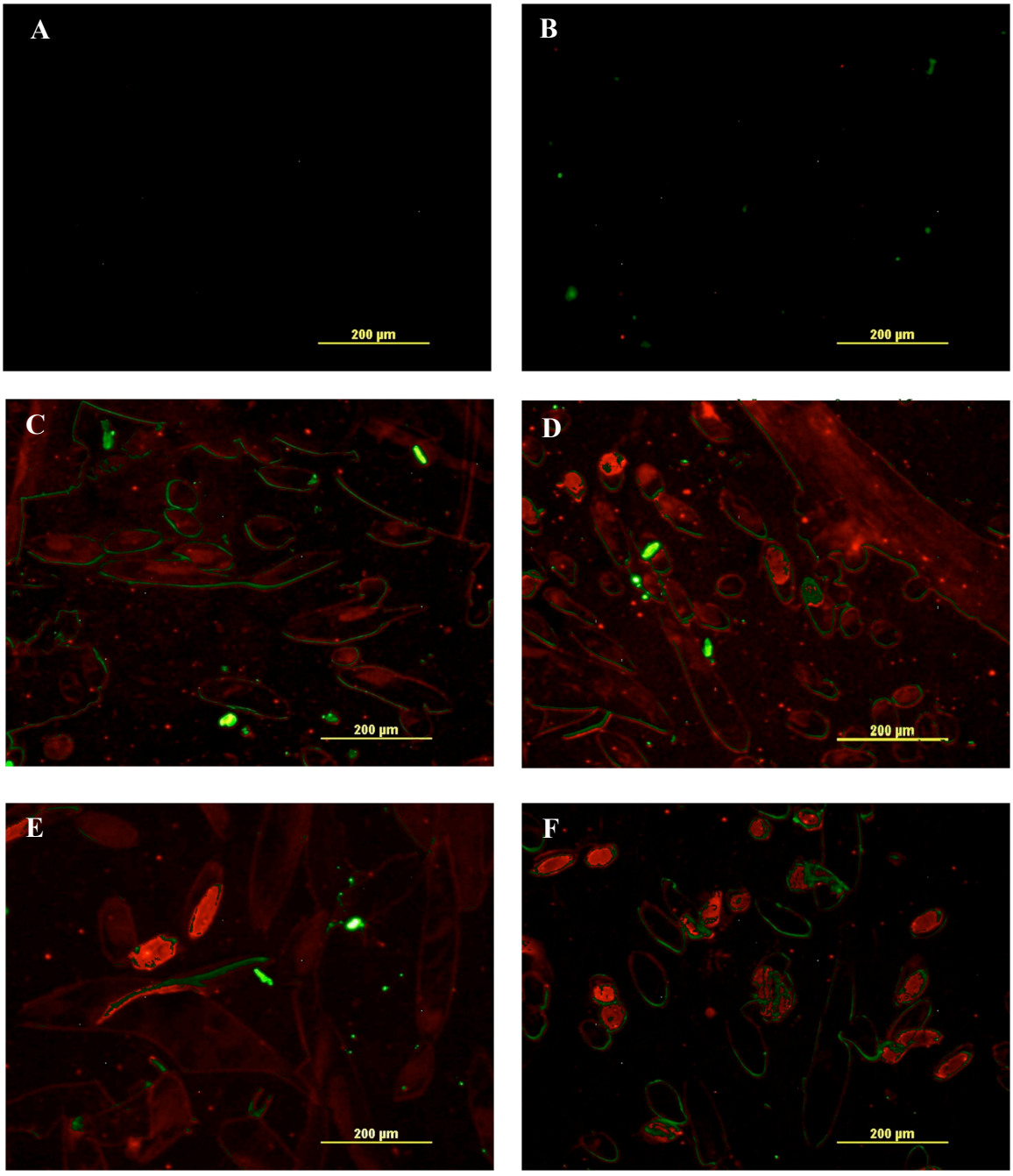

Figure 30. Shale amended biotarp prototype sections stained for Type II methanotrophs (green) using FISH and EPS (red) using Concanavalin A. **A)** Negative control biotarp section (no cells). **B)** Initial biotarp sample (fixed immediately after cell application). **C-F)** Biotarp layers from bottom to top, after 9 days incubation in a laboratory continuous flow chamber. All sections viewed at 100X magnification.

active biotarps treated with the methanotroph population. Negative control biotarp sections, which were incubated in the absence of cells, showed negligible fluorescence from hybridized Type I or II methanotroph RNA probes or from the lectin stain for EPS presence (Fig. 29A and Fig. 30A). This confirmed that in the absence of cells and EPS, there was no nonspecific binding of these molecules in active biotarp samples. When the biotarp prototype inoculated with the mixed methanotroph population but not incubated was stained, there was little EPS and few cells (Fig. 29 B and Fig. 30B). The EPS detected in these sections was likely carried-over from culture growth. When multiple fields were examined, cell aggregates appeared to be evenly distributed throughout the sections. The fiber definition seen in positive controls stained for EPS and for methanotroph coated fibers was not evident, indicating that there was no attachment and colonization of the geotextile immediately upon exposure to cells in NMS.

When a sample from an active biotarp was examined after its incubation in a continuous flow chamber, there was significant EPS accumulation in each layer of the prototype (Fig. 29 C-F and Fig. 30 C-F). Likewise, methanotrophic cells were present in all layers, and at much higher numbers than were observed in the biotarp prototype tested before chamber incubation. Growth was not confluent throughout a section, but the majority of the areas stained positive for methanotrophs co-localized with areas positive for EPS, suggesting that the polymer matrix was generated by associated cells. Furthermore, the shapes of the co-stained areas are consistent with the size and shape of fibers expected in the geotextile. Although most methanotrophs appeared to be attached and surrounded by EPS, both unattached cell aggregates and unpopulated EPS were observed.

There appeared to be more Type I methanotrophs in the middle two layers, relative to the outer layers, of the four layered biotarp. It was clear that all layers were colonized. There did not appear to be a difference in Type II methanotroph distribution. The uneven colonization may be due to the presence of other microbes or the simple result of methane "hotspots" that formed when injected gas followed a path of least resistance through the biotarp matrix. It is also possible that the shale placed between the two middle layers of the biotarp added methanotrophs, such that the layers adjacent to the shale were more highly populated. However, the shale methanotroph population contained both methanotroph types and the cell density should have increased in Type II samples as well. Since continuous flow chambers are not a sterile environment, some of the apparently cell-free EPS may have been due to the presence of other organisms. Similarly, the free cells may have been an artifact of the section preparation or a true phenomenon reflecting that not all methanotrophs were present as attached cells.

The hybridization of both Type I and II methanotroph RNA probes with cells in the mixed methanotroph population applied to the biotarp was consistent with diagnostic microarray results. These results detected the Type I genera *Methylobacter* and Type II genera *Methylosinus* and *Methylocystis* (Appendix A). Furthermore that co-staining technique help to elucidate the ways in which methanotrophs exist in EPS and the way EPS and cells are associated with the geotextile matrix. The results also demonstrate the elegant utility of combining EPS and bacteria-specific staining techniques. The combined staining system in this investigation was used to assess EPS and methanotrophic cell configurations in a geotextile material, but the methodology could be generalized to a variety of sessile cell systems.

A number of investigations have utilized methanotroph biofilms for various purposes (13, 111, 113, 273, 292, 302), however most investigators have assumed that the cells were attached and provided no evidence of material association. Clapp *et al.* (69) examined trichloroethylene removal by methanotrophs in a membrane bioreactor. Scanning electron microscopy was utilized to visualize methanotrophic cells as well as the biofilm. This investigation is the first reported use of fluorescent microscopy to visualize an active methanotroph population attachment to a material surface from a bioreactor system. This method allows for the simultaneous detection and visualization of methanotrophic cells and EPS. It was used to confirm that methanotrophs immobilized within a biotarp are associated with the geotextile fibers, likely attached to the fibers through the production of EPS. This evidence further supports the speculation that methanotrophs were adsorbed to the geotextile through the production of EPS.

CHAPTER 9: FIELD-TRIALS OF A METHANOTROPH IMMOBILIZED BIOTARP

Introduction

Global landfill methane emissions are estimated to be 14-40Tg/year (33, 35), making landfills a significant source of methane and a contributor to global climate change. Anaerobic organic waste decomposition in landfills yields methane at a rate about 257 L /kg wet refuse. Methane formation is facilitated by methanogenic microorganisms that cleave acetate into methane and carbon dioxide or reduce carbon dioxide and hydrogen (328). Landfill methane emission rates are variable, ranging from 0.0004 to 4000 g CH_4/m^2 day (36, 37, 41, 59, 79). The rate for a given site depends on biological, chemical, and physical processes occurring within the soil, and therefore, large rate variations can occur even at a single site (296).

Methanotrophic biocovers can mitigate landfill methane emissions. A biocover is an engineered cover designed to provide a hospitable environment for methanotrophic bacteria, which can consume methane. Biocovers are typically made with composted organic material, which offers a support structure for the bacteria as well as a permeable matrix for gases to enter and leave. Methanotrophs are aerobes, so they need access to atmospheric oxygen as well as methane coming from the decomposing waste layers.

A biocover consisting of shredded yard waste atop tire chips was investigated at the Outer Loop Landfill (Louisville, KY). Methane fluxes through a vegetated biocover were compared to those through a vegetated cover soil, and fluxes through the soil were

significantly higher than those through the biocover (19). A biofilter, which consisted of layered (top to bottom) humic topsoil (loamy sand) covered with grass vegetation, sand, gravel, crushed porous clay, and a final layer of gravel for water drainage, was used to remove methane from collected landfill gas. The gas was fed through the bottom of the biofilter, and methane removal was calculated at almost 100% (121). Humer and Lechner reported near total methane removal as well, when they tested a biocover composed of 0.9 m of sewage sludge compost or municipal solid waste compost placed atop 0.3 m of a coarse gravel gas distribution layer (157).

Together, these studies show that biotic treatment systems are viable tools for mitigating landfill methane emissions. Yet, they do not capture all the methane that is emitted from the open landfill cell during the time it is being filled. This study examines some prototype biotarps that aim to capture methane emitted from the open cell of a landfill when the landfill is not in service.

The concept of a biotarp to mitigate methane emissions from open landfill cells has been studied in the laboratory. Methanotrophic bacteria immobilized in a synthetic geotextile were tested in laboratory continuous flow chambers, where a multi-layered tarp removed 40%. Based on these laboratory findings, a field-scale biotarp prototype was designed, and its methane removal capacity was monitored on intermediate cover at a nearby landfill. Flux chambers were constructed and installed at the landfill site to monitor methane fluxes with and without the biotarps in place.

Materials and Methods

Culture Conditions

A mixed methanotrophic cell population, enriched and isolated from landfill cover soil as previously described in this investigation, was grown in Whittenbury's NMS (339) under a 10% methane-in-air headspace in 2 L gas tight flasks. Each flask was capped with a butyl rubber stopper fitted with a Swagelok cap containing a silicone septum. The methanotroph population was maintained at room temperature with constant stirring.

Flux Chamber Design

Flux chambers were used to measure methane flux from the landfill surface. Each of the six flux chambers consisted of two parts, as shown in Figure 31. The chamber base was constructed of a 40.6 cm diameter and 22.9 cm high, 3.175 mm thick, stainless steel cylinder. A 2.5 cm wide stainless steel ring was welded to the inside circumference of the cylinder, 7.6 cm from the base. A channel, 2.5 cm wide, was located at the top of each cylinder to accommodate a removable cover. The cover was made from a 3.175 mm thick, stainless steel bowl with a gas-tight septum inserted into the top of the dome. The septum was assembled from an open-cap stainless steel union tube fitting (Swagelok, Solon, OH) and a silicone septum was fitted into the cap to allow sampling with a gas-tight needle.

Evacuated Vial Preparation

Evacuated vials were used to collect gas samples from the flux chambers. The 20 mL serum bottles (Wheaton, Millville, NJ) were closed with a butyl rubber septum stopper (Bellco Glass Inc., Vineland, NJ) and secured with an aluminum cap. Vials were evacuated using a high vacuum pump (GEM 8990A,Welch), fitted with a digital vacuum gauge (DVG64, Omega) capable of measuring pressure values less than 200 mTorr. A

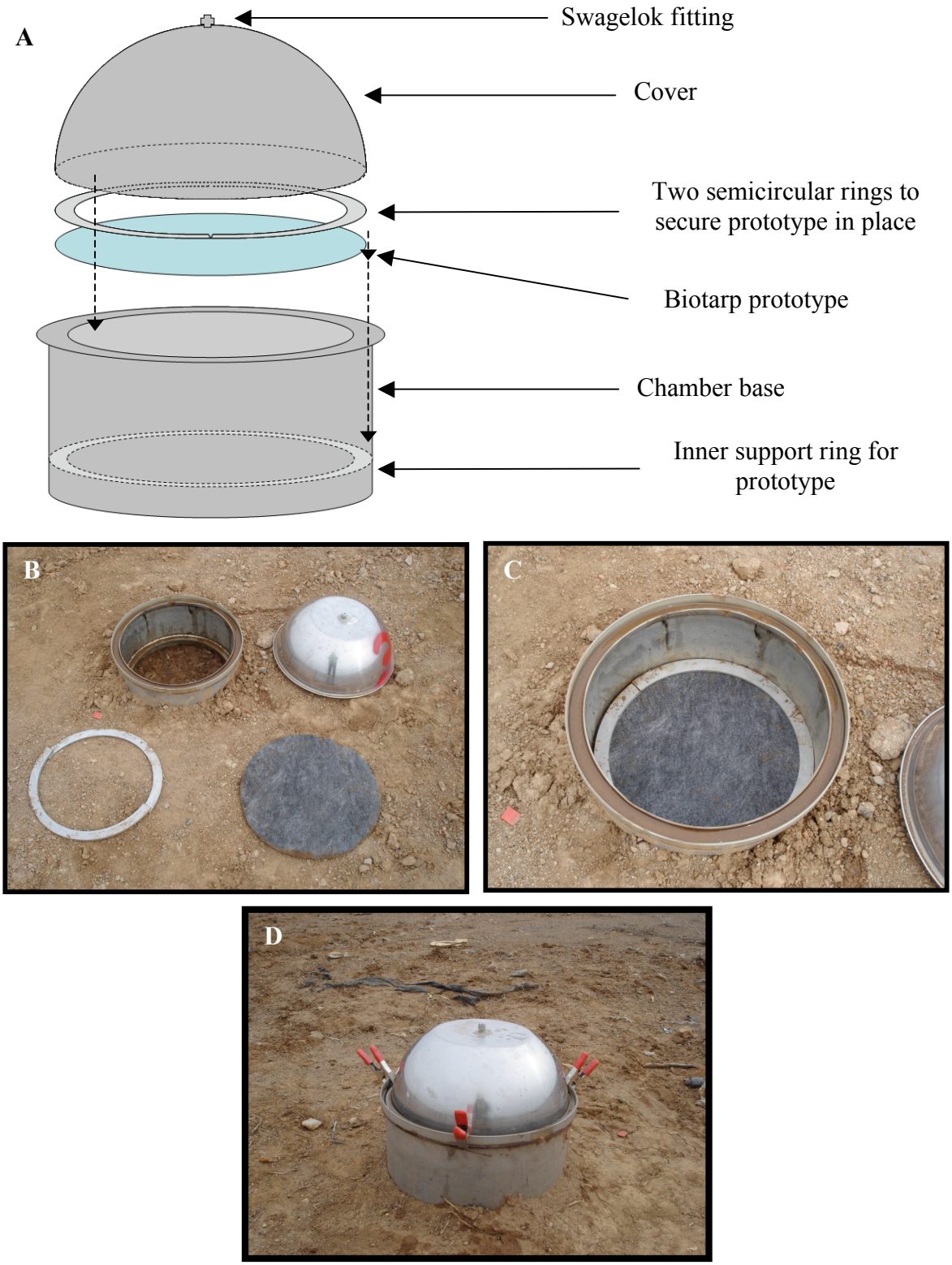

Figure 31. Flux chamber configuration. **A)** Diagram of flux chamber. **B)** Photo of flux chamber components. **C)** Photo of biotarp prototype in flux chamber. **D)** Photo of assembled flux chamber.

manifold was constructed to allow seven vials to be evacuated simultaneously. The manifold was constructed using 3.175 mm, stainless steel tubing, stainless steel union cross fittings, and stainless steel union tees (Swagelok, Solon, OH). Valves were included in the manifold to control the flow to each vial. The head of a 1 mL plastic syringe (Becton Dickinson & Co.) fitted with a 22G needle (Becton Dickinson & Co) was secured to the end of each manifold line. Vials were attached to the manifold by inserting the needle through the vial septum. A 50mTorr vacuum was applied to the vials and preliminary tests showed that they could reliably hold the vacuum for at least three days.

Field Biotarp Prototype Configuration and Preparation

A multilayer tarp was prepared by alternating layers of 40.64 cm diameter, washed and sterilized Ten Cate geotextiles IR26 and S1600. The IR26 had a fused side and a non-fused side. It was placed fused side up in the second and fourth (top) layers. The tarps were soaked in 1 L of an overnight methanotroph population for at least 10 min, removed, and allowed to drain until no further liquid dripped from them. Control biotarps were prepared with either sterile NMS or DI water.

A second prototype was assembled that consisted of the methanotroph-embedded multilayered biotarp, and a 0.33 cm thick layer of intermediate cover soil (Allied Waste landfill, Cabarrus County, NC) between the second and third layers. Approximately 400 g of landfill soil were enriched by incubation in a gas-tight jar under a 50% methane-in-air headspace for 2 days before the soil was added to the tarp. Negative controls consisted of a NMS-soaked four-layered biotarp without soil.

Compost additive tarps consisted of the multilayered tarp as described above with a 0.33 cm layer of finished compost (Compost Central Municipal Yard Waste

Composting Facility, Charlotte, NC) set between the third and fourth layer. Like the soil, the compost was first pre-incubated in a gas-tight jar with a 50% methane-in-air headspace for 2 days. The negative control tarp was a multilayer tarp prepared with sterile NMS only.

Shale was also examined as an additional biotarp additive. Shale Fines (Carolina Stalite) were sieved to produce a 2.00-4.76 mm particle size fraction that was washed with DI water and autoclaved. It was then pre-incubated in either excess solution of an overnight mixed methanotroph population or in sterile NMS for about 30 min. The biotarp was amended with the methanotroph-soaked shale, while the negative control tarps were amended with NMS-soaked shale. Like the soil and compost-amended tarps, a 0.33 cm layer of shale was placed in the middle of the four geotextile layers.

Landfill Gas Flux Measurements

Field trials were conducted at the Allied Waste landfill in Cabarrus County, NC. The study site was a section where intermediate soil cover was atop one year old municipal waste. The intermediate cover was composed of ~30 cm of clay topped with 30 cm of top soil. Six flux chambers (Fig. 31) were installed at random locations within a 20 ft x 20 ft area. The base of each chamber was set firmly into the ground, so that about 4 cm of the base depth was below grade. Additional soil was placed around the perimeter of the chamber and packed down tightly to seal the interface between the base and the surface.

The prototypes were placed atop the inside ring at the bottom of each base. Another stainless steel ring was placed on top of each prototype to secure it in place and prevent gas short circuiting. After each prototype was in place, the chamber lid was set in

a channel that ringed the top perimeter of the base, and four spring clamps were used to secure the cover to the base. Water was then poured into the channel to form a gas-tight seal between the lid and the base.

Immediately after preparing the seal, a gas sample was removed, and subsequently thereafter in 3 or 5 min intervals for 15 min. Gas samples were collected in 60 mL plastic syringes (Becton Dickinson, Franklin Lakes, NJ) equipped with a 22G needle and a gas-tight valve. The syringes were used to withdraw 50mL of gas from the flux chamber through the gas-tight septum, which was then injected into an evacuated vial. During sampling, the site temperature was measured and atmospheric pressure obtained from local meteorological data was recorded.

Landfill Gas Flux Determination

The methane concentration of each sample measured in the laboratory using gas chromatography. The methane concentration for each chamber was plotted over time, and linear regression was used to generate a regression coefficient (R^2). If the R^2 value was greater than 0.9, the best-fit line was considered acceptable, and the slope of the line was used to calculate the methane mass flow rate in ppm/min. The volumetric methane flux for a given chamber was calculated using the following equation (274):

$$J_{CH_4} = 1.44\frac{VMPc}{ART}$$

Where,

J = volumetric methane flux (g/m^2 day)

V = flux chamber volume above landfill surface (m^3)

M = molar mass of methane (g)

P = barometric pressure (atm)

c = methane concentration (ppm)

A = biotarp surface area (m^2)

R = ideal gas constant (L·atm/mol K)

T = air temperature (K)

Gas Chromatography

A gas chromatograph (SRI Instruments, 8610C) equipped with a CTR1 column (Alltech, Deerfield, IL) and a thermal conductivity detector (TCD) and flame ionization detector (FID) was used to measure the methane concentrations of injected field gas samples. Gas exiting the column flowed through the two detectors in series, with the helium carrier gas set at a flow rate of 20 cm^3/min. High methane concentrations were detected by TCD, with a detector temperature set to 100ºC. The injector and oven temperature were both maintained at 60°C. Low methane concentrations were detected by FID, which received both hydrogen set at a flow rate of 25 cm^3/min and ambient air (from an internal air compressor), set at a flow rate of 250 cm^3/min. A standard curve for TCD was generated using ultra high purity methane (National/Specialty Gases, Durham, North Carolina). Standard curves for FID were generated using 10% methane (nitrogen balance), 100ppm methane (nitrogen balance), and 10ppm methane (air balance) standards (Matheson Tri-Gas, Twinsburg, OH). A 5mL syringe (Becton Dickinson & Co.) fitted with a 22G needle (Becton Dickinson, Franklin Lakes, NJ) and a gas-tight valve was used to withdraw 2.5 mL of a gas sample from the vials. The samples were then injected into a 1 mL injection loop, which delivered the gas to the column. The manufacture's software package, Peak Simple 3.29, was used to plot and integrate peaks generated from both detectors.

Measurement of Ammonia in Landfill Gas Samples

A 4% (w/v) solution of boric acid was prepared and 25 mL aliquots placed in 25 mL gas-tight bottles. Standards were prepared by injecting known volumes of pure ammonia gas into the boric acid, which traps the ammonia to form an ammonium-borate complex. Ammonia was detected using the Hach Nitrogen Ammonia kit (Hach Method 10031), with some modifications. A 0.1 mL aliquot of the boric acid-ammonia solution was added to the Hach reagents, according to manufacturer's instructions, and incubated for 20 min. Sample absorbency were read at 425 nm and a standard curve constructed using duplicate samples of each standard amount. An R^2 value of one was considered acceptable.

Landfill gas samples were collected from two chambers and 1 mLwas injected into the boric acid as described. The absorbency measured, and using the standard curve, the concentration of ammonia was determined.

Statistical Analysis

Data were compared using linear regression, Student's t-test and One-way ANOVA with a Tukey's multiple comparison test. Statistical analysis was performed with Prism GraphPad software (GraphPad Software Inc., San Diego, CA).

Results and Discussion

Methane flux rates were measured at thre depths: (i) atop the bare refuse; ii) about 20 cm below the surface of the intermediate cover soil; and (iii) at the surface of the intermediate cover (Table 8). Over bare refuse, methane fluxes were highest, ranging from 420 to 5500 g/m^2 day. At 20 cm into the intermediate cover, fluxes ranged from 14 to 1300 g CH_4/m^2 day, while atop the intermediate cover soil, the fluxes ranged from 0.86

Table 8. Mean flux (g/m ^{2}day) from 6 chambers set in bare refuse, 20 cm into the intermediate cover, and atop intermediate cover.

Chamber		Bare Refuse Flux	20 cm Deep Flux	Intermediate Cover Flux
1	mean	1153.71	1320.84	6.37
	SD	536.31	995.18	4.87
2	mean	2596.15	537.27	24.48
	SD	1160.41	475.47	20.89
3	mean	5493.91	481.89	7.1
	SD	2554.27	361.05	6.1
4	mean	2675.8	334.91	39.4
	SD	1572.18	280.74	44.57
5	mean	4219.93	14.15	16.61
	SD	1911.72	8.98	7.47
6	mean	422.49	367.76	0.86
	SD	214.47	174.69	2.31

to 40 g CH_4/m^2 day. Considerable variation in methane fluxes within a specific site and between the six sites was found. The flux at one single location varied over two-fold, from 1830 g $CH_4/m^2 \cdot d$ to 4400 g $CH_4/m^2 \cdot d$ during one four week monitoring interval. Temperatures ranged from 8°C to 23°C over the course of the trials, and a relationship between temperature and methane flux has been well documented in landfills (41, 222, 224, 313), wetlands (97, 117, 345, 346) and rice paddies (68, 357). The decrease in methane flux with increasing temperatures is thought to result from higher methane

oxidizer activity at higher temperatures. The large spatial and temporal flux variations were observed here is also consistent with reports from other sites (1, 2, 33, 37-39, 79).

The decrease in methane flux with depth is due in part to the natural concentration gradients that result from multigas mixes, as atmospheric oxygen and nitrogen diffuse down into the soil, while carbon dioxide and methane are diffusing upward. The presence of resident methanotrophic bacteria in the intermediate soil cover also likely contributed to differences in flux with depth. These organisms are ubiquitous in soil, and their presence in intermediate cover soil would be very likely. Methanotrophs have been detected and/or isolated from final landfill cover soil samples (54, 166, 168, 308, 337, 347), however no attempts to isolated methanotrophs from intermediate cover have been reported. As the flux rates most similar to those found over open landfill cells (100-200 g CH_4/ m^2 day, Bogner, unpublished) corresponded best to those found at 20 cm below the intermediate cover here, all subsequent experiments were conducted at this depth.

While the addition of compost, landfill cover soil, and shale to the biotarp prototype yielded increased methane removal in laboratory continuous flow chambers, biotarp efficacy was not evident in the field. Soil amended biotarps, unamended biotarps, and negative control tarps with no methanotrophs added reduced methane fluxes by 94%, 88%, and 80% respectively (Fig. 32). Although there was a slight increase in methane removal by the soil amended biotarp, there was not a statistically significant difference between the three groups ($p<0.05$). The soil additive likely contained additional methanotrophs that could facilitate methane oxidation and therefore a higher flux reduction was measured. The soil also originated from the same site, thus this biotarp contained cells acclimated to landfill conditions.

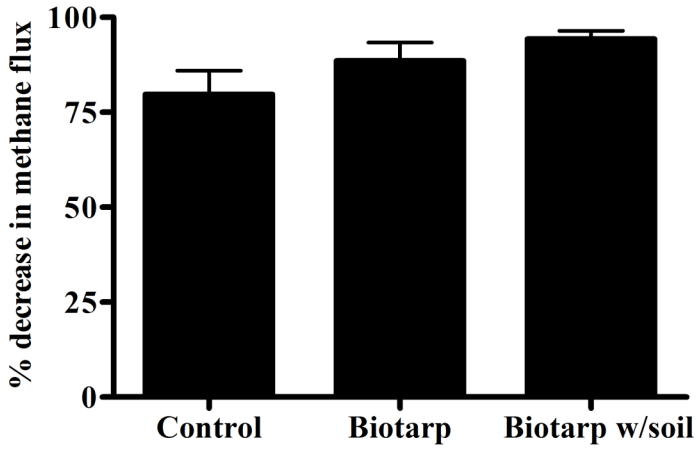

Figure 32. Methane flux reduction by a four layered methanotroph immobilized biotarp, a biotarp amended with enriched landfill cover soil, or a negative control tarp (NMS only). Error bars represent the standard error of the mean (SEM) of duplicate samples.

The addition of compost to the biotarp under field conditions appeared to lower the effectiveness of the biotarp. The unamended biotarp reduced methane flux by 67% , while the negative control yielded an 81% reduction. The biotarp amended with compost only reduced the flux by 53%. There was no statistically significant difference ($p>0.05$) between the three conditions (Fig. 33).

In trials with shale included in the biotarp, the methane flux reduction was 47%, which was slightly lower than the 55% reduction by the negative control (Fig. 34) containing not methanotrophs and no shale. Again, there was no statistically significant difference between the control and shale amended biotarp ($p>0.05$). The unamended biotarp was not included in this experiment so that a higher number of replicates could be

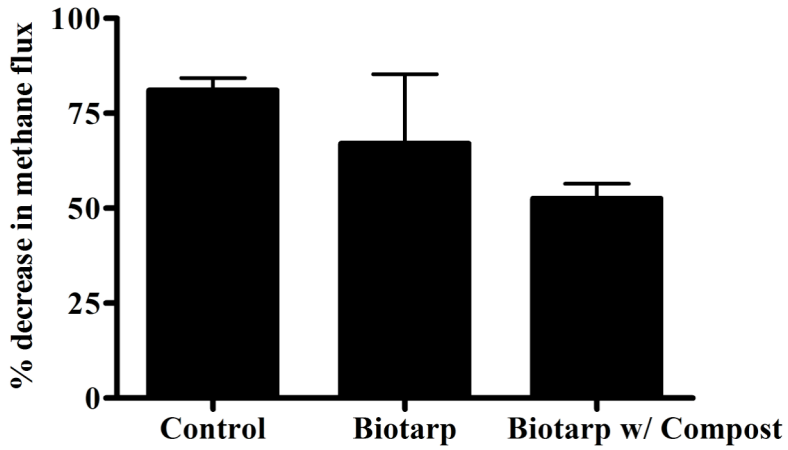

Figure 33. Methane flux reduction by a four layered methanotroph immobilized biotarp, a biotarp amended with compost, or a negative control tarp (NMS only). Error bars represent the standard error of the mean (SEM) of duplicate samples.

utilized. However, the increase in replicates did not reveal any statistically significant differences between the treatments.

Although the multi-layered biotarp prototypes, particularly those with a shale additive, were successful in methane removal under simulated landfill conditions in the laboratory, such success was not easily translated to field conditions. Overall, there was no difference between negative controls, the four layered biotarp configuration, and configurations with amendments. The lack of significance between any experimental condition and the negative control suggests that the biotarp prototypes are not effective at reducing methane emissions in the field, despite the good performance of biotarps in the laboratory and much poorer performance of control tarps in the laboratory chambers. The reductions accomplished by the negative controls in the field suggest that a wetted tarp without methanotrophs would reduce emissions. To determine the extent to which a moist tarp alone can reduce methane flux, dry four layered tarps and tarps saturated with

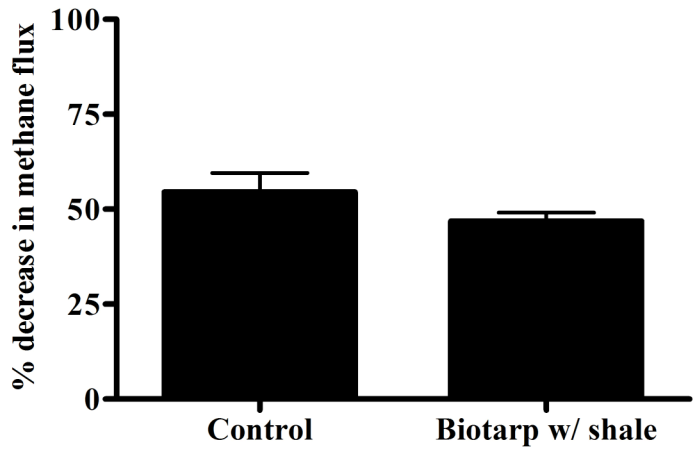

Figure 34. Methane flux reduction by a four layered methanotroph immobilized biotarp amended with shale or a negative control tarp (NMS only). Error bars represent the standard error of the mean (SEM) of three replicate samples.

dI water were compared in flux chambers. The average methane emissions reduction by the wet tarp was 89%, while the average flux reduction by the dry tarp was 63% reduction (Fig. 35). Methane reduction by the wet tarp was significantly higher ($p<0.05$) than the dry.

Taken together, these data suggest that the moisture on the biotarps contributed to methane flux reductions even in the absence of methanotrophic bacteria. The water may serve as a retardant to methane flow by physically blocking flow; by slowing the rate of gas diffusion through the pores of the geotextile where water is present; and by incorporating some methane that dissolves in the liquid.

There were a number of challenges present in the field that did not exist in the laboratory. The high day-to-day and even hour-to-hour variability in baseline fluxes from a given chamber made it difficult to compare trials from one treatment to another within a

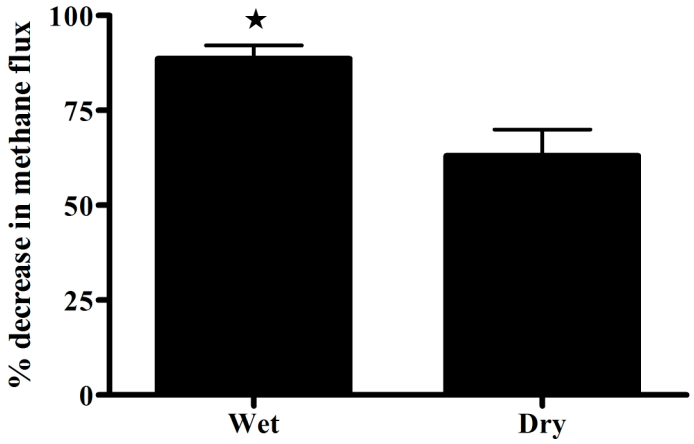

Figure 35**.** Methane flux through a wet and dry tarp. Error bars are the standard deviation of three replicates. ★ indicates a statistically significant difference ($p<0.05$).

given chamber or between chambers. The year over which these tests were conducted was a particularly wet one, and the opportunity to conduct field tests was reduced by the number of rainfall events that occurred during field testing. There may also be other volatile compounds present in the buried waste that were toxic to methanotrophs in the tarp.

Ammonia was detected in both flux chambers sampled, with concentrations ranging from 4-9 mg/L over a 15 min range (Fig. 36). Furthermore, the amount of ammonia present was similar at both chamber sites. These findings are noteworthy because ammonia is toxic to methanotrophs and may explain the poorer biotarp performance under field conditions. Ammonia is a known component of biogas and is produced from the decomposition of proteins (20). At low concentrations, it can be oxidized by methanotrophs due to the low specificity of the MMO enzyme (80, 159,

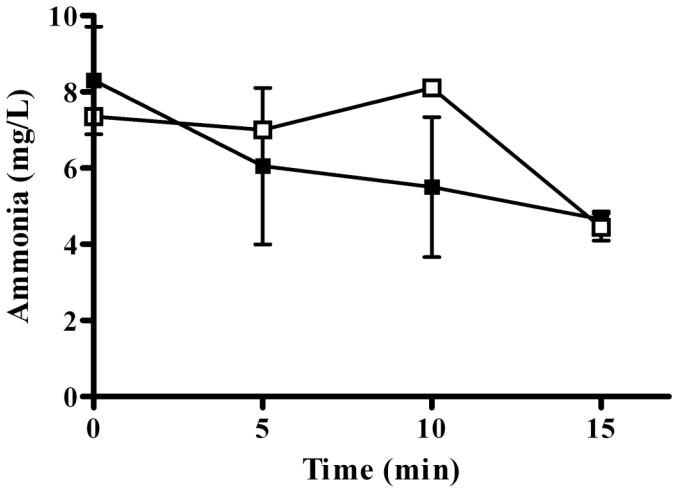

Figure 36. The ammonia concentration in landfill gas samples collected from two flux chambers (□ chamber 1 and ■ chamber 2) over a 15 minute period. Error bars represent the standard deviation of two replicate samples.

339). At high concentrations, however, ammonia can inhibit methane oxidation (85, 283). It is likely that ammonia may have inhibited oxidation by the laboratory and compost methanotrophs immobilized in the biotarp. The landfill soil amendment may have contained ammonia oxidizing microorganisms, which reduced ammonia levels locally within the biotarp and made methanotroph methane oxidation possible.

CHAPTER 10: SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

The purpose of this investigation was to develop a methanotroph embedded biotarp to function as a reusable, alternative daily cover that also mitigates early methane emissions from open landfill cells. To populate the biotarp and facilitate methane mitigation, methanotrophs were successfully enriched and isolated from landfill cover soil. Several isolation methods were explored, and all were challenging and time intensive. Furthermore, some methods were hindered by frequent heterotroph contamination. Isolation by adsorption onto a natural sponge and a synthetic geotextile were found to be the most successful. This technique likely exploited the natural adherent properties of methanotrophs, namely EPS. This method also probably minimized heterotroph interference because active methanotrophs were adsorbed from conditions that suited their growth.

Methane oxidation measurements and a diagnostic microarray confirmed that a robust population of methanotrophs was enriched from landfill cover soil. The microarray was sensitive down to the genera level, and only one non-methanotroph was isolated. Although further isolations and RNA sequencing would better characterize the population, this was not done because it was anticipated that biotarps would develop different population mixes in the field. Furthermore, characterization of landfill methanotroph populations had previously been reported by Wise *et al*. (347) and was beyond the scope of this investigation.

Three different immobilization techniques were investigated to embed methanotrophs into the biotarp. Cell entrapment in alginate beads and liquid-core gel capsules were evaluated because they would allow methanotrophic cells to be concentrated and maintained in a bead/capsule within the biotarp. Entrapment, particularly in liquid-core gel capsules, would also allow nutrients to be stored along with the methanotrophic cells. Through the use of automated bead synthesizing equipment, production could easily be scaled up to supply entrapped methanotrophs for commercial biotarp production. However, several problems with entrapment as an immobilization scheme were encountered. Both alginate beads and the gel capsules desiccated quickly when exposed to the open air and did not rehydrate when moistened. Furthermore, preliminary studies of methane flow through biotarp samples in the continuous flow chambers indicated that methane flow was not evenly distributed and methane "hotspots" were created in the tarps. Methanotrophs contained in beads or gel capsule would be unable to move to the high methane flow areas, which could reduce the overall effectiveness of the biotarp. The methane oxidation rates by entrapped cells were much lower compared to methanotrophs adsorbed to various materials, therefore no longer term methane oxidation studies of entrapped methanotrophs were conducted. However, other investigations have found that as the cell population expands within the bead, mass transfer resistance occurs (286, 287). Despite the problems found with cell entrapped in this investigation, it may be a feasible option using commercially available bead synthesizing systems. Such automated systems would allow multiple bead characteristics, such as diameter, wall thickness, pore size, core volume, etc, to be optimized for maximal methane oxidation capacity.

The highest methane oxidation rates measured during the survey of immobilization schemes occurred in cells adsorbed to a natural sponge, foam padding, and synthetic geotextile. Biomass accumulation measurements indicated that although methane oxidation was highest in samples containing the most water absorbent materials, it was not due to increased cell numbers. This suggests that the high surface area provided better methane exposure and/or materials chemical and physical properties promoted higher methane oxidation. Although the highest methane oxidation rates were observed with the sponge and foam padding, they were judged too bulky for field use. Adsorption was judged to be a very easy method for immobilizing methanotrophs. It would allow free cell movement throughout the tarp, an important feature for targeting higher cells densities around methane hotspots. However, because cells are not irreversible anchored within the biotarp, cell loss can occur. This was confirmed by stability assays that showed an approximately 70% loss after washing. Nevertheless, the remaining population was viable, and such washing may function to refresh the biofilm after heavy rainfall events.

The biotarp must be designed to target year round methane emissions from active landfill cells. There is very little basic research regarding the molecular and physiological responses of methanotrophs to temperature stress. Therefore, it was necessary to determine the methane oxidation response of immobilized methanotrophs at various temperate climate temperatures. Methane uptake rates increased with increasing temperatures, up to 35°C, and an optimal temperature range for this methanotroph population was evident. The finding of shifts in dominant methanotroph species and temperature optima with altered incubation temperatures suggests that biotarp

methanotrophs may acclimate to lower seasonal temperatures. Although acclimatization may occur, oxidation rates will likely continue to be lower at lower temperatures, as methanotrophic growth and likely enzymatic activity, would be lower as well.

As the biotarp will be in place during the evenings and weekends, methanotrophs in the tarp will encounter daily starvation periods. The data indicated that methane oxidation could be renewed after cells were methane starved; although the renewed rates declined the longer cells were starved. Oxidation rates also declined during a 12-hour "on-off" methane cycling regime. Positive control samples showed a similar, but delayed decline, indicating that factors other than methane starvation were contributing. Reports of methane starvation and renewed methane uptake in the literature vary, and the results of starvation studies in this investigation are not consistent with any previously reported findings. This confirms that the starvation response depends on many factors, including the methanotroph population composition, methane history, and environment. Furthermore, the decline in methane oxidation by controls that received methane continuously over five days suggests that either inorganic nutrient depletion or EPS accumulation may be contributing factors to the observed reduce methane uptake. It may be necessary to provide inorganic nutrients to biotarp in the form of a daily spray or in a slow-release capsule. EPS accumulation, which can limit oxygen and methane diffusion to the cells, may be reduced by rainfall events

The results of these environmental stress challenges suggest that low temperatures and methane starvation may reduce biotarp performance in field trials. It is possible that with continued exposure to field conditions, an initial inoculation of methanotrophs could yield an adapted population that could tolerate temperature and starvation stressors better

than the laboratory population. Furthermore, the washing out of cells could reduce EPS build-up within the biotarp and aid in sustained methane oxidation activity.

Evaluation of six different materials commonly used in cell immobilization applications indicated that a synthetic geotextile was most suitable as a biotarp component. A number of commercial and custom-manufactured geotextile materials were evaluated for their water hold capacity and ability to promote methane oxidation, as a part of the biotarp development. Of the nine geotextiles examined, Ten Cate Nicolon product materials S1600 and IR 26 were found to have both high water holding capacity and support high methane oxidation. Therefore, these materials were selected as components of a biotarp prototype. Evaluations of the various geotextiles demonstrated the importance of material properties that facilitate both high methane oxidation (a function of the water/methanotroph culture holding capacity) and high gas permeability.

Mass balance calculations were conducted for methane into and out of each continuous flow chamber. Preliminary experiments indicated that a multi-layered biotarp performed better than a single-layered biotarp, removing an average of 17% of the 20-25 g/m^2 day entering the bioreactor. Water condensation, oxygen consumption, and carbon dioxide production were also evident in chambers with active biotarps, while these changes were absent in control tarps. The methanotroph activity level in the chambers was a markedly different from batch studies. The difference was likely due to the residence time of the methane molecules with the cells. In batch, methanotrophic cells are surrounded by a given mass of methane for 24 hours. Under continuous flow, there is a finite time that a methane molecule has to diffuse into the cell. If diffusion does not

occur, the methane molecule escapes and the cell does not have another opportunity to oxidize that molecule.

In order to enhance methane uptake levels, various amendments were tested in four-layer biotarp prototypes. The amendments were landfill cover soil, compost, or shale which maintained more uniform methane oxidation rates over time and yielded average methane removal rates of 32%, 30%, and 40%, respectively. In addition to increasing gas detention within the biotarp, these amendments also introduced some additional methanotrophs, as well as changes in moisture conditions, other microbes and microbial products, and different surfaces for binding. Results from the continuous flow chambers revealed the need for increased gas detention time with the embedded cells. Gas distribution could also be examined in future prototypes to ensure that methane flow occurs uniformly across the biotarp to prevent the formation of methane hotspots. As increased methane removal by amended biotarps may be due, in part, to additional methanotrophs, promotion of growth within the biotarp could further increase removal rates. This could be achieved by supplying cells with inorganic nutrients or by increasing methane exposure. Methane oxidation rates can be increased by improving the biotarp design. Figure 36 illustrates important features of an ideal biotarp configuration. In this ideal design, the bottom layer should be composed of a highly gas permeable material that allows gases to enter, but not exit. The layer above that should consist of a material that promotes good gas distribution, such that the methane load is uniform across the biotarp. The inner layer should promote gas detention, methanotroph growth, and attachment; while the top layer should encourage methane detention, but oxygen penetration and carbon dioxide release.

Figure 37. Ideal biotarp prototype based on results from continuous flow chamber trials.

To confirm that methane oxidation was mediated by methanotrophs, and to visualize biofilm architecture in the geotextile, FISH was used to fluorescently label Type I and Type II methanotroph cells with type-specific RNA probes. The lectin, Concanavalin A was used to visualize EPS. Together, this staining technique clearly showed that the methanotrophs were associated with the EPS and that most appeared to be associated with the geotextile fibers due EPS production. Although, the depth of the cells could not be determined using standard fluorescent microscopy, the use of confocal microscopy would provide some insight into cell location. This information would be important in determining if reductions in methane oxidation rates were due to accumulation of EPS, in which case cells would be located under a thick EPS layer.

Examination of biotarp samples after incubation in the continuous flow indicated that both Type I and II methanotrophs, as well as EPS, coated geotextile fibers. This was consistent with diagnostic microarray analysis that showed both types of methanotrophs present. The density of methanotrophs on the geotextile fibers was lower than expected.

This indicates that the methanotroph load on the biotarp can be increased and methanotroph growth within the biotarp should be promoted.

Field tests of the biotarp prototypes used flux chambers set in intermediate cover at a local landfill. Baseline readings showed that a wide range of methane fluxes could be measured both at a given flux chamber site and between sites and may be confounding the biotarp performance. A reduction in methane flux was observed, relative to bare soil, when the prototypes were placed in the flux chambers; however there was no statistically significant difference between negative control tarps, the four-layered biotarp, or biotarp with any of the amendments. Additionally, ammonia was detected in the landfill gas, and is a known inhibitor of methanotroph methane oxidation. The addition of ammonia oxidizing microorganisms to the biotarp may be necessary to overcome ammonia-mediated methane oxidation inhibition.

The goal of this project was to determine the feasibility and to develop a methanotroph embedded biotarp. A method for immobilizing methanotrophs was identified and positive results were found using several biotarp prototypes in laboratory bioreactors. However, laboratory stress tests and field evaluations suggest that a number of biotarp features and properties need to be addressed, including methanotroph loading, nutrient supplementation, methane detention and distribution. Overall, the findings suggest that a methanotroph embedded biotarp appears to be a feasible strategy to mitigate methane emission from landfill cells. Further modification of the biotarp prototypes as recommended here, should facilitate increased methane removal under field conditions.

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APPENDIX A: METHANOTROPH DIAGNOSTIC MICROARRAY ANALYSIS