COMETABOLIC BIODEGRADATION OF GROUNDWATER CONTAMINANTS BY ACIDOPHILIC METHANOTROPHS

A Dissertation

by

YIRU SHAO

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Kung-Hui Chu
Committee Members,	Terry J. Gentry
	Yongheng Huang
	Jun Kameoka
Interdepartmental Program Chair,	Ronald Kaiser

December 2019

Major Subject: Water Management and Hydrology Sciences

ABSTRACT

Chlorinated solvents have been widely used in various industrial process. The wide applications and improperly disposed has resulted in groundwater and soil contaminated with these compounds. In contaminated groundwater, trichloroethylene (TCE) and dichloroethylene (DCE) are two commonly detected volatile organic compounds (VOCs) and 1,2,3-trichloropropane (TCP) is a common co-contaminant. These three contaminants are known or suspended carcinogens. Maximum contaminant levels for TCE and DCE in drinking water has been set by EPA. In 2009, the U.S. EPA listed TCP in the Drinking Water Contaminant Candidate List 3(CCL3) (EPA 2009). To protect public health, these compounds need to be removed from contaminated aquifers and soils.

While bioremediation technologies for chlorinated solvents have been successfully demonstrated in neutral pH aquifers, these technologies are often ineffective for remediating chlorinated solvents contamination in acidic aquifers (i.e., $pH < 5.5$). Acidophilic methanotrophs have been detected in several low pH environments, but their presence and potential role in chlorinated solvents degradation in acidic aquifers is unknown.

This study used stable isotope probing-based techniques to identify the presence and diversity of acidophilic methanotrophs in chlorinated solvent-contaminated acidic aquifers. By using TCE as a model groundwater contaminant, application of stable isotope probing-based technique has successfully identified active methanotrophs that were capable of degrading TCE in microcosms prepared from two low pH aquifer materials. A total of thirty-five clones of methanotrophs were derived from low pH microcosms in which methane and TCE degradation had been observed, with 29 clustered in γ-Proteobacteria and 6 clustered in α-Proteobacteria. None of the clones has a high similarity to known acidophilic

methanotrophs from other environments. The presence and diversity of particulate MMO and soluble MMO were also investigated. The *pmoA* gene was detected predominantly at one site, and the presence of a specific form of *mmoX* in numerous samples suggested that *Methylocella* spp. may be common in acidic aquifers. Finally, a methane-grown culture at pH 4 was enriched from an acidic aquifer and its ability to biodegrade various chlorinated ethenes was tested. Interestingly, the mixed culture rapidly degraded TCE and vinyl chloride, but not *cis*-1,2-DCE after growth on methane.

Research efforts also extended to examine the degradative ability of pure acidophilic methanotrophs to chlorinated solvents, with respect to degradation kinetics and transformation capacity(Tc), and effects of carbon substrates on the degradative enzyme expression. Two acidophilic methanotrophic strains were used as model microorganisms. Both strains were able to grow on multi-carbon sources, while only methane-grown cells showed non-specific enzyme activity based on naphthalene oxidation tests. Positive PCR results confirmed that *Methylocella tundrae* carried the cluster gene of propane monooxygenase and probably could use propane as the carbon source. While the strain grew well on isopropanol (an immediate product of propane oxidation), the strain grew poorly with propane. Interestingly, the isopropanol-grown cells showed negative results in naphthalene oxidation assay, suggesting that isopropanol might not be an inducer for propane monooxygenase. Methane-grown *Methylocella tundrae* and *Methylocystis bryophila* were able to degrade TCE and cis-1,2-DCE. Only *Methylocella tundrae* could degrade TCP. The results of this study suggest that aerobic biodegradation of TCE and other chlorinated solvents in low pH groundwater may be facilitated by methanotrophic

bacteria, and that there are potentially a wide variety of different strains that inhabit acidic aquifers.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor Kung-Hui Chu of Zachry Department of Civil and Environmental Engineering, Professor Terry J. Gentry of Department of Soil and Crop Sciences, Professor Yongheng Huang of Department of Biological and Agricultural Engineering, Professor Jun Kameoka of Department of Electrical & Computer Engineering and Professor Lawrence Dangott of Department of Biochemistry & Biophysics.

The data analyzed for Chapter 3 about the methane and TCE degradation in microcosms (Figure 3.1) and TCE, *cis*-1,2-DCE, VC degradation of enriched samples (Figure 3.7) were provided by Dr. Paul Hatzinger, Ms. Sheryl Streger and Ms. Rachael Rezes of APTIM Federal Services. The PCR sequencing samples were sent to Eton Bioscience, Inc to run.

All other work conducted for this dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by project of Strategic Environmental Research and Development Program (SERDP; Project ER-2531) and a fellowship from China Scholarship Council.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Dr. Kung-Hui Chu. It is her patience, her supervision and her great mentorship kept me pursuing this degree. Without her encouragement, I cannot finish my work. I appreciate every meeting with her and all the time she spent on me. She is a mentor in my life. I look forward her continuous mentorship. I also would like to thank committee members, Dr. Terry Gentry, Dr. Yongheng Huang, Dr. Jun Kameoka and Dr. Lawrence Dangott, for helping me with great comments, thoughts and advices of this research.

I must express my deep gratitude to Dr. Paul Hatzinger, Ms. Sheryl Streger and Ms. Rachael Rezes of APTIM Federal Services for their technical supports in the experiments and excellent suggestions in my research work.

I also give many thanks to my lab mates, Myung Hwangbo, Shih-Hung Yang, Fahad Asiri, Bilal Azeez, Dr. Po-Jung Huang, Dr. Theodore Alivio, Dr. Chih-Hung Chen, Ying Shi, Dr. Baixin Wang and Janessa Tran for their help, advice, support and encouragement. I enjoyed the lab work with you.

I am indescribably thankful for my parents and my friends, Dr. Yiwen Zhang, Dr. Xinyuan Yang, Dr. Xiaoyuan Xie and Yunye Zhu. They have supported me with unconditional love and never-ending encouragement. I was not able to do all of the graduate work without my family and friends.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

Page

1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

Chlorinated solvents were the compounds contain chlorides, which have been widely used in industrial process and were improperly disposed (Semprini 1995). The major compounds that were detected in groundwater include chlorinated alkanes, chlorinated alkenes and chlorinated aromatics (Frascari et al. 2015). In those contaminated groundwater, trichloroethylene (TCE) and dichloroethylene (DCE) are most frequently detected volatile organic compounds (VOCs) in aquifers (Zogorski et al. 2006). Their regulations in drinking water have been set by EPA in 1898 and 1992. In 2009 U.S. EPA listed 1,2,3-trichloropropane (TCP) in the Drinking Water Contaminant Candidate List 3 (CCL3) (EPA 2009). These three contaminants are known or suspended carcinogens (Frascari et al. 2015), and thus need to be removed from contaminated aquifers and soils.

These contaminants are biodegradable under aerobic and anaerobic conditions, and thus bioremediation has been recognized as an environmental friendly and economical technology for treating chlorinated solvent-contaminated groundwater and soils. While successful aerobic and anaerobic bioremediation of chlorinated solvents-contaminated aquifers under neutral pH conditions has been demonstrated (Adriaens and Grbic-Galic 1994, Broholm et al. 2005, DiSpirito et al. 1991, Fogel et al. 1986, Freedman and Gossett 1989, Kang et al. 2001, Kim et al. 2000, Little et al. 1988b, Middeldorp et al. 1999, Nelson et al. 1986, Tandoi et al. 1994, Tsien et al. 1989b, Uchiyama et al. 1989b, Vogel and McCARTY 1985, Wilson and Wilson 1985), these approaches, particularly anaerobic dechlorination, are not effective for acidic aquifers. Low pH groundwater

(pH <5.5) is common in many areas, including the Northern Atlantic Coastal Plain aquifer system in the US, which runs south from Long Island, New York through much of North Carolina(Denver et al. 2015). This aquifer system, which includes a number of large military facilities and urban areas, is also impacted by chlorinated volatile organic compounds (cVOCs) such as like perchloroethene (PCE) and TCE(Denver et al. 2015). The concentrations of TCE of three public-supply wells were greater than the maximum contaminant level (MCL) in drinking water. Adjusting pH in large dilute plumes is challenging and impractical, and current anaerobic biostimulation and bioaugmentation technologies are ineffective in these low pH environments (Vainberg et al. 2009, Yang et al. 2017).

Aerobic biodegradation of chlorinated solvents has been well studied, particularly through cometabolic degradation by methanotrophs (methane-oxidizing bacteria). Methanotrophs, ubiquitous in the environments, are a group of bacteria capable of expressing methane monooxygenase (MMO) to oxidize methane and many environmental pollutants, such as TCE (Oldenhuis et al. 1991, Wilson and Wilson 1985) and cis-DCE(Takeuchi et al. 2005). There are two different types of MMO, a soluble form (sMMO) and a membrane-bound particulate form (pMMO). The majority of methanotrophs contain pMMO only, some have both pMMO and sMMO, and only a few have sMMO only (DiSpirito et al. 1992, Fox et al. 1990, Hanson and Hanson 1996a, Koh et al. 1993b, Lee et al. 2006, Lontoh and Semrau 1998a, Oldenhuis et al. 1991). Compared to pMMO, sMMO has a broader substrate range and can degrade TCE more rapidly (Lee et al. 2006). TCE degradation by methanotrophs was first observed in 1985(Wilson and Wilson 1985) and later confirmed with *Methylosinus trichosporium* OB3b, a strain which could degrade TCE at rates exceeding 1.2 mmol/h per g (dry weight)(Tsien et al. 1989b). *Methylosinus trichosporium* OB3b possesses genes coding both pMMO and sMMO. With limited

copper, sMMO was dominant and was able to use TCE as a substrate.(Oldenhuis et al. 1989a) With high copper concentration, only pMMO was expressed and the affinity for TCE increased with increasing copper (Lontoh and Semrau 1998b).

Another methanotroph which degraded chlorinated solvents through pMMO is *Methylocystis* strain SB2. *Methylocystis* strain SB2 showed the ability to degrade vinyl chloride (VC), DCE, TCE and 1,1-trichloroethane (1,1,1-TCA) growing with methane and ethanol(Le and Coleman 2011). The methanotrophs growing simultaneously on methane and longer chain alkanes are facultative. This character of *Methylocystis* strain SB2 expands its presence in varies environment and improve its application in bioremediation(Jagadevan and Semrau 2013). However, there is comparatively little research on the impact of indigenous methanotrophic populations on the natural attenuation of cVOCs in aquifers (Wymore et al. 2007). Moreover, a vast majority of the methanotrophs that have been the subject of biodegradation research to date grow most effectively at neutral pH, with inhibition occurring below pH 5 (Dedysh et al. 1998a, Semrau et al. 2010).

In recent years, diverse acidophilic methanotrophs have been isolated from Sphagnum peat bogs where the pH ranges from 3.5 to 5 (Danilova et al. 2013, Dedysh et al. 2007, Dedysh et al. 2002, Dedysh et al. 2000, Dedysh et al. 1998a, Kip et al. 2011, Vorobev et al. 2011). Example organisms include *Methylocella palustris* (Dedysh et al. 2000), *Methylocella silvestris* (Dunfield et al. 2003) and *Methyloscella tundrae* (Dedysh et al. 2004*)* ,which is one of a few species containing only sMMO, and *Methylocapsa acidiphila* (Dedysh et al. 2002). Application of 16S rRNA-targeted fluorescent oligonucleotide probes has also lead to identification of other acidophilic methanotrophs(Dedysh et al. 2003), including *Methylocystis heyer (Dedysh et al. 2007), Methylosinus*(Kip et al. 2011), *Methyloferula stellate* (Vorobev et al. 2011), *Methylomonas*

paludis (Danilova et al. 2013) and *Methylocaldum sp.* BFH1 (Islam et al. 2016). All members of *Methylocella* are facultative. They not only can grow with C2-compounds, such as ethane, ethanol and acetate, but also C3 to C4 compounds, such as propane, pyruvate and succinate (Crombie and Murrell 2014). *Methylocapsa aurea* and some members of *Methylocystis* also show their ability in growing with acetate and methanol (Belova et al. 2013, Dunfield et al. 2010, Im and Semrau 2011, Leng et al. 2015).

The ability of utilizing alternative carbon source improves the adaption of acidophilic methanotrophs to different environment. However, some alternative carbon sources cannot be the inducer of MMOs expression, while MMOs are key enzymes to cometabolism(Rahman et al. 2011b). The degradation capabilities or prevalence of acidophilic methanotrophs in acidic aquifers is unknown. To explore the ability of acidophilic methanotrophs in bioremediation, studies about the MMOs constitutive expression growing with multi-carbon and contaminants degradation capabilities of acidophilic methanotrophs are needed more attention.

1.2. Goal, objectives, and hypotheses

The **overall goal** of this research is to examine the biodegradation potential of groundwater contaminants by acidophilic methanotrophs in acidic aquifers. Specifically, I will apply GC-FID/MS to monitor the degradation of chlorinated solvents for both microcosms and isolated strains, a stable isotope probing (SIP)-based method, called Q-FAST(Cho et al. 2013) to identify active methanotrophs in acidic microcosms (i.e., pH <5) , and molecular tools to determine the diversity of the acidophilic methanotrophic community and the catabolic genes encoding MMOs in relation to the chlorinated solvents degradation. To accomplish this goal, experiments will be performed to achieve three specific objectives as described below:

Objective 1: Evaluate methanotrophic bacterial communities capable of biodegrading TCE in acidic microcosms

Hypothesis: Acidophilic methanotrophs can degrade TCE thorough cometabolism in acidic aquifers.

Task 1a: Examine the ability of methanotrophs to degrade TCE in acidic microcosms.

Task 1b: Identify and characterize active acidophilic methanotrophs community in acidic groundwater microcosms.

Task 1c: Quantify genes encoding MMOs and correlate to TCE degradation.

Objective 2: Determine the types of oxygenases expressed by acidophilic methanotrophs grown with different multi-carbons

Hypothesis: Acidophilic mehanotrophs that can grow on multi-carbons such as acetate and propane can express different types of oxygenases (either sMMO, pMMO, or propane monooxygenase (PrMO)).

Task 2a: Examine the ability of *Methylocystis bryophila* and *Methylocella tundrae* to grow with multi-carbons, including acetate, propane and Isopropanol.

Task 2b: Evaluate the monooxygenases expression, including pMMO, sMMO and PrMO, of *Methylocystis bryophila* and *Methylocella tundrae* growing with different carbon sources.

Objective 3: Determine degradation kinetics of groundwater contaminants by oxygenase-expressed acidophilic methanotrophic strains

Hypothesis: The oxygenases, either MMOs or PrMO, expressed by facultative, acidophilic methanotrophs can cometabolically degrade groundwater contaminants such as TCE, *cis*-1,2-DCE, TCP.

Task 3a: Explore the potential of resting cells to degrade TCE, cis-1,2-DCE,1,2,3-TCP.

Task 3b: Study oxidation kinetics of different groundwater contaminants by MMOs or PrMO.

1.3. Dissertation overview

This dissertation consists of five chapters. The research overall goals, hypothesis and objectives are described in Chapter 1. Chapter 2 is the literature review of the four groundwater contaminants in this study, methanotrophs, acidophilic methanotrophs, facultative methanotrophs and types and regulation of oxygenases. Also, recent studies about applying methanotrophs into biodegradation of emergency contaminants are summarized. In Chapter 3, the application of stable isotope probing and other molecular tools to identify TCE-degrading microorganisms in acidic microcosoms is addressed. The research described in Chapter 3 has been published (Shao et al. 2019). The growth of two acidophilic methanotrophs *Methylocella tundrae* and *Methylocystis bryophila* with C2 and C3 carbon sources is studied and the biodegradation of TCE, cis-1,2-DCE, 1,2,3- TCP by acidophilic methanotroph *Methylocella tundrae* is examined in Chapter 4. In Chapter 5, the findings of this study are summarized, and the implications of the results of this work and future research directions are also discussed.

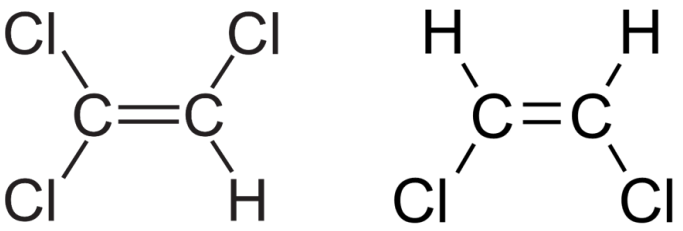
2. LITERATURE REVIEW

2.1. Groundwater contaminants

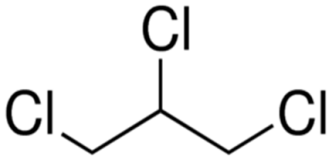
Trichloroethylene (TCE), a common degreasing agent used in mining, chemical production and electric utilities (EPA 2017), is one of the most commonly detected groundwater contaminants in the U.S. Exposure to TCE can impact the liver, kidneys, immune system, and the central nervous system (EPA 2016). TCE is a suspected carcinogen with a Maximum Contaminant Level (MCL) of 5μg/L for drinking water in the US. Approximately 9% of drinking water wells in the US are contaminated with TCE (EPA 2016), and 17% of the contaminated wells exceed the recommended MCL. *cis*-1,2-DCE is one degradation intermediate compounds during anaerobic biodegradation of TCE. Usually, *cis*-1,2-DCE was easy to degrade and the degradation efficiency was higher than TCE (Schäfer et al. 2003, Semprini et al. 1990, Wüst et al. 1999).

1,2,3-TCP used as a chemical intermediate in organic synthesis, as a solvent, and as an extractive agent. In addition to these intentional uses, TCP is produced in considerable amounts as a by-product from the manufacture of epichlorohydrin (Sconce 1962, Swanson 1999). 1,2,3-TCP causes cancer in laboratory animals and a potential carcinogen to human beings (Samin and Janssen 2012). 1,2,3-TCP has been detected in hundreds of surface water and drinking water sources, e.g., in the United States, at levels of 0.1-100 μg/l. Remediation of TCP-contaminated sites is difficult due to its persistent nature and its physiochemical properties, which causes spreading with flowing groundwater (Salter et al. 2010).

Figure 2.1. Structure of chlorinated solvents



TCE *cis*-1,2-DCE

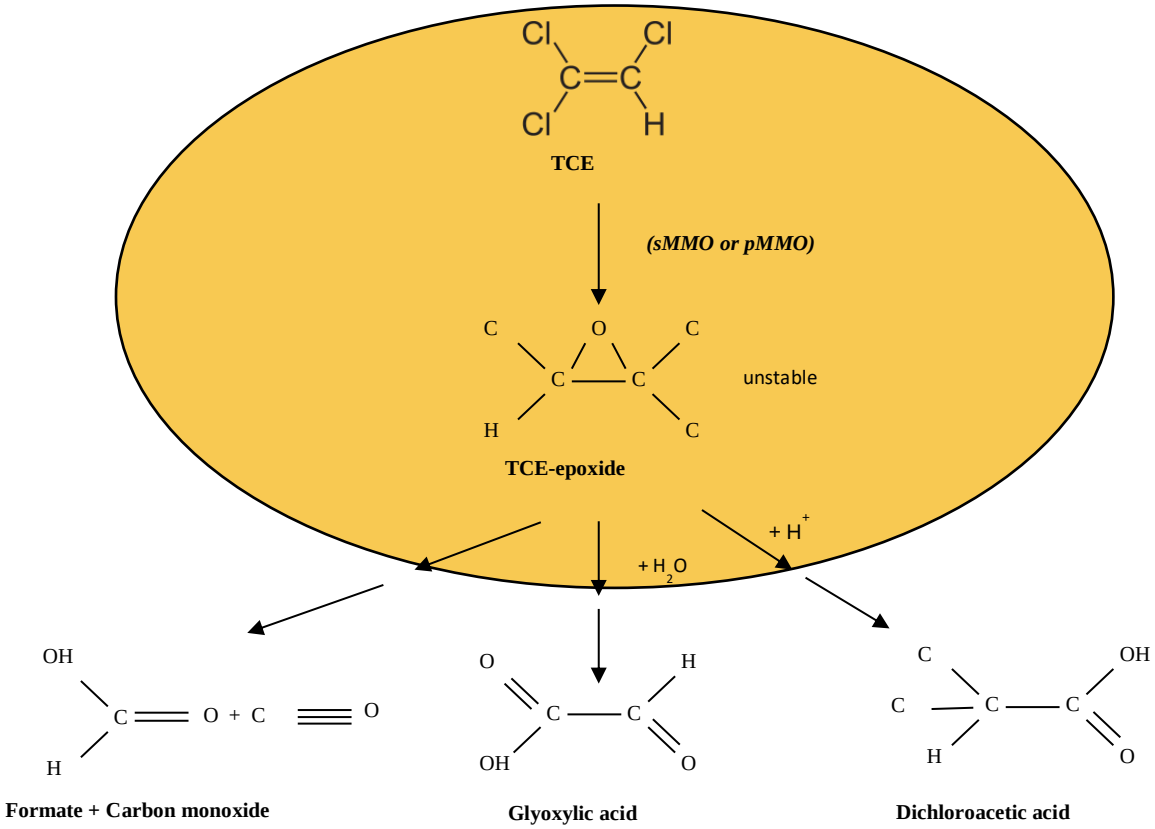


1,2,3-TCP

2.2. Biodegradation of groundwater contaminants

The aerobic process is called cometabolic degradation. Bacteria expressing oxygenase can oxidize the groundwater contaminants without gain energy. Many species are capable of degrading persistent contaminants, such as *Pseudomonas* and *Burkholderia*. Methanotrophs are the bacteria gained most study. Methanotrophs play very important role in bioremediation of TCE contaminated aquifers. In 1980s, researchers found that methane stimulated TCE degradation in aerobic sediments by mixed culture(Fogel et al. 1986, Wilson and Wilson 1985). In 1988, two pure methanotrophs capable of degrading TCE were isolated from groundwater samples(Little et al. 1988b). TCE is oxidized to TCE-epoxide by MMO. The spontaneous degradation of trichloroethylene epoxide can produce four products: formate, carbon monoxide, glyoxylate and dichloroacetate.

Figure 2.2. Cometabolic degradation pathway of TCE



2.3. Overview of phylogeny and physiology of methanotrophs

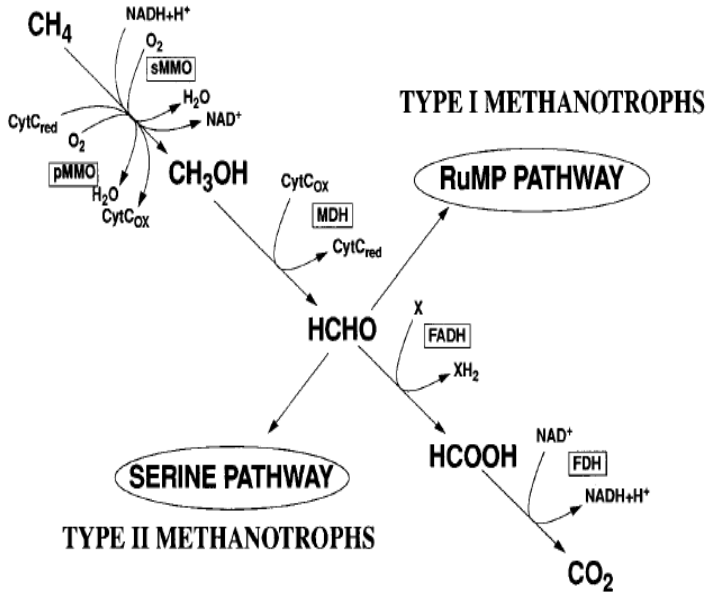
2.3.1. Early studies of methanotrophs

Methanotrophs, characterized by their ability to use methane as a sole carbon source, are phylogenetic diverse and widespread in the environment. Numerous neutrophilic methanotrophs (referred to strains survive in neutral environments) have been isolated from different environments including soils, sediments, wetlands, landfills, lakes, rice paddies. The neutrophilic methanotrophs are mainly belong to α–, β– or γ– proteobacteria in genus of *Methylobacterium, Methyloystis, Methylosinus, Methylobacterium, Methyococcus, Methylophilus, Methylomicrobium, Methylomonas and Methanomonas (Hanson and Hanson 1996b)*. Before late 1990's, only a few strains were isolated from pH extreme environments. *Methylomicrobium*

alcaliphilum is the first alkaliphilic methanotroph isolated from Tuva soda lakes (Khmelenina et al. 1997), and three acidophilic methanotrophs were isolated from Peat Wetlands (Dedysh et al., 1998a).

In the process of methane utilization by methanotrophs, methane is first oxidized to methanol, formaldehyde, formate and then to carbon dioxide for energy production (**Figure 2.3**). A fraction of formaldehyde is used for biosynthesis via one or both assimilation pathways: ribulose monophosphate (RuMP) pathway and serine pathway. In RuMP pathway, three moles of formaldehyde is combined with one mole of ribulose monophosphate to form cell biomass. Two enzymes, hexulose-6-phosphate synthase and hexulose-phosphate isomerase, are unique to this metabolism of one-carbon compounds by methanotrophs. In the serine pathway, two moles of formaldehyde and one mole of carbon dioxide are utilized to form a three-carbon intermediate. Additionally, three moles of ATP and two moles of NADH are involved in the reaction of serine pathway to provide energy and accept electron (Hanson and Hanson 1996b).

Figure 2.3. Pathways for the oxidation of methane and assimilation of formaldehyde. Abbreviations: CytC, cytochrome c; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase (adapted from Hanson and Hanson 1996b)



2.3.2. Acidophilic methanotrophs

The first evidence of the presence of acidophilic methanotrophs was reported by a study using 16S rRNA-based and mmoX-based (mmoX is a suprobes to detect methanotrophs in samples collected from an acidic peat environment (pH 3.6) in 1996 (McDonald et al. 1996). In 1998, three acidophilic methanotrophs were first isolated from acidic environments such as Peat Wetlands (Dedysh et al. 1998a). While researchers have never stopped research efforts on isolation of pure strains from acidic environments, only a few were isolated. This might be, in part, due to the use of growth medium that contains high mineral salts (1.5-3 g/L) (Dedysh, 2002). Most of known methanotrophs could not able to survive the extreme environment like Peat bogs which have a high acidity, a low buffer capacity and low temperature (Dedysh et al., 1998b). Successful isolations later occurred when low salt medium (containing 50 mg/L mineral salts) was used and incubation conditions were adjusted similarly to the acidic environments. Additional strains were then isolated from other acidic environments, such as *Sphagnum* peat bogs (Dedysh et al. 2002, Dedysh et al. 2000), and Sphagnum tundra peatlands (Dedysh et al. 2004).

Following changes of isolation procedure and medium, until now, ten pure acidophilic methanotrophs were isolated from other environments including forest soil, forest cambisol, and collapsed palsa soil (Danilova et al. 2013, Dedysh et al. 2007, Dedysh et al. 2004, Dedysh et al. 2015a, Dedysh et al. 2002, Dedysh et al. 2000, Dunfield et al. 2010, Dunfield et al. 2003, Vorobev et al. 2011). These pure strains have been also characterized in terms of their morphology, species, growth conditions, fatty acids and enzyme activities. The whole genome sequences of seven of the isolates have been reported (Dedysh et al. 2015b, Esson et al. 2016, Han et al. 2018, Kox et al. 2019, Miroshnikov et al. 2017, Oshkin et al. 2019, Ricke et al. 2005), providing basic information for further study. More discussion of these isolates is described below in Table 2.1 and section 4.

In recent years, the diversity and abundance of acidophilic methanotrophs were further explored through applications of molecular techniques based on 16S rRNA and/or functional genes. Detection of various novel clones revealed that acidophilic methanotrophs are more widely present in the environment than previously thought (Esson et al. 2016, Farhan Ul Haque et al. 2018, Kip et al. 2012, Kip et al. 2011).

2.3.3. Obligate methanotrophs vs. facultative methanotrophs

Obligate methanotrophs are referred to microorganisms grow only on C1 compounds such as methane or methanol. Researchers have been previously thought that methanotrophs were obligate, until the report of the first facultative methanotroph *Methylocella silvestris (Dunfield et al. 2003)*. Facultative methanotrophs are able to grow on multi-carbon compounds such as acetate, pyruvate, succinate, malate, ethanol in addition to methane (Dedysh et al. 2005). Following the first report of facultative methanotroph in 2003, more facultative methanotrophs, not only in the genre of *Methylocella* (Dedysh et al. 2004), but also in the genre of *Methylocystis* (Belova et al. 2013, Dedysh et al. 2007, Im and Semrau 2011) and *Methylocapsa* (Dunfield et al. 2010), were reported. Among all the facultative methanotrophs, the members of *Methylocella* are specially of interest due to their wider substrate range. *Methylocella* not only can grow with C2 compounds such as ethane, ethanol and acetate, but also with C3 to C4 compounds such as propane, pyruvate and succinate (Crombie and Murrell 2014). *Methylocapsa aurea* and some members of *Methylocystis* also show their ability to growth with acetate and methanol (Belova et al. 2013, Dunfield et al. 2010, Im and Semrau 2011, Leng et al. 2015). However, when grown with alternative carbon sources, some facultative methanotrophs could not express MMO constitutively or the expression of their MMO was repressed (Rahman et al. 2011b), suggesting that other

enzymes might involve in the oxidation of multi-carbon sources. Studies on facultative methanotrophs remain limited.

2.4. Oxygenases and their regulations

2.4.1. Methane monooxygenase (MMO)

Methane monooxygenases (MMOs) are enzymes responsible for the first step of methane oxidation methane, i.e., converting methane to methanol, which is a defining characteristic of methanotrophs (Hanson and Hanson 1996b). There are two forms of MMOs, particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO). pMMO, a membrane-associated protein, has a higher affinity toward methane than sMMO, a soluble cytoplasmic protein. Both enzymes require reducing equivalents and oxygen to carry out methane oxidation (Lawton and Rosenzweig 2016).

pMMO has a copper active site and highly express when copper is present at high concentrations. The 27 kDa subunit of the pMMO is encoded by *pmoA*, which is commonly used as a functional gene marker for methanotrophs. pMMO and ammonia monooxygenase (AMO) share high sequence identity, which showed they were evolutionarily closed related (Holmes et al. 1995), which is also can oxidize methane and transform methanol to CO_2 (Bédard and Knowles 1989). As the activity of purified pMMO activity decreased 10- to 100-folds, the 3D structure and mechanisms of pMMO was not understood thoroughly until year 2005(Kitmitto et al. 2005).

Before *Methylocella* (a genus belonging to acidophilic methanotrophs) was identified, pMMO was thought to present in all methanotroph and there is only one single type of pMMO. Recently, two isozymes of pMMO, pMMO1 and pMMO2, were found in the genus of

Methylocystis sp. (Baani and Liesack 2008). pMMO1 is a conventional pMMO with a low-affinity toward methane, while pMMO2 has a high-affinity toward methane. Most interestingly, pMMO2 was expressed constitutively and capable of oxidizing methane at low even trace levels (Baani and Liesack 2008). Most Type II methanotrophs (using serine pathway) possess pMMO2, Type I (using RuMP pathway) methanotrophs possess only pMMO1, not pMMO2. The presence of homologue monooxygenase may provide a growth advantage (or survival strategy) to bacteria under different environmental conditions. For example, the co-exist of pMMO1 and pMMO2 in *Methylocystis sp.* would allow the strain to grow in the environment with fluctuating methane concentrations, ranging from high to low (Baani and Liesack 2008).

Following purification and characterization of sMMO (Grosse et al. 1999a, Shen et al. 1997), the structure and mechanism of sMMO have been recently elucidated (Sirajuddin and Rosenzweig 2015). sMMO comprises three proteins, the hydroxylase (MMOH), the regulatory component (MMOB), and the reductase (MMOR) (Elango et al. 1997). sMMO is a soluble di-iron center monooxygenase (SDIMO), while the di-iron active site positioned in the α subunit of MMOH (Lee 2016). *mmoX*, which is treated as a marker of PCR based assays, encodes the α subunit of the MMOH in sMMO. Most methanotrophs only transcribe one copy of *mmoX*. Interestingly, *Methylosinus sporium* transcribed two copies of *mmoX* but only one copy was essential for sMMO activity (Ali et al. 2006). sMMO was thought not able to exist alone in methanotrophs, this believe was hold until *Methylocella* and *Methyloferula* were isolated (Dedysh et al. 2000, Vorobev et al. 2011).

2.4.2. Regulation of MMOs

2.4.2.1. Effects of metals on MMO expression

The expression of types of MMOs is regulated by copper. Accordingly, the copper levels have become an importance factor in the selection of dominant methanotrophs in different ecosystems. For methanotrophs that possess both sMMO and pMMO, the expression of sMMO is tightly controlled by the concentration level of copper. In these methanotrophs, sMMO is expressed under low copper conditions and is dramatically down-regulated in the presence of copper (Lawton and Rosenzweig 2016).

Several genes involved in regulation of sMMO expression. These genes include a σ^{N} promoter (*rpo*N), a regulatory gene (*mmo*R) and a gene encoding a GroEL homologue (*mmo*G). These genes were found in both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath). Yet, the precise function of these genes is not clear. However, the results of knock-out mutant strains indicated that *mmo*R and *mmo*G were depressed by copper, which caused no expression of sMMO (Csaki et al. 2003, Stafford et al. 2003).

Both *mmo*R and *mmo*G were also detected in *Methylocella silvestris* BL2 (an acidophilic methanotroph), with 47% amino acid identity to the *mmo*R of *Methylosinus trichosporium* OB3b and 33% identity to the *mmo*R of *Methylococcus capsulatus* (Bath), respectively (Theisen et al. 2005). Unlike the MMO regulatory mechanism in *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), the *mmo*R in *Methylocella silvestris* BL2 may not be the only regulatory protein in controlling the expression of the *mmo* operon. In *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath) genomes, the function of mmoR involves in activation or depression of the mmo genes in response to copper. Smirnova *et al*.(Smirnova and Dunfield

2018) also mentioned that mmoR was involved in protein-protein interactions with another protein to repress or active transcriptional activation of *mmo* genes.

2.4.2.2. Methanobactin

Methanobactins (mbns) produced by methanotrophs are copper-binding peptides (or called or chalkophores) that secreted into medium to recruit cupper. Its function is very similar to iron siderophores; methaobactins bind with Cu ions and then bring the Cu back to the cells. It was first found in *Methylosinus trichosporium* OB3b (Kim et al. 2004) and later found in *Methylomicrobium, Methylocystis* (Ul-Haque et al. 2015) and *Methylosinus* (Kenney et al. 2016a).

Mbns are high affinity copper-binding ligands but they can also bind with other metals (Baral et al. 2014, Xin et al. 2013). As copper will compete with other metals to bind with mbns, it has been hypothesized that mbns might also play a role in protection the microorganisms against toxicity of metal ions other than copper (Kenney and Rosenzweig 2018). Mercury is known to inhibit the activity of sMMO (Grosse et al. 1999b). Studies have reported that methanotrophs expressed methanobactin to bind with mercury that lead to lower toxicity to sMMO (Baral et al. 2014, Vorobev et al. 2013). Other metal like gold can compete with copper to bind mbns and limit the copper uptake, leading to promoton of sMMO expression (Kalidass et al. 2015). Recent study has shown that a system co-regulated mbn operon and sMMO operon, which is reciprocally regulated with the pMMO operon in *Methylosinus trichosporium* OB3b (Kenney et al. 2016b). In the medium containing low levels of copper, the expression of sMMO and mbns were higher than those with high levels of copper. It is also found that the exogenous addition of mbns would increase the expression of sMMO if the gene encoding mbns was disrupted (Ul-Haque et al. 2015).

It is unknown if mbns are produced in acidophilic methanotrophs as there was no reported on this topic. Acidophilic methanotrophs appear to be very sensitive to trace metals. Given the greater solubility (and by extension, availability) of Cu and other trace metals in acidic conditions, such active metals uptake mechanisms may not be necessary. The structure of mbn suggests that it can bind other metals, including mercury. External addition of mbns into medium have found to enhance the expression of sMMO but also to bind mercury with presence of copper by *Methylosinus trichosporium* OB3b (Vorobev et al. 2013), which suggesting that mbns are probably able to enhance the viability of acidophilic methanotrophs.

2.4.3. Other monooxygenases

Many homologue monooxygenases were detected in methanotrophs. In 2011, Tavormina *et al*. detected a sequence-divergent particulate monooxygenase (pXMO), a homologue of pMMO, from the genera *Methylomonas, Methylobacter and Methylomicrobium* (Tavormina et al. 2011). The function of pXMO has not been determined yet. The 16S-rRNA-based phylogenetic results showed this gene cluster was more closely related to AMO than to pMMO. However, the researchers were unable to find pXMO-harboring bacteria that can oxidize ammonia (Tavormina et al. 2011).

The genome of *Methylocystis bryophila* (a known acidophilic methanotroph) also contains a pXMO-like gene cluster that is similar to those in some gammaproteobacterial methanotrophs. *Methylocystis bryophila also* possess enzymes of pMMO1, pMMO2 and sMMO (Han et al. 2018). The members of genre *Methylocystis* expressing various monooxygenases are one of the frequently detected methanotrophs in environmental samples (Knief 2015). Possessing various

monooxygenases improves *Methylocystis* ability to survive with fluctuating methane concentrations as well as under copper or O_2 limitation (Han et al. 2018).

Analysis of two clusters of SDIMO in the genome of *Methylocella silvestris* suggests that one of the gene clusters encodes a PrMO and α subunit of the hydroxylase (*PrmA)*. PrMO expressed with the inducer of propane. In propane oxidation, both sMMO and PrMO were needed (Crombie and Murrell 2014). A recent study reported that a PrMO gene cluster was in megaplasmids of *Methylocella tundrae.* However, the expression of PrMO has not been demonstrated (Kox et al. 2019).

2.5. Acidophilic methanotrophic isolates and occurrences

2.5.1. Acidophilic methanotrophs isolates

Researchers have spent more than three decades to isolate acidophilic methanotrophs from environment. In 1985, a report describing an isolate belonging to the genus *Methylosinus* from an acidic peat lake (Dedysh et al. 1998b), but the ability of this isolate to grow at acidic pH values was not confirmed(Dedysh et al. 1998b). Later with modification of culturing conditions (Dedysh et al. 1998a), several acidophilic methanotrophs have been reported. Table 2.1 summarizes the characteristics of acidophilic methanotrophic isolates that have been reported. Most acidophilic methanotrophs were isolated from peat bogs, where methane is produced through anaerobic decay and peat moss could acidify its surroundings by taking up calcium and magnesium while releasing hydrogen ions. All of these acidophilic methanotrophs are gram-negative and have no flagellum and pili. These acidophilic methanotrophs are in the shape of rods or coccoid. The optimal growth temperature for these methanotrophs is below 30 °C, usually around 25 °C. Among these acidophilic methanotrophs isolates, species in the genre *Methylocella*, *Methylocystis* and

Methylocapsa aurea are facultative. *Methylocapsa aurea* only possesses pMMO but can use acetate as carbon source. *Methylomonas paludis* is the only one using RuMP pathway and belong to the class of γ-proteobacteria, while others belong to α-proteobacteria. *Methyloferula stellate* uses both serine and ribulose 1,5-bisphosphate (RuBP) pathway to assimilate carbon source. Two *Methylocystis* strains have a short generation time compared to other acidophilic methanotroph. The rapid growth of these *Methylocystis* strains may be due to that fact they process both pMMO and sMMO.

2.5.2. Journey of isolation of acidophilic methanotrophs from various environments

In 1998, Dedysh *et al.* reported successful isolated three acidophilic methanotrophs from peat bogs environments, when using a medium of low ionic strength. Based on the *mmo*X genes of these strains, these three strains were only moderately related to the known cluster of alpha-proteobacteria methanotrophs, i.e., *Methylosinus-Methylocystis* spp. Analysis of their 16S rDNA sequence revealed that these strains may evolve from the same ancestor with the acidophilic heterotrophic bacteria *Beijerinckia indica* and *Rhodopseudomonas acidophila* (Dedysh et al. 1998a).

Dedysh *et al.* later reported that these three acidophilic methanotrophic strains belonging to a new genus *Methylocella*, with *Methylocella palustris* was the first member in this genus. *Methylocella palustris* was found to only express sMMO. No products were observed in a PCR with pMMO-targeted primers; hybridization with a *pmoA* probe was also negative. As a result, the previous believe that all methanotrophs contained the *pmoA* genes and expressed pMMO was no longer valid (Dedysh et al. 2000).

In 2002, another species, *Methylocapsa acidiphila* $B2^T$, belonging to a novel genus, was isolated from an acidic *Sphagnum* peat bog. This bacterium belongs to the alpha-subclass of the proteobacteria and have 97.3% similarity to the 16S rRNA of *Methylocella palustris* K^T. However, there was only 7% of DNA-DNA hybridization value between strain *Methylocapsa acidiphila* $B2^T$ and *Methylocella palustris* K^T. *Methylocapsa acidiphila* $B2^T$ only expressed pMMO, which was different from *Methylocella palustris* K^T that only expressed sMMO (Dedysh et al. 2002). *Methylocapsa aurea* KYG^T is another acidophilic methanotroph in this genus. Different from *Methylocapsa acidiphila* $B2^T$ that is an absolute methanotroph, *Methylocapsa aurea* KYG^T was a facultative methanotroph. *Methylocapsa aurea* KYG^T could use acetate as carbon source but prefer methane. *Methylocapsa aurea* KYG^T was more sensitive to pH and salt concentrations in the medium. The optimum pH of *Methylocapsa aurea* KYG^T was in a narrow range of 6.0 to 6.2, and the strain could not survive in the environment of pH <5 (Dunfield et al. 2010). No study has reported the ecological role of *Methylocapsa aurea* KYG^T yet.

Methylocella silvestris was isolated from an acidic forest cambisol. This bacterium was morphologically and phenotypically similar to *Methylocella palustris* K^T. *Methylocella silvestris* possessed only sMMO and its expression is also affected by the concentration of copper in the growth medium. *Methylocella silvestris* can express more sMMO in medium containing 1µM copper than the medium containing 5nM copper (Theisen et al. 2005). While *Methylocella silvestris* can grow on acetate, the expression of *mmoX* was repressed (Rahman et al. 2011b). Mostly, *Methylocella silvestris* was the first methanotroph capable of using propane as carbon source while constitutively expressing sMMO (Crombie and Murrell 2014). The substrates that can be utilized by *Methylocella silvestris* has been expanded to 2-propanol, 1,2-propanediol,

acetone, methyl-acetate, acetol, glycerol, propionate, tetrahydrofuran, and gluconate, as well as the gaseous alkanes ethane and propane (Dunfield and Dedysh 2014).

Methylocella tundrae (Dedysh et al. 2004), with low pH tolerant and only possessing sMMO, is another *Methylocella* species. Genome analysis of *Methylocella tundrae* indicated that this bacterium carries a PrMO gene cluster in its megaplasmids (Kox et al. 2019). While two species in *Methylocella* possess PrMO allowing them to grow on multi-carbon submstrates, the PrMO gene cluster of *Methylocella silvertris* is in its genome but that of Methylocella tundrae is its megaplasmids. The reason caused this variation is unclear. A comparative genomic study of *Methylocella* showed their close relationship to *Beijerinckia indica*, which were acidophilic nitrogen fixing bacteria but non-methanotrophs (Tamas et al. 2014). *Methylocella* and *Beijerinckia* had been suggested to evolve from an obligate methanotroph which probably is a sort of degenerate methanotroph, which lost their specificity in methane oxidization (Dunfield and Dedysh 2014, Tamas et al. 2014).

Species in this genus *Methyloferula* are found to only possess sMMO. Unlike *Methylocella silvestris* that contains additional soluble diiron monooxygenas for propane oxidation, *Methyloferula stellata* is an obligate methanotroph. However, the close distance of 16S rRNA and sMMO sequence between two genera suggested that they are closely related.

Methylocystis possess both pMMO and sMMO and the expression of these MMOs was affected by the concentrations of copper. *Methylocystis heyri* and *Methylocystis bryophila* were two moderate acidophilic methanotrophs (optimum pH > 5.5) in this genus (Belova et al. 2013, Dedysh et al. 2007). These two species are also facultative methanotrophs and they can express

different forms of MMOs, explaining why they are wide spread in the environment (Han et al. 2018, Leng et al. 2015).

Methylomonas paludis was the first and the only acidophilic methanotrophs belonging to γ- *Proteobacteria* until now. It utilized RuMP pathway for carbon assimilation (Danilova et al. 2013). The absence of motility and the ability of growing at acidic sites made it different from other species in *Methylomonas.*

2.5.3. Molecular identification of acidophilic methanotrophs

Fluorescent oligonucleotide probes based on 16S rRNA (Bourne et al., 2000; Dedysh et al., 2003; Kalyuzhnaya et al., 2006), and PCR-based assays targeting 16S rRNA, pmoA, mmoX and methanol dehydrogenase gene, mxaF (Chen et al., 2008; Ghashghavi et al., 2017; Hutchens et al., 2004; Kip et al., 2011; Lau et al., 2007; Redmond et al., 2010) are commonly used to identify and investigate the present, diversity and abundance of acidophilic methanotrophs in different acidic environments. With the PCR-based assays, many uncultured clones were detected, demonstrating that widely existence and diversity of acidophilic methanotrophs in the environment (Chen et al. 2008, Kip et al. 2011, Knief 2015, McDonald et al. 1996). Recently, comparative genomic analysis provides insights into the study of acidophilic methanotrophs (Nguyen et al., 2018). Based on reconstructed genomes of uncultured bacteria, one can analysis the genomic features including genome sizes, G+C contents, and number of CDSs, and then compared to the reference genomes to identify the uncultured acidophilic methanotrophs in a given environmental sample (Nguyen et al. 2018).

Adapting the similar approach, we performed a comparative sequence analysis based 16S rDNA, *pmoA* and *mmoX* of isolated acidophilic methanotrophs shows the evolutionarily related

among these ten strains (Figures 2.2.-2.4.) Figure 2.2. and Figure 2.3. show the long distance between *Methylomonas paludis* and other acidophilic methanotrophs based on 16S rDNA and *pmoA* genes, respectively. Based on 16S rRNA sequences, *Methylomonas paludis* only has 80%-90%, homology to the acidophilic metahntorophic species in class α-proteobacteria. The *pmoA* gene sequence of *Methylomonas paludis* shows 71% homology to that of *Methylococcus capsulatus* which both species belong to the class of γ-proteobacteria. Species belonging to class α-proteobacteria have higher similarity (73%-87%) in *pmoA* gene. As shown in Figure 2.6, the *mmoX* genes of speices in class α-proteobacteria show 80%-86% similarity. These analyses showed that the genus of *Methylosinus* and *Methylocystis* are more closely related to each other. Similarly, *Methylocella* are closely related to. *Methyloferula.*

2.6. Role of Methanotrophs in degrading environmental pollutants

2.6.1. Cometabolic degradation of environmental pollutants by MMOs

While MMOs are expressed to oxidize methane, MMOs are also known for their ability to degrade a wide range of environmental pollutants through cometabolic reactions (Singh and Singh 2017). Cometabolic biodegradation is a biological process that microorganisms degrade non-growth contaminants using the enzymes that were expressed for the transformation of their growth substrates. The expressed enzymes commonly are non-specific, enabling the enzymes to catalyze both growth and non-growth substrates.

Monooxygenases including pMMO, sMMO and PrMO have been shown to degrade chlorinated solvents and release chloride (Anderson and McCarty 1997b, Little et al. 1988a). These monooxygenases were able to degrade benzene, toluene, ethylbenzene and xylene (BTEX), mainly benzene and toluene (He et al. 2018, Kulikova and Bezborodov 2000, Lee et al. 2011,

Wilkins et al. 1994). As shown in Table 2.2, MMOs are able to degrade chlorinated aliphatics such as trichlorethylene (TCE), trichloropropane (TCP), vinyl chloride (VC), and chloroform, aromatics such as BTEX, aromatic hydrocarbons and other organic pollutants. TCE, TCP, VC, and BTEX are common ground groundwater contaminants. TCP and 1,4- dioxane have been used as stabilizers for chlorinated solvents and are often coexist with TCE.

sMMO can degrade naphthalene, but pMMO cannot (Brusseau et al. 1990, Chang et al. 2002). sMMO and PrMO are able to oxidize several emerging contaminants, such as methyl tertiary-butyl ether (MTBE) (Hesselsoe et al. 2005, Steffan et al. 1997) and N-nitroso-dimethylamine (NDMA) (Sharp et al. 2005); sMMO can degrade some pharmaceuticals, such as sulfamethoxazole (Benner et al. 2015) and ibuprofen (Dawas-Massalha et al. 2014). Compared to sMMO and/or PrMO, pMMO is less active toward these contaminants and unable to degrade 1,4-dioxane or NDMA. Successful methanotrophic-based cometabolic degradation of these contaminants in contaminated sites (with neutral pH) have been reported {Frascari, 2015 #119;Semrau, 2011 #51;Pandey, 2014 #150}.

2.6.2. Implication and potential of acidophilic methanotrophs for bioremediation

As early as 1988, researchers found that methanotrophs biodegraded trichloroethylene (TCE) through cometabolic process that occurred when the organism is actively metabolizing a suitable growth substrate, such as methane or methanol (Little et al. 1988a). .In the following thirty years, researchers found the aerobic cometabolism was present in varies microorganisms, including *Pseudomonas butanovora* (Halsey et al. 2005, Hamamura et al. 1997), *Methylomonas methanica* (Koh et al. 1993a), *Mycobacterium vaccae JOB5* (Halsey et al. 2005, Hamamura et al. 1997, Wang and Chu 2017), *Nocardioides sp. CF8* (Halsey et al. 2005), *Rhodococcus*

aetherovorans (Frascari et al. 2006), *Methylosinus trichosporium OB3b* (Hamamura et al. 1997, Mahendra and Alvarez-Cohen 2006), *Burkholderia cepacia G4* (Mahendra and Alvarez-Cohen 2006), *Pseudomonas citronellolis* (Bravo et al. 2015), *Planococcus sp*. (Domaradzka et al. 2015), *Rhodococus jostii RHA1 (Wang and Chu 2017) etc*. Bacteria expression nonspecific monooxygenase, such as AMO (Ely et al. 1997, Zhou et al. 2019), PrMO (Wang and Chu 2017), butane monooxygenase (BMO) (Halsey et al. 2005, Hamamura et al. 1997) and alkane monooxygenase (AlkMO) (Bravo et al. 2015) are also able to biodegrade chloroform, TCE, 1,4-dioxane, MTBE and other pollutants. The efficiency of pollutant degradation by methanotrophs varies on the form and availability of MMOs. sMMO-expressing cells typically degrade more compounds than pMMO-expressing cells and degrade such compounds at faster initial rates (Alvarez-Cohen and McCarty 1991, Anderson and McCarty 1997b, Lee et al. 2006, Lontoh and Semrau 1998c, Shukla et al. 2009, Tsien et al. 1989a). Much of the early literatures focused on the utility of sMMO expressing methanotrophic community for polluted degradation (Alvarez-Cohen and McCarty 1991, Lee et al. 2006, McDonald et al. 1997b, Shigematsu et al. 1999). .However, more recent studies suggest that pMMO-expressing methanotrophs are more easily to survive in the condition with complex and high concentration of pollutants (Tavormina et al. 2011). It is thought that sMMO expressing methanotrophs accumulate toxic products faster and further pollutants oxidation is inhibited. The overall pollutant degradation by sMMO expressing methanotrophs will be less than that observed for pMMO-expressing microorganisms (Tavormina et al. 2011).

Accessing the potential of using acidophilic methanotrophs for bioremediation contaminated acidic environments has just begun in recent years. Two driving forces might be able to explain for this quest. First, acidic environments such as low pH groundwater (pH <5.5)

are common in many regions, including the Northern Atlantic Coastal Plain aquifer system in the US, which runs from the south of Long Island, New York to the majority parts of North Carolina (Denver et al. 2015). As many acidic aquifers are contaminated with chlorinated solvents and current bioremediation strategies that work only under neutral pH conditions, there is a urgent need to seek for alternative bioremediation strategies to remediate contaminated, acidic aquifers. The second driving force might be stepped from the recent findings of facultative methanotrophs. Among the acidophilic methanotrophs isolates, five of them are facultative. Unlike known neutrophilic methanotrophs, these acidophilic methanotrophs are more likely to present and/or survive in these acidic environments with and/or without methane and other multi-carbons. The big question is to determine if these acidophilic methanotrophs are present in the acidic environment and if they are able to express MMO for contaminant degradation.

Jeongdae Im *et al.* studied one methanotroph *Methylocystis* strain SB2, which was isolated from neutral medium. They found this bacterium constitutively expressed pMMO growing with ethanol and successfully degraded vinyl chloride (VC), trans-dichloroethylene (t-DCE), TCE and 1,1,1-trichloroethane (1,1,1-TCA) through cometobolism (Im and Semrau 2011, Jagadevan and Semrau 2013). Our recent study reported that active acidophilic methanotroph existed in acidic aquifers. Active methane-growing cultures in low pH groundwater have also showed their ability to biodegrade TCE. Using stable-isotope-probing technique, we detected much phylogenetically diverse active methanotrophs in low pH groundwater microcosms (Shao et al. 2019). Future studies about the biodegradation potential of mixed cultures and pure strains, especially acidophilic methanotrophs growing with alternative carbon sources and their utility in contamination sites are needed.

Table 2.1. Compounds degraded by monooxygenases

	Chlorinated aliphatic hydrocarbons				Aromatic hydrocarbons		Other compounds			
	TCE	TCP	vinyl chloride (VC)	chloroform	naphthalene	BTEX	1,4-dioxane	MTBE	NDMA	pharmaceut-icals
pMMO	(Anderson and McCarty 1997b)	N/A	(Anderson and McCarty 1997b)	(Im and Semrau 2011)	/	(Lee et al. 2011)	/	N/A	/	N/A
sMMO	(Little et al. 1988a)	(Bosma and Janssen 1998)	(Tsien et al. 1989a)	(Oldenhuis et al. 1989b)	(Brusseau et al. 1990)	(Wilkins et al. 1994)	(Mahendra and Alvarez-Cohen 2006)	(Hesselsoe et al. 2005)	(Sharp et al. 2005)	(Benner et al. 2015)
PrMO	(Wackett et al. 1989)	(Wang and Chu 2017)	(Wackett et al. 1989)	(Malachowsky et al. 1994)	N/A	(Kulikova and Bezborodov 2000)	(Mahendra and Alvarez-Cohen 2006)	(Steffan et al. 1997)	(Sharp et al. 2005)	N/A

/ —— not able to degrade; N/A —— no related literature

Table 2.2. Characteristics of isolated acidophilic methanotrophs

Strain Name	Source	Optimum pH	Optimum temperature	MMO expressed	Phenotype	Phylogenetic affiliation	Formaldehyde assimilation	Doubling time	Reference
Methylocella palustris	Sphagnum peat bogs	5.0-5.5	15-20°C	only sMMO	obligate	*α-Proteobacteria*	Serine	17-46 h	(Dedysh et al., 2000)
Methylocapsa acidiphila	Sphagnum peat bogs	5.0-5.5	20-24°C	only pMMO	obligate	*α-Proteobacteria*	Serine	15-40 h	(Dedysh et al., 2002)
Methylocella silvestris	Forest cambisol	5.5	15-25°C	only sMMO	facultative	*α-Proteobacteria*	Serine	2.1 d	(Dunfield et al., 2003)
Methylocella tundrae	Sphagnum tundra peatlands	5.5-6.0	15°C	only sMMO	facultative	*α-Proteobacteria*	Serine	18-45 h	(Dedysh et al., 2004)
Methylocystis heyri	Sphagnum peat-bog lake	5.8-6.2	25°C	both	facultative	*α-Proteobacteria*	Serine	7.7 h	(Dedysh et al., 2007)
Methylocapsa aurea	Forest soil	6.0-6.2	25-30°C	only pMMO	facultative	*α-Proteobacteria*	Serine	55.6 h	(Dunfield et al., 2010)
Methyloferula stellata	Sphagnum peat bogs	4.8-5.2	20-23°C	only sMMO	obligate	*α-Proteobacteria*	Serine and RuBP	66-200 h	(Vorobev et al., 2011)
Methylomonas paludis	Sphagnum peat bogs	5.8-6.4	20-25°C	only pMMO	obligate	*γ-Proteobacteria*	RuMP	13.8-17.3 h	(Danilova et al., 2013)

Table 2.2. Continued

Strain Name	Source	Optimum pH	Optimum temperature	MMO expressed	Phenotype	Phylogenetic affiliation	Formaldehyde assimilation	Doubling time	Reference
Methylocystis bryophila	Sphagnum peat-bog lake	6.0-6.5	25-30°C	both	facultative	*α-Proteobacteria*	Serine	11.5 h	(Belova et al., 2013)
Methylocapsa palsarum	Collapsed palsa soil	5.2-6.5	18-25°C	only pMMO	obligate	*α-Proteobacteria*	Serine	25 h	(Dedysh et al., 2015)

Figure 2.4. Phylogenetic tree based on 16S rDNA

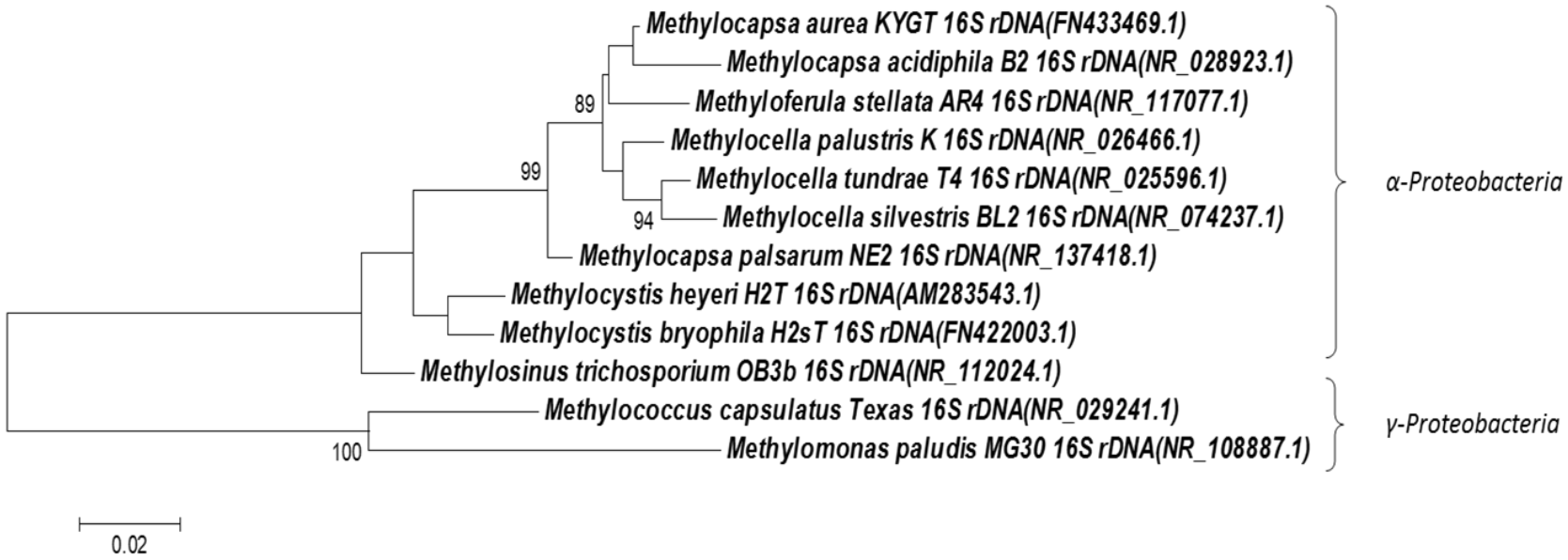


Figure 2.5. Phylogenetic tree based on *pmo*A

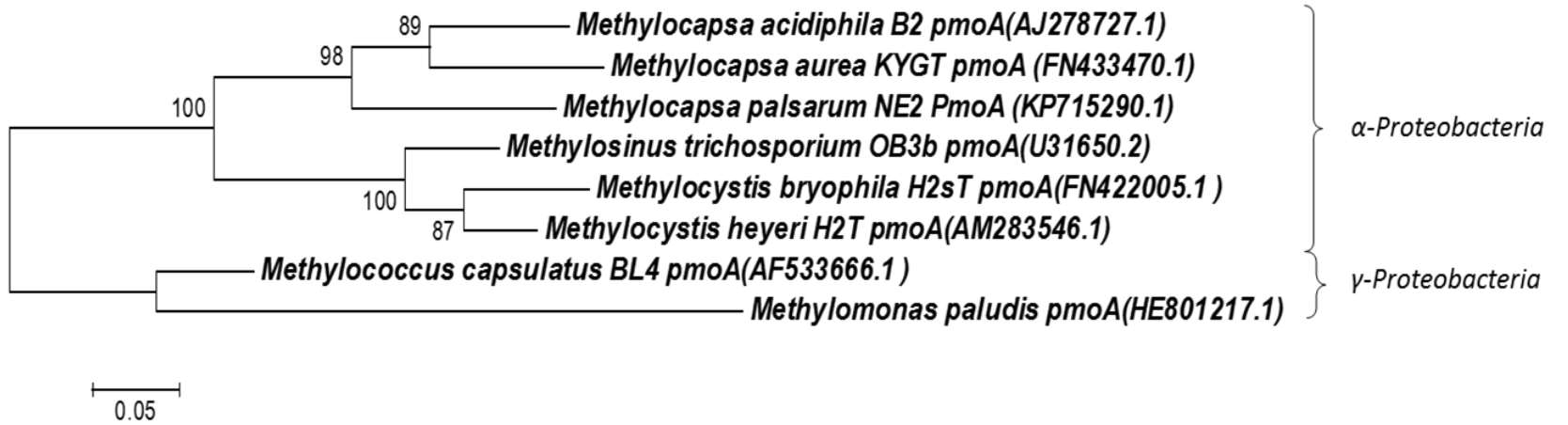
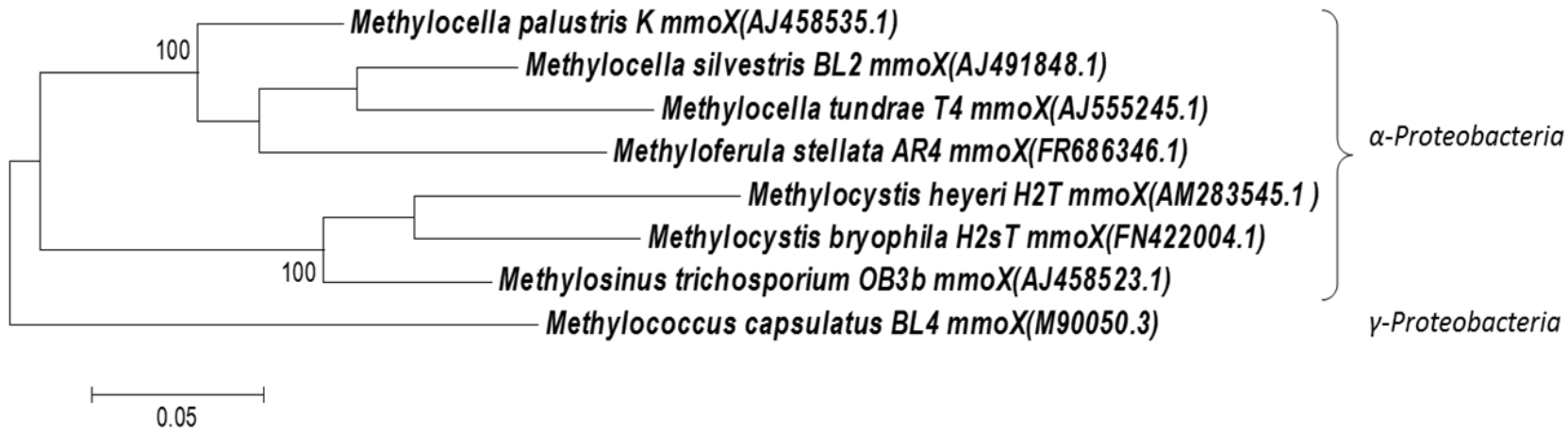


Figure 2.6. Phylogenetic tree based on *mmo*X



3. EVALUATION OF METHANOTROPHIC BACTERIA COMMUNITIES CAPABLE OF BIODEGRADATING TRICHLOROETHENE(TCE) IN ACID AQUIFERS*

3.1. Introduction

Trichloroethene (TCE), a common degreasing agent used in mining, chemical production and electric utilities (EPA 2017), is one of the most commonly detected groundwater contaminants in the US. Exposure to TCE can impact the liver, kidneys, immune system, and the central nervous system (EPA 2016). TCE is a suspected carcinogen with a Maximum Contaminant Level (MCL) of 5μg/L for drinking water in the US. Approximately 9% of drinking water wells in the US are contaminated with TCE (EPA 2016), and 17% of the contaminated wells exceed the recommended MCL.

Both aerobic and anaerobic biodegradation of TCE have been intensively studied during the past few decades (Alvarez-Cohen and Speitel 2001, Jablonski and Ferry 1992, Kim et al. 2010, Little et al. 1988a, Maymo-Gatell et al. 1997, Nelson et al. 1988, Nelson et al. 1986, Neumann et al. 1996, Oldenhuis et al. 1991, Terzenbach and Blaut 1994, Uchiyama et al. 1989a, b, Wild et al. 1996). This wealth of the knowledge has enabled successful bioremediation of TCE-contaminated aquifers under neutral pH conditions. However, low pH groundwater (pH <5.5) is common in many areas, including the Northern Atlantic Coastal Plain aquifer system in the US, which runs south from Long Island, New York through much of North Carolina (Denver et al. 2015). This aquifer system, which includes a number of large military facilities and urban areas, is also

* Reprinted with permission from "Evaluation of methanotrophic bacterial communities capable of biodegrading trichloroethene (TCE) in acidic aquifers" by Yiru Shao, Paul B. Hatzinger, Sheryl H. Streger et al, 2019. Biodegradation, volume 30, pp173-190, copyright(2019) by Springer.

significantly impacted by chlorinated volatile organic compounds (cVOCs) (Denver et al. 2015). Adjusting pH in large dilute plumes is challenging and impractical, and current anaerobic biostimulation and bioaugmentation technologies are ineffective in these low pH environments (Vainberg et al. 2009, Yang et al. 2017).

Methanotrophs, which are present in many different environments, are a group of bacteria capable of expressing methane monooxygenase (MMO) to oxidize methane and many environmental pollutants, including TCE (Oldenhuis et al. 1991, Wilson and Wilson 1985). MMO occurs in both a soluble form (sMMO) and a membrane-bound particulate form (pMMO). The majority of methanotrophs contain pMMO only, but some have both pMMO and sMMO, and a very small number have sMMO only (DiSpirito et al. 1992, Fox et al. 1990, Hanson and Hanson 1996a, Koh et al. 1993b, Lee et al. 2006, Lontoh and Semrau 1998a, Oldenhuis et al. 1991). Compared to pMMO, sMMO has a broader substrate range and can degrade TCE more rapidly (Lee et al. 2006). TCE degradation by methanotrophs was first observed in 1985 (Wilson and Wilson 1985) and later confirmed with *Methylosinus trichosporium* OB3b, a strain which can degrade TCE at rates exceeding 1.2 mmol/h per g (dry weight) (Tsien et al. 1989b). Although field trials have been conducted in which methane was applied *in situ* to stimulate pollutant biodegradation (Brockman et al. 1995, Pfiffner et al. 1997, Semprini et al. 1991), there is comparatively little research on the impact of indigenous methanotrophic populations on the natural attenuation of cVOCs in aquifers (Wymore et al. 2007). Moreover, a vast majority of the methanotrophs that have been the subject of biodegradation research to date grow most effectively at neutral pH, with inhibition occurring below pH 5 (Dedysh et al. 1998a, Semrau et al. 2010). As such, the potential importance of methanotrophs to the biodegradation of cVOCs or other pollutants in acidic aquifers has received little study.

In recent years, diverse acidophilic methanotrophs have been isolated from Sphagnum peat bogs where the pH ranges from 3.5 to 5 (Danilova et al. 2013, Dedysh et al. 2007, Dedysh et al. 2002, Dedysh et al. 2000, Dedysh et al. 1998a, Kip et al. 2011, Vorobev et al. 2011). Example organisms include *Methylocella palustris*[13] which is one of a few identified species containing only sMMO, and *Methylocapsa acidiphila* (Dedysh et al. 2002). Application of 16S rRNA-targeted fluorescent oligonucleotide probes has also lead to identification of other acidophilic methanotrophs (Dedysh et al. 2003), including *Methylocystis heyer* (Dedysh et al. 2007), *Methylosinus* (Kip et al. 2011), *Methyloferula stellate* (Vorobev et al. 2011), *Methylomonas paludis* (Danilova et al. 2013) and *Methylocaldum sp.* BFH1 (Islam et al. 2016). *Methylosinus* are the first acid-tolerant members of γ-*Proteobacteria* detected. Given that these acidophilic methanotrophs are capable of expressing MMOs, it is likely that they may be able to degrade TCE and other cVOCs via cometabolism; however, little is known about their degradation capabilities or their prevalence in acidic aquifers.

In this study, we applied a stable isotope probing (SIP)-based method, called Q-FAST (Cho et al. 2013), to identify active methanotrophs in acidic microcosms (i.e., pH <5) in which methane consumption and variable TCE degradation was observed. Molecular tools also were applied to determine the diversity of the acidophilic methanotrophic community and the catabolic genes encoding MMOs in relation to the TCE degradation observed in the microcosms. In addition, cultures capable of growing on methane were enriched from acidic aquifer samples and their ability to biodegrade various chlorinated ethenes was examined.

3.2. Materials and methods

3.2.1. Microcosm studies

Groundwater and aquifer sediments were collected from Joint Base McGuire-Dix-Lakehurst (JBMDL), NJ (Kirkwood formation) and from a US Navy site in Dahlgren, VA (Dahlgren). The Kirkwood aquifer has TCE in groundwater from a former parts washing operation. The natural groundwater pH in this region of the aquifer is $\leq$ 4.5. Aquifer solids were collected by direct push (Geoprobe) from 2.1 – 4.6 m below ground surface (BGS) and samples were transferred in the field from acetate sleeves to 1L glass screw-cap jars. Site groundwater was collected from a single well screened in the aquifer using low-flow sampling. At the Dahlgren site, which is not contaminated with TCE or any other cVOCs but has a natural pH of ~ 4.0 – 4.5, saturated aquifer material was collected from a depth of 1.4 - 1.7 m BGS using a hand auger, and placed in 1L glass jars. Groundwater was collected from a single well using low-flow sampling as for the Kirkwood site.

Samples were immediately placed in coolers on ice and hand delivered to APTIM's Lawrenceville, NJ laboratory. Aquifer solids and groundwater from each site were homogenized immediately prior to microcosm preparation. Microcosms were prepared in 160 mL serum bottles by adding 25 g site soil and 85 mL site groundwater. For each site, all replicate microcosms received 10% methane (v/v) in the headspace and a sterile aqueous solution of inorganic nutrients supplying 10 mg/L N from KNO_3 and 10 mg/L P from KH_2PO_4. Twelve of these microcosms were then amended TCE at ~ 250 - 500 µg/L, six of which also received mercuric chloride (300 mg/L) as a killing agent. Bottles were sealed with Teflon-lined butyl rubber septa and aluminum crimp caps, and incubated horizontally at 15^{o}C on a rotary shaker.

Aqueous samples were removed at selected time points through the septa using Gastight glass syringes and analyzed for cVOCs via EPA Method 8260B (gas-chromatography-mass spectrometry). The pH of the aqueous samples was analyzed using a microprobe or pH test strips. The liquid volume removed was replaced with sterile-filtered room air so that a vacuum was not created. Headspace samples were removed similarly and analyzed for oxygen and methane via EPA Method 3810, RSK-175 (gas chromatography). The aqueous methane concentration was calculated from headspace samples using Henry's Law.

In the Kirkwood microcosms, methane was re-added to bottles on days 20, 25, 33, and 36 of incubation. On day 33, half of the bottles (i.e., 3) in each treatment received 10 % ^{13}C-methane, (99 atom %; Sigma-Aldrich, Milwaukee, WI) while the other half continued to receive isotopically unenriched methane. Slurry samples (25 mL) were collected from all microcosms on Day 35, and the bottles were amended a second time with 10 % methane (^{13}C or unenriched as per the previous addition) on Day 36. A second set of slurry samples were collected from each bottle after 10-23 days of incubation. The slurry samples were preserved by freezing at -20^{o}C, and microbial community analysis was performed via SIP as described below.

The Dahlgren microcosms received additional unenriched methane day 54. On day 81, when this methane was depleted, duplicate bottles from the live treatment with TCE received ^{13}C-methane and duplicates continued to receive unenriched-methane. The pH in the bottles that were evaluated by SIP averaged ~ 4.4 SU. The pH in several of the live bottles with methane dropped to $\leq$ 2.0, and no loss of methane or TCE was detected in these samples over the course of the experiment, so these microcosms were excluded from the SIP study. The reason for this decline in pH is unclear, but may be a result of the low alkalinity of the samples. Samples for SIP and microbial community analysis were collected from the remaining microcosms 1 day, and then

again 2-3 days after ^{13}C-methane addition. Samples of the microcosm slurry were removed through the septa, frozen, and analyzed via SIP as for the Kirkwood samples.

3.2.2. Genomic DNA extraction and 13C-DNA and 12C-DNA separation

DNA in the frozen slurry samples were extracted using FastDNA spin kit for soil samples (MP Biomedical, Solon, OH) and the extracted DNA was separated into ^{13}C-DNA and ^{12}C-DNA fractions using equilibrium centrifugation in CsCl density gradients as described previously (Cho et al. 2015, Cho et al. 2016, Denver et al. 2015). The concentrations of the extracted DNA were determined using a NanoDrop ND-1000 Spectrophotometer (Fisher Scientific., Fair Lawn, NJ). Distribution of 16S rRNA gene copies in each DNA fraction was determined. The fractions showed the highest 16S rRNA gene copies were selected for further molecular analyses, including cloning and sequencing for bacterial identification and diversity of MMO, as well as real-time t-RFLP assay for microbial community structure profiling. The detailed description is available in appendix.

3.2.3. Detection of pmoA and mmoX genes

The extracted DNA and fractionated DNA were screened for the presence of pMMO and sMMO using primer sets specific for *pmo*A and *mmo*X (Dedysh et al. 2005, McDonald et al. 2008, Rahman et al. 2011a) genes, respectively. Two *pmo*A primer sets, a *pmoA* forward primer A189f (5'-GGNGACTGGGACTTCTGG-3') in combination with two different *pmoA* reverse primers mb661r (5'-CCGGMGCAACGTCYTTACC-3') and A682r (5'-GAASGCNGAGAAGAASGC-3'), were used. For sMMO detection, two different primer sets, *mmo*X (A-B) and *mmo*X (LF-LR) were used. The *mmoX (A*-B) primer set, forward *mmo*XA (5'-ACCAAGGARCARTTCAAG-3') and *mmo*XB (5'-TGGCACTCRTARCGCTC-3'), were designed from six known full *mmoX*

gene sequences; this primer set has been widely used to detect the presence of sMMO (Auman et al. 2000, Bussmann et al. 2006, Iguchi et al. 2011, Shukla et al. 2009). The *mmo*X(LF-LF) was originally designed for *Methylocella* genus-specific-sMMO (Rahman et al. 2011a). The forward primer *mmo*XLF (5'-GAAGATTGGGGCGGCATCTG-3') and reverse primer *mmo*XL (5'-CCCAATCATCGCTGAAGGAGT-3') were developed to amplify the *mmoX* genes in *Methylocella* spp. All primers were custom ordered from Integrated DNA Technologies, Inc. (Coralville, CA) and used in regular PCR amplifications. The 25-µL PCR reaction was prepared as follows: Each 25-µL PCR mixture contained 400 nM forward/reverse primers set and 12.5 µL of Taq DNA polymerase PCR mix buffer (Qiagen, Valencia, CA). The PCR amplification reactions were conducted in an automated thermal cycler (PCR Sprint system, Thermo Electron Corp. Waltham, MA). The thermal cycling protocol is as follows: 95 °C for 15 min; 35 cycles of 95 °C for 1 min, 55 °C for 45 sec, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The presence of PCR amplicons was determined using gel electrophoresis.

3.2.4. Analysis of active microbial community structure using real-time t-RFLP

To characterize active methane-oxidizing microbial community structure in each microcosm, real-time terminal restriction fragment length polymorphism (real-time-t-RFLP) assays were performed as described previously (Cho et al. 2013, Yu and Chu 2005). Briefly, a region of 16S rDNA sequence (352 bp long) was amplified with a fluorescence-labelled forward primer 16S1055f (5C'-hexachlorofluorescein-ATGGCTGTCGTCAGCT-3C'), a reverse primer 16S1392r (5C'-ACGGGCGGTGTGTAC-3C'), and a *Taq*man probe 16S*Taq*1115f (5C'-[6-carboxyfluorescein]-CAACGAGCGCAACCC-[6-carboxytetramethylrhoda mine]-3C'). The resulting PCR products were digested with MspI and the digested PCR products were analysed by

an ABI 3130xl Genetic Analyzer (DNA Technologies Core Lab, College Station, TX). Details of this procedure are provided in Appendix.

3.2.5. Cloning and sequencing

The identities of active methane-oxidizing microorganisms were determined based on the 16S rRNA gene sequences derived from the 13C-DNA fractions of the microcosms receiving 13C-methane. The PCR reactions for 16S rRNA gene amplification were performed as described by Roh et al., 2009. All DNA fragments samples were sent to Eton Biosciences Inc., which used ABI 3730xl DNA Sequencers to run sequencing. Phylogenetic analyses of aligned sequences were created using neighbor-joining approaching MEGA version 7.0.

3.2.6. Enrichment culture of methanotrophs from an acidic aquifer

A microbial enrichment with methane as a sole carbon source was initiated using site solids from Dahlgren. The enrichment was prepared by placing aquifer solids into ATCC Medium 2157 (*Methylocella* medium) at a ratio of 0.1 g soil per 10 mL medium in an Erlenmeyer flask. The medium was adjusted to pH 4 with phosphoric acid. Methane was provided in the headspace at 10% as the sole source of carbon and energy, with the balance of the headspace comprised of sterile-filtered room air. Once the culture grew turbid, it was passed at a ratio of 1 mL culture to 100 mL medium into fresh medium of the same type.

Following three successful passes into fresh medium, the ability of the enrichment culture to biodegrade TCE, *cis*-DCE (*cis*-1,2-dichloroethene; DCE) and vinyl chloride (VC) was examined. Cells were washed via centrifugation (6,000 RPM x 20 min) to remove any residual substrate gas, resuspended in fresh ATCC 2157 medium, and 12 mL at OD 0.2 was placed in a 15

mL serum vial which was then sealed with a Teflon-lined septum and aluminium crimp seal. Each vial then received a spike of the relevant chlorinated ethane (20 µM; from a reagent-grade stock). Samples were taken immediately after the initial contaminant spike (i.e., time zero), and at set timepoints thereafter and analyzed for cVOCs as described previously. Copper (Cu) as $CuCl_2$ was added at 1 µM to promote the production of pMMO, and formate was added at 20 mM as a source of reducing equivalents during culture growth. A second culture was grown with formate but without Cu to induce sMMO preferentially.

3.3. Results and discussion

3.3.1. Methane and TCE degradation in groundwater microcosms

Kirkwood

The initial pH in the Kirkwood microcosms was ~ 3.4. The pH in the microcosms rose somewhat over the course of this study, but was less than 4.0 in all bottles at the conclusion of the study (Appendix, Figure A1). The consumption of methane in the sample bottles was observed after the first week of incubation, with calculated aqueous concentrations declining from ~ 1,650 µg/L on Day 7 to < 1 µg/L on Day 19 (Figure 3.1.a). Methane was consumed at similar rates irrespective of the presence or absence of TCE. Headspace methane was re-added on Day 20, and three additional times after it was observed to have been depleted (days 20, 25, 33, and 36). In contrast, there was little (~12%) methane loss in the killed controls over the course of this study.

There was a lag period of ~ 3 weeks before TCE degradation was detectable in the Kirkwood bottles (Figure 3.1.c). TCE was reduced from 392 ± 40 µg/L to 74 ± 40 µg/L over the following 5 weeks. A first order rate constant of 0.050 day^{-1} was calculated for TCE (r^2 = 0.96)

from Day 21 to Day 55. TCE in the killed controls also declined somewhat over time, being reduced from 339 ± 71 µg/L to 267 ± 54 µg/L over 8 weeks of incubation. The reduction in TCE in the killed controls was likely due to volatile losses during sampling. The first order rate constant for the loss of TCE in the killed samples from Day 21 to Day 55 was 0.019 day^{-1} (r^2 = 0.95), or about 2.5 times lower than for the live samples. These data clearly indicate the biodegradation of both methane and TCE in microcosms with a starting pH of 3.4. It should also be noted that TCE degradation rates may have been significantly higher in the microcosms if methane was not re-added several times to the bottles, as methane is known to be a competitive inhibitor to TCE biodegradation by MMO enzymes (Oldenhuis et al. 1989c). The addition of methane multiple times was necesssary to increase the biomass of the methanotrophic community (required for SIP), but likely also served to decrease the observed TCE degradation rates. It is likely that such rates would be highest shortly after the methane was completely consumed.

Dahlgren

Methane was biodegraded within about 21 days in the Dahlgren microcosms with TCE added whose pH remained at ~ 4.4, with minimal losses observed in the killed controls. As noted previously, the pH in two of the live microcosms with TCE dropped to ~ 2, and these were excluded from the study because no loss of methane or TCE was apparent. Methane that was added to the remaining four live microcosms on day 54 was completely consumed within 8 days, and that added on day 81 was then consumed within 3 days (Figure 3.1.b). The increasing rate of methane consumption is consistent with increasing numbers of methanotrophs in the microcosms. The loss of TCE in the microcosms was somewhat inconsistent among replicate bottles, with individual values ranging from ~ 13% to > 77% lower than the mean of the killed controls at the conclusion of the study. The mean reduction of TCE in the live samples was 33%, from 261±22

µg/L to 175±60 µg/L compared to a decline of only 9% in the killed controls from 264±14 µg/L to 242±21 µg/L (Figure 3.1.d). However, these data and those of a previous study in which TCE biodegradation was monitored in microcosms from the same location that were prepared and incubated similarly with or without 10% methane also showed methane-enhanced degradation of TCE (Appendix, Figure A2).

The data collected from the two acidic aquifers clearly show the existence of methanotrophs in these locations and provide preliminary evidence that these methanotrophs can be stimulated to aerobically biodegrade TCE. Studies with ^{13}C-methane were conducted to identify active methanotrophs in these aquifer systems.

3.3.2. Shift of buoyant densities of DNA in 13CH4-receiving microcosms

Samples collected from ^{13}C-methane-receiving microcosms were used to extract DNA and the ^{13}C-DNA was separated from ^{12}C-DNA fraction using gradient ultracentifugation as described previously. Each DNA fraction was analysed for the buoyant density (BD) and the abundance of 16S rRNA gene copies (Figure 3.2.). As shown in Figure 3.2., the BD of the DNA from both the Dahlgren and Kirkwood microcosm samples increased; the curves shifted toward the right over time. For example, for Dahlgren samples collected 1 and 3 days after ^{13}C-methane was added, buoyant densities of the peaks shifted from 1.715g/ml (D1-XT-1) to 1.718 g/ml (D1-XT-3). For Kirkwood, samples were collected 2 and either 14, 21 or 23 days after initially spiking with ^{13}C-methane (which was added again 3 days after the initial spike). The abundance peak moved further in these samples than for Dahlgren likely because of increased incubation time and the fact that a second spike of ^{13}C-enriched methane was added. As an example, for one set of samples, the peak of 16s rRNA shifted from 1.706 (K5-XT-2) to 1.718 (K5-XT-23) (Fig 3.2.d). The overall results

suggest that ^{13}C in the labelled methane was successfully incorporated into the DNA of active methane-oxidizing bacteria. Thus, the DNA fractions corresponding to the peaks circled in Figure 3.2 were selected for further molecular analyses, including fingerprinting of the active methanotrophic microbial community structure and bacterial identification based on 16S rRNA genes.

3.3.3. Identification of active acidophilic methanotrophs in the microcosms

A total of thirty-five 16S rRNA clones, with 24 clones from Dahlgren microcosms and 11 clones from Kirkwood microcosms, were derived. The phylogenetic relationships between these clones and the known acidophilic methanotrophs are shown in Figure 3.3.. The known acidophilic methanotrophs are generally clustered in α-Proteobacteria, whereas our clones group in both the α- and γ- Proteobacteria. Two clones from Dahlgren (D4-XT1-clone2 and D4-XT3-clone7) and four clones from Kirkwood (K5-XT23-clone3, K5-XT23-clone1, K5-XT2-clone3 and K5-XT2-clone7) were in the α-Proteobacteria, but they appear not to be closely related to the known acidophilic methanotrophs. The rest of clones clustered in γ-Proteobacteria distant from known acid-tolerant species. The results are unexpected but interesting, suggesting that methanotrophs present in acidic aquifers may be phylogenetically diverse and different from those in other acidic environments. Future work is needed to isolate methanotrophs from acidic aquifers to better understand their potential role in cVOC biodegradation in these environments.

3.3.4. Prevalence of pMMO and sMMO in acidic groundwater slurry microcosms

To understand the distribution of MMO in the microcosms, genomic DNA extracted from all microcosms was screened for the presence of *pmo*A and *mmo*X genes encoding pMMO and sMMO, respectively, using regular PCR. Either *pmo*A or *mmo*X genes, or both, were detected in

a number of the live samples, but not in any of the killed controls (Table 3.1). The *pmoA* gene was detected in many Dahlgren samples, but only in few of Kirkwood samples. While negative results were observed using the primer set for *mmoX* (A-B), several positive PCR products were obtained using the primer set of *mmoX* (LF-LR). *Methylocella* are a distinct taxonomic cluster of acidophilic methanotrophs (Dedysh et al. 2004, Dedysh et al. 2005, Dedysh et al. 2000, Dunfield et al. 2003), which only express a form of sMMO. Primer set of *mmoX* (LF-LR) is specific to sMMO of the *Methylocella* genus. Several known sMMO-expressing *Methylocella palustris, Methylocella silvestris,* and *Methylocella tundra* can be detected by the *mmoX* (LF-LR) primers. A previous study reported that this primer set can also amplify a small fragment of *mmoX* gene from other methanotrophs, such as *Methylobacter, Methylocystis or Methylococcus*.(Rahman et al. 2011a). Thus, the positive results using this primer set suggested that *Methylocella* and/or *Methylocell*a genus-like sMMO were present in the Dahlgren microcosms. Similarly, positive PCR products were also detected in Kirkwood microcosms (Table 3.1), suggesting *Methylocella* or *Methylocella* genus–like methanotrophs were also present at this site. A recent study in our laboratory revealed for the first time that *Methylocella palustris* is capable of degrading TCE at pH 5 (Hatzinger and Chu 2017). An additional experiment showed that the bacterium can degrade more than 1 mg/L of TCE in 24 hr at a pH as low as 4, although rates were marginally slower than for pH 5 or 6 (data not shown).

Figure 3.4. shows the diversity of *pmoA* genes obtained from Dahlgren and Kirkwood samples. Interestingly, two different clusters were observed for samples from the two different locations. The *pmoA* genes derived from Dahlgren samples showed a closer relationship between *Methylomonas paludis* MG30 and *Methylocaldum* sp. BFH1 (Islam et al. 2016), which belongs to **γ-***Proteobacteria* and can only express pMMO (Danilova et al. 2013). Most *pmoA* clones from

Dahlgren are clustered in **γ**-*Proteobacteria*. *Methylomonas paludis* sp. nov. is the only known acid-tolerant *Methylomonas* expressing pMMO (Danilova et al. 2013). As shown in Figure 3.4., the *pmoA* clones derived from Dahlgren samples are very different from the *Methylomonas* genus.

The diversity of *mmoX* genes is presented in Figure 3.5. The *mmoX* clones derived from Dahlgren samples are more closely related to *Methylococcus*. Different from those observed in Dahlgren samples, three clones from Kirkwood are in clustered in *α-Proteobacteria* and they are more closely related to *Methylocystis*. Once again, all *mmoX* genes detected in this study are different from those of *Methylocella*, suggesting that other non-*Methylocella* strains might have similar *mmoX* genes.

3.3.5. Active acidophilic methanotrophic microbial community structure in TCE-degrading microcosms

Active acidophilic methanotrophic microbial community structures in acidic TCE-degrading microcosms were examined using real-time-t-RFLP. The DNA fractions corresponding to the peaks (circled) in Figure 3.2. were used as templates for the analysis. The profiles derived from these templates can be considered as active acidophilic methanotrophic communities in the microcosms. As shown in Figure 3.6., several ribotypes (represented as t-RFs), t-RFs = 97, 103,105,114,117 bp, were observed in all Dahlgren and Kirkwood samples. T-RFs = 77 and 83 bp were detected in the Dahlgren samples, while t-RF = 99 bp was detected only in the Kirkwood samples.

The t-RF of 105 bp observed in all the profiles corresponds to the theoretical t-RF of several known methanotrophs, including *Methylomonas, Methylocystis, Methylosinus,* and *Methylocella,* suggesting these methanotrophs might be present in acidic groundwater. *Methylomonas* can only

express pMMO, *Methylocella* can only express sMMO, *and Methylocystis* and *Methylosinus* can express both pMMO and sMMO depending on growth conditions (Lee et al. 2006, Ul-Haque et al. 2015). *Methylocella palustris* (Dedysh et al. 2000), *Methylocella silvestris* (Dunfield et al. 2003*), Methylocella tundrae* (Dedysh et al. 2004*), Methylocystis broyphila (*Belova et al. 2013*), Methylosinus* (Kip et al. 2011*) and Methylomonas palustis* (Danilova et al. 2013) were isolated from acidic bogs and peatlands, which are typically highly oligotrophic environments. The results suggest that these or similar methanotrophs may also be present in acidic groundwater aquifers. Strains BFH1 and BFH2 are thermophilic methanotrophs within genera *Methylococcus.*(Islam et al. 2016) belonging to class **γ-***Proteobacteria*. The strains BFH1 and BFH2 optimally grow at pH 5.5 to 6.0 and their theoretical t-RF is 83 bp. *Methylococcus capsulatus* Bath is a well-studied Type I methanotroph that can tolerate low pH; it also has a theoretical T-RF of 83 bp. Therefore, as the fragment of t-RF = 83 bp was observed in community profiles of Dahlgren samples, it is possible that BFH1 and BFH2-like methanotrophs are present at the Dahlgren site. Further sequencing and/or isolation work would be required to confirm these results.

3.3.6. Enrichment culture of methanotrophs from an acidic aquifer

For the Dahlgren site, an enrichment culture was obtained with methane at pH 4. In an attempt to selectively grow methanotrophs possessing pMMO, Cu was added to some of the enrichment media to specifically stimulate this enzyme while inhibiting production of sMMO. Other enrichments were maintained Cu-free to selectively stimulate organisms with sMMO. After multiple transfers, the cultures were evaluated for degradation of various cVOCs, including TCE, *cis*-DCE and VC.

The methane-enrichment culture was observed to biodegrade TCE and VC, but not *cis*-DCE in tests with or without Cu in the medium (Figure 3.7.). This is an interesting observation because previous studies with pMMO- and sMMO-containing methanotrophs suggest that *cis*-DCE is a substrate for each of these enzymes, although degradation rates may be somewhat slower with pMMO than for sMMO (Anderson and McCarty 1997a, Semrau et al. 2011). Thus, the results herein suggest that the specificity of degradation of cVOCs may differ under low pH conditions, and for cultures that thrive under these conditions, than for typical methanotrophs that grow optimally at neutral pH. Further studies are required to better understand the range of cVOCs degraded by methanotrophs typically found in acidic groundwater environments.

Figure 3.1. Methane (a,b) and TCE (c,d) degradation in acidic aquifer microcosms from Kirkwood and Dahlgren. The error bar showed the standard deviation of triplicate samples

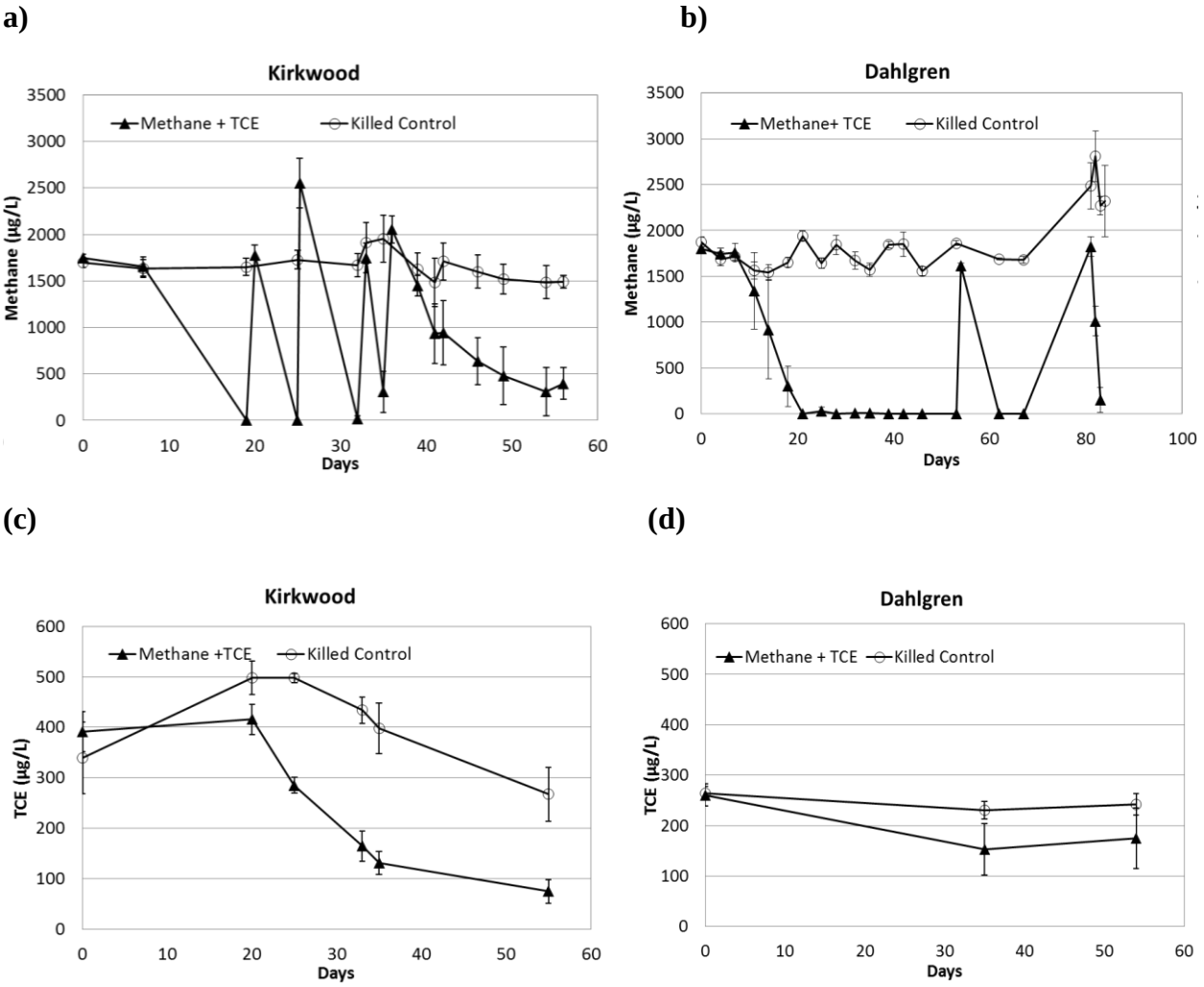


Figure 3.2. Relative abundance of 16S rRNA gene copies and gradient fractions of the day 1 sample (D1-XT-1) and day 3 sample (D1-XT-3) from Dahlgren Microcosm D1 (a); day 1 sample (D2-XT-1) and day 2 sample (D2-XT-2) from Dahlgren Microcosm D2 (b); day 2 sample (K4-XT-2) and day 21 sample (K4-XT-21) from Kirkwood Microcosm K4 (c); and day 2 sample (K5-XT-2) and day 23 sample (K5-XT-23) from Kirkwood Microcosm K5 (d)

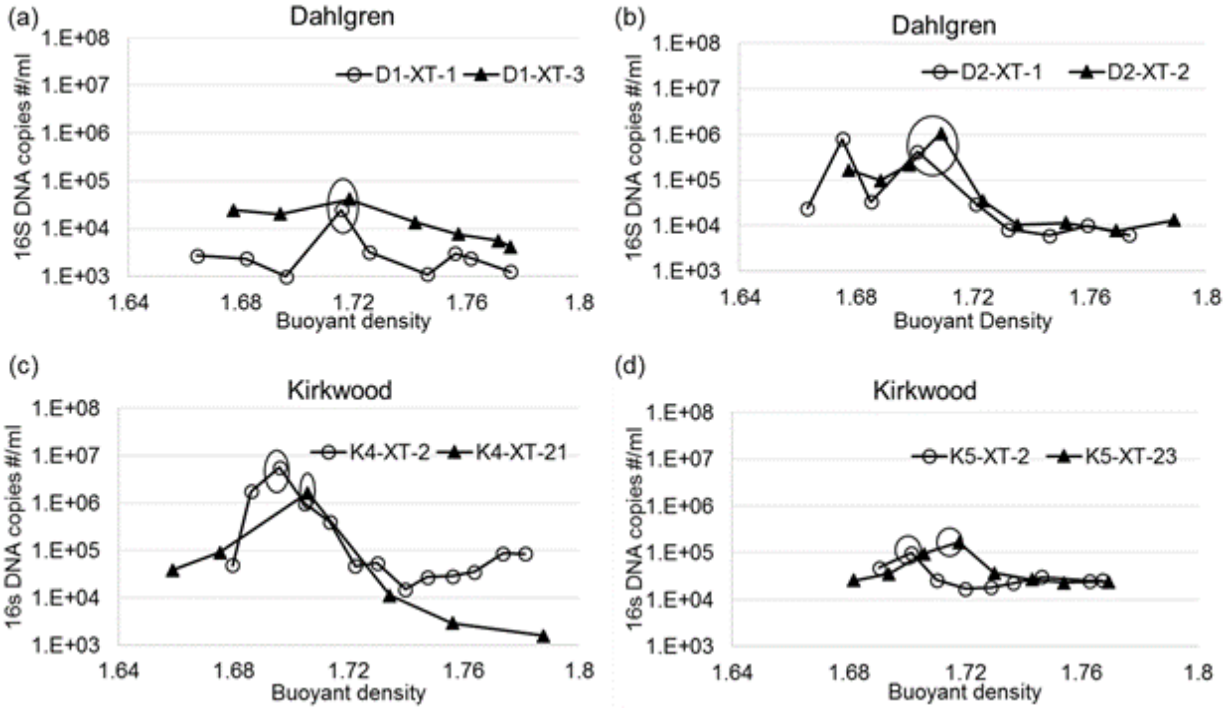


Figure 3.3. Phylogenetic tree based on 16s rRNA clones derived from acidic microcosms

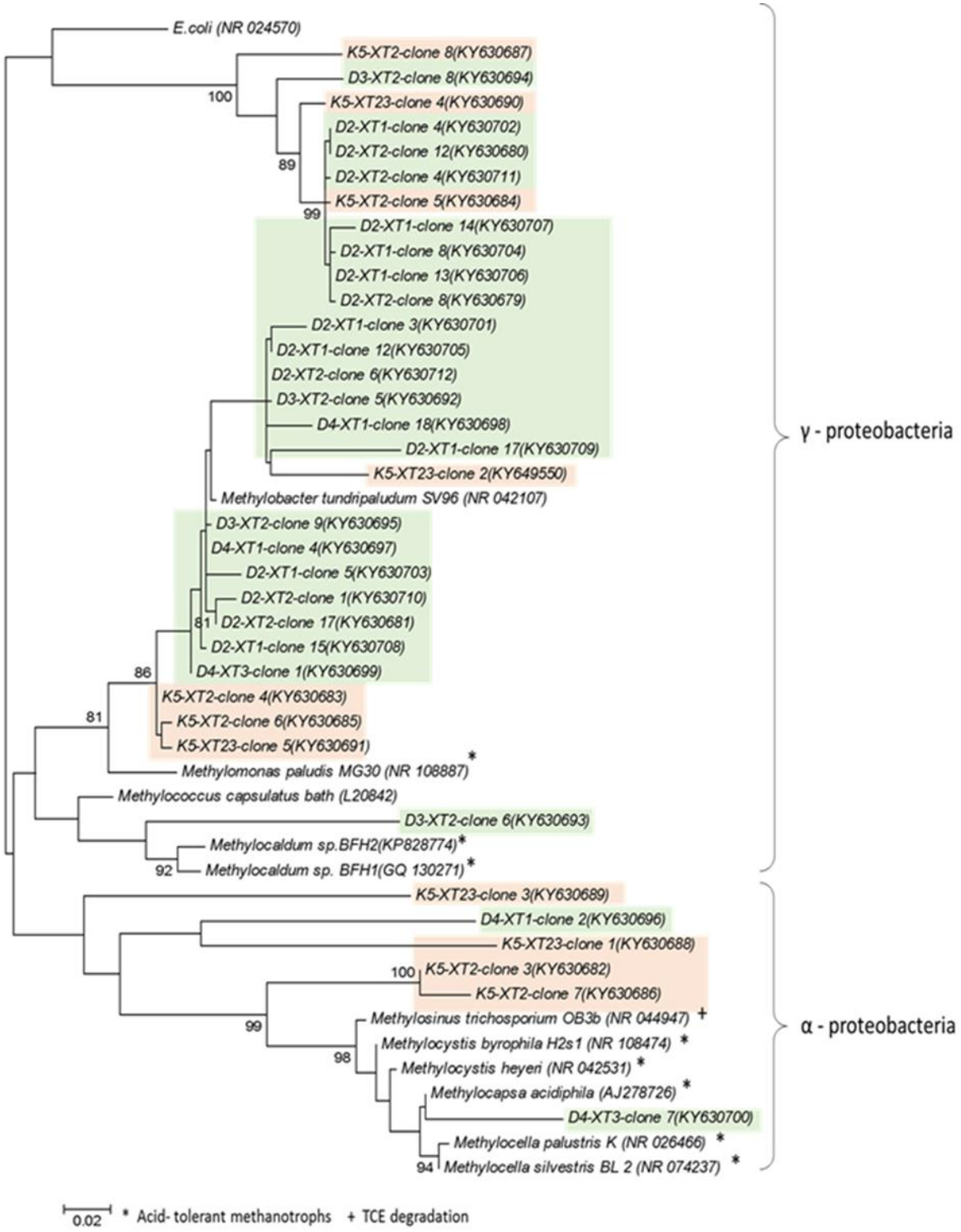


Figure 3.4. Phylogenetic tree based on *pmo*A

The bootstrap consensus tree inferred from 1000 replicates is presented. The percentage of replicate trees in which the associated sequence clustered together in the bootstrap test is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

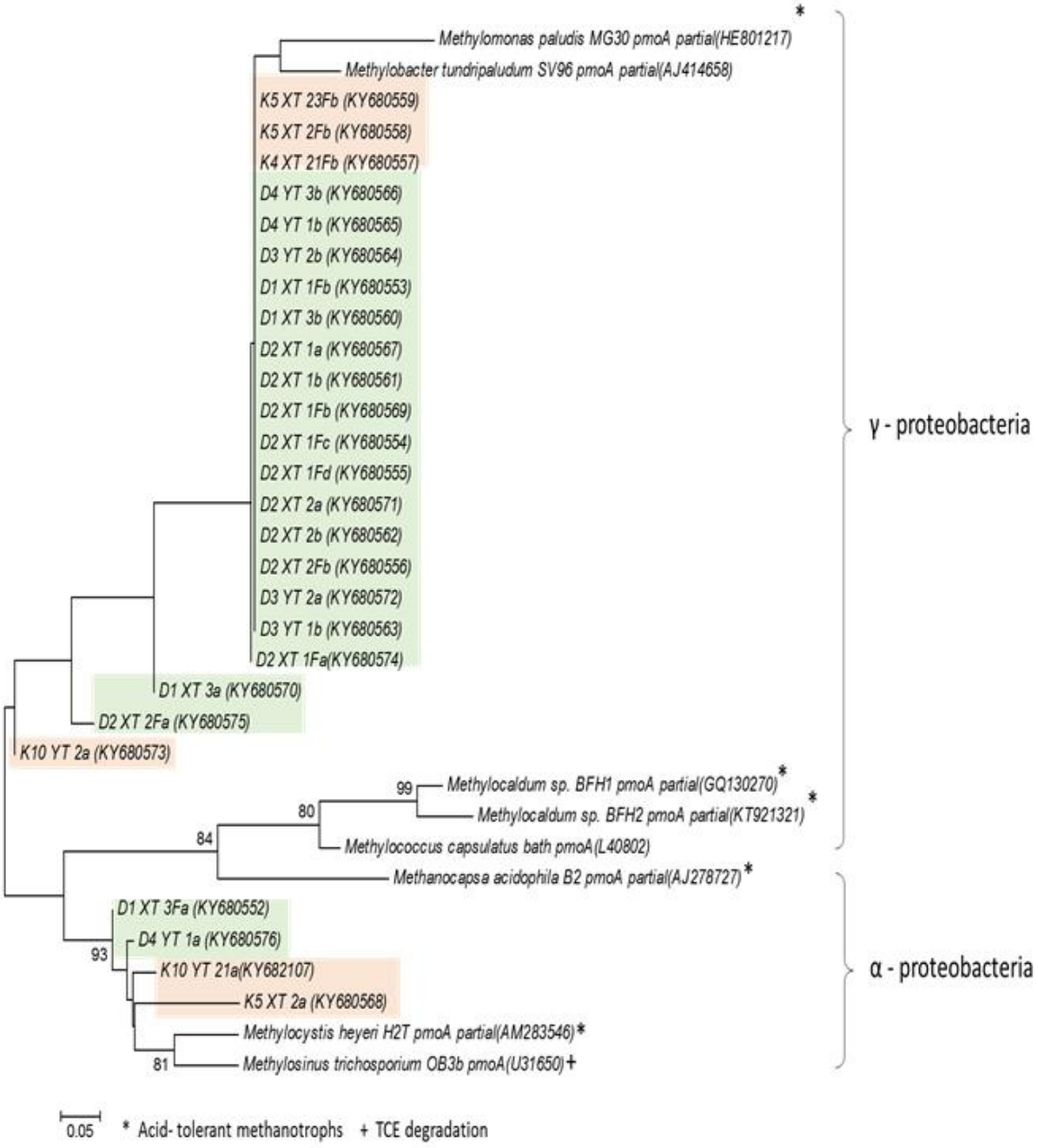


Figure 3.5. Phylogenetic tree based on *mmo*X(LF-LR)

The phylogenetic relations between samples and known acidophilic methanotrophs based on mmoX(LF-LR). mmoX(LF-LR) was designed from Methylocella silvestris BL2. BLAST results show that the primers also could be used to identify Methylocella palustris, Methylocella tundrae and Methylomonas sp. LC1. The mmoX genes in the samples from Dahlgren could be detected by this pair of primers. The phylogenetic tree shows that these genes are not closely related to Methylocella, but closed to Methylococcus capsulatus bath. They may be some uncultured acidophilic methanotrophs belong to other genre.

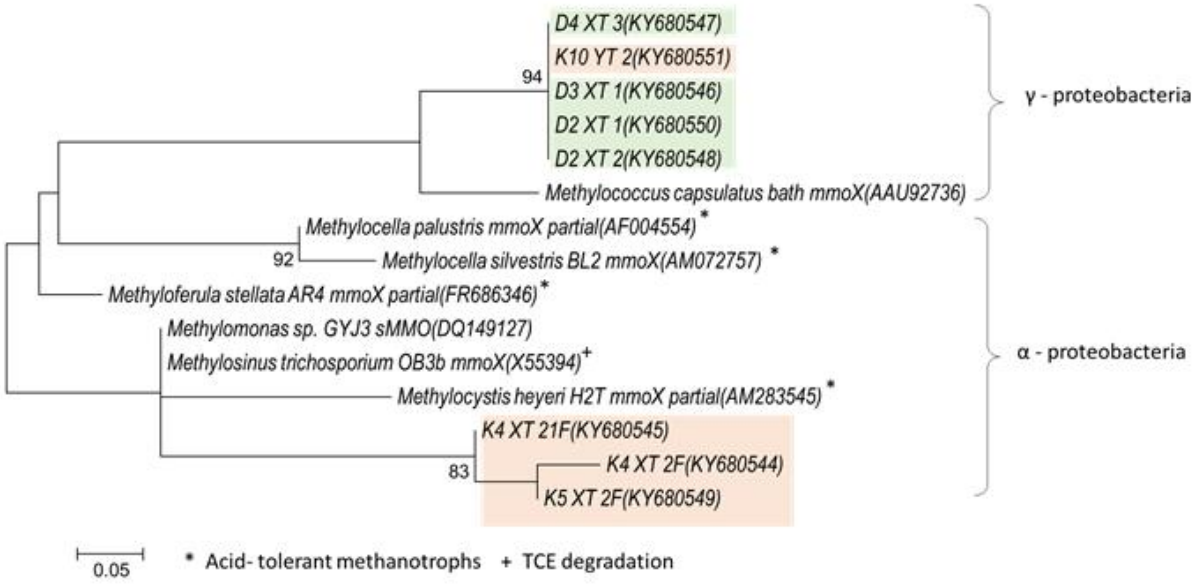
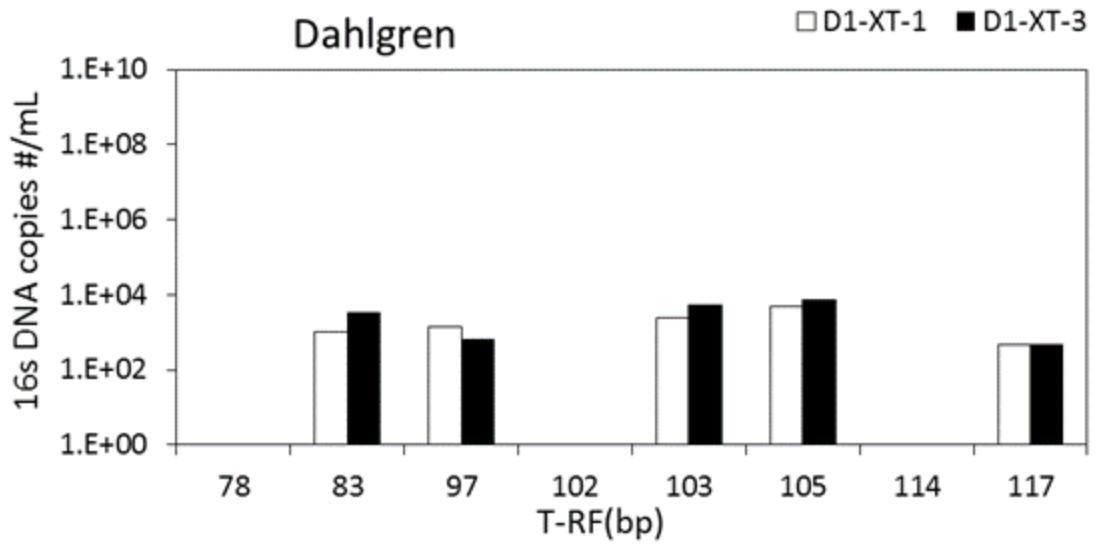


Figure 3.6. Changes of microbial community structure in TCE-degrading methanotrophic microcosms containing acidic aquifer samples from Dahlgren (a) and (b), and from Kirkwood (c) and (d)

(a)



(b)

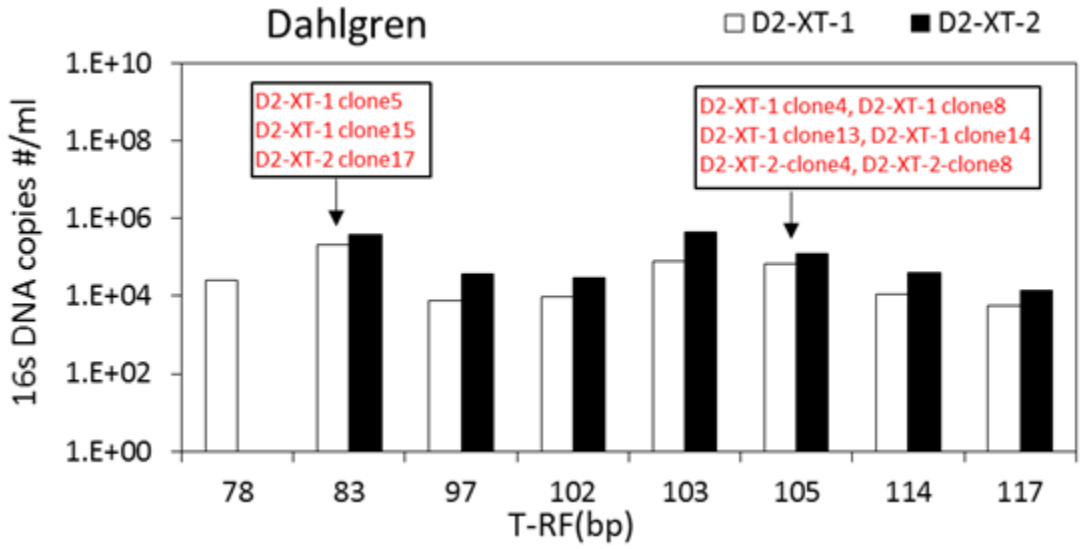
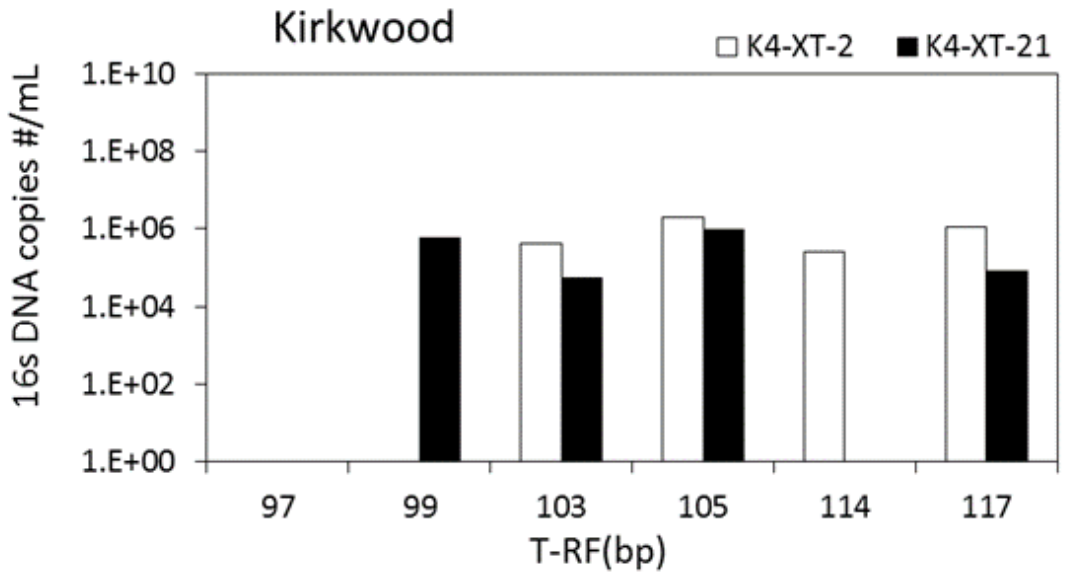


Figure 3.7. Continued

(c)



(d)

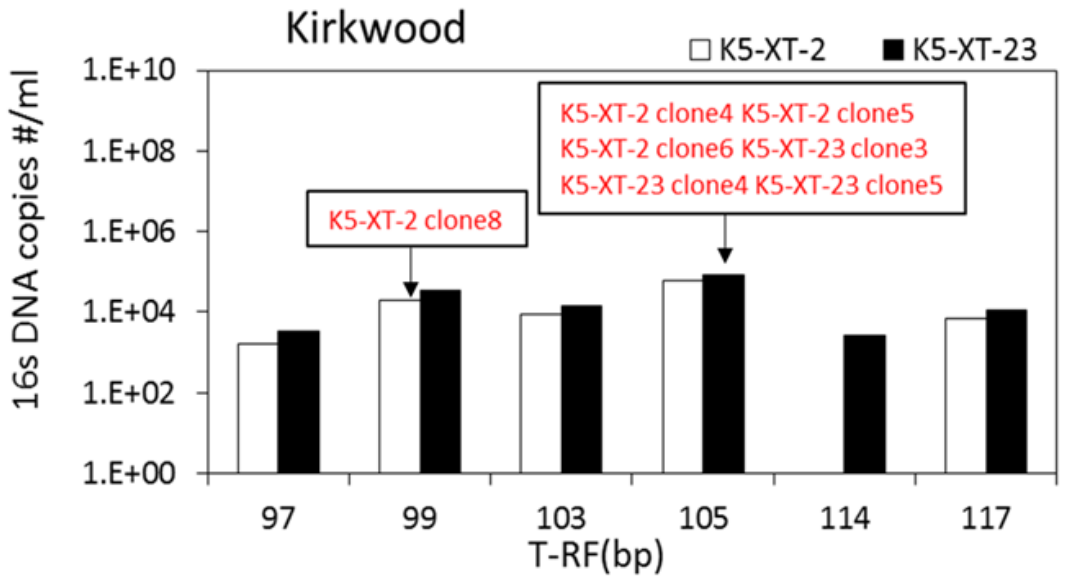


Figure 3.8. Biodegradation of TCE (top), cis-DCE (middle) and VC (bottom) at pH 4 by a methane-grown enrichment culture from the Dahlgren site. Values represent mean ± sd for duplicate samples. This culture was grown in the presence of 1 µM Cu, but similar results were obtained for a culture with no Cu. Control samples have no culture added

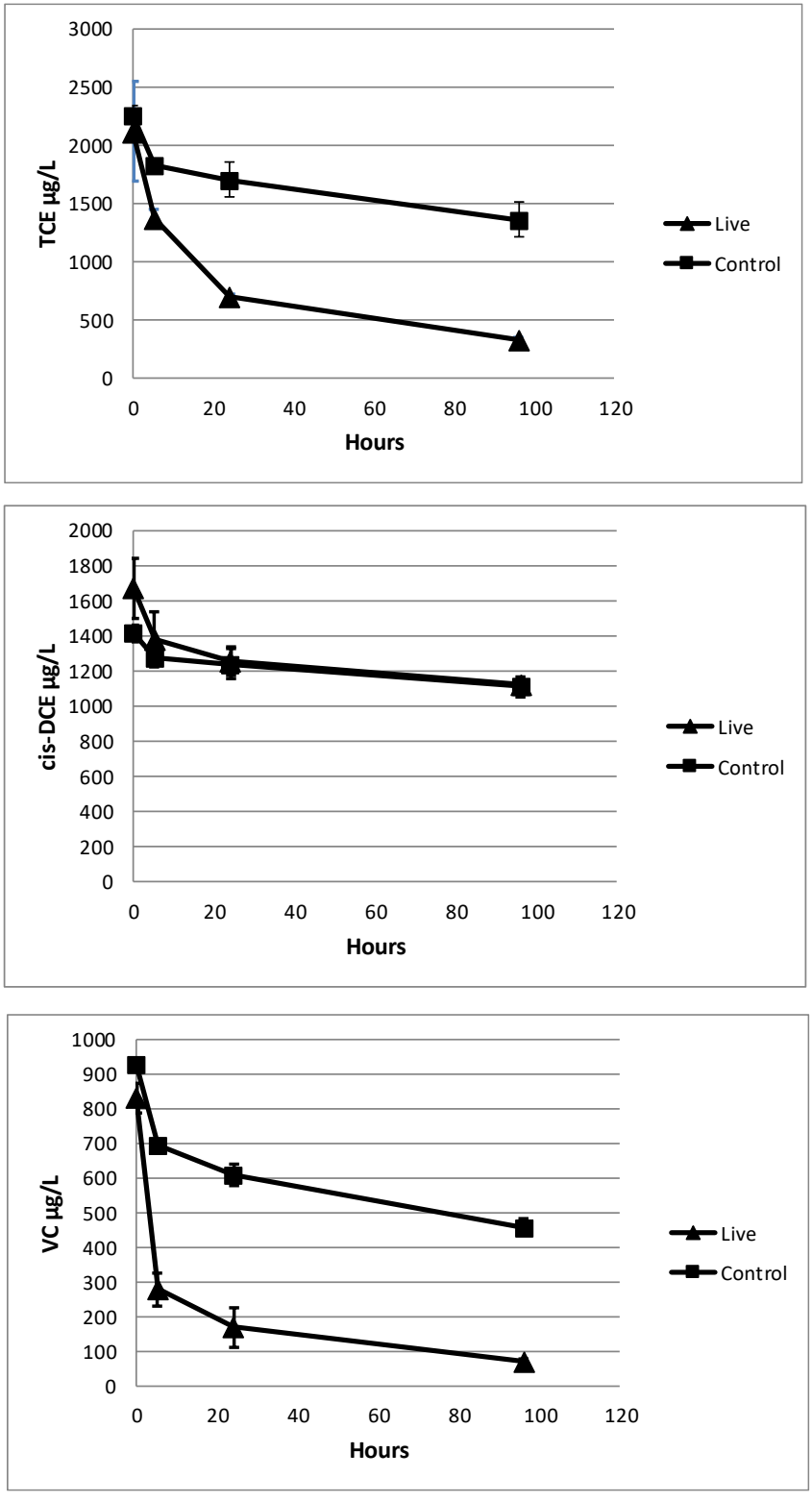


Table 3.1. Microcosm set up and sample naming, and detection of *mmoX* and *pmoA*

Microcosm Setup and naming				**Sample name**	***mmoX gene***		***pmoA gene***	
Ground-water [Dahlgren (D) or Kirkwood (K)]	**Micro-cosm #**	**Substrate amended** [$^{13}CH_4$ (X); $^{12}CH_4$ (Y); TCE (T)] Killed (Z)	**Sampling Time** [days after $^{13}CH_4$]		***mmoXL (F-R)***	***mmoX (A-B)***	***A189-mb661***	***A189-A682***
D	1	XT	1	D1-XT-1	-	-	-	-
			3	D1-XT-3	-	-	+	+
D	2	XT	1	D2-XT-1	+	-	+	+
			2	D2-XT-2	+	-	+	+
D	3	YT	1	D3-YT-1	+	-	+	+
			2	D3-YT-2	+	-	+	+
D	4	YT	1	D4-YT-1	-	-	-	+
			3	D4-YT-3	-	-	+	+
D	5	XTZ	1	D5-XTZ-1	-	-	-	-
			3	D5-XTZ-3	-	-	-	-
D	6	XTZ	1	D6-XTZ-1	-	-	-	-
			2	D6-XTZ-2	-	-	-	-
D	7	YTZ	1	D7-YTZ-1	-	-	-	-
			2	D7-YTZ-2	-	-	-	-
D	8	YTZ	1	D8-YTZ-1	-	-	-	-
			3	D8-YTZ-3	-	-	-	-
K	1	X	2	K1-X-2	-	-	-	-
			14	K1-X-14	-	-	-	-

Table 3.1. Continued

Microcosm Setup and naming				Sample name	*mmoX gene*		*pmoA gene*	
Ground-water [Dahlgren (D) or Kirkwood (K)]	**Micro-cosm #**	**Substrate amended** [$^{13}CH_4$ (X); $^{12}CH_4$ (Y); TCE (T)] Killed (Z)	**Sampling Time** [days after $^{13}CH_4$]		***mmoXL (F-R)***	***mmoX (A-B)***	***A189-mb661***	***A189-A682***
K	2	X	2	K2-X-2	-	-	-	-
			14	K2-X-14	-	-	-	-
K	3	X	2	K3-X-2	-	-	-	-
			14	K3-X-14	-	-	-	-
K	4	XT	2	K4-XT-2	+	-	-	-
			21	K4-XT-21	+	-	+	-
K	5	XT	2	K5-XT-2	+	-	+	+
			23	K5-XT-23	+	-	+	-
K	6	XT	2	K6-XT-2	-	-	-	-
			23	K6-XT-23	-	-	-	-
K	7	Y	2	K7-Y-2	-	-	-	-
			14	K7-Y-14	-	-	-	-
K	8	Y	2	K8-Y-2	-	-	-	-
			14	K8-Y-14	-	-	-	-
K	9	Y	2	K9-Y-2	-	-	-	-
			14	K9-Y-14	-	-	-	-
K	10	YT	2	K10-YT-2	-	-	+	-
			21	K10-YT-21	-	-	+	-
K	11	YT	2	K11-YT-2	-	-	-	-
			23	K11-YT-23	-	-	-	-

Table 3.1. Continued

Microcosm Setup and naming				**Sample name**	***mmoX gene***		***pmoA gene***	
Ground-water [Dahlgren (D) or Kirkwood (K)]	**Micro-cosm #**	**Substrate amended** [$^{13}CH_4$ (X); $^{12}CH_4$ (Y); TCE (T)] Killed (Z)	**Sampling Time** [days after $^{13}CH_4$]		***mmoXL (F-R)***	***mmoX (A-B)***	***A189-mb661***	***A189-A682***
K	12	YT	2	K12-YT-2	-	-	-	-
			23	K12-YT-23	-	-	-	-
K	14	XZ	2	K14-XZ-2	-	-	-	-
			21	K14-XZ-21	-	-	-	-
K	15	XZ	2	K15-XZ-2	-	-	-	-
			23	K15-XZ-23	-	-	-	-
K	16	YZ	2	K16-YZ-2	-	-	-	-
			14	K16-YZ-14	-	-	-	-
K	17	YZ	2	K17-YZ-2	-	-	-	-
			21	K17-YZ-21	-	-	-	-
K	18	YZ	2	K18-YZ-2	-	-	-	-
			21	K18-YZ-21	-	-	-	-

4. COMETABOLIC BIODEGRADATION OF GROUNDWATER BY ACIDOPHILIC FACULTATIVE METHANOTROPHS

4.1. Introduction

Chlorinated solvents are synthetic compounds and have been widely used as degreasing solvents, chemical intermediates, extraction solvents, or spotting agents in industry processes (Humans 2014). The wide applications of chlorinated solvents and improper disposal of the spent solvents wastes over past few decades has led to severe groundwater and soil contamination with chlorinated solvents (Amter and Ross 2001). Chlorinated solvents such as trichloroethylene (TCE) and dichloroethylene (DCE) are the most frequently detected volatile organic compounds (VOCs) in aquifers (Zogorski et al. 2006). 1,2,3-trichloropropane (TCP), as a stabilizer for chlorinated solvents, is also detected as a co-contaminant. TCE and cDCE are suspected carcinogens or carcinogens. Therefore, the US EPA has set the maximum contaminant levels (MCL) in drinking water for 0.005 mg/L of TCE and 0.07 mg/L of *cis*-1,2-DCE. In 2009, the U.S. EPA lists 1,2,3-trichloropropane (TCP) in the Drinking Water Contaminant Candidate List 3 (CCL3)(EPA 2009).

To protect human health, both chemical and biological methods have been applied to cleanup these contaminants in the environment (Bielefeldt et al. 1995, Cheng and Wu 2000, Halsey et al. 2005, Han et al. 1999). Three known biological methods that have been successfully applied for chlorinated solvents remediation are reductive dechlorination, aerobic oxidation and cometabolic biodegradation (Jesus et al. 2016). All these three biological processes work better under neutral conditions, but not effective for acidic environments. Chlorinated solvents that biodegraded via reductive dechlorination and aerobic oxidation are commonly served as substrates for degradative microorganism. Different from these two growth–linked biodegradation

processes, cometabolic biodegradation is a non-growth-linked process that bacteria use existing enzymes to degrade contaminant but gain no benefits from the degradation (Nzila 2013). Thus, cometabolic biodegradation presents several advantages over the growth-linked biodegradation processes, since cometabolic reactions can achieve higher degradation rates and are effective to degrade contaminants to very levels, and without produce and accumulate toxic byproducts that are commonly observed during reductive dechlorination) (Frascari et al. 2015).

Methane-oxidizing bacteria are most studied bacteria that have shown effective cometabolic degradation of persistent contaminants compared to those of many bacterial species (Li et al. 2016, Ryoo et al. 2000) (De Los Cobos-Vasconcelos et al. 2006, Kang and Doty 2014), Methane-oxidizing bacteria, also called methanotrophs, express methane monooxygenase (MMO) to oxidize methane. MMO can also oxidize many groundwater contaminants including chlorinated solvents like TCE. In 1980s, researchers found that methane stimulated TCE degradation in aerobic sediments (Fogel et al. 1986, Wilson and Wilson 1985). In 1988, two pure methanotrophs capable of degrading TCE were isolated from groundwater samples (Little et al. 1988b). Until now, pure methanotrophic strains showing ability to degrade TCE include *Methylosinus trichosporium* OB3b (Tsien et al. 1989a), *Methylomicrobium album* BG8 (Han et al. 1999), *Methylocystis sp.* strain M (McDonald et al. 1997a) and *Methylocystis* strain SB2(Kip et al. 2011). *Methylosinus trichosporium* OB3b and *Methylocystis sp.* strain M biodegraded TCE through soluble MMO (sMMO), while *Methylomicrobium album* BG8 and *Methylocystis* strain SB2 oxidize TCE through particulate MMO (pMMO). However, these strains are neutral methanotrophs and they are unable to survive in acidic environments where the pH is less than 5.5.

Acidophilic methanotrophs are a unique group of methane-oxidizing bacteria capable of expressing MMO to oxidize methane under low pH environments (pH < 5.5). However, their

ability to degrade groundwater contaminants in acidic aquifers has not been understood. Most interestingly, many of these acidophilic methanotrophs are also facultative, i.e., they are capable of growing on more than C_1 compounds (Belova et al. 2013, Crombie and Murrell 2014, Dunfield et al. 2010). The ability of acidophilic methanotrophs to survive in low pH environment and to using multi-carbons (such C2 or C3 carbons) suggest themselves as idea candidate for cometabolic degradation of TCE in acidic environments.

This study uses pure acidophilic methanotrophs to assess their ability to degrade groundwater contaminants under acidic conditions. *Methylocella tundrae* and *Methylocystis bryophila* are used as model strains in this study. *Methylocella tundrae,* isolated from acidic tundra peatlands, possess only sMMO (Dedysh et al. 2004), while *Methylocystis bryophila*, isolated from acidic *Sphagum* peat, can express both pMMO and sMMO (Belova et al. 2013). While these two strains were previously isolated, their ability to degrade chlorinated solvents, the ability to grow on multi-carbon substrates, and the effects of carbons sources on types of MMO expression have not been characterized. This study determined their growth curves with methane, and C2 and C3 carbons such as acetate and propane. The effects of growth substrates on the expression of MMO type were also investigated. Resting cell degradation tests were conducted to determine their degradation potential for trichlorethylene (TCE), cis-1,2-dichloetheylene (cis-1,2-DCE) and 1,2,3-trichloropropane (1,2,3-TCP).

4.2. Methods and materials

4.2.1. Chemicals

1,2,3-TCP (CAS#96-18-4, 99% purity) and cis-1,2-DCE (CAS#156-59-2, 97% purity) were purchased from Acros Organics (New Jersey, USA). TCE (CAS#79-01-6, 99+% purity, ACS

grade) and Fast Blue B Salt (CAS# 14263-94-6, dye content~95%) were purchased from Sigma-Aldrich(St. Louis, MO). Naphthalene (CAS# 91-20-3, 99+% purity) was purchased from Alfa Aesar (Haverhill, MA). Sodium acetate anhydrous (CAS#127-09-3, 99.0+% purity) was purchased from EMD (Chicago, IL). Isopropanol (CAS#67-63-0, ACS grade) was purchased from Macron Fine Chemical (Center Valley, PA). Methanol (CAS#67-56-1, Certified ACS) was purchased from Fisher Scientific (Hampton, NH). Propane (CAS# 74-98-6), was purchased from Coleman (Chicago, IL). Methane (CAS# 74-82-2, 99.99% purity) was purchased from GASCO (Oldsmar, FL). Saturated chlorinated solvent stock solutions in DI water were prepared freshly every two months.

4.2.2. Bacterial strain and culture conditions

Methylocella tundra (DSM 15673) and *Methylocystis bryophila* (DSM 21852) were purchased from German Collection of Microorganisms and Cell Cultures (DMSZ, Germany). Both bacteria were grown in M2 medium (Belova et al. 2013, Dedysh et al. 2004), except the trace element solution without adding $CuCl_2$. The final pH of M2 medium was 5.5-5.6. Glass bottles (120-ml) containing 20 mL of growth medium were used to achieve a headspace/liquid space ratio of 5:1(100 ml :20 ml). After inoculation, the bottles were sealed with rubber septa and crimp caps and methane or propane was added aseptically using a syringe equipped with a disposable filter (0.22 µm) to achieve a 10%-20% mixing ratio in the headspace. Methanol, isopropanol or sodium acetate solution was added to reach the initial concentrations of 0.5%(v/v), 0.5%(v/v) and 5µM, respectively. The bottles were incubated on a rotary shaker at 120 rpm at room temperature (22°C-23°C). Oxygen concentration, carbon dioxane concentration, and the cell density OD600 were monitored daily.

4.2.3. Naphthalene oxidation assay

Naphthalene oxidation assay is a simple colormetric assay to assess the non-specific oxygenase activity toward substrates. Non-specific oxygenases can oxidize naphthalene to naphthol as dead-end products which can react with Fast Blue B Salt to develop purple color. The assay was conducted as described by Chu and Alaverze-Cohen (Chu and Alvarez-Cohen 1998). Briefly, the OD600 of tested bacteria above 0.4 was used. Glass vials (5-ml) were amended with 1-ml of cell suspension, 20 µl of 2M sodium formate stock solution, and 1-ml of saturated naphthalene stock solution. For control, the naphthalene stock solution was replaced with 1-ml of DI water. The final concentration of formate is 20mM. The vials were incubated on a 37 °C incubator shake at 150 rpm for 1 hour. Following the incubation, 100µl of Fast Blue B Salt solution (2 mg/ml, prepared freshly) was added into the vial to establish color. The vials were shaken by hands for 10 seconds before measuring absorbance at OD535. Light pink to purple are considered as positive results.

4.2.4. Detection of *prm*A

Methylocella tundrae carries propane monooxygenase gene cluster in one of its megaplasmids (Kox et al. 2019). Primers sequences were designed with the Primer-Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the propane monooxygenase hydroxylase alpha subunit (*prm*A), accession number is LR536451. The primers used were as follows: *prm*A forward (5'-CACATAGCCCTGCTTTGACGA-3'); *prm*A reverse (5'-TGAATGGTGGAATGGCGGAA-3'). This pair of primers were used to amplify 615 base pair(bp) sequence. Another pair of primers were based on the promoter of *prm*A sequence of *Methylocella silvistris (Crombie and Murrell 2014)*. The primers PrPf (5′-

ACTCAATTGTCCGTTCCGTAACGCCTCTC-3′) and PrPr (5′-CGGCCGGCTGAGCTCCCGCTACGC-3′) were used to amplify the 1112 base pairs (bps) promoter. The designed primers were ordered from Integrated DNA Technologies, Inc. (Coralville, CA). The genomic DNA of bacterial strains used in this study were extracted using FastDNA spin kit (MP Biomedical, Solon, OH). The extracted DNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (Fisher Scientific., Fair Lawn, NJ). The PCR amplification was conducted in an automated thermal cycler (Bio-Rad, Hercules, CA). Each 25-µL PCR mixture contained 400 nM forward/reverse primers and 12.5 µL of Taq DNA polymerase PCR mix buffer (Qiagen, Valencia, CA). The thermal cycling protocol as follows: 95 °C for 15 min; 35 cycles of 95 °C for 30 sec, 52 °C for 1 min, and 72 °C for 2 min and a final extension at 72°C for 10 min. The presence of PCR amplicons was determined and visualized using gel electrophoresis.

4.2.5. Degradation tests using resting cells

Cells were harvested in mid to late exponential phase and checked for non-specific enzymatic activity using naphthalene oxidation assay. If the naphthalene oxidation results were positive, the cells were then used in the degradation experiments as described below. Prior to adding chlorinated solvents, the gaseous growth substrates (such as methane or propane) were removed. Biodegradation experiments were conducted in 26-mL vials containing 4 mL cell suspensions, 0.16 ml sodium formate (500 µM) and different initial chlorinated solvent concentrations at room temperature (22 °C to 23 °C). Formate was added to each vial to provide external reduce energy. Concentrations of chlorinated solvents were determined via headspace analysis using GC/FID. The results were used to calculate degradation kinetic and degradation capacity (see description below). Initial degradation rates were calculated by using at least three

measurements taken within the first 40 min of the experiment. The transformation capacities are calculated by subtracting the initial and final concentrations of chlorinated solvents over initial biomass, assuming that all the biomass was inactivated due to product toxicity after 24 h of reaction.

4.2.6. Determination of chlorinated solvent concentrations

The concentrations of TCE, 1,2-cis DCE and TCP in aqueous samples are determined by injection headspace samples (30 µL) into Gas Chromatography /Flame Ionization Detection (GC/FID). This method is based on the equilibrium of these three compounds between air and aqueous phases. The partition coefficient relating air and aqueous concentrations of a volatile substance is commonly referred to as Henry's constant. Henry's constant varies at different temperatures. Dimensionless Henry's constant Hc for TCE is 0.723, for 1,2-cis DCE is 0.167, and for TCP is 24.8. Calibration curves for each of chlorinated solvents were developed based the area of the corresponding peak and associated concentrations. The calibration curves were used to determine headspace concentrations which were then converted to aqueous concentrations using dimensionless Henry constants.

4.2.7. Kinetics parameters and transformation capacity (Tc)

Michaelis–Menten models, based on the enzyme kinetics without inhibition, was used to describe degradation kinetics of chlorinated solvents by acidophilic methanotrophs. The equation is expressed as below using degradation velocity (v, µmol/(mg·cell·day))), substrate concentrations with two kinetic parameters, Vmax and K_M.

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

where V_{max} represents the maximum velocity achieved by the system, at maximum (saturating) substrate concentrations. K_M (the Michaelis constant; sometimes represented as K_S instead) is the substrate concentration at which the reaction velocity is 50% of the V_{max}. [S] is the concentration of the substrate S. In this study, [S] are the concentrations of chlorinated solvents. To fit the model, 6 to 8 initial concentrations were used.

Transformation capacities (Tc, (mg·substrate)/(mg·cell)) were measured for the cometabolic substrates. Transformation capacities were calculated by subtracting the concentration of substrates remaining after 24 h experiment from the initial concentration and normalizing to inactivated cells during experiment.

$$Tc = \frac{S_t - S_0}{X_t - X_0}$$

Where S_0 and X_0 are the initial concentration of substrates (mg/L) and activated cells (mg/L), St and Xt are the final concentration of substrates (mg/L) and activated cells (mg/L).

4.3. Results and discussion

4.3.1. Growth of acidophilic methanotrophs with different carbon sources

Methylocystis bryophila were able to grow with different carbons sources and their growth courses were shown in the Figure 4.1. This figure presented the growth of bacteria in first 8 days. Growing with 20%(v/v) methane or 0.5% (v/v) methanol, *Methylocystis bryophila* entered exponential phase after three days. It took another four day to reach stationary phase. The OD_{600} (optical density) reached 0.25- 0.3. *Methylocystis bryophila* was not observed growth when 20%(v/v) propane or 0.5% (v/v) isopropanol were carbon sources in the first seven days.

Similar to *Methylocystis bryophila*, when supplied with methane and methanol, *Methylocella tundrae* took three days to enter exponential phase. When grown with 100 µM acetate or 0.5% (v/v) isopropanol, the lag phase would be shorter. After one day, growth of *Methylocella tundrae* was observed. After eight days of incubation, the OD_{600} reached 0.6, which was higher than the cells grown with methane, methanol and acetate. The reasons of the higher cell optical density when grown with isopropanol were unclear. It is possible that the strain has some enzymes that can catalyze isopropanol for cell growth. However, in the first eight days, cells growth with propane was not observed, although *Methylocella tundrae* was found to carry PrMO gene cluster in the megaplasmids (Kox et al. 2019). Whether propane or isopropanol could be an inducer for PrMO gene expression was unclear and no further experiments were performed to investigate this question in this study.

Figure 4.1. Growth curves of *Methylocystis bryophila* growing with different carbon sources

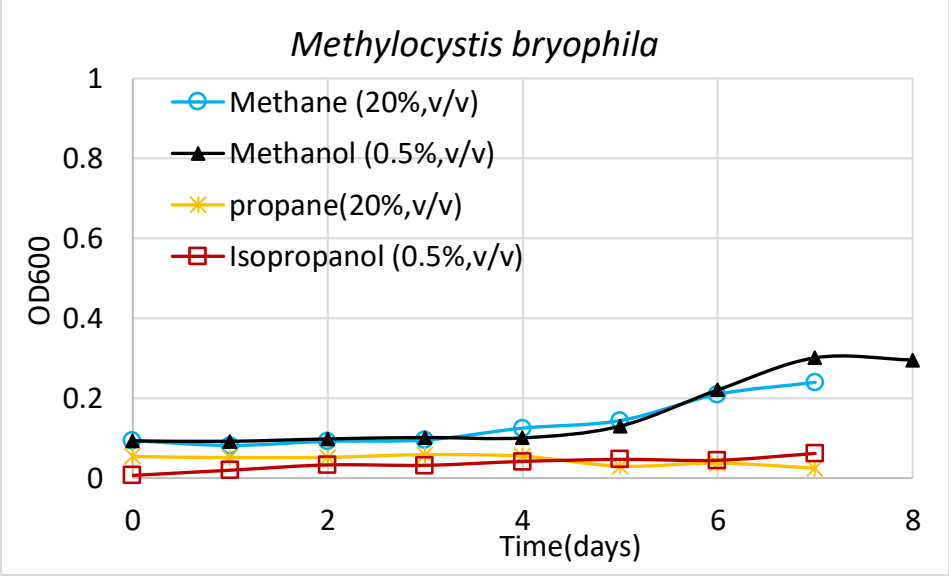
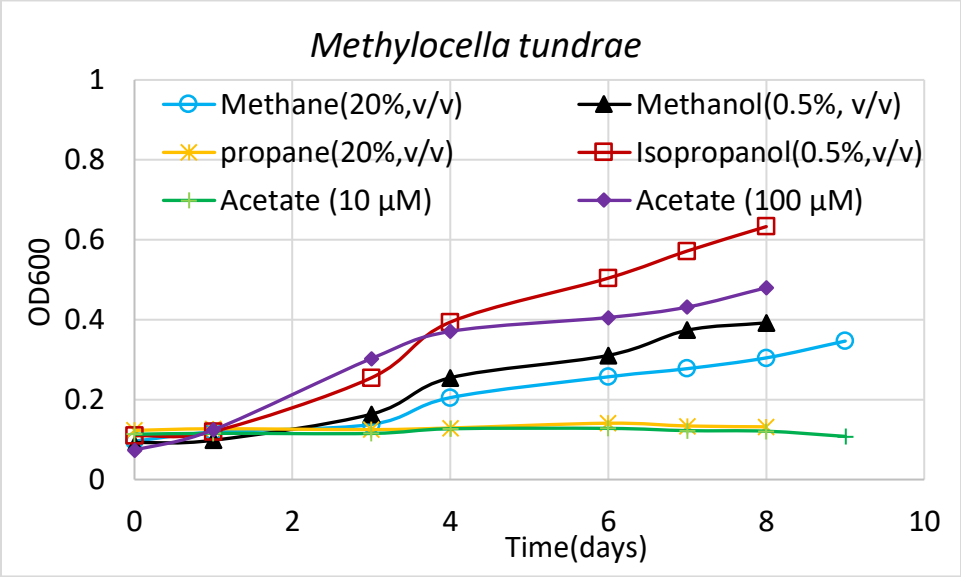


Figure 4.2. Growth curves of *Methylocella tundrae* growing with different carbon sources



4.3.2. Naphthalene oxidation results

Positive results of naphthalene oxidation assay only observed in methane-grown *Methylocystis bryophila* and *Methylocella tundrae.* Although cells could grow with methanol and isopropanol, the naphthalene oxidation assay results were negative, suggesting that non-specific enzymes were not expressed. *Methylocystis bryophila* could grow slowly with acetate at the concentration of 0.05 % (w/v) (Belova et al. 2013). In this study, the strain took 3-4 weeks to show sign of growth. Previous study of *Methylocystis bryophila* did not grow the cell with limited copper nor to use naphthalene oxidation assay to determine the expression of non-specific enzymes.

Methylocella tundrae grew with propane but poorly, where the OD600 increased from 0.1 to 0.15 after three weeks of incubation (data not shown). Negative naphthalene oxidation results

were also observed. However, the presence of PrMO gene in *Methylocella tundrae* was confirmed with positive PCR amplicons (Figure 4.3).

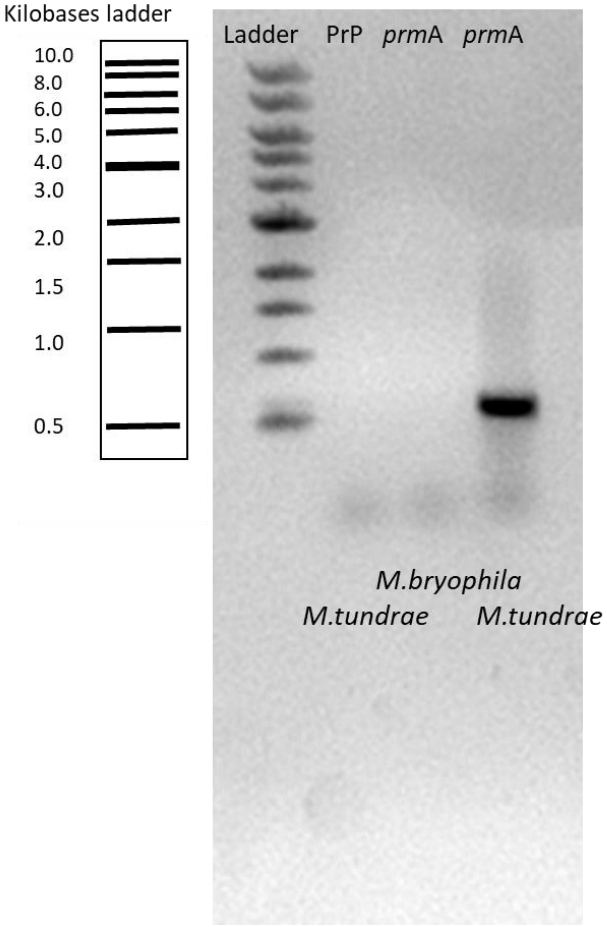
Table 4.1. Results of naphthalene oxidation assay

	Methylocystis bryophila				
Carbon source	Methane	Methanol	Acetate	Propane	Isopropanol
Growth	+	+	Slow growth	-	-
Naphthalene oxidation assay	+	-	/	-	-
	Methylocella tundrae				
Carbon source	Methane	Methanol	Acetate	Propane	Isopropanol
Growth	+	+	+	Little growth	+
Naphthalene oxidation assay	+	-	-	-	-

+ growth observed
- no growth observed
/ not mentioned in literature

The detection results of α subunit of PrMO genes(*prm*A*)* and propane monooxygenase promoter gene (PrP) in the genome of *Methylocella tundrae* and *Methylocystis bryophila* show in the Figure 4.3. The gel electrophoresis result confirms the present of *prm*A but not similar promoter genes in *Methylocella tundrae*. Whether the absence of propane monooxygenase promoter genes causes the PrMO fail to express is unclear, but it may be one reason that *Methylocella tundrae* cannot grow with propane effectively. There is no evidence that *Methylocystis bryophila* possesses similar PrMO with *Methylocella tundrae*.

Figure 4.3. Detection of PrP and *prm*A gene in *Methylocystis bryophila* and *Methylocella tundrae*



4.3.3. Degradation of chlorinated solvents by acidophilic methanotrophs

Figure 4.4 to 4.6 present the TCE, *cis*-1,2-DCE and TCP degradation by *Methylocystis bryophila* and *Methylocella tundrae.* With the expression of non-specific enzyme in *Methylocystis bryophila* and *Methylocella tundrae, the* removal efficiency of TCE and *cis*-1,2-DCE(in orange columns) are higher than the grown cells without express non-specific enzyme. This confirms that the non-specific enzyme, may be sMMO, oxidize the chlorinated solvents through cometabolism. The removal efficiency of TCE, cis-1,2-DCE and TCP by methane-grown *Methylocella tundrae* can reach above 50%, while removal efficiencies are only 10%-20% by methanol-grown

Methylocella tundrae. To *Methylocystis bryophila*, the difference of removal efficiency between methane-grown cells and methanol-grown cells is not that much. With or without expression of non-specific enzyme, TCE can be degraded by 20%-30%, while *cis*-1,2-DCE can be degraded by 40%-60%. The reason may be caused the by the expression of other enzymes. Literature shows that pMMO is also capable to degrade chlorinated ethenes (Yoon et al. 2011). *Methylocystis bryophila* possesses pMMO1, pMMO2 and a sequence-divergent particulate monooxygenase (pXMO), which may play roles in TCE and cis-1,2-DCE degradation.

Figure 4.4. TCE degradation by *Methylocystis bryophila* and *Methylocella tundrae*

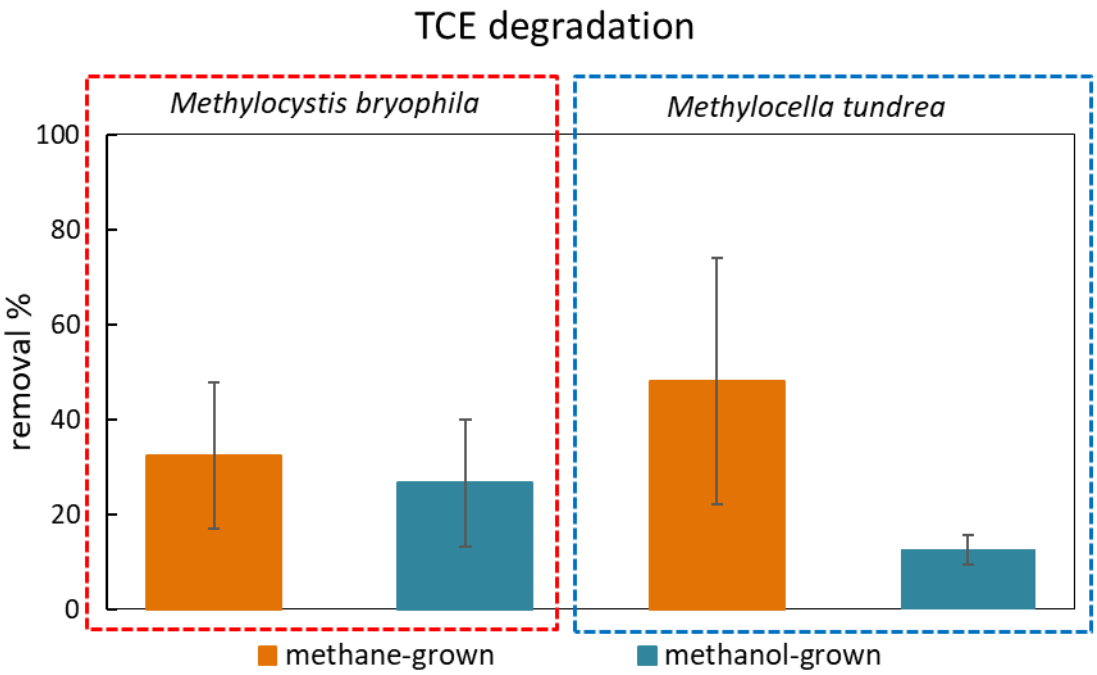


Figure 4.5. *cis*-1,2-DCE degradation by *Methylocystis bryophila* and *Methylocella tundrae*

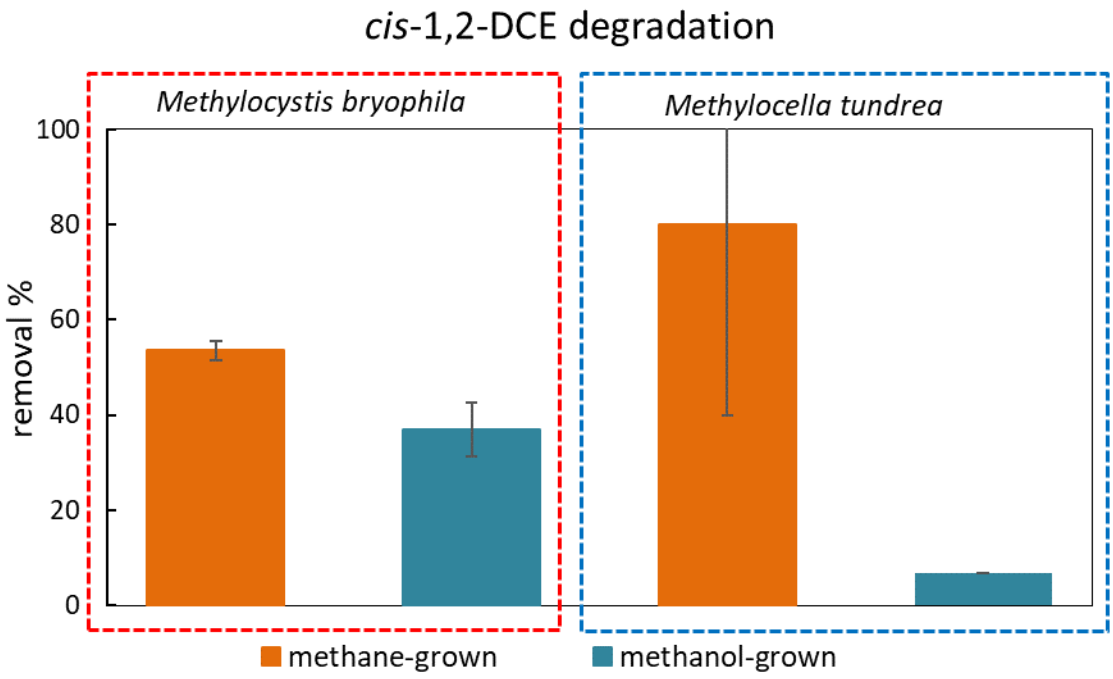
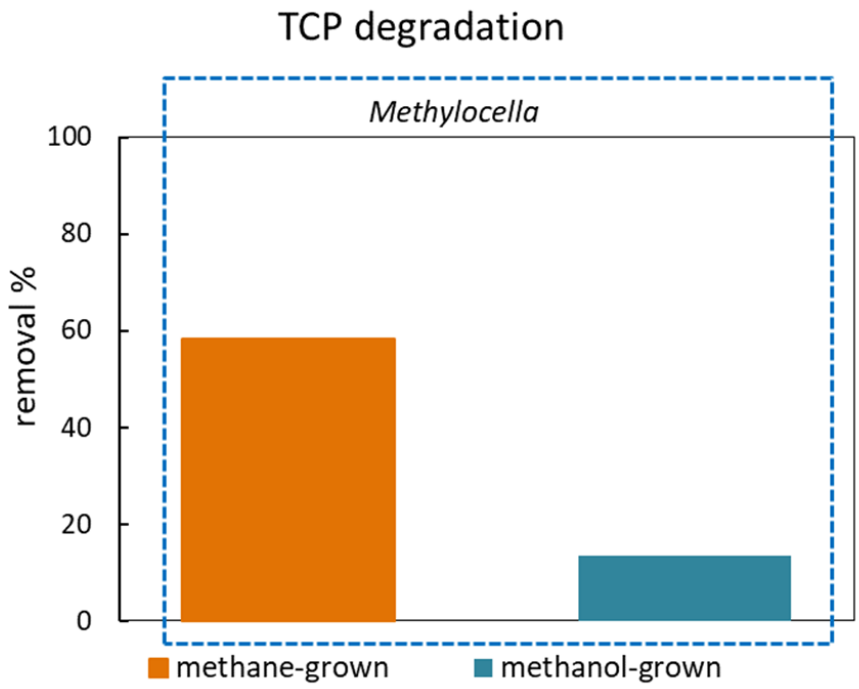


Figure 4.6. TCP degradation by *Methylocella tundrae*



4.3.4. Transformation capacities and kinetic model of chlorinated solvents

Table 4.2 presents the transformation capacities of three chlorinated solvents calculated based on the degradation by acidophilic methanotrophs. Compared with model strain, *Methylosinus trichosporium* OB3b, the Tc for TCE and *cis*-1,2-DCE are usually lower in two strains in this study, showing their ability in biodegradation chlorinated solvents are not that strong as *Methylosinus trichosporium* OB3b.

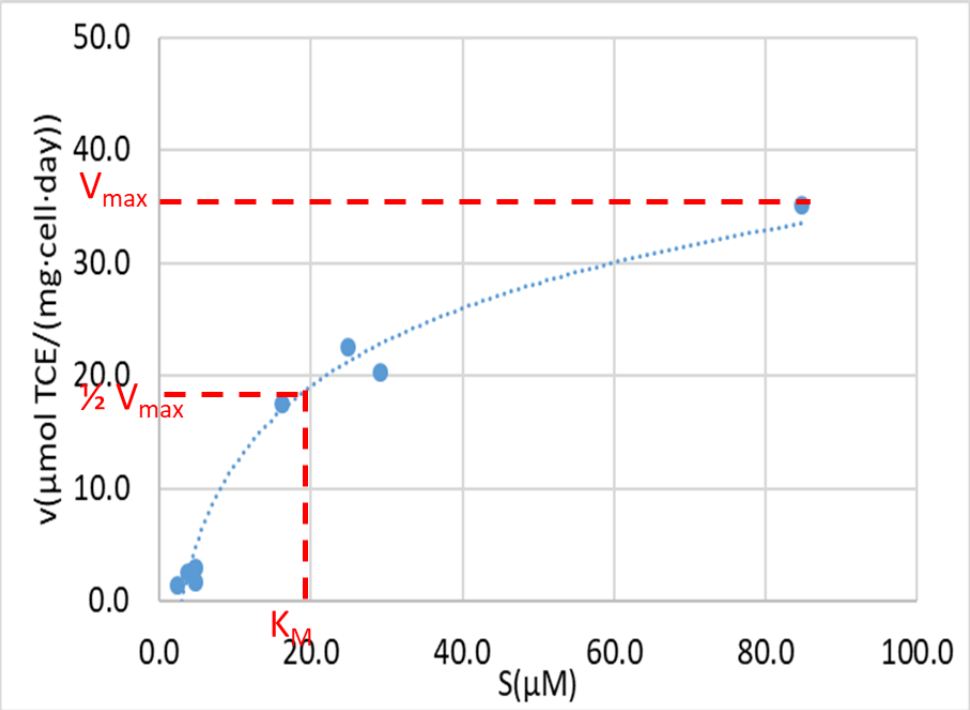
Table 4.1. Tc (μmol/(mg·cell)) of chlorinated solvents

	Methylocella tundrae	*Methylocystis bryophila*	*Methylosinus trichosporium* OB3b
	Tc μmol/(mg·cell)	Tc μmol/(mg·cell)	Tc μmol/(mg·cell)
TCE	0.53±0.036	0.40±0.062	ranging 0.42-1.42 *
cis-1,2-DCE	0.80±0.170	0.45±0.208	0.80-2.6 *
1,2,3-TCP	0.12±0.062	/	No related literature

*--- data from literature
/ --- no data

A range of aqueous concentrations of chlorinated solvents and their correspond initial degradation rates were measured to calculate the kinetics parameters V_{max} and K_M in the Michaelis-Menten model (Figure 4.7.). The V_{max} is 35.5 (μmol TCE/(mg·cell·day)), K_M is 62.0 μM TCE and the first-order rate constant K_M/V_{max} is 1.75.

Figure 4.7. Michaelis-Menten curve of TCE degradation by *Methylocella tundrae*



$$v = \frac{35.5[S]}{62.0+[S]}$$

5. SUMMARY, CONCLUSIONS AND FUTURE STUDIES

5.1. Summary and conclusions

The role of the methanotrophic community in the attenuation of pollutants in acidic aquifers has received very little study. The data of Chapter 3 presented herein indicate that that aerobic biodegradation of TCE and other chlorinated solvents in acidic groundwater may be facilitated by a wide range of methanotrophic bacteria that cluster within in the γ-Proteobacteria and a-Proteobacteria but are not necessarily closely related to known acidophilic methanotrophs, largely isolated from peat bogs. The results are unexpected but interesting, suggesting that many methanotrophs are present in the acidic groundwater aquifers and that they are more phylogenetically diverse than previously reported. In that reductive dechlorination does not generally occur at low pH (Vainberg et al. 2009; Yang et al. 2017), aerobic cometabolism may be particularly important, but currently underestimated as a mechanism of natural attenuation of cVOCs in both neutral pH and acidic aquifers. A recent study by Mills et al. (2018) confirmed the potential importance of cometabolic oxidation as a mechanism of TCE attenuation in groundwater. The authors added 14C-TCE to aerobic groundwater microcosms as a sensitive means to measure its cometabolism, and observed natural first order oxidation rates as high as 2.65 $year^{-1}$, with significant conversion of ^{14}TCE to $^{14}CO_2$. While this work did not target acidic groundwater or methanotrophs specifically, the locations of some of the groundwater samples (e.g., downgradient from landfills) would be expected to harbor significant populations of methanotrophic organisms. More importantly, the work provides further indication that cometabolic oxidation processes may be an important mechanism of cVOC loss in groundwater. Future studies in acidic aquifers are required to better understand the communities of methanotrophs in acidic groundwater aquifers,

the natural capabilities of these organisms to biodegrade cVOCs, and the potential to stimulate this process in situ for remediation purposes.

The study about pure acidophilic methanotrophs *Methylocella tundrae* and *Methylocystis bryophila* show that both acidophilic methanotrophs are facultative and able to use multi-carbon sources. *Methylocella tundrae* carrying the cluster gene of propane monooxygenase makes it probably grow with propane and one intermediate of propane oxidation, isopropanol. *Methylocella tundrae* was proved to grow well with isopropanol, but not showed positive results in naphthalene oxidation assay, suggesting that isopropanol might not be an inducer for propane monooxygenase. Methane-grown *Methylocella tundrae* and *Methylocystis bryophila* were able to degrade TCE and *cis*-1,2-DCE. Only *Methylocella tundrae* could degrade TCP. The results of this study provide the suggest that aerobic biodegradation of TCE and other chlorinated solvents in low pH groundwater may be facilitated by methanotrophic bacteria, and that there are potentially a wide variety of different strains that inhabit acidic aquifers.

5.2. Future studies

Although acidophilic methanotrophs have been detected for thirty years, little is known about this unique group of methanotrophs, in part, due to the challenges in isolation in early studies. Future studies are needed to further investigate physiological characteristics of these isolates, as majority of these isolates have not been fully characterized (Dedysh et al. 2015b, Han et al. 2018, Kox et al. 2019, Miroshnikov et al. 2017, Oshkin et al. 2019, Ricke et al. 2005). Given the importance of role of methanotrophs in carbon cycle, a better understanding of the diversity and distribution of the methanotrophic community in both acidic and non-acidic environments continues is warranted. Study on the bioconversion of methane to methanol (Mardina et al. 2016,

Patel et al. 2016, Patel et al. 2019, Patel et al. 2018, Patel et al. 2017) highlighted the need for exploring the potential applications of acidophilic methanotrophs to this emerging area.

As mentioned earlier, only limited studies explored the application of acidophilic methanotrophs for pollutant degradation. To fully maximize the use of acidophilic methanotrophic activity for pollutant degradation, several questions as described below need to be answered.

First, while genomic information of several acidophilic methanotrophs has been recently revealed, the effects of carbon sources on types of MMOs expression by these microorganisms (particularly facultative acidophilic methanotrophs) remain unclear. Studies using proteomic and/or metabolomic approaches might be able to shed light on these unknowns. Additionally, as the expression of pMMO and sMMO is regulated by the concentration of copper, and the acidophilic methanotrophs might have additional regulation systems, it is necessary to better understand the bioavailability of Cu in acidic environment where low pH means higher solubility of other metal compounds. Will other metal ions also present in the acidic environments? Will their presence affect the bioavailability of copper? Mostly, several acidophilic methanotrophs are facultative, but they may not express MMOs while growing on other carbon sources. For example, *Methylocella silvestris* expressed sMMO when grown with propane (Crombie and Murrell 2014), while repressed the transcription of sMMO while grown with acetate (Rahman et al. 2011b). The expression of MMOs will affect the oxidation of methane and other pollutants. Thus, a better understanding of interactions of substrate utilization and their MMO expression is the key knowledge for us to better assess greenhouse gases, and to formulate a better strategy to maximizing cometabolic biodegradation for environmental pollutants in acidic environments.

Second, there is a great need to isolate more acidophilic methanotrophs from the environments. While molecular techniques have found powerful and revealed the wide presence of acidophilic methanotrophs, only isolates can enable us to study their unknown metabolic pathways and physiological properties. Thus, continue efforts on enrichment and isolation of pure strains of acidophilic methanotrophs can't be over emphasized.

Finally, how to implement acidophilic methanotrophs for in-situ pollutant degradation in acidic environments is another important question to be addressed. Available studies on biodegradation ability of acidophilic methanotrophs are very limited. Mixture of contaminants are commonly in contaminated acidic environments. Thus, it is important to determine the range of substrates that acidophilic methanotrophs can cometabolically degraded. There is also a gap between the lab scale experiments and in situ bioremediation. Degradation rates of individual contaminants have been recently reported (Semrau 2011); however, effects of contaminant mixtures and factors affecting contaminant degradation remain unknown. Currently, most results of biodegradation were gained from microcosms and lab experiments. Can these microorganisms also work well in situ? Will facultative acidophilic methanotrophs be more useful than obligate ones during in situ bioremediation or natural attenuation? Will facultative methanotrophs be stimulated via addition of multi-carbon sources? If so, can they out compete endogenous heterotrophic microorganisms. There are all important questions to be addressed in the future for realization of using acidophilic methanotrophs for effectively remediating contaminants in acidic environments.

REFERENCES

1. Adriaens, P. and Grbic-Galic, D. (1994) Cometabolic Transformation of Mono- and Dichlorobiphenyls and Chlorohydroxybiphenyls by Methanotrophic Groundwater Isolates. Environmental science & technology 28(7), 1325-1330.

2. Ali, H., Scanlan, J., Dumont, M.G. and Murrell, J.C. (2006) Duplication of the mmoX gene in Methylosinus sporium: cloning, sequencing and mutational analysis. Microbiology 152(10), 2931-2942.

3. Alvarez-Cohen, L. and McCarty, P. (1991) Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. 57(1), 228-235.

4. Alvarez-Cohen, L. and Speitel, G.E. (2001) Kinetics of aerobic cometabolism of chlorinated solvents. Biodegradation 12(2), 105-126.

5. Amter, S. and Ross, B. (2001) Was Contamination of Southern California Groundwater By Chlorinated Solvents Foreseen? Environmental Forensics 2(3), 179-184.

6. Anderson, J.E. and McCarty, P.L. (1997a) Transformation yields of chlorinated ethenes by a methanotrophic mixed culture expressing particulate methane monooxygenase. Applied and Environmental Microbiology 63(2), 687-693.

7. Anderson, J.E. and McCarty, P.L. (1997b) Transformation yields of chlorinated ethenes by a methanotrophic mixed culture expressing particulate methane monooxygenase. Appl. Environ. Microbiol. 63(2), 687-693.

8. Auman, A.J., Stolyar, S., Costello, A.M. and Lidstrom, M.E. (2000) Molecular characterization of methanotrophic isolates from freshwater lake sediment. Applied and Environmental Microbiology 66(12), 5259-5266.

9. Baani, M. and Liesack, W. (2008) Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in Methylocystis sp. strain SC2. Proceedings of the National Academy of Sciences of the United States of America 105(29), 10203-10208.

10. Baral, B.S., Bandow, N.L., Vorobev, A., Freemeier, B.C., Bergman, B.H., Herdendorf, T.J., Fuentes, N., Ellias, L., Turpin, E., Semrau, J.D. and DiSpirito, A.A. (2014) Mercury binding by methanobactin from Methylocystis strain SB2. Journal of Inorganic Biochemistry 141, 161-169.

11. Bédard, C. and Knowles, R. (1989) Physiology, biochemistry, and specific inhibitors of CH4, NH4+, and CO oxidation by methanotrophs and nitrifiers. Microbiological reviews 53(1), 68-84.

12. Belova, S.E., Kulichevskaya, I.S., Bodelier, P.L. and Dedysh, S.N. (2013) Methylocystis bryophila sp. nov., a facultatively methanotrophic bacterium from acidic Sphagnum peat, and emended description of the genus Methylocystis (ex Whittenbury et al. 1970) Bowman et al. 1993. International journal of systematic and evolutionary microbiology 63(3), 1096-1104.

13. Benner, J., De Smet, D., Ho, A., Kerckhof, F.-M., Vanhaecke, L., Heylen, K. and Boon, N. (2015) Exploring methane-oxidizing communities for the co-metabolic degradation of organic micropollutants. Applied Microbiology and Biotechnology 99(8), 3609-3618.

14. Bielefeldt, A., Stensel, H. and Strand, S. (1995) Cometabolic degradation of TCE and DCE without intermediate toxicity. Journal of Environmental Engineering 121(11), 791-797.

15. Bosma, T. and Janssen, D. (1998) Conversion of chlorinated propanes by Methylosinus trichosporium OB3b expressing soluble methane monooxygenase. Applied microbiology and biotechnology 50(1), 105-112.

16. Bravo, A.L., Sigala, J.C., Le Borgne, S. and Morales, M. (2015) Expression of an alkane monooxygenase (alkB) gene and methyl tert-butyl ether co-metabolic oxidation in Pseudomonas citronellolis. Biotechnology Letters 37(4), 807-814.

17. Brockman, F.J., Payne, W., Workman, D.J., Soong, A., Manley, S. and Hazen, T.C. (1995) Effect of gaseous nitrogen and phosphorus injection on in situ bioremediation of a trichloroethylene-contaminated site. Journal of Hazardous Materials 41(2), 287-298.

18. Broholm, K., Ludvigsen, L., Jensen, T.F. and Østergaard, H. (2005) Aerobic biodegradation of vinyl chloride and cis-1, 2-dichloroethylene in aquifer sediments. Chemosphere 60(11), 1555-1564.

19. Brusseau, G.A., Tsien, H.-C., Hanson, R.S. and Wackett, L.P. (1990) Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1(1), 19-29.

20. Bussmann, I., Rahalkar, M. and Schink, B. (2006) Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. FEMS Microbiology Ecology 56(3), 331-344.

21. Chang, S.W., Hyman, M.R. and Williamson, K.J. (2002) Cooxidation of naphthalene and other polycyclic aromatic hydrocarbons by the nitrifying bacterium, Nitrosomonas europaea. Biodegradation 13(6), 373-381.

22. Chen, Y., Dumont, M.G., Neufeld, J.D., Bodrossy, L., Stralis-Pavese, N., McNamara, N.P., Ostle, N., Briones, M.J.I. and Murrell, J.C. (2008) Revealing the uncultivated majority: combining DNA stable-isotope probing, multiple displacement amplification and metagenomic analyses of uncultivated Methylocystis in acidic peatlands. Environmental Microbiology 10(10), 2609-2622.

23. Cheng, S.-F. and Wu, S.-C. (2000) The enhancement methods for the degradation of TCE by zero-valent metals. Chemosphere 41(8), 1263-1270.

24. Cho, K.-C., Lee, D.G., Fuller, M.E., Hatzinger, P.B., Condee, C.W. and Chu, K.-H. (2015) Application of ^{13}C and ^{15}N stable isotope probing to characterize RDX degrading microbial communities under different electron-accepting conditions. Journal of Hazardous Materials 297(0), 42-51.

25. Cho, K.-C., Lee, D.G., Roh, H., Fuller, M.E., Hatzinger, P.B. and Chu, K.-H. (2013) Application of 13 C-stable isotope probing to identify RDX-degrading microorganisms in groundwater. Environmental pollution 178, 350-360.

26. Cho, K.C., Fuller, M.E., Hatzinger, P.B. and Chu, K.H. (2016) Identification of groundwater microorganisms capable of assimilating RDX-derived nitrogen during in-situ bioremediation. Science of the Total Environment 569-570, 1098-1106.

27. Chu, K.-H. and Alvarez-Cohen, L. (1998) Effect of Nitrogen Source on Growth and Trichloroethylene Degradation by Methane-Oxidizing Bacteria. Applied and Environmental Microbiology 64(9), 3451-3457.

28. Crombie, A.T. and Murrell, J.C. (2014) Trace-gas metabolic versatility of the facultative methanotroph Methylocella silvestris. Nature 510(7503), 148-151.

29. Csaki, R., Bodrossy, L., Klem, J., Murrell, J.C. and Kovacs, K.L. (2003) Genes involved in the copper-dependent regulation of soluble methane monooxygenase of Methylococcus capsulatus (Bath): cloning, sequencing and mutational analysis. Microbiology 149(7), 1785-1795.

30. Danilova, O.V., Kulichevskaya, I.S., Rozova, O.N., Detkova, E.N., Bodelier, P.L., Trotsenko, Y.A. and Dedysh, S.N. (2013) Methylomonas paludis sp. nov., the first acid-tolerant member of the genus Methylomonas, from an acidic wetland. International journal of systematic and evolutionary microbiology 63(6), 2282-2289.

31. Dawas-Massalha, A., Gur-Reznik, S., Lerman, S., Sabbah, I. and Dosoretz, C.G. (2014) Co-metabolic oxidation of pharmaceutical compounds by a nitrifying bacterial enrichment. Bioresource Technology 167, 336-342.

32. De Los Cobos-Vasconcelos, D., Santoyo-Tepole, F., Juarez-Ramirez, C., Ruiz-Ordaz, N. and Galindez-Mayer, C.J.J. (2006) Cometabolic degradation of chlorophenols by a strain of Burkholderia in fed-batch culture. Enzyme and microbial technology 40(1), 57-60.

33. Dedysh, S.N., Belova, S.E., Bodelier, P.L., Smirnova, K.V., Khmelenina, V.N., Chidthaisong, A., Trotsenko, Y.A., Liesack, W. and Dunfield, P.F. (2007) Methylocystis heyeri sp. nov., a novel type II methanotrophic bacterium possessing 'signature'fatty acids of type I methanotrophs. International journal of systematic and evolutionary microbiology 57(3), 472-479.

34. Dedysh, S.N., Berestovskaya, Y.Y., Vasylieva, L.V., Belova, S.E., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Liesack, W. and Zavarzin, G.A. (2004) Methylocella tundrae sp. nov., a novel methanotrophic bacterium from acidic tundra peatlands. International journal of systematic and evolutionary microbiology 54(1), 151-156.

35. Dedysh, S.N., Didriksen, A., Danilova, O.V., Belova, S.E., Liebner, S. and Svenning, M.M. (2015a) Methylocapsa palsarum sp. nov., a methanotroph isolated from a subArctic discontinuous permafrost ecosystem. International journal of systematic and evolutionary microbiology 65(10), 3618-3624.

36. Dedysh, S.N., Dunfield, P.F., Derakshani, M., Stubner, S., Heyer, J. and Liesack, W. (2003) Differential detection of type II methanotrophic bacteria in acidic peatlands using newly developed 16S rRNA-targeted fluorescent oligonucleotide probes. FEMS Microbiology Ecology 43(3), 299-308.

37. Dedysh, S.N., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Liesack, W. and Tiedje, J.M. (2002) Methylocapsa acidiphila gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. International journal of systematic and evolutionary microbiology 52(1), 251-261.

38. Dedysh, S.N., Knief, C. and Dunfield, P.F. (2005) Methylocella species are facultatively methanotrophic. Journal of Bacteriology 187(13), 4665-4670.

39. Dedysh, S.N., Liesack, W., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Bares, A.M., Panikov, N.S. and Tiedje, J.M. (2000) Methylocella palustris gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. International journal of systematic and evolutionary microbiology 50(3), 955-969.

40. Dedysh, S.N., Naumoff, D.G., Vorobev, A.V., Kyrpides, N., Woyke, T., Shapiro, N., Crombie, A.T., Murrell, J.C., Kalyuzhnaya, M.G., Smirnova, A.V. and Dunfield, P.F. (2015b) Draft Genome Sequence of Methyloferula stellata AR4, an Obligate Methanotroph Possessing Only a Soluble Methane Monooxygenase. Genome announcements 3(2), e01555-01514.

41. Dedysh, S.N., Panikov, N.S., Liesack, W., Großkopf, R., Zhou, J. and Tiedje, J.M. (1998a) Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. Science 282(5387), 281-284.

42. Dedysh, S.N., Panikov, N.S. and Tiedje, J.M. (1998b) Acidophilic Methanotrophic Communities fromSphagnum Peat Bogs. Applied and environmental microbiology 64(3), 922-929.

43. Denver, J.M., Ator, S.W., Fischer, J.M., Harned, D.C., Schubert, C. and Szabo, Z. (2015) Water Quality in the north Atlantic Coastal Plain Surficial Aquifer System, Delaware, Maryland, New Jersey, New York, North Carolina, and Virginia, 1988-2009. . https://pubs.usgs.gov/circ/1353/]., U.C.O. (ed).

44. DiSpirito, A.A., Gulledge, J., Shiemke, A.K., Murrell, J.C., Lidstrom, M.E. and Krema, C.L. (1991) Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs. Biodegradation 2(3), 151-164.

45. DiSpirito, A.A., Gulledge, J., Shiemke, A.K., Murrell, J.C., Lidstrom, M.E. and Krema, C.L. (1992) Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs. Biodegradation 2(3), 151-164.

46. Domaradzka, D., Guzik, U., Hupert-Kocurek, K. and Wojcieszyńska, D. (2015) Cometabolic degradation of naproxen by Planococcus sp. strain S5. Water, Air, & Soil Pollution 226(9), 297.

47. Dunfield, P.F., Belova, S.E., Vorob'ev, A.V., Cornish, S.L. and Dedysh, S.N. (2010) Methylocapsa aurea sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus Methylocapsa. International journal of systematic and evolutionary microbiology 60(11), 2659-2664.

48. Dunfield, P.F. and Dedysh, S.N. (2014) Methylocella: a gourmand among methanotrophs. Trends in microbiology 22(7), 368-369.

49. Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A. and Dedysh, S.N. (2003) Methylocella silvestris sp. nov., a novel methanotroph isolated from an acidic forest cambisol. International Journal of Systematic and Evolutionary Microbiology 53(5), 1231-1239.

50. Elango, N., Radhakrishnan, R., Froland, W.A., Wallar, B.J., Earhart, C.A., Lipscomb, J.D. and Ohlendorf, D.H. (1997) Crystal structure of the hydroxylase component of methane monooxygenase from Methylosinus trichosporium OB3b. Protein science : a publication of the Protein Society 6(3), 556-568.

51. Ely, R.L., Williamson, K.J., Hyman, M.R. and Arp, D.J. (1997) Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects, and bacterial response. Biotechnology and bioengineering 54(6), 520-534.

52. EPA (2009) Drinking Water Contaminant Candidate List 3.

53. EPA (2016) Fact Sheet on Trichloroethylene (TCE).

54. EPA (2017) TRI Releases for Trichloroethylene in the 2015 TRI National Analysis-Industry Sector.

55. Esson, K.C., Lin, X., Kumaresan, D., Chanton, J.P., Murrell, J.C. and Kostka, J.E. (2016) Alpha- and Gammaproteobacterial Methanotrophs Codominate the Active Methane-Oxidizing Communities in an Acidic Boreal Peat Bog. Applied and Environmental Microbiology 82(8), 2363-2371.

56. Farhan Ul Haque, M., Crombie, A.T., Ensminger, S.A., Baciu, C. and Murrell, J.C. (2018) Facultative methanotrophs are abundant at terrestrial natural gas seeps. Microbiome 6(1), 118.

57. Fogel, M.M., Taddeo, A.R. and Fogel, S. (1986) Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Applied and environmental microbiology 51(4), 720-724.

58. Fox, B.G., Borneman, J.G., Wackett, L.P. and Lipscomb, J.D. (1990) Haloalkene oxidation by the soluble methane monooxygenase from Methylosinus trichosporium OB3b: mechanistic and environmental implications. Biochemistry 29(27), 6419-6427.

59. Frascari, D., Pinelli, D., Nocentini, M., Fedi, S., Pii, Y. and Zannoni, D. (2006) Chloroform degradation by butane-grown cells of Rhodococcus aetherovorans BCP1. Applied Microbiology and Biotechnology 73(2), 421-428.

60. Frascari, D., Zanaroli, G. and Danko, A.S. (2015) In situ aerobic cometabolism of chlorinated solvents: A review. Journal of Hazardous Materials 283, 382-399.

61. Freedman, D.L. and Gossett, J.M. (1989) Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Applied and environmental microbiology 55(9), 2144-2151.

62. Grosse, S., Laramee, L., Wendlandt, K.-D., McDonald, I.R., Miguez, C.B. and Kleber, H.-P. (1999a) Purification and Characterization of the Soluble Methane Monooxygenase of the Type II Methanotrophic BacteriumMethylocystis sp. Strain WI 14. Applied and Environmental Microbiology 65(9), 3929-3935.

63. Grosse, S., Laramee, L., Wendlandt, K.-D., McDonald, I.R., Miguez, C.B. and Kleber, H.-P. (1999b) Purification and Characterization of the Soluble Methane Monooxygenase of the Type II Methanotrophic BacteriumMethylocystis sp. Strain WI 14. Applied and environmental microbiology 65(9), 3929-3935.

64. Halsey, K.H., Sayavedra-Soto, L.A., Bottomley, P.J. and Arp, D.J. (2005) Trichloroethylene degradation by butane-oxidizing bacteria causes a spectrum of toxic effects. Applied Microbiology and Biotechnology 68(6), 794-801.

65. Hamamura, N., Page, C., Long, T., Semprini, L. and Arp, D.J. (1997) Chloroform cometabolism by butane-grown CF8, Pseudomonas butanovora, and Mycobacterium vaccae JOB5 and methane-grown Methylosinus trichosporium OB3b. Appl. Environ. Microbiol. 63(9), 3607-3613.

66. Han, D., Dedysh, S.N. and Liesack, W. (2018) Unusual genomic traits suggest Methylocystis bryophila S285 to be well adapted for life in peatlands. Genome Biology and Evolution, evy025-evy025.

67. Han, J.I., Lontoh, S. and Semrau, J.D. (1999) Degradation of chlorinated and brominated hydrocarbons by Methylomicrobium album BG8. Archives of Microbiology 172(6), 393-400.

68. Hanson, R.S. and Hanson, T.E. (1996a) Methanotrophic bacteria. Microbiol Rev 60(2), 439-471.

69. Hanson, R.S. and Hanson, T.E. (1996b) Methanotrophic bacteria. Microbiol. Mol. Biol. Rev. 60(2), 439-471.

70. Hatzinger, P.B. and Chu, K.H. (2017) Role of Acidophilic Methanotrophs in Long Term Natural

71. Attenuation of cVOCs

72. He, R., Su, Y., Ma, R.-C. and Zhuang, S. (2018) Characterization of toluene metabolism by methanotroph and its effect on methane oxidation. Environmental Science and Pollution Research 25(17), 16816-16824.

73. Hesselsoe, M., Boysen, S., Iversen, N., Jørgensen, L., Murrell, J.C., McDonald, I., Radajewski, S., Thestrup, H. and Roslev, P. (2005) Degradation of organic pollutants by methane grown microbial consortia. Biodegradation 16(5), 435-448.

74. Holmes, A.J., Costello, A., Lidstrom, M.E. and Murrell, J.C. (1995) Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS microbiology letters 132(3), 203-208.

75. Humans, I.W.G.o.t.E.o.C.R.t. (2014) Trichloroethylene, Tetrachloroethylene, and Some Other Chlorinated Agents. IARC monographs on the evaluation of carcinogenic risks to humans 106, 1.

76. Iguchi, H., Yurimoto, H. and Sakai, Y. (2011) Methylovulum miyakonense gen. nov., sp. nov., a type I methanotroph isolated from forest soil. International journal of systematic and evolutionary microbiology 61(4), 810-815.

77. Im, J. and Semrau, J.D. (2011) Pollutant degradation by a Methylocystis strain SB2 grown on ethanol: bioremediation via facultative methanotrophy. FEMS Microbiology Letters 318(2), 137-142.

78. Islam, T., Torsvik, V., Larsen, Ø., Bodrossy, L., Øvreås, L. and Birkeland, N.-K. (2016) Acid-Tolerant Moderately Thermophilic Methanotrophs of the Class Gammaproteobacteria Isolated From Tropical Topsoil with Methane Seeps. Frontiers in Microbiology 7, 851.

79. Jablonski, P.E. and Ferry, J.G. (1992) Reductive dechlorination of trichloroethylene by the CO-reduced CO dehydrogenase enzyme complex from Methanosarcina thermophila. FEMS Microbiology Letters 96(1), 55-59.

80. Jagadevan, S. and Semrau, J.D. (2013) Priority pollutant degradation by the facultative methanotroph, Methylocystis strain SB2. Applied Microbiology and Biotechnology 97(11), 5089-5096.

81. Jesus, J., Frascari, D., Pozdniakova, T. and Danko, A.S. (2016) Kinetics of aerobic cometabolic biodegradation of chlorinated and brominated aliphatic hydrocarbons: A review. Journal of Hazardous Materials 309, 37-52.

82. Kalidass, B., Ul-Haque, M.F., Baral, B.S., DiSpirito, A.A. and Semrau, J.D. (2015) Competition between metals for binding to methanobactin enables expression of soluble methane monooxygenase in the presence of copper. Applied and environmental microbiology 81(3), 1024-1031.

83. Kang, J., Lee, E. and Park, S. (2001) Co-metabolic biodegradation of trichloroethylene by Methylosinus trichosporium is stimulated by low concentrations methane or methanol. Biotechnology letters 23(22), 1877-1882.

84. Kang, J.W. and Doty, S.L. (2014) Cometabolic degradation of trichloroethylene by Burkholderia cepacia G4 with poplar leaf homogenate. Canadian journal of microbiology 60(7), 487-490.

85. Kenney, G.E., Goering, A.W., Ross, M.O., DeHart, C.J., Thomas, P.M., Hoffman, B.M., Kelleher, N.L. and Rosenzweig, A.C. (2016a) Characterization of methanobactin from Methylosinus sp. LW4. Journal of the American Chemical Society 138(35), 11124-11127.

86. Kenney, G.E. and Rosenzweig, A.C. (2018) Methanobactins: maintaining copper homeostasis in methanotrophs and beyond. Journal of Biological Chemistry, jbc. TM117. 000185.

87. Kenney, G.E., Sadek, M. and Rosenzweig, A.C. (2016b) Copper-responsive gene expression in the methanotroph Methylosinus trichosporium OB3b. Metallomics 8(9), 931-940.

88. Khmelenina, V.N., Kalyuzhnaya, M.G., Starostina, N.G., Suzina, N.E. and Trotsenko, Y.A. (1997) Isolation and Characterization of Halotolerant Alkaliphilic Methanotrophic Bacteria from Tuva Soda Lakes. Current Microbiology 35(5), 257-261.

89. Kim, H.J., Graham, D.W., DiSpirito, A.A., Alterman, M.A., Galeva, N., Larive, C.K., Asunskis, D. and Sherwood, P.M. (2004) Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria. Science 305(5690), 1612-1615.

90. Kim, S., Bae, W., Hwang, J. and Park, J. (2010) Aerobic TCE degradation by encapsulated toluene-oxidizing bacteria, Pseudomonas putida and Bacillus spp. Water Science and Technology 62(9), 1991-1997.

91. Kim, Y., Arp, D.J. and Semprini, L. (2000) Chlorinated solvent cometabolism by butane-grown mixed culture. Journal of environmental engineering 126(10), 934-942.

92. Kip, N., Fritz, C., Langelaan, E.S., Pan, Y., Bodrossy, L., Pancotto, V., Jetten, M.S.M., Smolders, A.J.P. and Op den Camp, H.J.M. (2012) Methanotrophic activity and diversity in different Sphagnum magellanicum dominated habitats in the southernmost peat bogs of Patagonia. Biogeosciences 9(1), 47-55.

93. Kip, N., Ouyang, W., van Winden, J., Raghoebarsing, A., van Niftrik, L., Pol, A., Pan, Y., Bodrossy, L., van Donselaar, E.G. and Reichart, G.-J. (2011) Detection, isolation, and characterization of acidophilic methanotrophs from Sphagnum mosses. Applied and environmental microbiology 77(16), 5643-5654.

94. Kitmitto, A., Myronova, N., Basu, P. and Dalton, H. (2005) Characterization and Structural Analysis of an Active Particulate Methane Monooxygenase Trimer from Methylococcus capsulatus (Bath). Biochemistry 44(33), 10954-10965.

95. Knief, C. (2015) Diversity and Habitat Preferences of Cultivated and Uncultivated Aerobic Methanotrophic Bacteria Evaluated Based on pmoA as Molecular Marker. Frontiers in microbiology 6(1346).

96. Koh, S.-C., Bowman, J.P. and Sayler, G.S. (1993a) Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, Methylomonas methanica 68-1. Appl. Environ. Microbiol. 59(4), 960-967.

97. Koh, S.C., Bowman, J.P. and Sayler, G.S. (1993b) Soluble Methane Monooxygenase Production and Trichloroethylene Degradation by a Type I Methanotroph, Methylomonas methanica 68-1. Appl Environ Microbiol 59(4), 960-967.

98. Kox, M.A., Haque, M.F.U., van Alen, T.A., Crombie, A.T., Jetten, M.S., den Camp, H.J.O., Dedysh, S.N., van Kessel, M.A. and Murrell, J.C. (2019) Complete Genome Sequence of the Aerobic Facultative Methanotroph Methylocella tundrae Strain T4. Microbiology resource announcements 8(20), e00286-00219.

99. Kulikova, A. and Bezborodov, A. (2000) Oxidation of organic compounds by Rhodococcus erythropolis 3/89 propanomonooxygenase. Prikladnaia biokhimiia i mikrobiologiia 36(3), 267-271.

100. Lawton, T.J. and Rosenzweig, A.C. (2016) Methane-Oxidizing Enzymes: An Upstream Problem in Biological Gas-to-Liquids Conversion. Journal of the American Chemical Society 138(30), 9327-9340.

101. Le, N.B. and Coleman, N.V. (2011) Biodegradation of vinyl chloride, cis-dichloroethene and 1,2-dichloroethane in the alkene/alkane-oxidising Mycobacterium strain NBB4. Biodegradation 22(6), 1095-1108.

102. Lee, E.-H., Park, H. and Cho, K.-S. (2011) Effect of substrate interaction on oxidation of methane and benzene in enriched microbial consortia from landfill cover soil. Journal of Environmental Science and Health, Part A 46(9), 997-1007.

103. Lee, S.-W., Keeney, D.R., Lim, D.-H., Dispirito, A.A. and Semrau, J.D. (2006) Mixed pollutant degradation by Methylosinus trichosporium OB3b expressing either soluble or particulate methane monooxygenase: can the tortoise beat the hare? Applied and Environmental Microbiology 72(12), 7503-7509.

104. Lee, S.J. (2016) Hydroxylation of methane through component interactions in soluble methane monooxygenases. Journal of Microbiology 54(4), 277-282.

105. Leng, L., Chang, J., Geng, K., Lu, Y. and Ma, K. (2015) Uncultivated Methylocystis Species in Paddy Soil Include Facultative Methanotrophs that Utilize Acetate. Microbial Ecology 70(1), 88-96.

106. Li, S., Wang, S. and Yan, W. (2016) Biodegradation of methyl tert-butyl ether by co-metabolism with a Pseudomonas sp. strain. International journal of environmental research and public health 13(9), 883.

107. Little, C.D., Palumbo, A.V., Herbes, S.E., Lidstrom, M.E., Tyndall, R.L. and Gilmer, P.J. (1988a) Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. 54(4), 951-956.

108. Little, C.D., Palumbo, A.V., Herbes, S.E., Lidstrom, M.E., Tyndall, R.L. and Gilmer, P.J. (1988b) Trichloroethylene biodegradation by a methane-oxidizing bacterium. Applied and environmental microbiology 54(4), 951-956.

109. Lontoh, S. and Semrau, J.D. (1998a) Methane and Trichloroethylene Degradation by Methylosinus trichosporium OB3b Expressing Particulate Methane Monooxygenase. Applied and Environmental Microbiology 64(3), 1106-1114.

110. Lontoh, S. and Semrau, J.D. (1998b) Methane and Trichloroethylene Degradation by Methylosinus trichosporium OB3b Expressing Particulate Methane Monooxygenase. Applied and environmental microbiology 64(3), 1106-1114.

111. Lontoh, S. and Semrau, J.D. (1998c) Methane and Trichloroethylene Degradation byMethylosinus trichosporium OB3b Expressing Particulate Methane Monooxygenase. Appl. Environ. Microbiol. 64(3), 1106-1114.

112. Mahendra, S. and Alvarez-Cohen, L. (2006) Kinetics of 1,4-Dioxane Biodegradation by Monooxygenase-Expressing Bacteria. Environmental science & technology 40(17), 5435-5442.

113. Malachowsky, K.J., Phelps, T.J., Teboli, A.B., Minnikin, D.E. and White, D.C. (1994) Aerobic Mineralization of Trichloroethylene, Vinyl Chloride, and Aromatic Compounds by Rhodococcus Species. Applied and Environmental Microbiology 60(2), 542-548.

114. Mardina, P., Li, J., Patel, S.K., Kim, I.-W., Lee, J.-K. and Selvaraj, C. (2016) Potential of immobilized whole-cell Methylocella tundrae as a biocatalyst for methanol production from methane. J. Microbiol. Biotechnol 26(7), 1234-1241.

115. Maymo-Gatell, X., Chien, Y.-t., Gossett, J.M. and Zinder, S.H. (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276(5318), 1568-1571.

116. McDonald, I., Uchiyama, H., Kambe, S., Yagi, O. and Murrell, J. (1997a) The soluble methane monooxygenase gene cluster of the trichloroethylene-degrading methanotroph Methylocystis sp. strain M. Appl. Environ. Microbiol. 63(5), 1898-1904.

117. McDonald, I.R., Bodrossy, L., Chen, Y. and Murrell, J.C. (2008) Molecular ecology techniques for the study of aerobic methanotrophs. Applied and Environmental Microbiology 74(5), 1305-1315.

118. McDonald, I.R., Hall, G.H., Pickup, R.W. and Colin Murrell, J. (1996) Methane oxidation potential and preliminary analysis of methanotrophs in blanket bog peat using molecular ecology techniques. FEMS microbiology ecology 21(3), 197-211.

119. McDonald, I.R., Uchiyama, H., Kambe, S., Yagi, O. and Murrell, J.C. (1997b) The soluble methane monooxygenase gene cluster of the trichloroethylene-degrading methanotroph Methylocystis sp. strain M. Applied and Environmental Microbiology 63(5), 1898-1904.

120. Middeldorp, P.J., Luijten, M.L., Pas, B.A.v.d., Eekert, M.H.v., Kengen, S.W., Schraa, G. and Stams, A.J. (1999) Anaerobic microbial reductive dehalogenation of chlorinated ethenes. Bioremediation Journal 3(3), 151-169.

121. Miroshnikov, K.K., Didriksen, A., Naumoff, D.G., Huntemann, M., Clum, A., Pillay, M., Palaniappan, K., Varghese, N., Mikhailova, N., Mukherjee, S., Reddy, T.B.K., Daum, C., Shapiro, N., Ivanova, N., Kyrpides, N., Woyke, T., Dedysh, S.N. and Svenning, M.M. (2017) Draft Genome Sequence of Methylocapsa palsarum NE2(T), an Obligate Methanotroph from Subarctic Soil. Genome announcements 5(24), e00504-00517.

122. Nelson, M., Montgomery, S. and Pritchard, P. (1988) Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Applied and Environmental Microbiology 54(2), 604-606.

123. Nelson, M.J., Montgomery, S., O'neill, E. and Pritchard, P. (1986) Aerobic metabolism of trichloroethylene by a bacterial isolate. Applied and Environmental Microbiology 52(2), 383-384.

124. Neumann, A., Wohlfarth, G. and Diekert, G. (1996) Purification and characterization of tetrachloroethene reductive dehalogenase from Dehalospirillum multivorans. Journal of Biological Chemistry 271(28), 16515-16519.

125. Nguyen, N.-L., Yu, W.-J., Gwak, J.-H., Kim, S.-J., Park, S.-J., Herbold, C.W., Kim, J.-G., Jung, M.-Y. and Rhee, S.-K. (2018) Genomic Insights Into the Acid Adaptation of Novel Methanotrophs Enriched From Acidic Forest Soils. Frontiers in microbiology 9(1982).

126. Nzila, A. (2013) Update on the cometabolism of organic pollutants by bacteria. Environmental Pollution 178, 474-482.

127. Oldenhuis, R., Oedzes, J.Y., Van der Waarde, J. and Janssen, D.B. (1991) Kinetics of chlorinated hydrocarbon degradation by Methylosinus trichosporium OB3b and toxicity of trichloroethylene. Applied and Environmental Microbiology 57(1), 7-14.

128. Oldenhuis, R., Vink, R., Janssen, D.B. and Witholt, B. (1989a) Degradation of chlorinated aliphatic hydrocarbons by Methylosinus trichosporium OB3b expressing soluble methane monooxygenase. Applied and environmental microbiology 55(11), 2819-2826.

129. Oldenhuis, R., Vink, R., Janssen, D.B. and Witholt, B. (1989b) Degradation of chlorinated aliphatic hydrocarbons by Methylosinus trichosporium OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55(11), 2819-2826.

130. Oldenhuis, R., Vink, R.L., Janssen, D.B. and Witholt, B. (1989c) Degradation of chlorinated aliphatic hydrocarbons by Methylosinus trichosporium OB3b expressing soluble methane monooxygenase. Applied and Environmental Microbiology 55(11), 2819-2826.

131. Oshkin, I.Y., Miroshnikov, K.K. and Dedysh, S.N. (2019) Draft Genome Sequence of Methylocystis heyeri H2(T), a Methanotroph with Habitat-Specific Adaptations, Isolated from a Peatland Ecosystem. Microbiology resource announcements 8(29), e00454-00419.

132. Patel, S.K., Mardina, P., Kim, S.-Y., Lee, J.-K. and Kim, I.-W. (2016) Biological methanol production by a type II methanotroph Methylocystis bryophila. J. Microbiol. Biotechnol 26(4), 717-724.

133. Patel, S.K.S., Jeon, M.S., Gupta, R.K., Jeon, Y., Kalia, V.C., Kim, S.C., Cho, B.K., Kim, D.R. and Lee, J.-K. (2019) Hierarchical Macroporous Particles for Efficient Whole-Cell Immobilization: Application in Bioconversion of Greenhouse Gases to Methanol. ACS Applied Materials & Interfaces 11(21), 18968-18977.

134. Patel, S.K.S., Kumar, V., Mardina, P., Li, J., Lestari, R., Kalia, V.C. and Lee, J.-K. (2018) Methanol production from simulated biogas mixtures by co-immobilized Methylomonas methanica and Methylocella tundrae. Bioresource Technology 263, 25-32.

135. Patel, S.K.S., Singh, R.K., Kumar, A., Jeong, J.-H., Jeong, S.H., Kalia, V.C., Kim, I.-W. and Lee, J.-K. (2017) Biological methanol production by immobilized Methylocella tundrae using simulated biohythane as a feed. Bioresource Technology 241, 922-927.

136. Pfiffner, S.M., Palumbo, A.V., Phelps, T.J. and Hazen, T.C. (1997) Effects of nutrient dosing on subsurface methanotrophic populations and trichloroethylene degradation. Journal of Industrial Microbiology and Biotechnology 18(2), 204-212.

137. Rahman, M.T., Crombie, A., Chen, Y., Stralis-Pavese, N., Bodrossy, L., Meir, P., McNamara, N.P. and Murrell, J.C. (2011a) Environmental distribution and abundance of the facultative methanotroph Methylocella. The ISME journal 5(6), 1061.

138. Rahman, M.T., Crombie, A., Moussard, H., Chen, Y. and Murrell, J.C. (2011b) Acetate repression of methane oxidation by supplemental Methylocella silvestris in a peat soil microcosm. Applied and Environmental Microbiology 77(12), 4234-4236.

139. Ricke, P., Kube, M., Nakagawa, S., Erkel, C., Reinhardt, R. and Liesack, W. (2005) First genome data from uncultured upland soil cluster alpha methanotrophs provide further evidence for a close phylogenetic relationship to Methylocapsa acidiphila B2 and for high-affinity methanotrophy involving particulate methane monooxygenase. Applied and Environmental Microbiology 71(11), 7472-7482.

140. Ryoo, D., Shim, H., Canada, K., Barbieri, P. and Wood, T.K. (2000) Aerobic degradation of tetrachloroethylene by toluene-o-xylene monooxygenase of Pseudomonas stutzeri OX1. Nature Biotechnology 18(7), 775-778.

141. Salter, A.J., Johnson, R. and Tratnyek Paul, G. (2010) Degradation of 1, 2, 3-trichloropropane by zero-valent zinc: Laboratory assessment for field application, pp. 24-27.

142. Samin, G. and Janssen, D.B. (2012) Transformation and biodegradation of 1, 2, 3-trichloropropane (TCP). Environmental Science and Pollution Research 19(8), 3067-3078.

143. Schäfer, D., Köber, R. and Dahmke, A. (2003) Competing TCE and cis-DCE degradation kinetics by zero-valent iron—experimental results and numerical simulation. Journal of Contaminant Hydrology 65(3-4), 183-202.

144. Sconce, J.S. (1962) Chlorine: its manufacture, properties and uses, Reinhold Pub. Corp.

145. Semprini, L. (1995) In situ bioremediation of chlorinated solvents. Environmental Health Perspectives 103(Suppl 5), 101-105.

146. Semprini, L., Hopkins, G.D., Roberts, P.V., Grbic-Galic, D. and McCarty, P.L. (1991) A Field Evaluation of In-Situ Biodegradation of Chlorinated Ethenes: Part 3, Studies of Competitive Inhibition. Ground Water 29(2), 239-250.

147. Semprini, L., Roberts, P.V., Hopkins, G.D. and McCarty, P.L. (1990) A Field Evaluation of In-Situ Biodegradation of Chlorinated Ethenes: Part 2, Results of Biostimulation and Biotransformation Experiments. Groundwater 28(5), 715-727.

148. Semrau, J. (2011) Bioremediation via Methanotrophy: Overview of Recent Findings and Suggestions for Future Research. Frontiers in microbiology 2(209).

149. Semrau, J.D., DiSpirito, A.A. and Vuilleumier, S. (2011) Facultative methanotrophy: false leads, true results, and suggestions for future research. FEMS Microbiology Letters 323(1), 1-12.

150. Semrau, J.D., DiSpirito, A.A. and Yoon, S. (2010) Methanotrophs and copper. FEMS Microbiology Reviews 34(4), 496-531.

151. Shao, Y., Hatzinger, P.B., Streger, S.H., Rezes, R.T. and Chu, K.-H. (2019) Evaluation of methanotrophic bacterial communities capable of biodegrading trichloroethene (TCE) in acidic aquifers. Biodegradation 30(2), 173-190.

152. Sharp, J.O., Wood, T.K. and Alvarez-Cohen, L. (2005) Aerobic biodegradation of N-nitrosodimethylamine (NDMA) by axenic bacterial strains. Biotechnology and Bioengineering 89(5), 608-618.

153. Shen, R.-n., Yu, C.-l., Ma, Q.-q. and Li, S.-b. (1997) Direct Evidence for a Soluble Methane Monooxygenase from Type I Methanotrophic Bacteria: Purification and Properties of a Soluble Methane Monooxygenase fromMethylomonassp. GYJ3. Archives of Biochemistry and Biophysics 345(2), 223-229.

154. Shigematsu, T., Hanada, S., Eguchi, M., Kamagata, Y., Kanagawa, T. and Kurane, R. (1999) Soluble methane monooxygenase gene clusters from trichloroethylene-degrading Methylomonas sp. strains and detection of methanotrophs during in situ bioremediation. Applied and Environmental Microbiology 65(12), 5198-5206.

155. Shukla, A.K., Vishwakarma, P., Upadhyay, S., Tripathi, A.K., Prasana, H. and Dubey, S.K. (2009) Biodegradation of trichloroethylene (TCE) by methanotrophic community. Bioresource technology 100(9), 2469-2474.

156. Singh, J. and Singh, D.P. (2017).

157. Sirajuddin, S. and Rosenzweig, A.C. (2015) Enzymatic oxidation of methane. Biochemistry 54(14), 2283-2294.

158. Smirnova, A.V. and Dunfield, P.F. (2018) Differential Transcriptional Activation of Genes Encoding Soluble Methane Monooxygenase in a Facultative Versus an Obligate Methanotroph. Microorganisms 6(1), 20.

159. Stafford, G.P., Scanlan, J., McDonald, I.R. and Murrell, J.C. (2003) rpoN, mmoR and mmoG, genes involved in regulating the expression of soluble methane monooxygenase in Methylosinus trichosporium OB3b. Microbiology 149(7), 1771-1784.

160. Steffan, R.J., McClay, K., Vainberg, S., Condee, C.W. and Zhang, D. (1997) Biodegradation of the gasoline oxygenates methyl tert-butyl ether, ethyl tert-butyl ether, and tert-amyl methyl ether by propane-oxidizing bacteria. Applied and Environmental Microbiology 63(11), 4216-4222.

161. Swanson, P.E. (1999) Dehalogenases applied to industrial-scale biocatalysis. Current Opinion in Biotechnology 10(4), 365-369.

162. Takeuchi, M., Nanba, K., Iwamoto, H., Nirei, H., Kusuda, T., Kazaoka, O., Owaki, M. and Furuya, K. (2005) In situ bioremediation of a cis-dichloroethylene-contaminated aquifer utilizing methane-rich groundwater from an uncontaminated aquifer. Water research 39(11), 2438-2444.

163. Tamas, I., Smirnova, A.V., He, Z. and Dunfield, P.F. (2014) The (d)evolution of methanotrophy in the Beijerinckiaceae--a comparative genomics analysis. The ISME journal 8(2), 369-382.

164. Tandoi, V., DiStefano, T.D., Bowser, P.A., Gossett, J.M. and Zinder, S.H. (1994) Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. Environmental science & technology 28(5), 973-979.

165. Tavormina, P.L., Orphan, V.J., Kalyuzhnaya, M.G., Jetten, M.S.M. and Klotz, M.G. (2011) A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. Environmental Microbiology Reports 3(1), 91-100.

166. Terzenbach, D.P. and Blaut, M. (1994) Transformation of tetrachloroethylene to trichloroethylene by homoacetogenic bacteria. FEMS Microbiology Letters 123(1-2), 213-218.

167. Theisen, A.R., Ali, M.H., Radajewski, S., Dumont, M.G., Dunfield, P.F., McDonald, I.R., Dedysh, S.N., Miguez, C.B. and Murrell, J.C. (2005) Regulation of methane oxidation in the facultative methanotroph Methylocella silvestris BL2. Molecular Microbiology 58(3), 682-692.

168. Tsien, H.-C., Brusseau, G.A., Hanson, R.S. and Waclett, L. (1989a) Biodegradation of trichloroethylene by Methylosinus trichosporium OB3b. Appl. Environ. Microbiol. 55(12), 3155-3161.

169. Tsien, H.-C., Brusseau, G.A., Hanson, R.S. and Waclett, L. (1989b) Biodegradation of trichloroethylene by Methylosinus trichosporium OB3b. Applied and environmental microbiology 55(12), 3155-3161.

170. Uchiyama, H., Nakajima, T., Yagi, O. and Tabuchi, T. (1989a) Aerobic degradation of trichloroethylene at high concentration by a methane-utilizing mixed culture. Agricultural and Biological Chemistry 53(4), 1019-1024.

171. Uchiyama, H., Nakajima, T., Yagi, O. and Tabuchi, T. (1989b) Aerobic degradation of trichloroethylene by a new type II methane-utilizing bacterium, strain M. Agricultural and Biological Chemistry 53(11), 2903-2907.

172. Ul-Haque, M.F., Kalidass, B., Vorobev, A., Baral, B.S., DiSpirito, A.A. and Semrau, J.D. (2015) Methanobactin from Methylocystis sp. Strain SB2 Affects Gene

Expression and Methane Monooxygenase Activity in Methylosinus trichosporium OB3b. Applied and environmental microbiology 81(7), 2466-2473.

173. Vainberg, S., Condee, C.W. and Steffan, R.J. (2009) Large-scale production of bacterial consortia for remediation of chlorinated solvent-contaminated groundwater. Journal of Industrial Microbiology and Biotechnology 36(9), 1189-1197.

174. Vogel, T.M. and McCARTY, P.L. (1985) Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Applied and environmental microbiology 49(5), 1080-1083.

175. Vorobev, A., Jagadevan, S., Baral, B.S., DiSpirito, A.A., Freemeier, B.C., Bergman, B.H., Bandow, N.L. and Semrau, J.D. (2013) Detoxification of mercury by methanobactin from Methylosinus trichosporium OB3b. Applied and environmental microbiology, AEM. 01673-01613.

176. Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F. and Dedysh, S.N. (2011) Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase. International journal of systematic and evolutionary microbiology 61(10), 2456-2463.

177. Wackett, L.P., Brusseau, G.A., Householder, S.R. and Hanson, R.S. (1989) Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Applied and Environmental Microbiology 55(11), 2960-2964.

178. Wang, B. and Chu, K.-H. (2017) Cometabolic biodegradation of 1,2,3-trichloropropane by propane-oxidizing bacteria. Chemosphere 168, 1494-1497.

179. Wild, A., Hermann, R. and Leisinger, T. (1996) Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene. Biodegradation 7(6), 507-511.

180. Wilkins, P.C., Dalton, H., Samuel, C.J. and Green, J. (1994) Further Evidence for Multiple Pathways in Soluble Methane-Monooxygenase-Catalysed Oxidations from the Measurement of Deuterium Kinetic Isotope Effects. European Journal of Biochemistry 226(2), 555-560.

181. Wilson, J.T. and Wilson, B.H. (1985) Biotransformation of trichloroethylene in soil. Applied and environmental microbiology 49(1), 242.

182. Wüst, W.F., Köber, R., Schlicker, O. and Dahmke, A. (1999) Combined zero-and first-order kinetic model of the degradation of TCE and cis-DCE with commercial iron. Environmental science & technology 33(23), 4304-4309.

183. Wymore, R.A., Lee, M.H., Keener, W.K., Miller, A.R., Colwell, F.S., Watwood, M.E. and Sorenson, K.S. (2007) Field Evidence for Intrinsic Aerobic Chlorinated Ethene

Cometabolism by Methanotrophs Expressing Soluble Methane Monooxygenase. Bioremediation Journal 11(3), 125-139.

184. Xin, J.-y., Cheng, D.-d., Zhang, L.-x., Lin, K., Fan, H.-c., Wang, Y. and Xia, C.-g. (2013) Methanobactin-Mediated One-Step Synthesis of Gold Nanoparticles. International Journal of Molecular Sciences 14(11), 21676-21688.

185. Yang, Y., Capiro, N.L. and Marcet, T.F. (2017) Organohalide Respiration with Chlorinated Ethenes under Low pH Conditions. 51(15), 8579-8588.

186. Yoon, S., Im, J., Bandow, N., DiSpirito, A.A. and Semrau, J.D. (2011) Constitutive expression of pMMO by Methylocystis strain SB2 when grown on multi-carbon substrates: implications for biodegradation of chlorinated ethenes. Environmental Microbiology Reports 3(2), 182-188.

187. Yu, C.-P. and Chu, K.-H. (2005) A quantitative assay for linking microbial community function and structure of a naphthalene-degrading microbial consortium. Environmental Science & Technology 39(24), 9611-9619.

188. Zhou, L.-J., Han, P., Yu, Y., Wang, B., Men, Y., Wagner, M. and Wu, Q.L. (2019) Cometabolic biotransformation and microbial-mediated abiotic transformation of sulfonamides by three ammonia oxidizers. Water research 159, 444-453.

189. Zogorski, J.S., Carter, J.M., Ivahnenko, T., Lapham, W.W., Moran, M.J., Rowe, B.L., Squillace, P.J. and Toccalino, P.L. (2006) Volatile organic compounds in the nation's ground water and drinking-water supply wells. US Geological Survey Circular 1292, 101.

APPENDIX A[*]

Supporting Information of Section 3: methods, Table A1, Figures A1, A2 and A3.

- 13C-DNA and 12C-DNA separation.
- Analysis of Active Microbial Community Structure using real-time t-RFLP.
- Cloning and sequencing.
- Table A1. Primer information for detecting *mmo*X and *pmo*A genes.
- Figure A1. pH in the Kirkwood microcosms over time.
- Figure A2. Biodegradation of TCE in microcosms prepared from acidic aquifer samples collected from the Dahlgren site.

13C-DNA and 12C-DNA separation. Briefly, the extracted DNA (1 µg) was loaded into 3.5-mL Beckman centrifuge tubes containing 1.0 g/mL CsCl in TE buffer solution. All tubes were sealed and centrifuged using a desk-top Beckman Coulter TLX-120 Optima Ultracentrifuge in a TLA 100.3 rotor at 70,000 rpm at 20°C for 24 h. A fraction recovery system (Beckman Coulter, CA) was used to collect 13C- and 12C-DNA fractions. The syringe pump (Model 7801001, Fisher Scientific, Fair Lawn, NJ) at a flow rate 250 µL/min was used to dispense mineral oil into the top of the tube and collected the 20 fractions of 100 µL from the bottom of the tube. The buoyant density of each DNA fraction was determined based on the refractive index that was measured by a Reichert AR200 digital refractometer (Depew, NY). The DNA fractions were extracted and recovered via ethanol precipitation. The recovered DNA were stored at -20 °C before use.

[*] Reprinted with permission from "Evaluation of methanotrophic bacterial communities capable of biodegrading trichloroethene (TCE) in acidic aquifers" by Yiru Shao, Paul B. Hatzinger, Sheryl H. Streger et al, 2019. Biodegradation, volume 30, pp173-190, copyright(2019) by Springer.

Analysis of Active Microbial Community Structure using real-time t-RFLP. The initial copies in samples were determined on the basis of standard curves using plasmid no. 931 that carries a partial 16S rRNA gene for Nitrospira (GenBank accession number AF420301). The PCR products were excised from a 0.9% agarose gel in 1% TAE buffer, recovered and purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The purified PCR products were then digested with restriction enzyme *Msp*I (Promega Corp., Madison, WI), precipitated with ethanol, and resuspended in molecular-biology grade water. The lengths of T-RFs of digested PCR products were automatically determined on an ABI 3130xl Genetic Analyzer (DNA Technologies Core Lab, College Station, TX) by comparing internal standards (Mapmarker ROX-400 standards) using the Peak Scan 2.0 version. Genomic DNA extracted from microcosms receiving unlabelled methane were used as templates for determining overall microbial community structures. The 13CDNA fractions from 13C-methane-amended microcosms were used as templates to determine the active methane-oxidizing microbial community structure. The real-time PCR reactions were performed using Bio-Rad iQ5 multicolor Real-Time PCR detection System (Hercules, CA). The results of terminal fragments were analyzed with online program MiCA3.

Table A1. Primer information for detecting *mmo*X and *pmo*A genes

Primers	**Enzyme**	**Annealing temperature**	**PCR products length(bps)**
mmoX(A-B)	sMMO	60	1230
mmoX(LF-LR)	sMMO	58	450
pmoA(A189-mb661)	pMMO	54	500
pmoA(A189-A682)	pMMO	56	525

Cloning and sequencing. Briefly, the amplified fragments of the 16S rRNA genes were cloned into the vector pCR4-TOPO using a TA Cloning Kit (One Shot TOP10 Competent cells) (Invitrogen, Carlsbad, CA). Colonies with inserts were verified by PCR with primers M13F (5´-GTAAAACGACGGCCAG-3´) and M13R (5´-CAGGAAACAGCTATGAC-3´), and screened on 0.9 % agarose gels (Fisher Scientific, Fair Lawn, NJ). For each 13C-DNA fraction sample, seventy clones were randomly selected from the plates. The amplified 16S rRNA were purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA), followed by digestion with enzymes, HhaI and HaeIII (Promega Corp., Madison, WI). The clones with unique restriction fragment length polymorphism (RFLP) patterns on 4% metaphor agarose gels were then selected for sequencing. The selected clones were grown overnight in 5 mL of Luria-Bertani (LB) broth with 50 mg/L kanamycin before use for plasmid extraction. The plasmids were extracted using a plasmid purification Kit (QIAGEN, Valencia, CA). The inserted sequences were determined by Eton Bioscience Inc. Phylogenetic analyses of aligned sequences were created using neighborjoining software MEGA version 7.0.

Figure A1. pH in the Kirkwood microcosms over time.

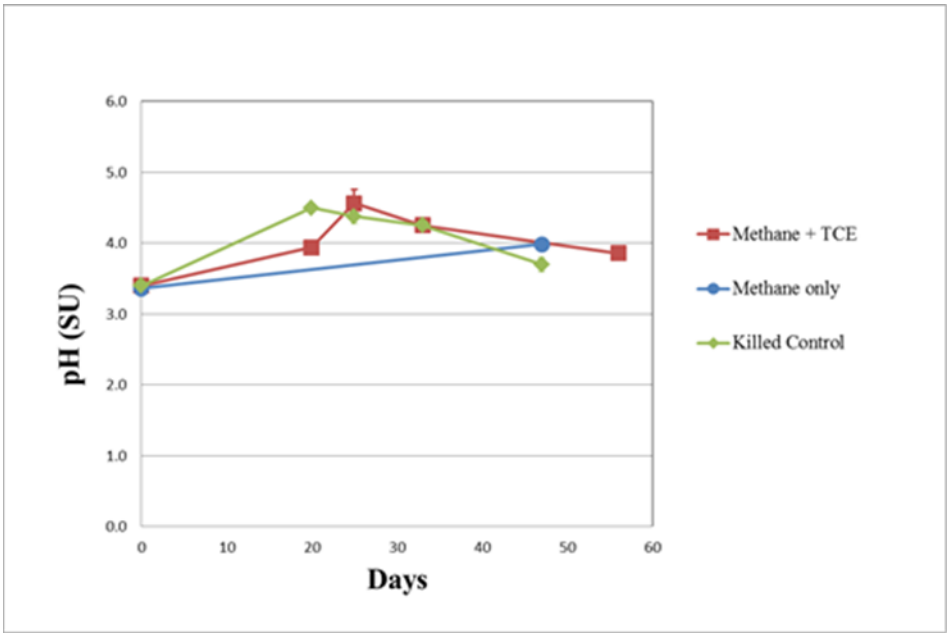


Figure A2. Biodegradation of TCE in microcosms prepared from acidic aquifer samples collected from the Dahlgren site