# BIO-TARP: DEVELOPING A METHANOTROPHIC ALTERNATIVE DAILY COVER TO REDUCE LANDFILL METHANE EMISSIONS

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SUMMARY: We are developing a bioactive alternative daily cover, a "bio-tarp", to mitigate methane ($CH_4$) emissions during the active life of a landfill cell, prior to placement of final cover. A prototype is currently under development, supported by static and dynamic laboratory testing of alternative materials with embedded methanotrophic microorganisms. Progress to date has included laboratory-scale testing of a variety of suitable immobilization techniques and development of a mixed methanotrophic culture isolated from landfill cover samples. Microarray analysis with methanotroph-specific probes confirmed the presence of a mixture of methanotrophs consisting mostly of *Methylobacter* and *Methylocystis* species. Future phases of this project will include field testing of prototype materials as well as the development of conceptual designs and proposed operational strategies.

## 1. BACKGROUND

Landfills are the largest source of anthropogenic $CH_4$ emissions in the U.S. (U.S. Environmental Protection Agency, 2007). Field data (unpublished) have shown that substantial $CH_4$ generation and emissions can occur before engineered gas extraction is economically feasible. Methanotrophs are indigenous aerobic microorganisms in landfill cover soils that can oxidize $CH_4$ and thereby provide a complementary biological mechanism to reduce emissions. Even though both landfill $CH_4$ emissions and oxidation rates can vary by more than 6 orders of magnitude (Bogner, *et al.*, 1997), field data have confirmed that methanotrophs are capable of substantial reductions in $CH_4$ emissions in both conventional covers and engineered "biocovers" (Huber-Humer, 2004). In addition,

methanotrophs are capable of reducing emissions of non-methane hydrocarbons (Scheutz *et al.*, 2003).

Federal law in the U.S. requires that daily cover be placed over open landfill cells at the end of each working day. At some sites, approved alternative daily cover (ADC) materials are being used where appropriate local soils are not available, less expensive alternatives (such as garden waste compost) are readily available, or the ADC can cost-effectively gain additional air space for site operators. Tarps are one type of ADC which maximizes airspace because the tarps are placed at the end of the working day and removed the next morning.

In this three-year project supported by the U.S. Dept. of Energy, we are developing a methanotrophic ADC, a "bio-tarp", to reduce early $CH_4$ emissions from landfilled waste through the presence of an active methanotrophic population. The bio-tarp could be removed and re-activated as needed, and it would provide a portable emissions reduction strategy for areas undergoing active filling or requiring a short-term temporary cover over a period of weeks or months. We are focusing on a strategy of cell immobilization using adsorption to various candidate biotarp materials. Cell immobilization is a well-established technique that has been utilized with many cell types in many diverse applications; immobilization involves the fixation of cells, including microorganisms, to a supporting material (Fenice *et al.*, 2000). This project is focusing on adsorption, which is one of the simplest immobilization techniques, and one which allows the natural development of biofilms with minimal effects on normal cell activity. Bacterial adsorption to both organic supporting materials (Liu *et al.*, 1998, Fenice *et al.*, 2000, Lante *et al.*, 2000, Pattanasupong *et al.*, 2004, Yang *et al.*, 2004) and inorganic supports (Kovalenko & Sokolovskii, 1992, Kotha, *et al.*, 1998, Bonin *et al.*, 2001) has been reported. Methanotrophs are known to produce biofilm (Whittenbury *et al.*, 1970, Hou *et al.*, 1979), and in one instance, they were found attached to stone masonry (Kussmaul, *et al.*, 1998). In previous studies, methanotroph biofilm formation has been used successfully to attach cells to diatomaceous earth and granulated activated carbon in an attached-film, fluidized-bed (MAFFB) reactor system (Fennell 1992).

The goal of this project is to develop and field test one or more prototype bio-tarps to replace traditional soil cover or ADC. This paper focuses on laboratory tests that were conducted during Year 1 of this project in which the potential to immobilize methanotrophs by adsorption was tested with a variety of candidate tarp materials.

## 2. METHODS

Landfill soil enrichment. Samples of conventional landfill cover soil were locally collected (Hilger & Humer, 2003). After sieving, 50 g replicate subsamples were placed in four gas-tight, 1 L glass jars. Two jars were maintained with a headspace of 9% $CH_4$ in air while the other two were maintained at 45% $CH_4$ in air (v/v).

Cell immobilization on candidate support materials. Five types of support materials were tested for their ability to maintain a population of adsorbed $CH_4$ oxidizers: natural sponge (Florida Sponge, Pinellas Park, FL); non-woven polypropylene geotextile (Ten Cate

Nicolon, Pendergast, GA); plastic trickling filter media; polycarbonate membrane (GE Osmonics, Minnetonka, MN); and glass beads (200-300-mm dia). Each support was incubated for nine days with 20 g of enriched soil in gas-tight 100 mL glass jars after adjusting the soil moisture content to 15%.±0.5%. The jars were sealed with metal chemostat port fittings (Bellco, Vineland, NJ) overtopped with white septum caps. Duplicate tests of each support material were conducted in parallel with control jars that contained enriched soil only. All tests were conducted under a 20% $CH_4$ (v/v) headspace monitored using gas chromatography (GC).

Rates of $CH_4$ oxidation by cells on support media. After incubation with the enriched landfill soil, the supports were subdivided and re-incubated in either 10 mL of Whittenbury's Nitrate Mineral Salts (NMS) (Whittenbury, *et al.*, 1970) or 10 mL of sterile soil slurry. Both incubations were maintained for 20 days in 100 mL gas-tight jars with a 20% $CH_4$ (v/v) headspace. The headspace gas concentrations were measured every two days using GC to determine $CH_4$ uptake rates. After the initial 20 day incubation, the NMS incubation was continued for a total of 7 weeks to assess the capacity of the cells for sustained $CH_4$ uptake. The NMS was replenished every 10 days.

To determine whether the supports influenced $CH_4$ oxidation rates, each material was incubated with 10 mL of liquid mixed culture developed from the spent NMS collected after the previous experiment. The tests were conducted in gas-tight jars for 17 days under a 20% $CH_4$ (v/v) headspace. Then the supports were used to determine the biomass accumulation and moisture-holding capacity (described below). Also, the cell concentration of the liquid mixed culture was measured by plating serial dilutions of the culture on NMS agar plates and counting the colony forming units (CFUs)/mL.

Isolation of a methanotroph population. The spent media was collected from the NMS incubations described above, pooled, and diluted 1:10 in NMS. Twenty mL liquid cultures were maintained at room temperature in 100 mL gas-tight jars with 20% $CH_4$ headspace and continuous shaking In addition, a membrane isolation technique was employed, modified from Svenning et al. (2003). A slurry was prepared with 3g of landfill soil and 27 mL of a 0.9% NaCl solution mixed for 15 min and then settled for 30 min. The settled landfill slurry was then serially diluted through $10^{-5}$, and 100 µL portions of each dilution were spread on sterile 100 mm, 0.2 µm polycarbonate filters (GE Osmonics Minnetonka, MN). . Each polycarbonate filter was then placed atop an NMS agar plate, which allowed the liquid to be absorbed while the bacterial cells remained on the filter. An 80 g portion of non-landfill soil (commercial potting soil) was mixed with 100 mL of deionized (DI) water and subdivided into 10 mL portions in sterile Petri dishes. Then the filters were removed from the agar and placed atop the soil slurries. The Petri dishes were closed and incubated at room temperature for 47 days in anaerobic jars with a 50% (v/v) $CH_4$ headspace, exchanged every three days.

DNA isolation and microarray analysis. A mixed methanotroph population was grown in liquid culture overnight before DNA was extracted using a DNeasy Tissue Kit (Qiagen, Inc.). A DNA microarray analysis was conducted by L. Bodrossy of the Austrian Research Center as described in Stralis-Pavese, *et al.* (2004). Briefly, the *pmoA*/*amoA* genes were amplified from the sample and RNA transcript was obtained. The purified RNA was fragmented and tested for hybridization with a variety of molecular probes. Hybridized slides were scanned, and the results were normalized to a positive control.

PCR for methanotrophs. DNA was harvested from pure cultures collected from the membrane isolation by boiling cells in 1 mL of DNase/RNase-free water for 5 min. Cells were then spun down and the pellet resuspended in 1 mL of DNase/RNase-free water. Polymerase chain reaction (PCR) was used to amplify the DNA, as described by Wise et al. (1999), and the PCR products were visualized on a 1.5% agarose gel.

Determination of biomass accumulation on support media. Biomass accumulation was determined by weighing dry supports before and after their use in an oxidation trial. At the end of a trial, the supports were dried at 105°C for 1 h then cooled in a desiccator before weighing. Drying and weighing were continued until there was no further weight change, and the dry biomass weight was calculated.

Gas chromatography (GC). A Shimadzu GC-14A equipped with a CTR-1 column (Alltech, Deerfield, IL) and a thermal conductivity detector was used for $CH_4$ analysis. The He carrier gas flow rate was 60 $cm^3$ $min^{-1}$, the detector temperature was 75ºC, and the injector and oven temperatures were both maintained at 60ºC. Standard calibration curves were generated using ultra-high purity $CH_4$ (National Welders, Augusta, GA).

## 3. RESULTS AND DISCUSSION

Enrichment under both 9% and 45% (v/v) $CH_4$ in air yielded methane oxidation activity, with rates 10-fold higher under the 45% $CH_4$ headspace compared to the 9% $CH_4$ headspace (data not shown). Composited soil from both incubations was used for subsequent experiments, which were conducted at a mid-range of 20% headspace $CH_4$.

The first attempts to adsorb methanotrophs from enriched soil onto a variety of candidate support matrices showed that the sponge and geotextile supports in the soil enhanced $CH_4$ uptake over control jars with soil only (Fig. 1), suggesting that the supports had a positive effect on uptake capacity. The jars with glass beads and trickling filter media had $CH_4$ uptake rates comparable to the controls. After the supports were removed from the enriched soil and incubated with a small amount of liquid NMS media, $CH_4$ uptake continued, albeit at a lower rate. The lower rate was likely due to differences in cell number, as the number of organisms in enriched landfill soil amended with a support was far greater than the number of cells on the support in NMS. Methane oxidation in jars with support media and NMS but no soil is significant because it indicated that methanotrophs must have adsorbed to the supports and traveled with them to subsequent NMS or soil slurry incubations. The soil slurry incubations offered no advantages over the defined NMS media with respect to methanotroph growth or oxidation rates, indicating that there was no critical nutritional element present in the soil that the NMS media was not providing

Continued incubation of the supports in NMS but no soil was continued for a total of seven weeks (Fig. 2). The highest $CH_4$ uptake rates continued to be observed in jars with the natural sponge and geotextile. Unlike the experiments in enriched soil, however, the polycarbonate membrane performed as well as the beads and trickling filter during these long-term incubations. In addition to showing that long-term activity by adsorbed methanotrophs could be sustained, these trials showed increases in $CH_4$ uptake rates relative to those measured in the initial days of the incubation, suggesting that the cell population expanded and colonized the supports.

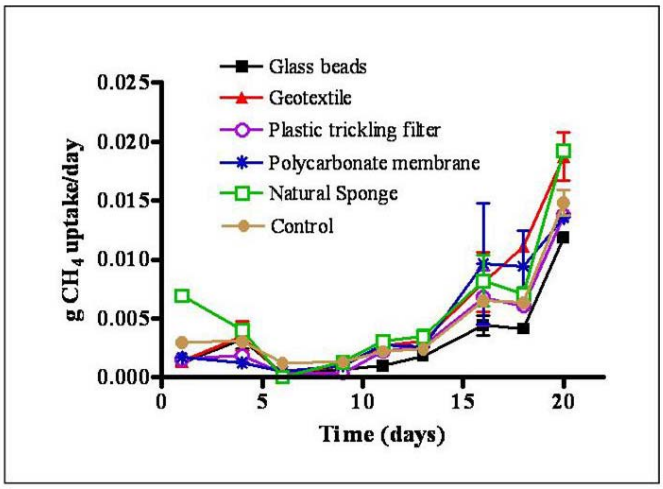


**Figure 1. Relative $CH_4$ uptake by methanotroph enriched landfill soil amended with various supports. Error bars represent he SD of duplicate samples.**

The mixed methanotroph culture created from spent NMS and subjected to microarray analysis (Fig. 3), was found to have two predominant genera -- *Methylobacter* and *Methylocystis*. These results are consistent with other reports of limited genera being found together (Auman *et al.*, 2000, Dedysh *et al.*, 2001, Kolb *et al.*, 2003, Bodelier *et al.*, 2005, Bodrossy *et al.*, 2006), and of reports that this particular combination was found in environmental samples (Wise *et al.*, 1999).

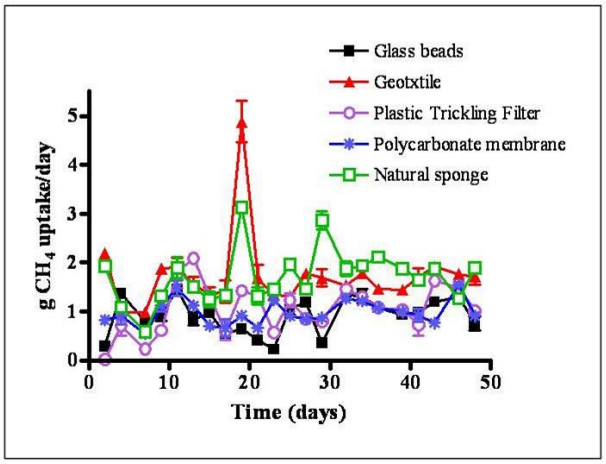


**Figure 2. The maintenance of $CH_4$ uptake by supports incubated in NMS for 7 weeks. Error bars represent the SD of duplicate samples.**

*Methylobacter* species belong to Type Ia methanotrophs, a subgroup of Type I that includes those genera that grow optimally under mesophilic conditions (Bodrossy, *et al.*, 2006), while *Methylocystis* is a Type II methanotroph. These methanotroph types generally have very different optimal growth conditions, with Type I methanotrophs dominating in low $CH_4$, high $O_2$ environments and Type II methanotrophs under the

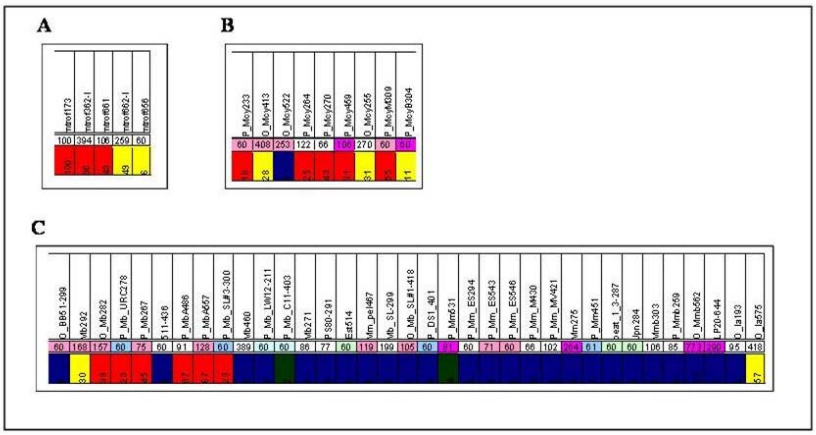


**Figure 3. Selected microarray analysis of the mixed methanotroph population. ■ indicates a signal >20% (positive), ■ indicates a signal > 5% (positive), ■ indicates a signal > 2.5% (unsure) and, ■ indicates no signal (negative). Panel A shows methanotrophic specific probes, B shows *Methylocystis* specific probes, and C shows *Methylobacter* specific probes.**

opposite conditions (Amaral & Knowles, 1995, Henckel, *et al.*, 2000). This diversity may reflect methanotrophs from different strata of the landfill cover or just a typical mixture present in soil. Subsequent analyses of the mixed culture also confirmed, as expected, that non-methanotrophic organisms (heterotrophs) were also present. For example, one non-methanotroph strain was isolated and identified by genetic sequencing to be *Acinetobacter* genomospecies 3. Also, among the methanotrophs isolated with the membrane technique, four were confirmed by 16s rRNA PCR to be type II methanotrophs (Fig. 4).

Thus, initial trials showed that $CH_4$ oxidation activity could be detected in soil and on physical supports incubated with the soil. Further, the supports were able to transfer that activity, denoting that some level of cell attachment was occurring. If nutrient supplementation was provided, attached cells could sustain $CH_4$ uptake over prolonged incubations. A mixed culture developed from biomass on the supports was shown to contain several genera of methanotrophs.

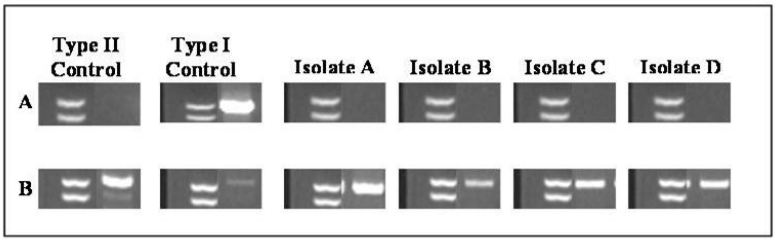


**Figure 4. PCR for methanotroph specific 16S rRNA sequences. Panel A represents amplification of Type I sequences and Panel B represents amplification od Type II sequences.**

Subsequent experiments were performed to quantify the performance of a given number of cells on different support types. Supports tested after previous incubations with soil may have transferred different numbers of organisms to the incubations in NMS. When a standardized volume of a mixed methanotroph culture was inoculated at 9.0 x $10^7$ cfu/ mL onto each support type and incubated under 20% headspace $CH_4$, the jars

containing geotextile and natural sponge had an average consumption rate of 1.5g $CH_4$/d in 17-day batch experiments (Fig. 5). Measures of biomass accumulation and water-holding capacity of the supports after the 17 day incubation (Fig. 6) suggested that the high porosity and water-holding capacities of the sponge and geotextile materials were important for enhanced $CH_4$ oxidation activity. Relatively high $CH_4$ uptake rates were also observed in samples containing the polycarbonate membrane. This membrane has previously been shown to be useful for isolating methanotrophs from soil (Svenning, *et al.*, 2003) and for mechanical trapping of cells in the membrane pores (El Kirat *et al.*, 2005). The $CH_4$ uptake rates for incubations with the other candidate support materials did not differ from the unamended controls, suggesting that these materials did not increase methanotrophic activity.

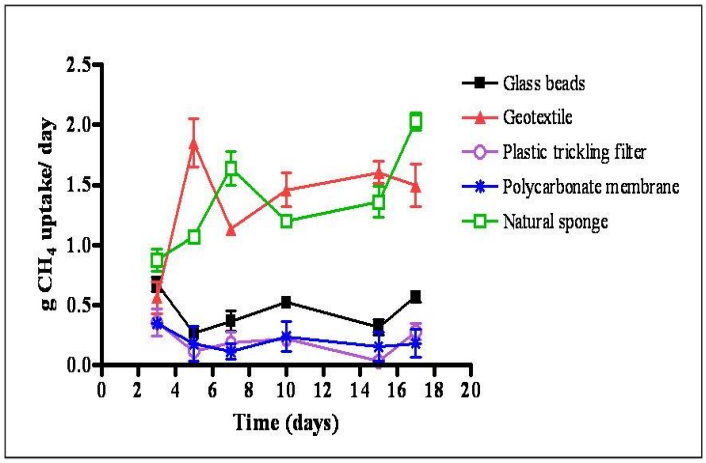


**Figure 5. Relative performance of a standardized mixed methanotroph culture absorbed to different matrices. Error bars represent the SD of duplicate samples.**

Based on these results, ongoing investigations of the suitability of sponge and geotextile materials for methanotroph adsorption are assessing uptake rates in continuous flow chambers with a steady $CH_4$ flux. Fluorescent *in-situ* hybridization studies are also underway to examine the nature of the biomass adsorption on the matrices. In combination with experiments to evaluate the stability of cell sorption to the support media, we expect the results to improve our understanding of cell fixation during precipitation or other physical disturbances. Similar tests are also being conducted on layered composite materials. Finally, the performance of the mixed methanotroph culture under temperature, nutrient, and moisture stress is also being evaluated.

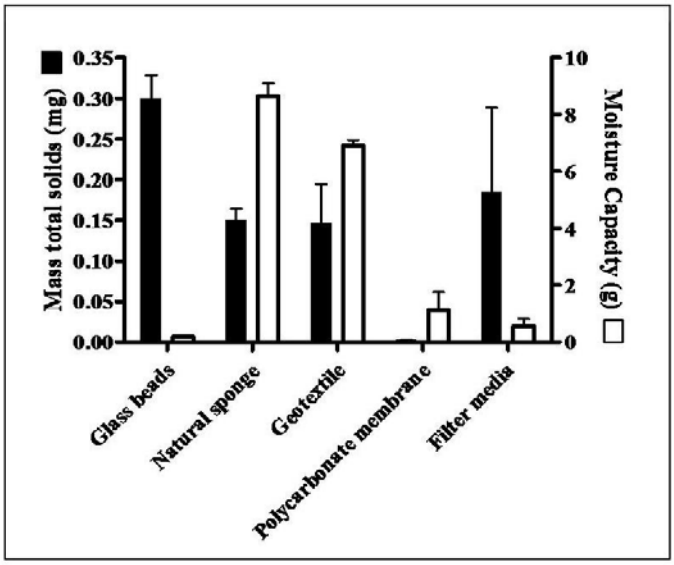


**Figure 6. Measurement of biomass and moisture holding capacity of each support material. Error bars represent the SD of duplicate samples.**

Although the ultimate colonizers of a field biotarp may differ from the mixed culture developed to date, these experiments demonstrate that methanotrophs will tend to adhere readily to materials that could be used for landfill ADC. Other more sophisticated immobilization techniques may offer additional options and performance benefits. In addition to these fundamental microbiology considerations, our research team is also considering operational strategies to maintain high rates of microbial $CH_4$ uptake with biotarps. Field testing of prototype biotarps is scheduled to begin in late 2007.

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