INCREASING METHANE CONSUMPTION IN AGRICULTURAL SOILS BY USE OF BACTERIAL INOCULA

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ABSTRACT

INCREASING METHANE CONSUMPTION IN AGRICULTURAL SOILS
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Methane (CH_4) is 25 times more effective than carbon dioxide at trapping infrared radiation over a 100 year period and is the second most significant source of radiative forcing in Earth's atmosphere. The largest biological sink is through oxidation by aerobic soil microbes, termed methanotrophs, which can be impacted by land management such that both methanotroph diversity and CH_4 consumption decrease by 70% when forests are converted to row-crop agriculture. In this study, the potential of a methanotrophic soil inoculum to enhance methane consumption was investigated in both microcosm and pilot-scale field experiments.

Mixed methanotrophic enrichment cultures were obtained from native forest soil and consist primarily of *Methylocystis* and *Methylosinus* species. Application of mixed methanotrophic enrichments significantly increased rates of methane consumption in agricultural soil microcosms. In preliminary field trials, methanotroph-inoculated sites demonstrated a 4-fold increase in total methane consumed over a 7 day period, as compared to uninoculated sites. Subsequent studies will focus on optimization of cultivation and soil inoculation methods, with the aim of increasing the magnitude and duration of *in situ* methane flux. These experiments serve as a starting point for a bioengineering solution to the effects of agriculture on climate change and the global methane budget.

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CHAPTER 1

Literature Overview

Methane as a Greenhouse Gas

Methane (CH_4) is the second most important of the long-lived greenhouse gasses, with 21-25 times the global warming potential (GWP) of carbon dioxide (CO_2) over a 100-year period. Primarily due to its high radiative forcing, CH_4 contributes about 30% of total greenhouse warming (Shindell et al. 2009; B. K. Singh et al. 2010). In addition to being a significant contributor of radiative forcing, atmospheric methane concentrations have increased 150% over pre-industrial measurements, making CH_4 an important target for mitigation (Solomon et al. 2007). Though methane emissions slowed in the early 2000s, most recent measurements indicate renewed increases observed at all monitoring stations, with a current global atmospheric mixing ratio of 1.8 ppmv (Rigby et al. 2008).

Methane is emitted from both anthropogenic activities and natural environments. Natural methane production is primarily due to anaerobic decomposition of organic materials by methanogenic archaea that are found in wetlands, contributing about 80% of annual natural methane emissions. Additional natural sources include the hindgut of termites, and geologic emissions such as mud volcanoes or fossil fuel deposits (Denman et al. 2007). Anthropogenic sources, which are estimated to contribute over 50% of total global methane emissions, include landfills, enteric fermentation in ruminant livestock, rice cultivation, manure management, and natural gas and petroleum systems (Solomon et al. 2007).

Reaction with hydroxyl radicals in the troposphere accounts for 90% of the total methane removed from the atmosphere (Denman et al. 2007). The only terrestrial methane sink is through

biological oxidation by methanotrophic bacteria, which are estimated to contribute 3-6% of the total global methane sink (Jay s Singh 2011). Though accurate assessment has proven difficult due to the many factors contributing to atmospheric methane concentration, recent studies have estimated the global methane imbalance at 2-6% annually, indicating a net increase in atmospheric methane (Denman et al. 2007; Solomon et al. 2007; Neef et al. 2010). Any small changes in methane production or consumption can have a great impact on atmospheric methane, and thus global warming, due to the narrow methane budget

Methane oxidizing bacteria

Aerobic methanotrophs are gram-negative bacteria distinguished by their ability to use methane as a sole source of both carbon and energy. They include members of the *Verrucomicrobia* and *Proteobacteria* phyla, and have been detected in a range of environments. Oxidation of methane to methanol is accomplished through the enzyme methane monooxygenase (MMO), which is a defining characteristic of aerobic methanotrophs, along with their elaborate internal membrane structures (Holmes et al. 1995). Methanol is then converted to formaldehyde, which is an important intermediate for anabolism and catabolism (Figure 1-1) (Whittenbury et al. 1970). The fate and treatment of formaldehyde is a distinguishing factor for the major phylogenetic groups of methanotrophs: type I, type II, and type X. Type I methanotrophs are members of the gamma-proteobacteria phylum, and use the ribulose monophosphate (RuMP) pathway for carbon assimilation. They are also distinguished by the bundles of parallel internal membranes whereas type II methanotrophs (alpha-proteobacteria) have membrane structures that are arranged around the perimeter of the cell and assimilate carbon through the serine pathway (Tavormina et al. 2011; Siegbahn et al. 1998). The type X

distinction has been given to methanotrophs such as *Methylococcus capsulatus*, which utilize both RuMP and serine pathways (Colby & Dalton 1976; Strom et al. 1974)

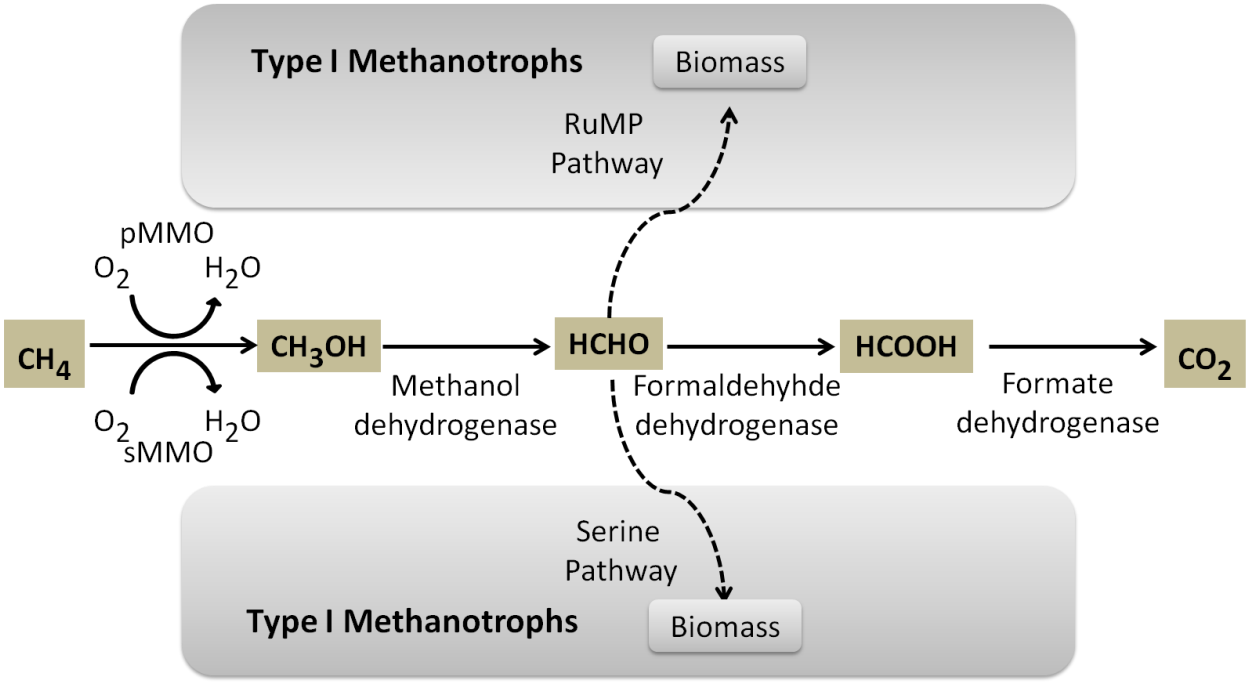

Figure 1-1. Pathways for methane oxidation and assimilation of formaldehyde in both type I and type II methanotrophs. The key enzyme, MMO, is available in either particulate membrane-bound (pMMO) or soluble, cytoplasmic (sMMO) forms. (Modified from R. S. Hanson & T. E. Hanson 1996). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

The enzyme MMO is exhibited as either a particulate, membrane bound form (pMMO) or a soluble, cytoplasmic form (sMMO) and methane oxidizing bacteria may contain one or both enzymes (Tavormina et al. 2011; Holmes et al. 1995). Particulate methane monooxygenase is encoded by the operon *pmoCAB* and is found in nearly all methanotrophs (Gilbert et al. 2000; Holmes et al. 1995). The only exception is *Methylocella* species, which only contain sMMO (Lawrence & Quayle 1970). The β subunit, and active site of MMO, is encoded by the gene *pmoA* which is conserved among aerobic methanotrophs, and whose sequence similarity correlates to that of 16S rDNA (Jensen et al. 2000; Heyer et al. 2002; Degelmann et al. 2010). The *pmoA* gene has been used as a functional marker gene for the detection and classification of

methanotrophs both in culture and in the environment (Stralis-Pavese et al. 2011; Holmes et al. 1999). This is additionally beneficial because *pmoA* is not constrained by phylogeny, so environmental methanotrophs that are outside of known phylogenetic groups may be detected by the presence of the gene (Wise et al. 1999).

Since their first isolation in 1906, methanotrophs have been well studied in culture, but isolates are limited to a subset of the total diversity detected by culture-independent methods (McDonald et al. 2008; Kolb et al. 2005). Furthermore, Michaelis-Menton kinetics of CH_4 oxidation in many cultivated methanotrophs reported enzyme affinity values above 1μM, whereas those measured in soils that consume atmospheric methane were in the nanomolar range (Martin Bender & Ralf Conrad 1995; Martin Bender & Ralf Conrad 1992; M. Bender & Ralf Conrad 1993). This indicates that most bacteria in culture are not representative of those that consume methane at atmospheric levels in soil, termed "high-affinity" methanotrophs (Amaral et al. 1998). Culture-independent surveys of atmospheric oxidizing soil communities have attributed high affinity methane oxidation to several clades of methanotrophs, most of which have no cultured representatives. As reviewed by (Kolb 2009), Upland Soil Cluster α were detected in 73% of all soils investigated and *Methylocystis* species were detected in 44%.

Some high affinity methanotrophs have been successfully isolated, including *Methylocystis* and *Methylosinus* species, by enrichment at an ultra low methane concentration (<270 ppmv) which excludes low affinity oxidizers not capable of growth on methane at atmospheric levels (P. F. Dunfield et al. 2002; P. F. Dunfield et al. 1999). These isolates demonstrate MMO enzyme affinity values comparable to those seen in soils and are able to consume methane at atmospheric (1.8 ppmv) concentrations (P. F. Dunfield et al. 1999; Kravchenko et al. 2009; P. F. Dunfield & Ralf Conrad 2000). Other isolation attempts have

focused on capturing numerically dominant soil methanotrophs, using a dilution-extinction technique to enrich and isolate novel type I and type II strains (P. F. Dunfield & Ralf Conrad 2000; Button et al. 1993). Studies involving these high affinity isolates and strains have shown that methane oxidation rates are dependent on factors such as pH, temperature, and nitrogen source as well as methane availability (M. Bender & Ralf Conrad 1993; Martin Bender & Ralf Conrad 1992; Martin Bender & Ralf Conrad 1995; Knief & P. F. Dunfield 2005). Though much has been learned from available methanotroph isolates, those thought to be responsible for high affinity oxidation in soil, and thus able to affect atmospheric methane concentrations, are not yet well understood. Further efforts are needed to isolate these important environmental strains and to understand the part they play in the global methane cycle.

The Effect of Land Management

Several studies have investigated the impact of land management on the structure and function of soil methanotroph communities. At the Kellogg Biological Station Long Term Ecological Research Experiment (KBS-LTER), molecular surveys of methanotroph communities were conducted across a gradient of land management intensities (Suwanwaree & G. Philip Robertson 2005; G. P. Robertson 2000). In these soils, both methanotroph richness and methane consumption rates were 7-fold lower at sites where deciduous forest had long ago been converted to row crop agriculture, in comparison to mature secondary deciduous forest sites in the vicinity (Levine et al. 2011; Levine 2009). Furthermore, both methane flux and methanotroph richness recovered concurrently over a successional gradient as agricultural sites were abandoned (Figure 1-2). Studies at the KBS-LTER have further demonstrated that lands abandoned from agriculture are able to recover methanotroph diversity as well as methane flux. At these study sites, methanotroph richness and functioning return to that of a mature native forest when agriculture

has been abandoned for approximately 80 years (Levine et al. 2011; Levine 2009). This is consistent with other studies in a variety of environments, where recovery of methane consumption in soil is estimated to require 100 years (K. A. Smith et al. 2000).

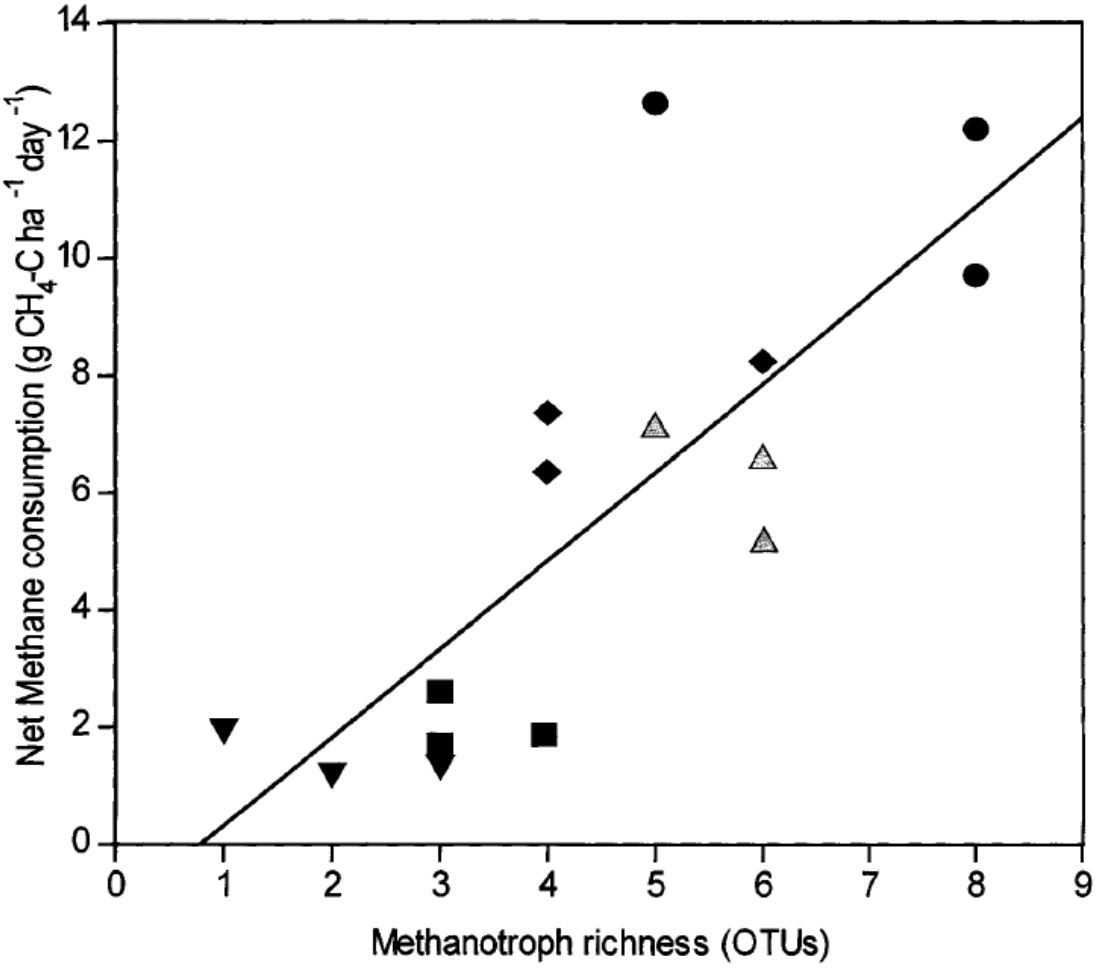

Figure 1-2. Methanotroph richness is correlated with methane consumption of soils at KBS-LTER, as demonstrated by the linear relation of summer methane consumption (June-August) to corresponding methanotroph richness based on tRFLP analysis of the *pmoA* gene (Linear regression, r^2=0.62, p<0.001). Symbols represent a gradient of land management practices, including historically tilled agricultural fields (▼), early successional plant communities on lands that had been abandoned from agriculture for 20 years (■), mid successional plant communities on historically tilled (▲) or never tilled (◆) and a late successional deciduous forest (●) (modified from Levine et al. 2011).

Reduced rates of soil methane oxidation upon land conversion to agriculture has been seen in temperate, tropical, and boreal forests as well as temperate and tropical grasslands,

tundra, and even desert ecosystems (reviewed in (K. A. Smith et al. 2000)). Several causes for this phenomenon have been proposed, though the precise mechanisms involved are not completely understood. One important factor for survival and growth of methanotrophs in soil is the availability of methane and oxygen through diffusion. These can be limited by soil factors such as bulk density and water-filled pore space, which are both affected by agriculture (Nauer & Schroth 2010; Bárcena et al. 2011; Jang et al. 2006). When soil is compacted, as with long term tillage practices, soil macropores are lost and gas diffusivity is reduced, thus limiting the ability of methanotrophs to access atmospheric methane and oxygen (M. Bender & Ralf Conrad 1993; Kumaresan et al. 2011).

Another important factor, particularly in intensive agricultural cropping systems, is the use of nitrogen-based fertilizer. The enzyme MMO, required for the initial oxidation step of methane to methanol, is evolutionarily related to ammonia monooxygenase (AMO), and they share some substrate affinity (Holmes et al. 1995). If nitrogen is introduced to soils in the form of ammonia (NH_4^+), it can act as a competitive inhibitor of methane oxidation (Mohanty et al. 2006). Long term nitrogen fertilization is known to alter the structure of methanotroph communities (Maxfield et al. 2011; Gulledge et al. 2004). Addition of NH_4^+ to forest and successional sites at KBS-LTER has been shown to cause an reduction in methane oxidation rates (Suwanwaree & G. Philip Robertson 2005). Similar experiments at Rothamsted Research Station (Hertfordshire, UK) demonstrated that the use of farmyard manure as fertilizer resulted in a shift of methanotroph community from type II to type I, though did not alter CH_4 consumption rates (Maxfield et al. 2011).

Application of Bacteria to Soil

Several types of soil bacteria, among them aerobic methanotrophs, have been studied with respect to bioremediation for many years. Due to the broad substrate specificity of MMO, methanotrophs are able to co-metabolize toxic compounds such as halogenated hydrocarbons (Trotsenko & Murrell 2008), and have for this reason been used in both bioreactors and in soil as a means of bioremediation or biotransformation (Chang & C.S. Criddle 1997; J. T. Wilson & B. H. Wilson 1985; Shukla et al. 2009; Oremland et al. 1994; Forrester et al. 2005). For the purpose of bodegradation of pollutants, such as halogenated hydrocarbons, environmental methanotrophs can be stimulated by pumping methane through soil columns, enhancing the existing population's ability to degrade pollutants through co-metabolism (Chang & C.S. Criddle 1997; Oremland et al. 1994).

The addition of beneficial bacterial inocula has also been used to either enhance nitrogen fixation, as in root nodules of leguminous plants, or as a biocontrol agents to competitively inhibit colonization by a virulent strain, as some *Pseudomonas* species are used (Aggarwal & Goyal 2008; Dyke & J I Prosser 2000; Date 2001). Inoculation of rhizosphere soil is often successful, and conditions for colonization are favorable. Other soils or surfaces, however may have a number of adverse biotic and abiotic factors that typically result in a decline of the population size and activity of inoculated cultures, termed soil microbiostasis (Ho & Ko 1985). This is in part due to the physiological traits that an inoculant may possess and in part due to soil edaphic factors such as pH, moisture, nutrient availability, and temperature (van Elsas et al. 1998; Evans et al. 1993).

For inoculation of bacteria into soil, it is often beneficial to include a carrier substrate that is added to the soil along with the inoculum, which can be applied either as a liquid culture

or as lyophilized cells (Date 2001; Dyke & J I Prosser 2000). To this end, a myriad of carrier substances have been investigated, from natural substances such as peat, clay, sterile soil, and biochar to numerous synthetic molecules that can be used to coat seeds before planting (Albareda et al. 2008; Dyke & J I Prosser 2000; Daza et al. 2000; Lehmann et al. 2011). Physical structure of the carrier may provide an increased surface area on which the inoculum may be absorbed which may also serve to increase diffusion, a feature particularly beneficial to methanotrophic inoculum (Heijnen et al. 1992; Date 2001). Alternatively, cells can be encapsulated or immobilized in a polymer such as agar, agarose, gelatin, or polyurethane which can form a protective barrier between microbes and external stressors, and can also provide a nutrient source for the inoculum (Albareda et al. 2008; John et al. 2011). Due to the wide variety of inocula, carrier substrates, and destination soils, it is likely that the optimal combination will need to be tailored to each situation to provide the optimal survival and functioning of microbial inocula.

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CHAPTER 2

INCREASING METHANE CONSUMPTION IN AGRICULTURAL SOILS BY USE OF BACTERIAL INOCULA

Abstract

Methane oxidation by bacteria in well drained soils is a key process in the regulation of atmospheric concentrations of this significant greenhouse gas. Both methane consumption and the diversity of the methanotroph community are significantly reduced by the conversion of native ecosystems to row-crop agriculture. In this study, we investigated the potential to mitigate the effects of agriculture on the global methane budget through the inoculation of agricultural soils with methanotrophic bacteria. We established methods for enrichment of methane-oxidizing bacteria and application of enrichment cultures to soils at the Kellogg Biological Station Long Term Ecological Research site (Michigan, USA). In laboratory scale soil microcosms, inoculation with methanotroph cultures resulted in a significant increase in the methane consumption rates of agricultural soils. Methanotroph enrichment cultures applied to soils in the field resulted in a 4-fold increase in total methane consumed during the 7-day pilot experiment, as compared to uninoculated plots. As the world's population becomes increasingly dependent on agriculture for food and fuel, it is important to consider the implications of changing land use for the global methane budget and consequently for climate change. Application of methanotrophic bacteria to soils presents a potentially viable near-term to help mitigate the effects of agriculture on global warming.

Introduction

Methane (CH_4) is a major driver of greenhouse warming, contributing 30% of the total net anthropogenic radiative forcing (Solomon et al. 2007; Ralf Conrad 1996). The mixing ratio of methane in the atmosphere has increased from the pre-industrial 0.715 ppmv to current levels

of 1.78 ppmv primarily due to anthropogenic sources such as agriculture (including rice paddies) and fossil fuel production (Denman et al. 2007; Anon 2010; Bousquet et al. 2006). The major biological sink for methane is through oxidation by aerobic soil microbes, termed methanotrophs, which account for up to 6% of total global methane consumption (Dörr, Glaser, & Kolb, 2009; Jay Singh, 2011). Aerobic methanotrophs are a phylogenetically diverse group of organisms comprised primarily of alpha- and gamma-proteobacteria, that are characterized by the ability to use CH_4 as a sole source of carbon and energy (Whittenbury et al. 1970; R. S. Hanson & T. E. Hanson 1996). They are traditionally divided into subgroups based on internal membrane structures and carbon assimilation pathways. Type I methanotrophs use the ribulose monophosphate (RuMP) pathway, type II use the serine pathway, and type X use both the serine and the RuMP pathways (R. S. Hanson & T. E. Hanson 1996).

The initial oxidation step is catalyzed by the enzyme methane monooxygenase (MMO) which has been found in two forms. The most common form is membrane-bound particulate methane monooxygenase (pMMO). It is present in all methanotroph species with the exception of the genus *Methylocella,* which contains the soluble form sMMO (Theisen et al. 2005). The active site of pMMO, encoded by the gene *pmoA,* is highly conserved among methanotrophs and the molecular phylogeny of *pmoA* mirrors that of 16S rDNA (Holmes et al. 1995; Kolb et al. 2003). Therefore PCR amplification and sequencing of the *pmoA* gene has proved a useful tool for the detection and phylogenetic analysis of methanotrophs from the environment (McDonald et al. 2008). The most commonly used primers (A189f/A682r) have been previously evaluated at the Kellogg Biological Station's Long Term Ecological Research study site in southwestern Michigan (KBS-LTER) (Levine 2009).

Most methanotroph isolates have been unable to consume methane at atmospheric levels in a laboratory setting, and do not demonstrate the high substrate affinity necessary to be responsible for oxidation of atmospheric methane (Whittenbury et al. 1970; Trotsenko & Murrell 2008). Not until recently have strains of high-affinity methanotrophs been isolated and atmospheric methane oxidation been demonstrated in culture (P. F. Dunfield et al. 1999; Kravchenko et al. 2009; Kolb 2009). These isolates do not represent the most active or numerically dominant methane-oxidizing bacteria in forest soils that are capable of high-affinity methane oxidation, as determined by stable isotope studies (Morris et al. 2002; Dumont et al. 2011; Kolb et al. 2005). It is hypothesized that an as-yet-uncultured subset of the methanotroph community is responsible for much of the consumption of atmospheric CH_4, whereas those methanotrophs with lower substrate affinity remain dormant between relatively brief periods of elevated CH_4 concentrations in soil (Knief et al. 2003; Murrell & Jetten 2009).

Methanotrophs are found in all environments with an air-methane interface, but it is only in well-drained soils that they produce a measurable net methane uptake. Soil methanotroph community is significantly affected by land management practices (Menyailo et al. 2008; Dörr et al. 2009). Several studies have evaluated the impact of land management actions such as deforestation, afforestation, and row-crop agriculture on the community structure and function of methane oxidizing bacteria in soil (Nazaries et al. 2011; Dörr et al. 2009; Menyailo et al. 2010). At the KBS-LTER, conversion of native deciduous forests to row-crop agriculture resulted in a 7-fold decrease in methane consumption and a corresponding decrease in methanotroph species richness (Levine et al. 2011; Levine 2009). This loss in ecosystem services in direct relation to an altered microbial community suggests an opportunity for remediation through the addition of methanotrophic bacteria to soil.

In the past, methanotrophs were often studied for their potential role in bioremediation because the wide substrate specificity of MMO enables them to co-metabolize halogenated hydrocarbon pollutants in soil or groundwater (J. T. Wilson & B. H. Wilson 1985; Shukla et al. 2009). It has also been proposed that methanotrophs could act as a biological tool to mitigate methane emissions from landfills or coal mines (Solomon et al. 2007; Huber-Humer et al. 2008; Nikiema et al. 2007). In this study, we evaluated the potential for increasing methane consumption by adding methane oxidizing bacteria to agricultural soil either in laboratory microcosm experiments or in field trials. Methanotrophs were enriched from forest soils, where the highest methanotroph richness and methane consumption rates have been observed. They were inoculated into nearby agricultural soils where the native methanotroph community structure and function had been diminished, thereby helping to recover the soil's original methane oxidation capability, one of the ecosystem services that was lost upon conversion to agriculture.

MATERIALS AND METHODS

Site description

This study was conducted on soils from the W. K. Kellogg Biological Station's Long Term Ecological Research Site (KBS LTER, Michigan State University, Hickory Corners, MI), sampled at both the main cropping experiment and the biodiversity gradient experiment. Further soil and site descriptions can be found at http://lter.kbs.msu.edu. From the main cropping system experiment both Treatment 1 (T1; a corn-soybean-wheat rotation with standard tillage and chemical inputs) and DF (never-tilled late-successional deciduous forest) sites were studied (Table 2-1). Microcosm and *in situ* bacteria application experiments were conducted on

biodiversity gradient treatment B10- a corn-soybean-wheat rotation with standard tillage and no chemical inputs during 2011, a year when corn was grown.

Abbreviation	KBS Experiment	Site Description
DF	LTER Main Site	Late successional deciduous forest, never tilled, no fertilization
T1	LTER Main Site	Corn-soybean-wheat rotation, conventional fertilization, chisel plowed
B10	Biodiversity Gradient	Corn-soybean-wheat rotation, no fertilization inputs, chisel plowed

Table 2-1. Description of the soils used in this study and their respective experiments at the Kellogg Biological Station.

Methanotroph enrichments

Cultures of methane oxidizing bacteria were enriched from DF and T1 soils under a variety of headspace CH_4 concentrations. Molecular surveys of methanotroph and total bacterial communities in these and other KBS soils are described by (Levine et al. 2011). Fresh soil cores (2.5 x 10 cm) were collected in triplicate, pooled, sieved through 4 mm mesh then transported to the laboratory on ice.

Primary enrichment cultures were obtained by adding 0.1 g of source soil to 10 ml of modified carbon-free MBL medium (CF-MBL, see Appendix B) in a 120 ml serum vial capped with a butyl rubber septum and incubated at 25°C, shaking at 200 rpm. Enrichments were obtained by initial incubations under atmospheres with 200 ppm, 10,000 ppm, or 100,000 ppm CH_4 (v/v in air). Headspace methane concentrations were regularly monitored using a Shimadzu GC-2014 Gas Chromatograph equipped with a flame ionization detector (GC-FID). Each culture was allowed to consume 90% of headspace methane two times before it was subcultured at a 1:20 dilution in fresh medium. To refresh headspace methane, vials were opened in a biosafety

cabinet for one hour then recapped. 250 ml samples of headspace were then withdrawn through a syringe and replaced with ultra high purity CH_4 (> 99.999% methane) (Airgas, Inc., PA, USA). Stable enrichment cultures were stored in aliquots at -80°C in CF-MBL with 20% glycerol for subsequent use.

After enrichments had been passaged several times, methane concentrations were increased to 1% or 10% (v/v in air) to facilitate faster and denser growth. Each enrichment culture was later screened for growth at low methane by incubating 10 ml of culture under a 50 ppmv CH_4 headspace in a 120 ml serum vial. Cultures that were able to grow and consume methane at this low level were considered to have a high affinity methane monooxygenase.

Enrichment Name	Initial CH_4 (% v/v)	Source Soil	Collection Date	Initial N Source	Growth at 50 ppm CH_4
E1DF-10	10	DF	28-Feb-10	NH_4Cl	+
E2T1-10	10	T1	28-Feb-10	NH_4Cl	-
E3DF-1	1	DF	12-Dec-10	KNO_3	+
E4DF-1	1	DF	27-Mar-10	KNO_3	-
E5DF-10	10	DF	27-Mar-10	KNO_3	-
E6DF-1	1	DF	27-May-10	KNO_3	+
E7DF-1	1	DF	13-Apr-11	KNO_3	-
E8DF-1	1	DF	13-Apr-11	KNO_3	-
E9DF-0.02	0.02	DF	13-Apr-11	KNO_3	+
E10DF-0.02	0.02	DF	13-Apr-11	KNO_3	+
E11T1-1	1	T1	29-Jun-10	KNO_3	+

Table 2-2. Summary of mixed methanotrophic enrichments obtained from KBS-LTER soils. DF denotes late successional deciduous forest, and T1 denotes row crop agricultural management with corn/soybean/wheat rotation and standard chemical inputs. (Concentrations of KNO_3 and NH_4Cl were 10 mM)

Scale-up enrichments (1-10 L volumes) were obtained by growing batch cultures under an atmosphere of 10-20% CH_4 (v/v in air) that was bubbled through CF-MBL medium at approximately 0.5 L h^{-1} with constant mixing. Cultures were incubated at 25° for 4-6 days until turbid. Prior to soil inoculation cultures were concentrated by centrifugation for 20 min at 5,000 x g then resuspended in fresh CF-MBL to the desired concentration.

Molecular Characterization

Molecular surveys of each enrichment culture were performed through *pmoA* clone library analysis as described in (Levine et al. 2011) with some modifications. DNA was extracted using the UltraClean Microbial DNA Isolation kit (MoBio, Carlsbad, CA, USA), following manufacturer instructions. Fragments of *pmoA* were amplified using primers A189 (5'-GGNG ACTGGGACTTCTGG-3') and A682 (5'-GAASGCNGAGAAGAASGC-3') (Holmes et al. 1995) in three PCR reactions as described in (Levine et al. 2011). These were then pooled to minimize amplification bias prior to purification and digestion with restriction endonuclease PflF1, a six-base cutter, with restriction sites in *amoA* but not in *pmoA*. Digestion products were separated by gel electrophoresis and the 500 bp band containing the un-cut *pmoA* sequences was retrieved using the Wizard® Gel Extraction kit (Promega Corp., WI, USA). Clone libraries of *pmoA* amplicons were created using TOPO®-TA pCR4 cloning kit (Invitrogen Corp., CA, USA) according to manufacturer's instructions. Clones were selected and inserts were amplified by PCR amplification at M13 primer sites, screened for size by gel electrophoresis, then selected clones were sequenced at Michigan State University on an ABI 3730 Genetic Analyzer (Applied Biosystems Inc., CA, USA). Sequences were aligned and trimmed using BioEdit (T. A. Hall 1999), and molecular phylogeny was analyzed using ARB (W. Ludwig et al. 2004).

Isolation and identification of heterotrophs

Subsamples of methane-consuming enrichment cultures were spread onto CF-MBL plates (see Appendix B for recipe) for isolation and identification of cultivable members. Culture aliquots were removed from actively-growing enrichments, serially diluted to 10^{-5}- 10^{-8} in fresh CF-MBL, then 100 μl was spread onto CF-MBL plates and incubated under 10% CH_4 atmosphere for 3-7 days. Colonies were isolated through repeated streak-plates, then colony PCR was performed using 16S rDNA primer set 8F, 1492R (Lane 1991; Weisburg et al. 1991). Sequences were generated at Michigan State University's Research Technology Support Facility, as described above, then submitted to the Ribosomal Database Project Classifier (Q. Wang et al. 2007) for phylogenetic identification.

Soil Microcosms

All microcosm experiments were conducted using soil cores collected from Treatment B10 plots. For initial microcosm experiments, two soil cores of 2.5 cm diameter were collected from the top 10 cm of soil at three replicate sites then pooled, homogenized through a 4 mm mesh sieve and dried at 55°C. A 10 g sample of this soil was added to 120 ml serum vials and re-wetted with 2 ml of either methanotroph culture or sterile liquid medium, resulting in 20% soil moisture. Vials were incubated at 25°C in gastight boxes through which humidified air was flushed at a rate of 1 L h^{-1}. For consumption measurements, vials were closed and headspace methane concentrations were measured as described below.

Intact soil microcosm experiments were performed to assess the behavior of methanotroph enrichment cultures when applied to minimally-disturbed native soil cores (6.5 x 5 cm). Cores were collected from three replicate plots of corn/soy/wheat (KBS Biodiversity plots

117, 209, and 320) and inserted into wide-mouth 1 pint glass Mason jars which were incubated in gastight boxes through which 1 L h^{-1} humidified air was flushed. Cores were allowed to equilibrate for 2 days at 25°C before inoculation. For headspace sampling, lids were fitted with untreated red rubber septa from 5 ml blood collection tubes (Becton-Dickinson, New Jersey, USA). Lids were closed at time zero and headspace gas samples were taken every 90 min for 4.5 hours. Linear regressions were calculated to determine methane consumption rates.

Field Scale Application

Field trials of methanotroph application were conducted in triplicate on KBS Biodiversity Gradient corn/soy/wheat rotation plots 117, 209, and 320. Static flux chambers (30 cm plastic buckets cut to 10 cm height) were installed in pairs, 2 m apart in each plot. Soil was allowed to equilibrate in the laboratory for 2 days before inoculation. Inocula consisted of cultures E1DF-10, E3DF-1, E6DF-1, and E9DF-0.02 (Table 2-2) pooled in equal volumes. Each had been grown and concentrated 20x as described above, then resuspended in CF-MBL. In each chamber 200 ml of either mixed methanotroph inoculum or sterile growth medium was added to the soil surface by spraying.

Methanotroph inocula were allowed to soak into the soil surface for one hour. Lids were then closed and headspace samples were collected at 0, 30, 60, and 120 min. Gas samples were collected in 3 ml vacutainer vials (BD, NJ, USA) that had previously been flushed and pressurized with ultra high purity nitrogen (>99.999%, Airgas Inc., PA, USA). At each time point, vials were flushed with 5 ml of the gas sample then pressurized with an additional 5 ml of the sample. For gas analysis, 200 µl subsamples were removed and analyzed in duplicate by GC-FID, as described below. Flux chambers remained in the ground and open for the duration of the experiment, and were only closed for the two hours during which gas flux was measured.

Methane Analysis

Methane concentrations were determined by injection of duplicate 200 µl samples into a Shimadzu 2012 Gas Chromatograph (Shimadzu Scientific Instruments, Inc., MD) equipped with a flame ionization detector (GC-FID) set to 100°C and Porapak N 80/100, mesh 3 mm I.D. x 1m and Porapak Q 80/100 mesh, 3 mm x 1 m I.D. packed columns (Sigma Aldrich Inc., MO, USA). Oven temperature was set to 50°C, and injector temperature to 60°C. GC Solutions software (Shimadzu, Inc.) was used to calculate peak areas and methane concentrations. Methane standards were prepared in the laboratory by diluting UHP- CH_4 in a balance of UHP-N_2, then following sample injection procedures to create standard curves with $r^2 > 0.99$. Gas standards were made and stored alongside all field samples to decrease any storage effect, and curves were re-calibrated with each sample run.

RESULTS

Methane-oxidizing behavior of enrichment cultures

Methanotroph enrichments were obtained by cultivation under methane headspace ratios much lower than those that are conventionally used, such as those described for methanotroph isolation by Whittenbury et al. (1970). From the multiple enrichment strategies explored (Table 2-2) eight methanotroph enrichment cultures were obtained: E1DF-10, E2T1-10, E3DF-1, E4DF-1, E6DF-1, E9DF-0.02, E10DF-0.02, and E11T1-1. Each of these cultures demonstrated the capacity to oxidize methane when grown in liquid culture, at both their respective enrichment CH_4 concentrations and at the higher (1-10% CH_4) levels used to promote robust growth. Additionally, each culture was able to recover from storage at -80°C in 20% glycerol, and continued to oxidize methane (not shown).

Each culture was screened for high-affinity methane oxidation capability, defined as the ability to consume methane at concentrations that are below 200 ppmv, by monitoring for growth and consumption of ultra-low methane concentrations in CF-MBL medium. Enrichments were grown in duplicate under 50 ppmv headspace methane and monitored for growth and methane consumption over ten days of culture. Six of the eight enrichments tested demonstrated significant methane consumption ($p < 0.05$), as determined by simple linear regression (Figure. 2-1). The four enrichments designated with dashed lines were selected for later microcosm and field inoculation experiments. These were chosen from among the high affinity cultures to include the largest diversity of *pmoA* sequences available (see below).

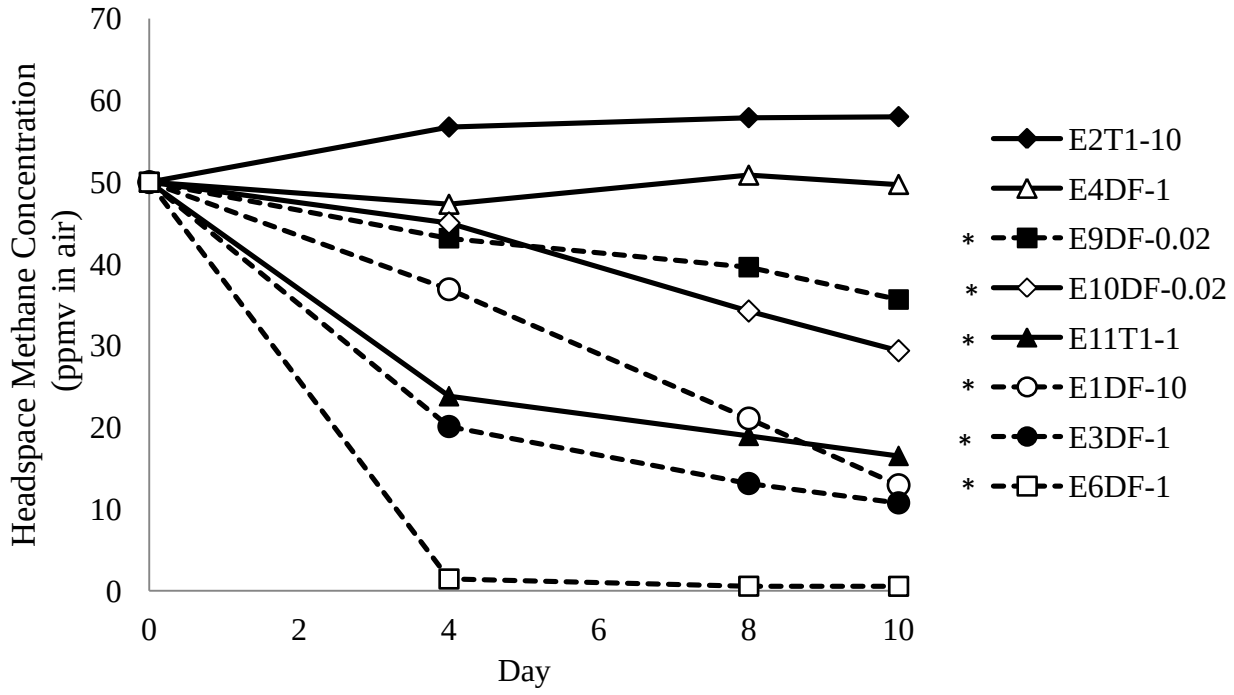


Figure 2-1. Mixed methanotroph enrichments were screened for consumption of methane at 50 ppmv in liquid medium CF-MBL. Data provided are representative measurements for each of the eight cultures screened. Dashed lines indicate the four cultures that were used in later microcosm and field application experiments. (Cultures whose slope differs significantly from zero are marked with a *, based on simple linear regression. The slope of E6DF-1 was calculated based on the linear segment, day 0-4.)

Phylogenetic Analysis of Enrichments

Methanotroph enrichment cultures were characterized through sequencing *pmoA* clone libraries. A phylogenetic tree comprising clones from enrichment sequences, as well as those of previously collected environmental sequences from KBS-LTER and published reference *pmoA*s, was created in ARB using a Neighbor-joining method. All culture conditions yielded *pmoA*s that cluster closely with *Methylocystis* and *Methylosinus* spp. Previous molecular surveys of KBS-LTER sites DF and T1 contained no detectable sequences of this lineage, despite using comparable methods for sample collection and processing, PCR amplification and sequencing (55, 56).

Each enrichment culture contained one or more *pmoA* sequences closely related to *Methylocystis* sp. or *Methylosinus* sp., the methanotrophs most often isolated in similar cultivation attempts (Wise et al. 1999; Whittenbury et al. 1970; Svenning et al. 2003; P. F. Dunfield et al. 2002; P. F. Dunfield et al. 1999; Kravchenko et al. 2009). Sequences related to *pmoA2*, which encodes a constitutively expressed high affinity pMMO (Baani & Liesack 2008; P. F. Dunfield et al. 2002), were also found in enrichment cultures except E1DF-10 and E4DF-1. Enrichments E2T1-10, E3DF-1, E9DF-0.02, E10DF-0.02 contain *pmoA2* sequences that form a distinct clade, separate from those that have been previously sequenced.

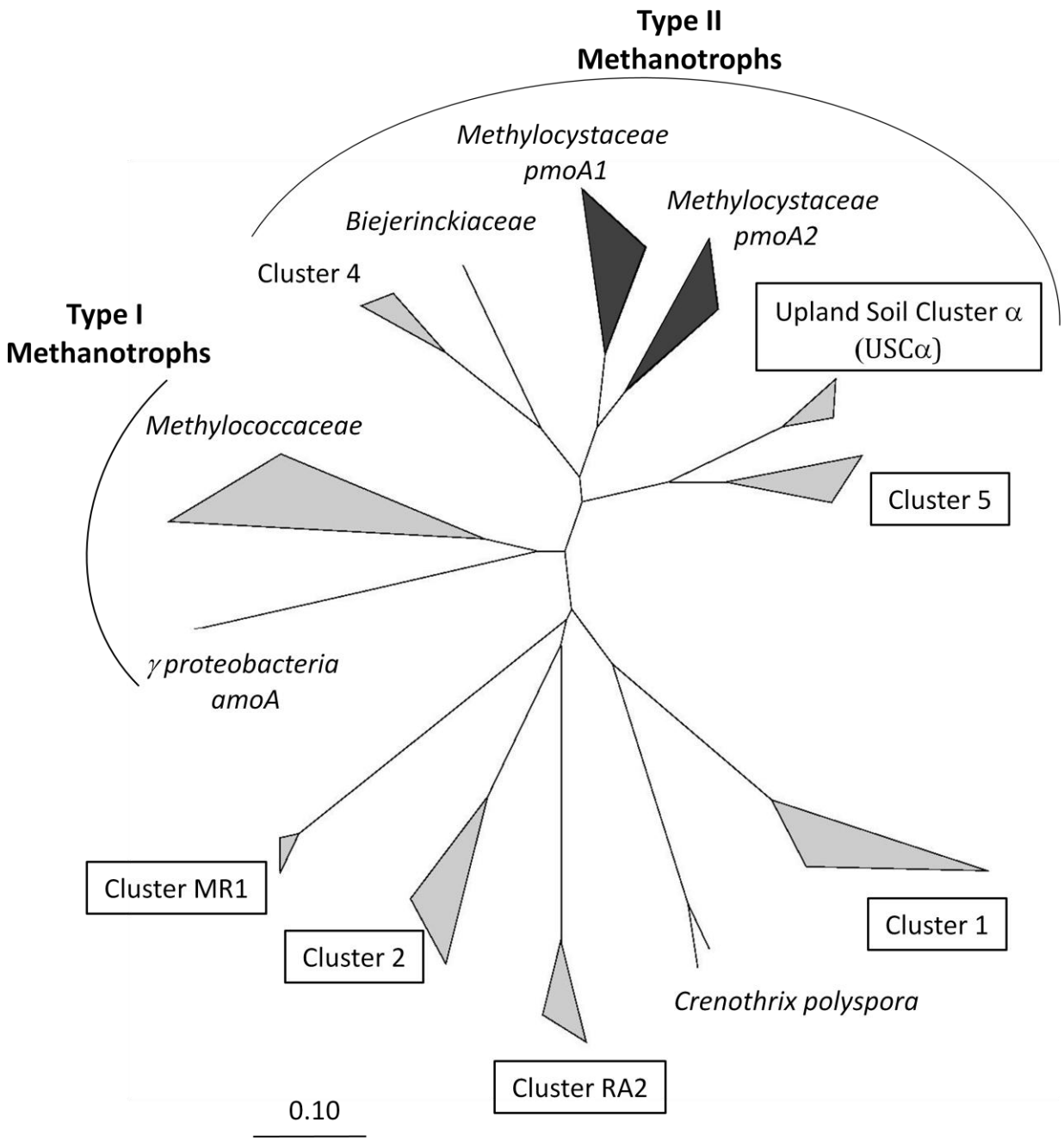


Figure 2-2. Phylogenetic tree of most commonly detected methanotrophs in upland soils, based on partial sequences (164 amino acids) of PmoA using the Neighbor –joining method as implemented in ARB (W. Ludwig et al. 2004). Darkened groups are those that were obtained in enrichment cultures in this study. Groups whose names are outlined by boxes represent those that were detected in previous molecular surveys of KBS-LTER soil (Levine et al. 2011; Levine 2009)

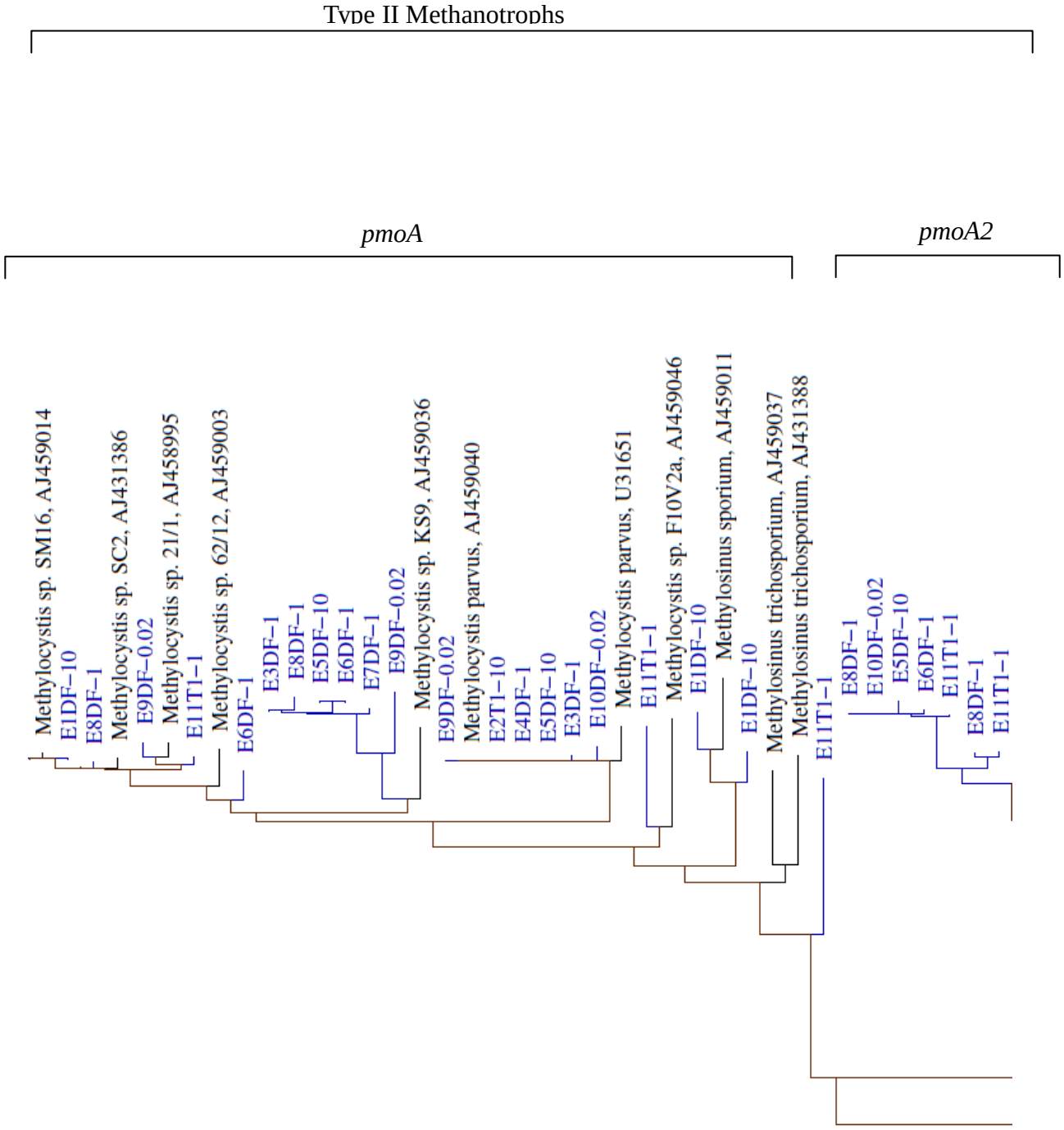
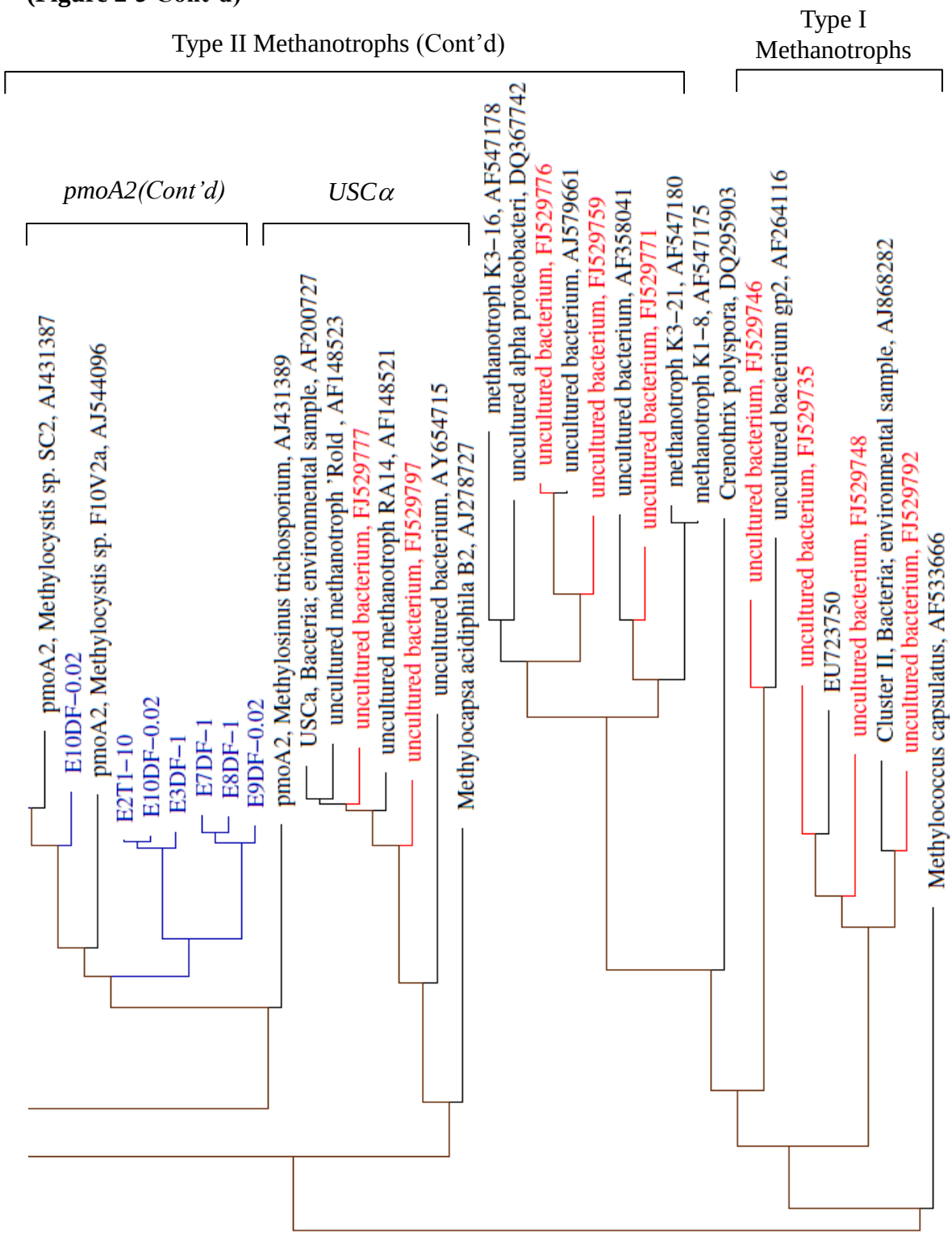


Figure 2-3. Phylogenetic tree of representative partial *pmoA* sequences (495 base pairs) that were collected from methanotroph enrichment cultures in this study (blue), in previous surveys of KBS-LTER sites (red) (Levine et al. 2011; Levine 2009) and selected reference sequences obtained from GenBank (black) followed by their respective accession numbers. The scale bar corresponds to 10 substitutions per 100 nucleotide sites (evolutionary distance).

(Figure 2-3 Cont'd)



Soil Microcosm Experiments

Initial microcosm experiments were conducted by inoculating sieved, dried agricultural soil from KBS Biodiversity Gradient plot B10 with enrichment culture E3DF-1. When this soil was amended with increasing concentrations of inoculum, methane consumption increased in a dose-dependent manner (Figure 2-4). On the first day post-inoculation, mean methane flux rates were not detectable for soil to which only sterile medium was added. When enrichment E3DF-1 was concentrated to 4x, 16x, and 64x culture conditions, initial consumption rates were -10.86 ± 0.67, -24.30 ± 3.07, and -57.00 ± 3.69 ng CH_4 gdw soil $^{-1}d^{-1}$, respectively (based on linear regression of headspace methane concentrations).

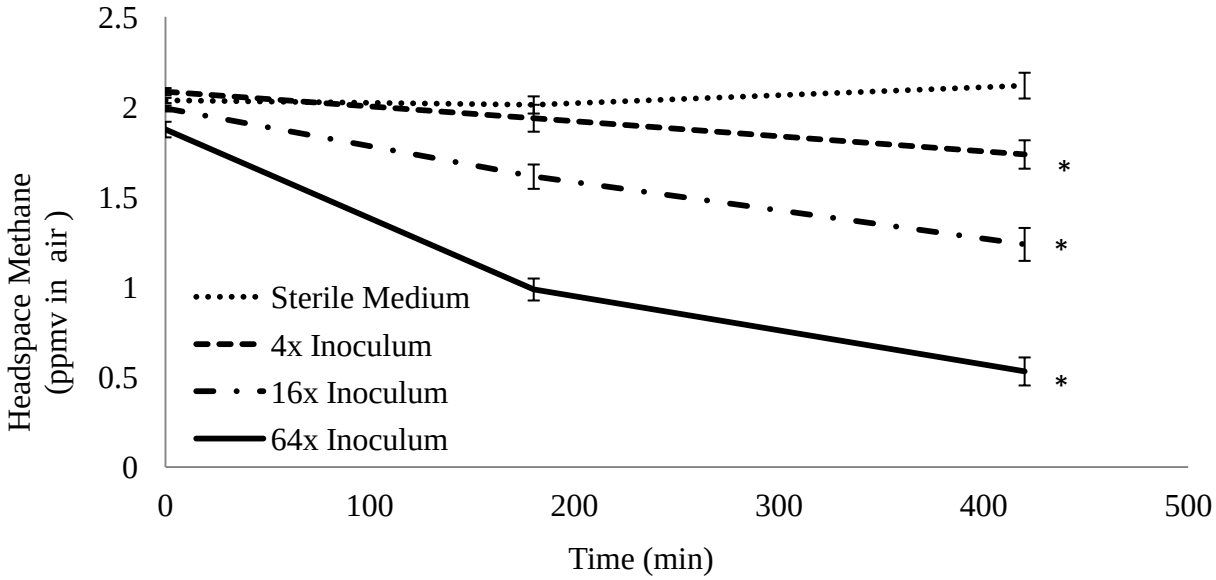


Figure 2-4 Methanotroph enrichment E3DF remained active after addition to sieved dried agricultural soil microcosm (20% final soil moisture). Initial methane consumption occurs in a dose-dependent manner, increasing with the concentration of cells added to soil. Each point represents the mean of 3 replicates, ± standard error. (Significant methane consumption based on simple linear regression is indicated with a *, where $p > 0.05$)

When inoculated with mixed methanotroph enrichments, intact soil microcosms of cores collected from site B10 showed a significant increase in methane flux. E1DF-10, E3DF-1, E6DF-1, and E9DF-0.02 cultures, when pooled in equal ratios and concentrated to 20x, caused a

significant increase ($p < 0.005$, based on paired t-tests within days) in consumption compared to microcosms treated with sterile culture medium CF-MBL (Figure 2-5). The increase of approximately 15 ng CH_4 cm^{-2} soil day^{-1} was observed for the first three days, after which it dropped by 50%. In total, a statistically significant increase in CH_4 consumption was measured for 9 days post-inoculation. Over the 18-day duration of the experiment, a significant increase of 8.408 ± 1.939 ng CH_4 cm^{-2}soil day^{-1} was observed for inoculated microcosms ($p = 0.005$, based on a repeated-measures ANOVA, accounting for day and treatment effects on variance).

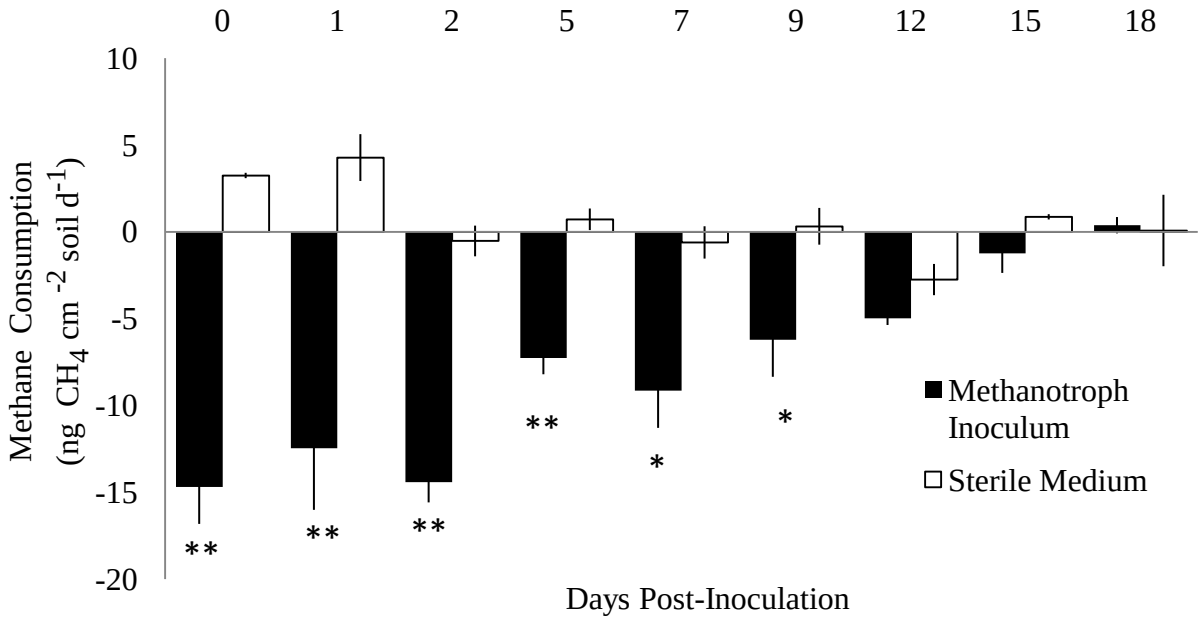


Figure 2-5. Intact soil microcosms inoculated with mixed methanotroph enrichment cultures exhibit increased methane consumption rates for approximately 9 days post-inoculation. Values are means ± standard error of three biological replicates. Methanotroph inoculum consists of enrichments E1DF-10, E3DF-1, E6DF-1, and E9DF-0.02 mixed in equal volumes and concentrated to 20x culture conditions. (** $p<0.005$, * $p<0.05$)

On day 7 of this experiment, half of the technical replicates were supplemented with nutrients to see if the highest methane flux could be recovered after the 2-fold decrease seen after 5 days. Nutrient amendment was added in the form of 2x concentrated liquid MBL medium

sprayed onto the soil surface. However, it was insufficient to recover the loss in methane consumption measured after day 5 (Figure 2-6). There was no significant change in methane flux between amended and unamended microcosms whether or not methanotroph inoculum was present, as determined by a repeated-measures ANOVA (p=0.77).

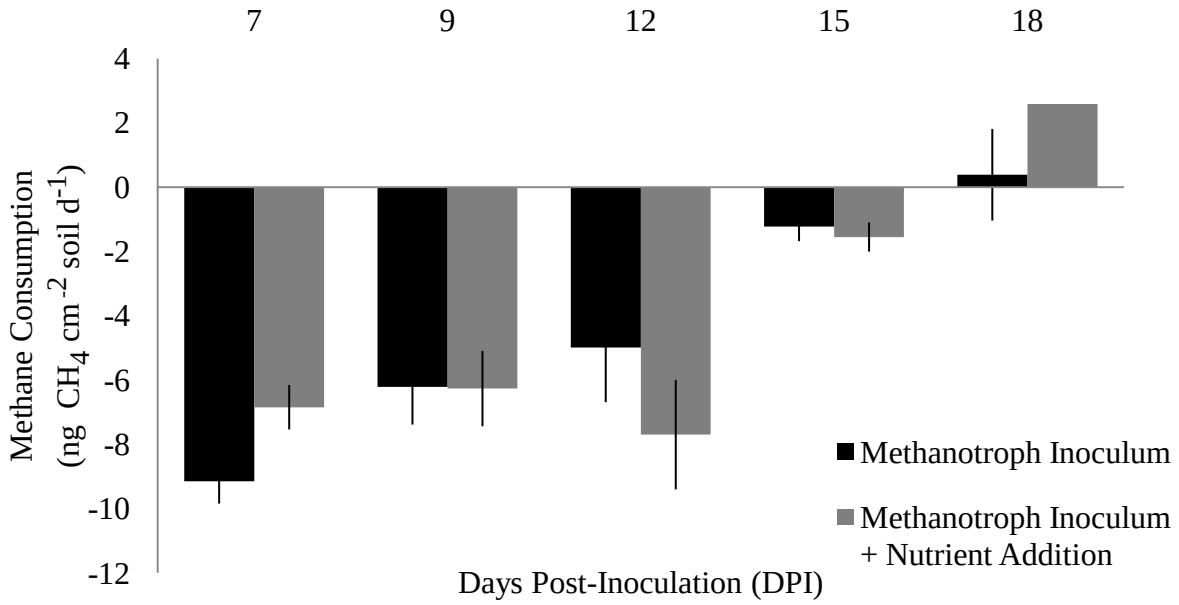


Figure 2-6. The addition of nutrients to intact soil microcosms in the form of 2 ml of 2x concentrated liquid MBL culture medium (Nichols 1973) was insufficient to restore methane consumption when provided on day 7, after a 2 fold decrease from initial rates had been observed (Figure 2-4). Values are means ± standard error of three biological replicates. Methanotroph inoculum consisted of enrichments E1DF-10, E3DF-1, E6DF-1, and E9DF-0.02 mixed in equal volumes and concentrated to 20x culture conditions.

Field scale inoculation

Preliminary field trials were conducted to mirror the conditions tested in laboratory microcosm studies, using the same mixed methanotroph enrichment cultures E1DF-10, E3DF-1, E6DF-1, and E9DF-0.02 that had been concentrated 20x. When compared over the 7-day experiment, methanotroph treated sites consumed significantly more methane than uninoculated sites (p =0.004). Treated soils consumed a mean of13.503 ±1.966 ng CH_4 cm^{-2} more than untreated soils

over the 7 days measured (based on repeated-measures ANOVA, accounting for variance due to day and plot).

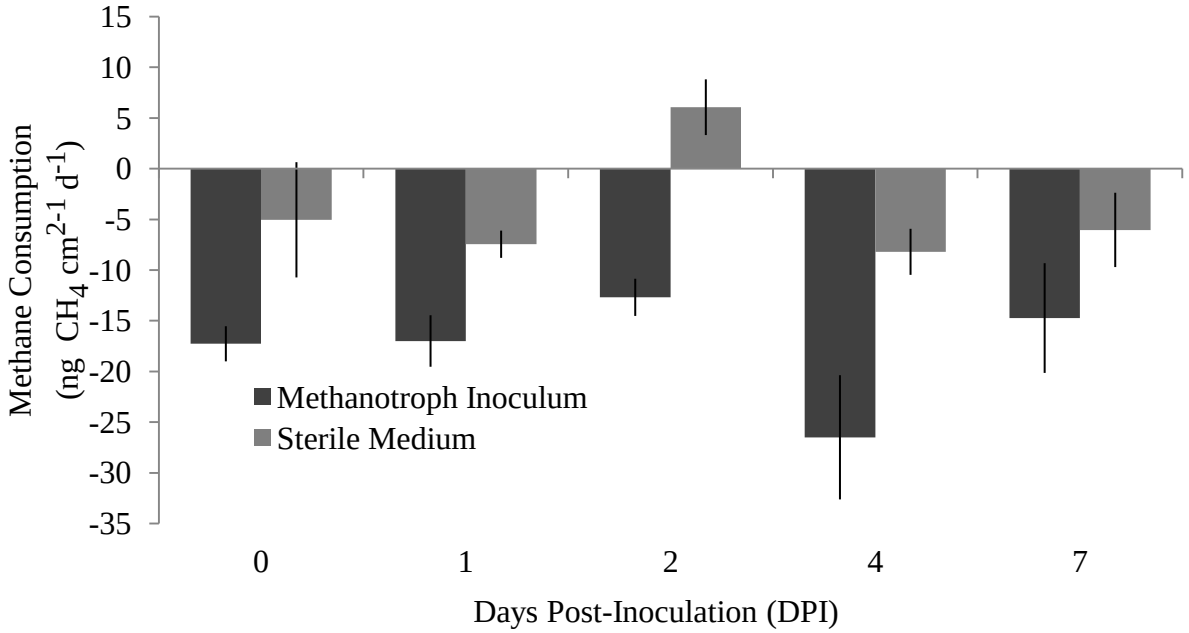


Figure 2-7. Preliminary field-scale inoculation of soils in a corn/soybean/wheat rotation at the KBS Biodiversity Gradient. Inoculum consists of mixed methanotroph enrichments E1DF-10, E3DF-1, E6DF-1, and E9DF-0.02 combined in equal volumes, concentrated by centrifugation to 20x culture conditions. Values represent mean ± standard error of three replicate plots (plots 117, 209, and 320).

DISCUSSION

Methanotroph Enrichments

Methane-oxidizing enrichment cultures were grown under a variety of initial methane concentrations using either agricultural or forest soil inocula. The *pmoA* sequences obtained from these cultures represented a different subset of methanotrophs than those of culture-independent surveys of the same soils (Figure 2-3). Previous molecular surveys indicated that KBS-LTER soils contain methanotrophs from clades that remain largely uncultured (Figure 2-2) (Levine 2009; Levine et al. 2011). The present study showed that methanotrophic enrichments from the same soils resulted in communities that are heavily skewed toward *Methylocystis* and

Methylosinus species, suggesting that while they were not found in molecular surveys, these taxa are present at these sites. They have been detected in 50% (*Methylocystis)* and 20% (*Methylosinus)* of analyzed methane-consuming forest soils (Kolb 2009). It may be that in KBS soils, these two methanotroph genotypes are present below detectable limits when PCR-based targeted metagenomics techniques are used.

The skew toward *Methylocystis* and *Methylosinus* also highlights cultivation bias and shows that predominant cultivation techniques are insufficient to capture the available methanotroph diversity (Vorob'ev & S. N. Dedysh 2008; Cébron et al. 2007). The most numerically dominant methanotrophs in soils that are capable of consuming atmospheric levels of methane have largely eluded cultivation attempts (reviewed by(Kolb 2009)). Upland Soil Cluster α is detected in 80% of all forest soils that consume atmospheric methane and is thought to be one of the key methanotrophs responsible for net CH_4 uptake in soils. This clade was notably absent from enrichment cultures though it was detected previously in soil from KBS-LTER (Figure 2-2) via targeted metagenomics surveys (Levine et al. 2011). One possible cause for the cultivation bias toward type II methanotrophs is the ratio of available oxygen to methane. When oxygen is limiting but methane is abundant, methanotroph communities are skewed toward type II; environments with high oxygen and low methane levels favor type I (Amaral et al. 1995; Bussmann et al. 2006). Nutrient concentrations may also play a role in the enrichment bias seen here and in other studies. Type II methanotrophs were numerically dominant in dilute minimal medium but only type I were enriched with undiluted mineral salts medium (Wise et al. 1999). Future attempts to obtain enrichments that better represent the microbial assemblage of source soils would benefit from a dilution-extinction cultivation technique, which may increase

the likelihood of obtaining methanotrophs that are abundant in KBS soils (Pfluger et al. 2011; D. W. Graham et al. 1993; McDonald et al. 1996).

Mixed methanotroph cultures–as opposed to a single environmental isolate–were chosen for the soil inoculum for several reasons. First, it was not initially apparent which of the enriched methanotroph strains would be best able to oxidize atmospheric methane when added to agricultural soils. As demonstrated in plant communities, increased species richness has been linked to both productivity and stability of the community (Isbell et al. 2011; D. Tilman & Pacala 1993). Second, mixed enrichment cultures also contained some heterotrophs that coexist and were subcultured along with methane oxidizing bacteria. These include *Pseudomonas, Acinetobacter,* and *Variovorax* species, among others (see Table A-1). *Variovorax* specifically has been demonstrated to increase methane oxidation rates in co-culture with high affinity methanotrophs, though the mechanism is unknown, as *Variovorax* is unable to oxidize methane (P. F. Dunfield et al. 1999). It may be that these heterotrophs are able to increase methane consumption in co-culture by removing inhibitory compounds such as reactive oxygen species. Therefore, we chose to use mixed-species enrichment cultures for soil inoculation to both increase the breadth of methanotroph diversity, and to cultivate any mutualistic relationships. It is possible that the isolation of high affinity methanotrophs is particularly challenging due to similar mutualistic relationships between methanotrophs and other soil heterotrophs (van der Ha et al. 2011).

Soil Microcosms

Preliminary microcosm experiments demonstrated the ability for methane oxidizing enrichment cultures to consume CH_4 when added to agricultural soil (Figure 2-4). The dose dependent increase in methane consumption with added methanotroph enrichment lends further

evidence to support a biological basis for the additional CH_4 consumed. Interestingly, though the methanotroph culture was concentrated in 4-fold intervals, methane consumption rates only increased 2.3- fold from 4x to 16x inoculum, and 2.1- fold from 16x to 64x inoculum concentrations. This discrepancy could indicate a potential upper limit for the addition of methanotroph culture to soil. Most likely, the diminishing returns are due to a resource limitation, which could be addressed in future studies by providing a source of nutrients or increasing CH_4 diffusion through the use of a substrate carrier with a greater surface area. To some degree, the nutrient-limitation hypothesis was tested (Figure 2-6), by the addition of concentrated MBL medium to intact soil core microcosms. Though no significant increase in CH_4 consumption rates was seen, nutrient addition may have occurred too late to restore methane oxidation activity, or been deficient in the necessary nutrient that had been depleted in the soil.

Intact Soil Microcosms

Addition of mixed methanotroph enrichment to intact agricultural soil microcosms provided the opportunity to assay for potential interactions between methanotrophs and biotic or other edaphic factors that may inhibit methane oxidation *in situ*. Because soil cores were minimally perturbed upon collection, then stored at 25°C for only two days prior to inoculation, native populations of microbes or micro-fauna should have remained intact and viable within the microcosms. The significant increase in CH_4 consumption following their addition indicates methanotrophs are viable and active for 9 days in the presence of soil biota, and thus are not likely victims of predation or phage during this time period.

After day 9, microcosms treated with methanotroph cultures no longer presented a significant increase in CH_4 consumption (Figure 2-5). This could be due to a phenomenon termed soil microbiostasis, which is described as the inhibition of growth or survival of bacteria introduced to soil (reviewed in (Ho & Ko 1985). This effect has been attributed primarily to a nutrient limitation in soils where non-native bacteria are added, as well as several other biotic and abiotic factors (J. van Veen et al. 1997). In laboratory microcosm experiments factors such as temperature, UV damage, and soil moisture were controlled to limit their negative effects on methanotrophic inocula. However, other factors such as diffusion of methane and nutrient or niche availability are targets for optimization in future experiments.

Preliminary field trial

Field-scale soil inoculations, unlike those in microcosms, did not show a significant increase in daily CH_4 consumption as a result of added methanotroph enrichments (Figure 2-6). This was probably in part due to the noise inherent to sample collection and measurement. Due to the increased scale of field inoculation, replication was reduced to only three treated and three untreated plots. Transfer of gas samples from field flux chambers to the lab for sampling by GC-FID results in a much greater degree of variability than the microcosm sampling scheme Although methanotroph inoculation did not yield statistically significant rates when examined on a per-day basis, total methane consumed over the 7 day experiment was significantly greater in treated plots. It also appears that there may be an increase in methane consumption simply due to the addition of sterile medium to these soils, resulting in higher levels of background CH_4 consumption. Future experiments should incorporate an unaltered control, to account for the effects of nutrient addition without methanotrophs present. Although this initial field experiment

showed mixed results, the encouraging results from the lab experiments suggest further research is justified, and that the use of methanotroph cultures to enhance methane consumption in agricultural soils remains a potentially viable bioengineering option to help mitigate global climate change.

APPENDICES

APPENDIX A

Heterotropic Bacteria Isolated from Methanotroph Enrichment Cultures

Enrichment Culture	Isolated Heterotroph (RDP Classification)
E1DF-10+	*Pseudomonas, Pimelobacter, Shinella, Variovorax*
E2DF-10	*Pseudomonas, Variovorax, Pimelobacter, Thermosulfidibacter*
E3DF-1+	*Pseudomonas, Angulomicrobium, Alkanindiges, Pseudomonas, Acinetobacter, Novosphingobium*
E4DF-1	*Pseudomonas*
E5DF-10	*Variovorax, Bosea*
E6DF-1+	*Acinetobacter, Azomonas, Sphingobium, Pseudomonas*
E7DF-1	*Pseudomonas*
E8DF-1	*Pseudomonas, Rhizobium*
E9DF-0.02+	*Acinetobacter*
E10DF-0.02	*Pseudomonas*
E11T1-1	*Pseudomonas, Variovorax, Stenotrophomonas*

Table A-1. Heterotrophic bacteria isolated from methanotroph enrichment cultures through repeated streak plating. 16S rDNA was amplified by colony PCR using the 8F/1492R primer set, sequenced, and analyzed using the Ribosomal Database Project's RDP Classifier. Enrichment cultures that were combined for use in microcosms and field applications are indicated by a +.

APPENDIX B

Supplementary Methods

MEDIA RECIPES

Carbon-Free MBL Medium

Component	Final Concentration
Na_2HPO_4	1 mM
KNO_3	10 mM
Na_2SO_4	1 mM
100X Freshwater Base	1X
1000X Trace Elements	1X
100 X Phosphate Buffer (pH 5.2)	1X

MBL Medium

Component	Final Concentration
Na_2HPO_4	1 mM
KNO_3	10 mM
Na_2SO_4	1 mM
100X Freshwater Base	1X
1000X Trace Elements	1X
100 X Phosphate Buffer (pH 5.2)	1X

Filter Sterilized Ingredients	
1000X Vitamin Solution	1X
Cyanocobalamin	100 mg

STOCK SOLUTIONS

100X Phosphate Buffer pH 5.2 (per 100 ml)	
Component	100X Stock Concentration (g)
NaH_2PO_4	11.3
Na_2HPO_4	4.7

100X Freshwater Base

Component	Final Concentration (mM)
NaCl	17.1
$MgCl_2$	1.97
$CaCl_2$	0.15
KCl	6.71

1000 X Trace Elements Solution

Component	Final Concentration (μM)
20 mM HCL	20
$FeSO_4$	7.5
H_3BO_3	0.48
$MnCl_2$	0.5
$CoCl_2$	6.8
$NiCl_2$	1
$CuCl_2$	12 nM
$ZnSO_4$	0.5
Na_2MoO_4	0.15
$NaVO_3$	2
Na_2WO_4	75 nM
Na_2SeO_3	23 nM

1000X Vitamin Solution

Component	Final Concentration (μg/ml)
Riboflavin	1
Biotin	0.3
Thiamine HCl	1
L-Ascorbic acid	1
D-Ca-Pantothenate	1
Folic Acid	1
Nicotinic Acid	1
4-Aminobenzoic Acid	1
Pyrodixine	1
Lipoic Acid	1
NAD	1
Thiamine Pyrophosphate	1

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CHAPTER 3

Future Directions

Methanotrophic enrichment cultures used in this study, while demonstrating a promising capability to oxidize atmospheric methane in lab culture as well as preliminary field application, are not yet fully described. Obtaining isolates of the individual members in these mixed enrichment cultures would allow for the characterization of each strain present. Methanotrophic isolates would provide flexibility in creating mock communities that could be adapted to unique soil types to provide for maximum CH_4 consumption and 50noculums longevity in soils. Isolates of the heterotrophs present in co-culture would also be highly beneficial due to the potential mutualistic relationship between methanotrophs and some heterotrophs. As in the case of *Variovorax paradoxus,* further study is needed to determine the mechanisms involved in the effects of co-cultured heterotrophs on methane oxidation (P. F. Dunfield et al. 2002)

The current breadth of cultured or isolated methanotrophic bacteria is limited to a subset of the diversity that evidently exists in soils. Many of the high-affinity methanotrophs thought to oxidize atmospheric methane have thus far eluded isolation attempts (Kolb 2009; Kolb et al. 2005). Such a discrepancy is also true in this study, where most of the surveyed methanotroph types were not present in enrichment cultures (Figures 2-1 & 2-2) (Levine et al. 2011; Levine 2009). Further work toward enrichment and isolation of native methanotrophs is necessary to begin to study the physiology and enzyme kinetics of the strains that play a major role in the global methane balance. An increased diversity of strains comprising the methanotrophic consortium may also provide a more stable and active soil 50noculums *in situ* (Isbell et al. 2011; D. Tilman & Pacala 1993; Date 2001).

To this end, several enrichment and isolation techniques may be employed. Some environmental bacteria are difficult to culture on surfaces, and thus a challenge for isolation, therefore dilution-extinction techniques may be used to isolate numerically dominant methanotrophs (Button et al. 1993). Membrane systems have also been successful for isolating methanotrophs on a less well defined medium, where soil extract is used instead of traditional culture medium (Svenning et al. 2003). These types of alternative culturing methods may be the key to isolation of currently elusive groups such as the upland soil clusters α and γ which have been detected at numerous soil sites, but have yet to be isolated.

It is unknown which members of the current methanotrophic enrichments are active in soil, nor is it apparent whether the methane consumption measured is largely attributable to one or all members of this community. To begin to answer these questions, stable isotope probing experiments could be performed, where microcosms are incubated under a headspace of ^{13}C-labeled methane. Both DNA and RNA could then be extracted, and the labeled nucleic acids separated and sequenced, which will indicate those methanotrophs that are actively oxidizing methane once they have been added to soil. It is possible that the active subset of the inoculum community may consist of one or all of the included strains, and may also change over time. This information would be highly useful in the efficient creation of mock communities, and could also shed light on the activity and interactions of native methanotroph communities by determining how different subsets react to stressors such as moisture, pH, or temperature.

Future studies will also focus on the fate and activity of methanotroph inocula after they have been applied to soil. Current results demonstrate methane oxidation for 9 days in microcosms, which is not yet a practical duration for a larger scale application. Use of a microbial carrier system is one method shown to improve viability and activity of bacteria added

to soil (Daza et al. 2000; Albareda et al. 2008; Dyke & J I Prosser 2000). As nutrient availability is a known challenge in soil ecosystems, carrier substrates may also serve as a nutrient source by soaking or mixing the carrier with additional nutrients. Intact soil microcosms are an ideal means to evaluate different carrier materials and delivery methods and their effect on methane oxidation rates and duration. Methanotroph delivery and activity will be assessed for lyophilized cells as well as liquid culture, as dried cell material would be ideal for large scale transport and field applications.

In the long term, an in depth knowledge of the physiology and behavior of soil methanotrophs could inform land management practices, with a focus on maintaining methane consumption and methanotroph communities. It is already known that the form and amount of nitrogen-based fertilizers can have a marked impact on the soil methanotroph community on long and short term scales (Mohanty et al. 2006; Gulledge et al. 2004). Additionally, agricultural practices such as tillage alter the soil pore space, which ca-n alter the exchange of gases such as methane and oxygen between soils and overlying air, thus affecting the activity of methanotrophs and other soil bacterial communities (Suwanwaree & G. Philip Robertson 2005).

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