Republic of Iraq

Ministry of Higher Education

and Scientific Research

Al-Nahrain University,

College of Science,

Biotechnology Department

Investigation of Methane Oxidizers Community in Soil by Using Denaturant Gradient Gel Electrophoresis

A Thesis

Submitted to the Council of College of Science, Al-Nahrain University, in Partial Fulfillment of the Requirements for the Degree of Master in Science, Biotechnology

By:

Samer Imad Al-Saffar

B.Sc. in Biotechnology, Al-Nahrain University 2009

Supervised By:

Prof. Majid Hussien Al-Jailawi

Assist. Prof. Ali Abd Al-Hafedh Ibrahim

December, 2013 Sufar, 1435

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and gratitude to:

- Professor Majid Al-Jailawi for all his teachings, motivation, and guide throughout my whole time at the department of Biotechnology.
- Dr. Ali A. Ibrahim for his continuous support, invaluable advice, and for all the troubles I put him through.
- Professor Warren Dick, at the Ohio State University, for accepting me at his lab and providing me with a chance to acquire tremendous experience and knowing him as the generous man he is; without his aid, this work would have never been done.
- Zaid Al-Temimi whom I consider nothing less than a brother.
- Ahmed Khamis for making my stay in the United States less lonely.
- Subbu Kumarappan, Nathan Crook, Qichun Zhang, Aditi Sengupta, and all my other friends at OSU for helping me adjust to life at US and for all those Walmart trips.
- All my friends inside and outside Al-Nahrain University for their support and for being there when I needed them the most.
- Last, but not least, the staff of Biotechnology department/Al-Nahrain University who not only they were part of my academic study but also, throughout the years, we have become a family.

Summary

Methanotrophs in soil serves as a major atmospheric methane sink, and soil management has a direct impact on the diversity of microorganisms in soil. This study was set to identify methanotrophs in soil under different management systems. The study was conducted at the Ohio Agricultural Research and Development Center (OARDC), Ohio State University (OSU), Wooster, Ohio, US as part of their long continuous research in No-Till production. Twenty-four samples were collected during winter (February, 2012) from different ecological sites; (1) no-tillage, (2) tillage, (3) grassland, and (4) forest. Direct DNA extraction enabled specific amplification of *pmoA*, encoding a subunit of the particulate methane monooxygenase encoding gene, and the 16S rRNA genes of methanotrophs for direct identification.

Primers targeting *pmoA* and 16S rRNA genes of both type-I and type-II methanotrophs were all tested via *in silico* polymerase chain reaction (PCR). The primers which gave best results and were chosen for this study are the A189f-A682r and A189f-mb661 primer sets for specific amplification of *pmoA*; and type IF-type IR and type IIF-type IIR for the amplification of the 16S rRNA genes of type-I and type-II methanotrophs, respectively. PCR was successful in amplifying all targeted genes. The utilized primers along with the thermocycling conditions were optimized for analysis by denaturant gradient gel electrophoresis (DGGE). A semi-nested approach proved to be more efficient in obtaining better amplicons. Bands from DGGE profile were all purified from gel, re-amplified, re-resolved in gel to assess bands purity. The resultant bands were purified and sent for sequencing.

The retrieved sequences were all aligned with matched database sequences, and by utilizing bioinformatics tools, the sequences were grouped based on bootstrapping method. Profile of the functional gene were able to retrieve diverse groups of methanotrophs, including *Methylobacter, Methylomonas, Methylocystis, Methylomicrobium, Methylococcus,* in addition to a number of uncultured methanotrophs. The primers used to amplify the 16S rRNA genes were able to detect; *Methylomonas, Methylomicrobium, Methylosarcina, Methylobacter, Methylococcus,* and *Methylocaldum* within type-I category, and *Methylosinus, Methylocystis, Methylocapse,* and *Methylocella* within type-II category, respectively. These results were all depicted as phylogenetic trees.

Further analysis of methanotrophs using terminal restriction fragment length polymorphism (T-RFLP) technique by targeting *pmoA* gene was attempted. Electropherograms were successfully generated with four four-base cutter enzymes. The lack of a functional gene-specific database has led to not getting meaningful informations regarding those data. However, these data, along with other collected data, is considered valuable resources toward creating a database for this gene.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BLAST	Basic Local Alignment Search Tool
DDBJ	DNA Databank of Japan
DGGE	Denaturant Gradient Gel Electrophoresis
EBI	European Bioinformatics Institute
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
FISH	Fluorescence *in situ* Hybridization
INSD	International Nucleotide Sequence Database
IUPAC	International Union of Pure and Applied Chemistry
MEGA	Molecular Evolutionary Genetics Analysis
MMO	Methane Monooxygenase
MOB	Methane Oxidizing Bacteria
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PLFA	Phospholipids-derived Fatty Acids
pmoA	Particulate Methane Monooxygenase A
qPCR	Quantitative PCR
RDP	Ribosomal Database Project
SDS	Sodium Dodecyl Sulphate
SIP	Single Isotope Probing
TAE	Tris, Acetic Acid, EDTA
TEMED	Tetramethylethylenediamine
T-RFLP	Terminal Restriction Fragment Length Polymorphism

Chapter One

Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

Increases in the abundance of atmospheric greenhouse gases (GHGs) since the industrial revolution are the result of human activity and are largely responsible for the observed increases in global temperature (Forster *et al.*, 2007). Methane (CH_4) is a potent greenhouse gas that absorbs terrestrial radiation more effectively than does carbon dioxide. Although the current concentration of methane is much lower than the concentration of carbon dioxide (CO_2) in the atmosphere, methane is estimated to contribute about 26 times that of CO_2 on similar mass basis to climate change (IPCC, 2001). During the past century, methane has accounted for 15 to 25% of the thermal trapping while carbon dioxide has contributed 60%. Reductions in methane emissions would be 20 to 60 times more effective in reducing the potential warming of the Earth's atmosphere over the next century than would equivalent molar reductions in CO_2 emission (NOAA/AGGI, 2012). Microbial processes are the main method for methane production and consumption, and only three key functional groups of microorganisms of limited diversity regulate the fluxes of methane on earth, namely the aerobic methanotrophic bacteria, the methanogenic archaea, and their close relatives, the anaerobic methanotrophic archaea (Knittel and Boetius, 2009).

Soil serves as a major sink for atmospheric methane, with soil microbes playing a crucial role (Holmes *et al.*, 1999). The fourth assessment report of Intergovernmental Panel on Climate Change (IPCC) estimates that soils represent a methane sink of around 30 million tonnes per year (IPCC, 2007).

Methanotrophs, or methane-oxidizing bacteria (MOB) present in aerobic soil, serve as the only known biological sink for atmospheric CH_4. These methanotrophs use methane monooxygenases (MMOs) to catalyze the oxidation of CH_4 and are grouped into "type-I" and "type-II" categories based on the oxidation pathway followed (McDonald *et al.*, 2008). These MMOs demonstrate a remarkably low substrate specificity, which results in a fortuitous metabolism of a large number of compounds. Nitrogen fertilization is one key factor inhibiting CH_4 oxidation of soils used for agriculture. This is mainly because methanotrophic bacteria and ammonia oxidizers are very similar in the way they oxidize CH_4 and NH_3, respectively. Inhibition of CH_4 oxidation by nitrogen compounds is of great importance for the ecology of methanotrophic bacteria in arable, grassland and forest soils (Dai *et al.*, 2013). Therefore, it has been widely stated that conversion from conventional to reduced or no-tillage agriculture could have a favorable impact on atmospheric concentrations of GHGs by promoting the storage of soil carbon (West and Post, 2002). Agriculture, amongst other land-use practices, impacts the rate of CH_4 oxidation, with a number of studies indicating that varying tillage practices, undisturbed grasslands and pristine forests have different rates of CH_4 oxidation (Hütsch, 2001; Livesley *et al.*, 2007).

Two types of the methane monooxygenase enzyme exist, the particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO) and methanotrophs either possess one or both enzymes (Chen *et al.*, 2007), though pMMO is more common (Murrell *et al.*, 2000). Culture-based techniques have been found to be too selective to give a comprehensive and authentic picture of the entire microbial community as it has been estimated that the majority (over 99%) of bacteria in nature cannot be cultivated by using traditional techniques (Rondon *et al.*, 2000). Therefore, identification of methanotrophs in soils is often performed by the cultivation-independent

detection of a fragment of *pmoA*, a gene encoding the active-site subunit of particulate MMO. Sequence-based *pmoA* phylogeny correlates well with 16S rRNA-based phylogeny, so *pmo*A sequences can be assigned to specific genera or even species of methanotrophs (Kolb *et al.*, 2003). The *pmoA* gene is thus represents an excellent functional gene marker and has been widely used to characterize methanotrophic communities in soils that consume atmospheric methane (Reay *et al.*, 2001; Kong *et al.*, 2013), as well as to characterize the nature and abundance of methanotrophic communities in different soils (Ke, 2013).

The objective of the current study was to identify the general diversity of methanotrophic bacteria in different soil types; under long-term (48-50 years) (1) no-tillage, (2) plow-tillage, (3) grasslands and (4) forests. It is part of a study designed for developing a rapid method for detection of methanotrophs in soil using specific FISH (fluorescent *in-situ* hybridization) probes.

To achieve this objective, soil samples were collected from no-till corn fields, tilled corn fields, grass land and forest using a randomized sampling design. Using these soils, we will target marker genes that are responsible for methane oxidation in methanotrophs. Since the majority of microbes in soil are uncultivable, soil DNA will be extracted directly, followed by PCR amplification of *pmoA* and 16S rRNA of both type-I and type-II methanotrophs genes. The amplicons will be subjected to DGGE profiling, followed by results analysis. A better understanding about the presence of methanotrophic bacteria in soils under contrasting tillage/management systems will lead to a better ways to affect the concentration of CH_4 in the atmosphere.

1.2 Literature Review

1.2.1 Triplett van Doren Project

The Triplett van Doren research project was initiated more than fifty years ago by establishing small corn/soybean plots at Ohio State University's Ohio Agricultural Research and Development Center (OSU/OARDC), Wooster, OH, USA. Nowadays, about 3.7 million acres (~40% of all cropland) are in no-till production in Ohio. The OARDC no-till plots were established by The Ohio State University soil physicist Dave Van Doren and weed scientist Glover Triplett. They had observed that corn planted into no-till fields performed better than those that grew in tilled fields. It has been hypothesized that no-till management is necessary for sustainable crop agriculture. This conservation tillage management practice can reduce soil erosion, increase microbial diversity and activity, enhance soil productivity by increasing carbon storage and organic matter, decrease dependency on fossil fuels and minimize water, nutrient, and pesticide runoff. Today, the Wooster no-till plots, and those established at the OARDC Northwest and Western agricultural research stations are considered to be the longest continuously maintained no-till plots in the world (Triplett and Dick, 2008; Derpsch, 2012).

No-till, the practice of leaving residue on the soil surface instead of plowing it under, is considered one of the most important innovations in US history that revolutionized agriculture. In 1984, the task of maintaining and studying the no-till plots was passed to Warren A. Dick. In 2007, Dick received the Ohio No-Till Award for Education and Research, presented to individuals who have played a major role in the development of no-till in Ohio, and he continue to provide new discoveries and valuable research data till this day (Triplett and Dick, 2008; Derpsch, 2012).

1.2.2 Methane Oxidizing Bacteria

Methane-oxidizing bacteria (MOB), or methylotrophs, encompass both; the methanotrophs, a unique group of methylotrophic bacteria which utilize methane as their sole carbon and energy source (Murrell, 1994; Hanson and Hanson 1996), and the ammonia-oxidizing bacteria (Holmes *et al.*, 1999). These organisms have been isolated from a wide variety of environments including soils (Whittenbury *et al*, 1970), sediments (Smith *et al*., 1997), landfills (Wise *et al*., 1999), forests (Kolb *et al*., 2005), groundwater (Fliermans *et al*., 1988), seawater (Holmes *et al*., 1995), peat bogs (McDonald *et al*., 1996; Ritchie *et al*, 1997; Dedysh, *et al*., 1998), hotsprings (Bodrossy *et al*. 1995 and 1997), plant rhizosphere (Gilbert *et al*., 1998), salt reservoirs (Khmelenina *et al*, 1996) and the Antarctic (Bowman *et al*., 1997).

Methanotrophs were initially grouped according to their morphology, type of resting stage, intra-cytoplasmic membrane structure and physiological characteristics (Whittenbury *et al*, 1970). Phylogenies based on 16S rRNA genes sequence analysis showed that MOB form distinct lineages in the gamma subclass of the class proteobacteria (type-I MOB) and the alpha subclass of the proteobacteria (type-II MOB) (Heyer *et al*., 2002; Semrau *et al*., 2010). The two types of methanotrophs can be distinguished on the basis of biochemical and ultrastructural features (Bowman *et al*., 1993). Subsequent studies has further clarified these phylogenetic relationships and defined eight genera of methanotrophs, namely *Methylococcus*. *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylocaldum*, *Methylosphaera*, *Methylocystis* and *Methylosinus*. These genera are divided into two distinct physiological groups. Type-I methanotrophs (*Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylocaldum*, *Methylosphaera*) assimilate formaldehyde produced from the oxidation of methane (via methanol) using the ribulose-monophosphate pathway, have cellular membranes that are composed of predominantly 16-

carbon fatty acids and possesses bundles of intra-cytoplasmic membranes. Type-II methanotrophs (mainy *Methylocystis* and *Methylosinus*) utilize the serine pathway for formaldehyde assimilation, have intra-cytoplasmic membranes arranged around the periphery of the cell and contain predominantly 18-carbon fatty acids (Trotsenko and Khmelenina, 2002; Semrau *et al.*, 2008). Members of the genus *Methylococcus* possess a combination of characteristics of both type I and type II methanotrophs (Hanson and Hanson, 1996).

Methanotrophic members of the recently described, extremely acidophilic, phylum of bacteria, *Verrucomicrobia*, have been isolated from volcanic areas. They do not contain intracellular membrane structures, and their biochemistry and physiology still need to be further studied (Hou *et al.*, 2008; Op den Camp *et al.*, 2009; Khadem *et al.*, 2010).

In addition to their role in atmospheric methane mitigation, methanotrophs have been widely investigated for *in situ* bioremediation due to their ubiquity and their ability to degrade halogenated hydrocarbons through the activity of MMOs (Lee *et al.*, 2006). Methane oxidizing bacteria have been found to have an important role in the biodegradation of chlorinated hydrocarbons such as trichloroethylene, dichloroethane, and chloroform (Hanson and Hanson, 1996). Chlorinated ethenes are synthetic compounds with no identified natural sources and are commonly used in various industrial practices including degreasing operations, dry cleaning, dying, and textile production (Bakke *et al.*, 2007). Despite their widely perceived carcinogenicity (Bolt, 2005; Scott and Chiu, 2006), there have been significant historical releases to the environment and as a result, these compounds are often detected in substantial concentrations in subsurface soils and groundwater (Westrick *et al.*, 1984). The reductive *in situ* application of anaerobic biodechlorination has been limited as this process does not result in complete dechlorination and thus,

can lead to accumulation of compounds such as TCE, cis-dichloroethylene (c-DCE), trans-dichloroethylene (t-DCE), and VC (Maymo-Gatell *et al.*, 1999).

Direct aerobic biodegradation of chlorinated compounds by bacteria has been widely examined as an alternative to anaerobic bacterial degradation (Verce *et al*., 2000; Coleman *et al*., 2002). There are also as well as many bacterial strains that co-oxidize these compounds (Futamata *et al.*, 2001). Methanotrophs are capable of degrading these pollutants via co-oxidation, and due to their omnipresence in various environments, have been widely applied to sites polluted with chlorinated ethenes to stimulate decontamination (Semrau *et al.*, 2010).

1.2.3 Methane Monooxygenase

Methane-oxidizing bacteria are able to utilize methane as a sole source of carbon and energy for growth (Hanson and Hanson, 1996). These bacteria play an important role in the global methane cycle by oxidizing CH_4 released by methanogens in freshwater sediments and wetlands and thus mitigate the global warming effect of this greenhouse gas (Conrad, 1996). The first step in CH_4 oxidation, the conversion of methane to methanol, is carried out by the methane monooxygenase enzyme (Hütsch, 2001):

$$CH_4 + O_2 + NADPH \rightarrow CH_3\text{-}OH + H_2O + NADP$$

This enzyme exists in two forms, a particulate, membrane-associated form (pMMO) and a cytoplasmic, soluble form (sMMO). The two forms of the enzyme differ in their structure, kinetic properties, and in the range of substrates which are utilized (Murrell *et al*., 2000). Only a restricted number of MOB species possess sMMO, while almost all MOB possess pMMO. *pmo*A gene is present in all known methanotrophs, with the exception of *Methylocella* genus

(Theisen *et al.*, 2005). In MOB that harbor both forms of MMO, sMMO is synthesized under copper-deficient conditions, while in the presence of even a minuscule amount of available Cu(II) (0.85 to 1.0mol/g [dry weight] of cells) only pMMO is synthesized (Hakemian and Rosenzweig, 2007).

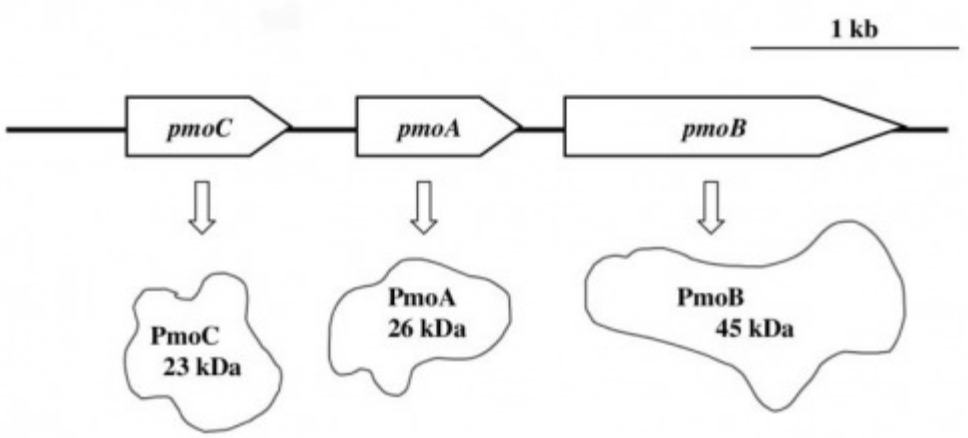


Figure 1-1: Particulate methane monooxygense (pMMO) operon (Murrell *et al.*, 2000).

The pMMO gene cluster (Figure 1-1) consists of three consecutive open reading frames (*pmoC*, *pmoA*, and *pmoB*) in both type-I MOB (Stolyar *et al*., 1999) and type II MOB (Gilbert *et al*., 2000). The *pmo* genes from *Methylococcus capsulatus* (str. Bath) are transcribed into a single 3.3-kb polycistronic mRNA (Nielsen *et al*., 1997). *pmo*A is presumed to contain the active site because it has been shown to be specifically labeled by [^{14}C]-acetylene, a suicide substrate for MMO (Zahn and DiSpirito, 1996). Thus, the *pmo*A gene was shown to be an excellent phylogenetic marker for methanotrophs (Dumont and Murrell, 2005a). The type-I MOB *Methylococcus capsulatus* Bath and *Methylomicrobium album* BG8 (Semraue *et al*., 1995; Stolyar *et al*., 1999), as well as the type II organisms *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (Gilbert *et al*., 2000), have been shown to contain duplicate copies of the *pmo* operon. The sequences of the duplicate *pmoCAB* gene clusters however are nearly identical.

The sMMO operon is composed of a three component hydroxylase $(\alpha\beta\gamma)_2$ encoded by *mmo*XYZ, respectively, a reductase encoded by mmoC, and a regulatory protein, protein B, encoded by mmoB (Ali *et al.*, 2006). The *mmo*X gene encodes the active site of the conserved subunit (α) of the hydroxylase component of the sMMO and has been used previously as a marker for sMMO (Auman *et al.*, 2000).

An interesting similarity is found between particulate methane monooxygenase and ammonia monooxygenase, as they are evolutionarily related enzymes despite their different physiological roles in these bacteria. *Nitrosococcus oceonus amoA*, for example, showed higher identity to *pmoA* sequences from other members of the gamma-proteobacteria than to *amoA* sequences (Holmes *et al.*, 1995).

1.2.4 Molecular Techniques Utilized in the Study of Methanotrophs

Researchers worldwide have been interested in studying the differences and nature of microbial composition within various environmental samples (Amann *et al.*, 1995). Since the vast majority of soil bacteria cannot be cultured via traditional laboratory techniques and must be identified using molecular methods, successful characterization of microbial communities is therefore often dependent on DNA that is extracted from the environment (Rappe and Giovannoni, 2003). The application of metagenomics, i.e. the culture-independent extraction and subsequent analysis of genomic DNA from the environment, has greatly expanded our knowledge of the diversity of microbes within mixed populations (Beja *et al.*, 2002; Elshahed *et al.*, 2008) and microbial protein families (Chen and Murrell, 2010). Metagenomics approaches have now been applied to a variety of environments, from the human gut

microbiome to soils (Tringe *et al.*, 2005; Gill *et al.*, 2006; Li *et al.*, 2008), and from the deep sea to the indoor atmosphere (Martin-Cuadrado *et al.*, 2007; Tringe *et al.*, 2008). By using these techniques, diverse enzymes and their encoding genes have been identified (Schmeisser *et al.*, 2007). With such studies, various microorganisms in those environments along with their metabolic and environmental functions have also been known.

In the last few decades, cultivation-independent molecular methods have been applied widely to investigate microbial diversity and quantify predominant organisms in natural microbial communities (Neufeld and Mohn, 2006). And the methanotrophs diversity has been studied in different environments using the polymerase chain reaction (PCR) (Uz *et al*., 2003) and denaturing gradient gel electrophoresis (Bodelier *et al*., 2005). Similarly, to investigate the biodiversity of methanotrophic communities, one can use PCR with primers targeting the 16S rRNA gene or functional genes like the methane monooxygenase gene *pmoA* (Conrad, 2007; McDonald *et al.*, 2008; Tavormina *et al.*, 2010).

1.2.4.1 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) has proven to be one of the most popular methods for determination of microbial diversity (Muyzer and Smalla, 1998; Yu and Morrison, 2004). DGGE is a molecular fingerprinting method that separates PCR-generated DNA products derived from environmental samples directly without the need for laborious processes such as culturing (Leser *et al.*, 2002) or cloning procedures (Hall, 2007; Medini *et al.*, 2008; Schuster, 2008). The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequences that represent many of the dominant microbial organisms. However, since PCR products from a given

reaction are of similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of the DNA (Fromin *et al.*, 2002; Brons and van Elsas, 2008).

During electrophoresis, PCR products encounter increasingly higher concentrations of chemical denaturant, such as urea and formamide, as they migrate through a polyacrylamide gel. In addition, the gel should be run at a high temperature, usually 60 °C. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Once denatured, the PCR products could continue running through the gel as single-stranded DNA, but the fragments have to remain precisely where they denatured. To achieve this, a so-called GC-clamp is attached, to prevent complete denaturing (Rettedal *et al.*, 2010). This GC-clamp is a string of 40–60 nucleotides composed only of guanine and cytosine and is attached to one of the PCR primers. PCR with a GC clamp results in a product with one end having a very high denaturing temperature. A PCR product running through a DGGE gel will, therefore, denature partially while the GC-clamp remains double stranded. The fragment will form a Y-shaped piece of DNA that will stick firmly at its position on the gel. Differing sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands (Figure 1-2). Each band theoretically representing a different bacterial population present in the community (Muyzer and Smalla, 1998; Temmerman *et al.*, 2003; Ercolini, 2004; Vanhoutte *et al.*, 2005).

DGGE has been used to analyze DNA from a range of environments such as soil, oceans, dental flora, the human gastrointestinal tract, and skin have revealed a bacterial diversity much higher than previously speculated (Janssen,

2006; Ley *et al.*, 2006; Azam and Malfatti, 2007; Fierer *et al.*, 2010; Kolenbrander *et al*., 2010).

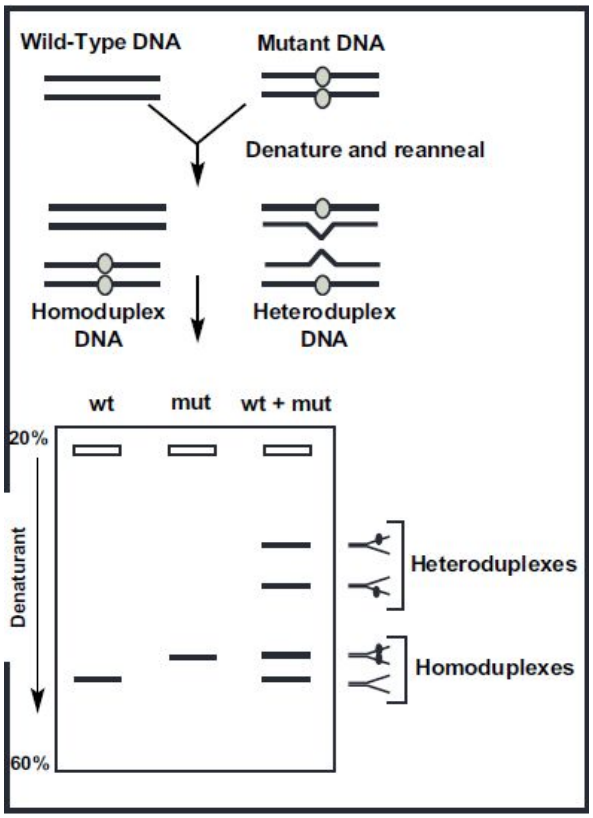


Figure 1-2: An example of wild-type and mutant DNA fragments that were denatured and re-annealed to generate four fragments; two heteroduplexes and two homoduplexes run on a parallel denaturant gradient gel. The melting behavior of the heteroduplexes is altered so that they melt at a lower denaturant concentration than the homoduplexes and can be visualized on a denaturant gradient gel even if the difference is a single nucleotide change (Muyzer *et al.*, 1993)

1.2.4.2 Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) analysis of PCR-amplified genes is another widely used fingerprinting technique when it comes to diversity studies. This analysis is based on the restriction endonuclease

digestion of fluorescently end-labeled PCR products. The digested product is mixed with a DNA size standard, itself labeled with a distinct fluorescent dye, and the fragments are then separated by capillary or gel electrophoresis using an automated sequencer. Upon analysis, only the terminal end-labeled restriction fragments are detected. An electropherogram is produced, which shows a profile of the microbial community as a series of peaks of varying height (Tiquia, 2010). The first application of *pmo*A T-RFLP was reported by Horz *et al.* (2001), and it has been widely used in a number of subsequent studies (Horz *et al.*, 2002; Bussman *et al*., 2004; Horz *et al.*, 2005).

1.2.4.3 Fluorescence *in situ* Hybridization Technique

Fluorescence *in situ* hybridization (FISH) targeting the 16S rRNA gene has been used to identify (Eller *et al.*, 2001) and enumerate (Dedysh *et al.*, 2001; Dedysh *et al.*, 2003) methanotrophs using 16S rRNA probes. Oligonucleotide probes can be developed based on an extensive target-gene sequence database (Dedysh *et al.*, 2003), and the probe is introduced into the cells via electroporation (Shao *et al.*, 1995). The cells are then passed through a flow cytometer, an instrument where cells are aligned hydro-dynamically by an entrainment fluid into a very narrow stream onto which several powerful laser light sources are focused. Each time a particle passes through the beam; it scatters light in a way depending on the refractive index, size, and shape of the particle, the light pulses are converted into digital signals that can be processed by a computer (Marie *et al.*, 2005).

By using specific probes, FISH technique provide a rapid method for determining the presence of methanotrophs in soil, and a study by Kubota *et al.* (2006) has linked the use of functional genes in methanogens with FISH to identify active methanotrophs.

1.2.4.4 Stable Isotope Probing (SIP)

Although there are several DNA-based approaches that have given insight into the diversity of methanotrophs present in the environment, analysis of relevant functional communities for methane oxidation is still a challenge, as DNA could be stable in resting cells and even dead cells (Lindahl, 1993). Efforts have been made to identify active methanotrophs in the environment (Radajewski *et al*., 2000; Dumont and Murrell, 2005a). Therefore, DNA-stable isotope probing is a powerful tool for analyzing the active populations in environmental samples, as only active cells will assimilate the ^{13}C-labelled substrate (Radajewski *et al*., 2000; Radajewski *et al*., 2003; Cébron *et al*., 2007; Ruo *et al.*, 2012).

SIP is a method that attempts to link the identity of an organism with its biological function under conditions approaching those *in situ* (Radajewski *et al.*, 2000; Radajewski *et al.,* 2003). Addition of ^{13}C-labelled substrate to an environmental sample results in ^{13}C-labelling of actively dividing bacteria when the ^{13}C-labelled substrate is used as a carbon source. The microorganism's DNA therefore becomes heavier and can be separated by CsCl density gradient centrifugation from ^{12}C-DNA of bacteria which have not assimilated the labeled substrate (Dumont and Murrell, 2005b; McDonald *et al.*, 2005).

1.2.4.5 Phospholipids

Methanotrophs contain unique phospholipids-derived fatty acids (PLFAs) (Bowman *et al.*, 1991). The measurement of these signature PLFAs has been widely used to estimate the biomass distribution of type I and II methanotrophs in environments well supplied with methane (Bowman *et al.*, 1991; Borjesson *et al.*, 1998).

The use of $^{13}CH_4$ to isotopically label the PLFAs of methanotrophs in a soil increased the sensitivity of detection of the PLFAs and provided evidence of methane assimilation at true atmospheric concentrations (Bull *et al.*, 2000). The incorporation of ^{13}C into PLFAs has been used in other studies of atmospheric methane oxidation (Knief *et al.*, 2003; Maxfield *et al.*, 2006), with both studies suggesting the presence of novel type-I and -II methanotrophs. ^{13}C-labeled PLFA analyses were also used to study methanotrophs in high-methane environments, including landfill cover soils (Crossman *et al.*, 2004), acidic peatland soils (Chen *et al.*, 2008), and freshwater sediment (Boschker *et al.*, 1998).

1.2.4.6 Quantitative PCR

Quantitative real-time polymerase chain reaction (qPCR) is a method used to determine the concentration of target DNA in environmental DNA extracts. It is basically a PCR technique in which the primer used is labeled with a fluorescent dye and the reaction is monitored as it progresses in *real time*; this allows amplifying and simultaneously quantifying the targeted DNA molecule (Becker *et al.*, 2000; Raeymaekers, 2000). Quantitative PCR has been used for quantification of microorganisms in environmental samples, by targeting either 16S ribosomal DNA (rDNA) or functional marker genes. The use of *pmoA* gene has already been utilized in a number of studies (Becker *et al.*, 2000; Suzuki *et al.*, 2000; Hermansson and Lindgren, 2001; Mygind *et al.*, 2001; Stubner, 2002).

1.2.4.7 Microarray

DNA microarray (microchip, biochip, or gene chip) technology allows the parallel analysis of highly complex gene mixtures in a single assay and thus

symbolizes the post-genomic era of high-throughput science. Although microarrays initially emerged as tools for genome-wide expression analysis and are nowadays routinely used for this purpose, they are also increasingly being developed for diagnostic applications, drug development, comparative and functional genomics studies, and various other fields. Microbial diagnostic microarrays (MDMs) consist of nucleic acid probe sets, with each probe being specific for a given strain, subspecies, species, genus, or higher taxon (Bodrossy and Sessitsch, 2004). The first MDM to target methanotrophs was a prototype functional gene array that targeted genes involved in nitrogen cycling, including nitrite reductase (*nirS* and *nirK*), ammonia monooxygenase (*amoA*), and particulate methane monooxygenase (*pmoA*) genes (Wu *et al.*, 2001). That study indicated the potential of microarrays for revealing functional gene composition in natural microbial communities, and a newer version of this array was published by He *et al.* (2007). Another MDM was specifically developed for the detection and community analysis of methanotrophs (Bodrossy *et al.*, 2003); the microarray consisted of 59 oligonucleotide probes designed and fully validated against the *pmoA* genes of all known methanotrophs and *amoA* of the ammonia-oxidizing, nitrifying bacteria. The probes applied on this array were short oligonucleotides (i.e., 18 to 27 nucleotides) and were, in most cases, able to determine nucleotide discrimination. The potential of the *pmoA* microarray was tested with environmental samples, and the results were in close agreement with those of clone library sequence analysis (Bodrossy *et al.*, 2003). The microarray was then applied successfully to analyse the methanotroph communities in landfill cover soils (Stralis-Pavese *et al.*, 2004; Čébron *et al.*, 2007).

Later, an mRNA-based application of MDMs was successfully tested using a *pmoA* microarray for methanotrophs (Bodrossy *et al.*, 2006; Chen *et al.*, 2008) and may provide additional information on composition and functioning of microbial communities provided by DNA-based microarrays. The *pmoA*

MDM has been upgraded and the latest version is comprised of up to 138 probes (Kip *et al.*, 2011).

1.2.5 Bioinformatics Resources

The national institute of health (NIH) defines bioinformatics as the field concerned with research, development, or application of computational tools and approaches for expanding the use of biological, medical, or behavioral data, including those to acquire, store, organize, archive, analyze, or visualize such data. Computational biology, as a branch of bioinformatics, refers to the development and application of data-analytical and theoretical methods, algorithms, mathematical modeling and computational simulation techniques to the study of biological systems (NIH website, 2012). This section focuses on introducing the resources utilized in this study.

1.2.5.1 Biological Databases

Over time, many biological databases have been developed. These databases are libraries of life sciences information, e.g. collected from scientific experiments, published literature, high throughput experiment technology and computational analyses. Of various biological databases available for research, GenBank is one of the largest and oldest biological databases. It contains all publicly available DNA sequences; it is massive and doubles in size about every 15 months (Wilson, 2002). GenBank is one of three primary sequence databases run as part of an international collaboration between data collection centers including the National Center for Biotechnology Information (NCBI), the European Molecular Biology Laboratory (EMBL), and the DNA Databank of Japan (DDBJ). Sequence submissions are independently updated every 24 hours

at all three centers, and records are distributed in a common format among all three databases (Figure 1-3). Therefore, a query to any one of the databases will produce results derived from submissions made at all three centers (Karsch-Mizrachi, 2011).

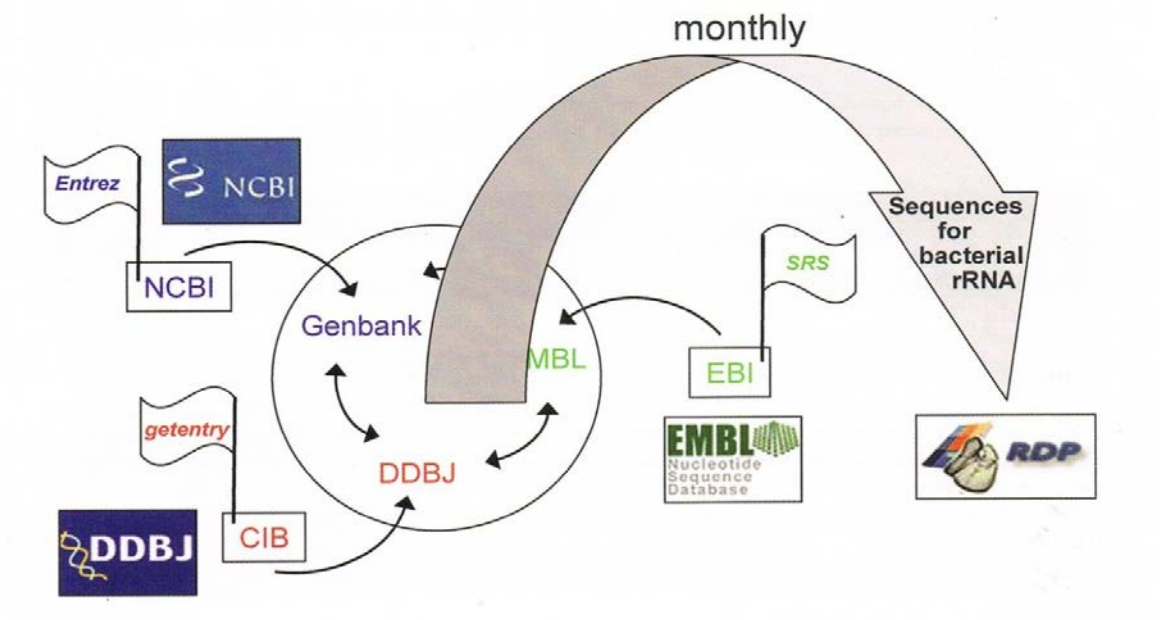


Figure 1-3: International Nucleotide Sequence Database (INSD) collaboration. Data flow among the three data collection centers (NCBI, EMBL, and DDBJ), and from INSD to RDP. This illustration is a courtesy of Erin Sanders (Sanders and Miller, 2010).

1.2.5.2 NCBI-BLAST

One application for local DNA sequence alignments is in database searches. The basic local alignment search tool or BLAST (Altschul *et al.*, 1997) is a widely used search tool that is available for searching query sequences against massive genetic databases such as GenBank (Benson *et al.*, 2009). The NCBI-BLAST algorithm conducts its search through the GenBank via a secondary database linked to all the sequence information found in GenBank. This secondary database is organized by taking smaller sequences

"words" of each GenBank record. BLAST also chops the query sequence into all possible defined sizes and compares these to the GenBank database words. These words may vary in length, ranging from 7-11 nucleotides (blastn) to as large as 28-64 nucleotides (megablast). In general, increasing the word size tends to retrieve faster, more identical results, albeit of less distant relationships. A third option is (discontiguous megablast), which is designed to find database sequences that are similar, but not identical, to a search query (Ma *et al.*, 2002).

The BLAST program begins by finding identical words between the secondary database and a query search and aligns them. The neighborhood is extended from the query word in both directions, and continues until a maximal local alignment length is achieved. A search hit that results from this optimal local alignment of maximal length is called a high-scoring segment pair (HSP). The HSP is then subjected to a number of statistical processes to determine several crucial values before NCBI-BLAST reports to the user. Of these, the "Expect" or E-value, specifies whether the alignment represents a biological relationship or is simply due to random chance. The lower the E-value the more significant is the HSP. The other value determined is called the bit score, which is a normalized value calculated by BLAST from the maximum nominal score for an entire alignment. The sum of bit scores (max scores) of all HSPs found in a single database entry is called the total score. A total score is an informative parameter for entries in which more than one HSP may be present. After determining these values, HSPs results are then returned to the user in a pairwise alignment form (Baxevanis, 2005).

1.2.5.3 Ribosomal Database Project (RDP)

Ribosomal Database Project (RDP-II) is a massive database aimed specifically towards the analysis of ribosomal DNA (rDNA). It contains

hundreds of thousands of ribosomal sequences submitted by various researchers from all over the world. RDP acquires bacterial rDNA sequences every month from the International Nucleotide Sequence Database (INSD), i.e. NCBI (GenBank), DDBJ, and EMBL (Figure 1-2) (Cole *et al.*, 2005). RDP-II provides several tools to make a meaningful comparative analysis of new sequences with the database based on secondary-structure alignments. Of these tools, the SeqMatch tool, can be used to find the nearest neighbors or the closest matching sequences within the database to a query sequence. SeqMatch resembles BLAST in that it uses "words" to search a query against the database. Furthermore, sequences are also pre-aligned to a secondary-structure model since rRNA, an essential component of ribosomes, forms extensive and predictable secondary structures. SeqMatch is thought to be more accurate than BLAST at finding closely related rRNA gene sequences (Cole *et al.*, 2005). The Classifier, another tool at RDP-II, allows one to classify sequences at different taxonomic levels (Wang *et al.*, 2007). This tool uses the NCBI database as a source and the classification scheme in *Bergey's Manual* in order to assign "words" to a particular taxonomic group (Garrity *et al.*, 2004). For primer studies, the Probe Match tool can be used to test universal primers against its own database (Mao *et al.*, 2012).

1.2.5.4 Multiple Sequence Alignment

Obtaining an accurate alignment is the first and most important step in constructing a phylogenetic tree, which is used to depict evolutionary relationships between and among sequences. A sequence alignment is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence for functional, structural, or evolutionary relationships (Mount, 2004). Multiple alignments are often used in identifying

conserved sequence regions across a group of sequences hypothesized to be evolutionarily related (Elias and Isaac, 2006). Aligned sequences of a given query set of nucleotide or amino acid residues are typically represented as rows within a matrix. Mismatches can be interpreted as point mutations and gaps as indels (i.e. insertion or deletion mutations) and are inserted between the residues so that identical or similar characters are aligned in successive columns (Ng and Henikoff, 2001).

Multiple sequence alignment is an extension of pairwise alignment to incorporate more than two sequences at a time. Pairwise alignment is a method used to find the best-matching piecewise alignments, whether it is global (i.e. align every residue in every sequence, e.g. the Needleman-Wunsch algorithm) or local (i.e. align regions with larger sequence context, e.g. the Smith-Waterman algorithm), of two query sequences (Mount, 2004). There are three primary methods of producing pairwise alignments. One is the dot-matrix plot that is a simple, graphical but time-consuming method for analyzing on a large scale. It is useful for identifying certain sequence features such as insertions, deletions, or inverted repeats but it is limited to two sequences (Wild and Seber, 2000). A second method is dynamic programming. This method involves a substitution matrix to transform one sequence into another using edit operations that replace, insert, or remove an element. Each operation has an associated score, and the goal is to find the sequence of edits with the lowest total score (Chao, 2005). The third method is the word method(s) whereby identify a series of short subsequences "words" in the query sequence are identified and then matched to candidate database sequences. This is especially useful in large-scale database searches. Word methods are best known for their implementation in the database search tools FASTA and the BLAST family (Mount, 2004).

Clustal is a widely used multiple sequence alignment computer program (Chenna *et al.*, 2003). There are three main variations. ClustalW has a command

line interface (Larkin *et al.*, 2007) and ClustalX: has a graphical user interface (Thompson *et al.*, 1997). Clustal Omega is the latest addition to the Clustal family. It has a command line interface and it offers a significant increase in scalability over previous versions, allowing hundreds of thousands of sequences to be aligned in only a few hours (Sievers *et al.*, 2011).

1.2.5.5 Phylogenetic Tree

Evaluating the evolutionary tree, or phylogeny, is a formal study of organisms and their evolutionary history with respect to each other. Phylogenetic trees are most commonly used to depict the relationships that exist between species. In particular, they clarify whether certain traits are homologous (found in the common ancestor as a result of divergent evolution) or homoplasy (or sometimes referred to as analogous, a character that is not found in a common ancestor but whose function developed independently in two or more organisms, known as convergent evolution). The depiction of the tree can be achieved with the aid of computational algorithms, methods and programs that make various phylogenetic analyses (Strait and Grine, 2004).

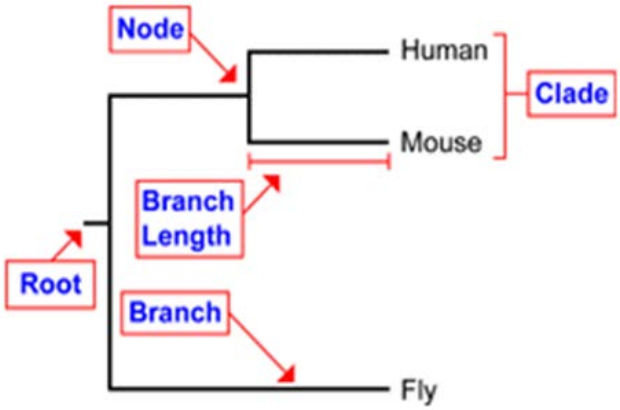


Figure 1-4: A simplified depiction showing the most common terms used in phylogenetic trees (NCBI handbook, 2002).

The term topology is used to refer to the shape of the tree, while evolutionary distance is a measure of the differences that distinguish organisms. In general, the tree topology consists of branches connecting two terminal nodes. Each terminal node represents a gene or an organism referred to as a taxon, sometimes referred to as a clade (Figure 1-3). The branch length between two nodes is proportional to the number of changes that have occurred in the branch as a function of time (Higgs and Attwood, 2005).

Once a proper alignment of the given sequences is established, several statistical processes should be applied to optimize the tree as much as possible before it can be described as reliable. A fundamental unit of a phylogenetically informative tree construction is bipartition. This is a term that refers to two internal nodes connected via a branch. There are several ways to test whether a bipartition is robust. An accepted method to assess this is called bootstrapping (Hall, 2008). In general, bootstrapping is a statistical procedure in which raw data are resampled numerous times to estimate optimum parameters such as the mean, the average value within a distribution of values or the variance, the deviation from an expected value which captures the degree to which a distribution spread out within a particular sample. This procedure emphasizes values that are common and deemphasizes rarely observed data. In such case, the raw data being resampled are the alignment, and the parameters could be the topology and branch length (Wilkinson *et al.*, 2007).

The next step is choosing a method to calculate a phylogeny. Several methods have been devised for evolutionary analysis. Parsimony is a statistical tree-searching method in which many trees are constructed and then a criterion is applied to the resulting trees to allow the selection of the “best” tree that meets the criterion. The best or “most parsimonious” tree is the one with the minimum number of changes for every possible topology (Jaynes and Bretthorst, 2003). The maximum-likelihood method is the most popular

alternative to parsimony that uses statistical techniques for inferring probability to the possible phylogenetic trees. The model consists of distance and topology. Maximum-likelihood methods find the single model that exhibits the highest likelihood of any of the models (Holder and Lewis, 2003; Felsenstein, 2004).

Distance-based methods, on the other hand, use pairwise distances and data clustering techniques to build the most likely tree relating a group of taxa. The distances are used to determine the topology of the tree and are calculated using a specified model of evolution (Saitou and Nei, 1987; Studier and Keppler, 1988). Neighbor-joining is an example of a distance-based method, which provides a measurement of the amount of evolutionary change between any two sequences since divergence from a common ancestor (Mount, 2004; Mihaescu *et al.*, 2009). It is much less computationally intensive than either parsimony or likelihood-based methods. Neighbor-joining is one of the most widely used methods for building phylogenetic trees, since it is used to construct a single tree that best summarizes the relationships among taxa rather than building all possible trees and then evaluating each one by some certain criterion (Gascuel and Steel, 2006; Didelot, 2010).

MEGA 5 (Molecular Evolutionary Genetics Analysis) is the most widely used computer software to build phylogenetic trees using the maximum-likelihood or Neighbor-Joining methods (Tamura *et al.*, 2011).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments were used in the study of methanotrophs in soil:

Equipment	**Origin**
ABI PRISM 3100xl Genetic Analyzer	Applied Biosystems, US
Autoclave	Market Forge, US
Chromato-Vue Transilluminator	Ultra Violet Products inc., US
DCode Universal Mutation Detection system	BioRad, US
DGGE, Gradient Delivery System	BioRad, US
Equatherm Water Bath	Curtin Matheson Scientific inc., US
Freezer (-20^{o}C)	Frigidiare, US
Fridge (4^{o}C)	Frigidiare, US
Gel Logic 200 Imaging System	Kodak, US
Horizontal Vortex	Fisher Scientific, US
LabMniTM Mini Centrifuge	Southwest Science, US
Magnetic Stirrer	Fisher Scientific, US
Microfuge	Beckman Coulter, US
Microwave Oven	Kenmore, US
Midigel Electrophoresis system “Horizontal”	Fisher Biotech, US
NanoDrop ND 1000 Spectrophotometer	Thermo Scientific, US
PCR Workstation	AirClean Systems, US
Sensitive Balance	Mettler Toledo, US
Thermal Cycler PTC-100	MJ Research inc., US
Thermolyne Vortex Maxi Mix II	Thermo Scientific, US

2.1.2 Reagents

The following reagents were used in the study of methanotrophs in soil:

Material	**Origin**
Acetic acid-glacial, acrylamide/bis-acrylamide [37.5:1] 40%, agarose, ammonium persulfate, bromophenol blue, EDTA disodium salt dihydrate, ethanol (95%), formamide, nuclease-free H_2O, urea, xylene cyanol	Fisher Scientific, US
Ethidium bromide	Sigma-Aldrich, Germany
Tris-base, DNA ladder 100-bp	Promega, US
Tetramethylethylenediamine (TEMED)	OmniPur, US

2.1.3 Buffers and Solutions

2.1.3.1 TAE Buffer (50x) (Ausubel *et al.*, 2002)

This buffer was made by dissolving 242 g of Tris-base into 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA (pH=8), and adjusting the final volume to 1 L with double deionized (DDI) H_2O. The solution was mixed, autoclaved for 20 min and stored at room temperature.

A running buffer of 1x TAE was prepared by diluting 50x TAE solution with DDI H_2O in a 1:50 ratio.

2.1.3.2 EDTA Solution (Green and Sambrook, 2012)

EDTA solution was prepared by adding 186.1 g of EDTA disodium salt dihydrate into 800 ml DDI H_2O and stirring vigorously on a magnetic stirrer. Since EDTA does not dissolve till the pH approaches 8.0, NaOH was added to the solution. The addition was continued till all the EDTA was dissolved. The volume was adjusted to 1 L with DDI H_2O.

2.1.3.3 Ammonium Persulfate (10%): (Ausubel *et al.*, 2002)

This solution was prepared by dissolving 0.1 g of ammonium persulfate in 1.0 ml of DDI H_2O, and then storing the solution at -20 ^{o}C for about a week.

2.1.3.4 DCode Dye Solution (Ausubel *et al.*, 2002)

The dye solution used with the DCode system was prepared by dissolving 0.05 g of both bromophenol blue and xylene cyanol in 10 ml of 1x TAE buffer. The solution was stored at room temperature.

2.1.3.5 DGGE Denaturing Solution (Muyzer *et al.*, 1993)

The denaturing solution used with a 6% acrylamide gel was prepared by mixing 15 ml of 40% acrylamide/bis-acrylamide (37.5:1) and 2 ml of 50x TAE buffer, and then adding formamide and urea at the appropriate concentrations. The volume was adjusted to 100 ml using DDI H_2O. The solution was stored at 4 ^{o}C in brown bottles for approximately 1 month. The amounts of formamide and urea added are dependent upon the required concentrations of the high and low denaturing solutions. The amounts used for various types of denaturing solutions are as follows:

	Concentration of Denaturant Solution (%)										
Denaturant	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Formamide (ml)	0	4	8	12	16	20	24	28	32	34	40
Urea (g)	0	4.2	8.4	12.6	16.8	21	25.2	29.4	33.6	37.8	42

2.1.3.6 Ethidium Bromide (10mg/ml) (Green and Sambrook, 2012)

This solution was prepared by dissolving 0.1 g of ethidium bromide into 10 ml of DDI H_2O using a magnetic stirrer. The solution was stored in the dark at room temperature.

2.1.3.7 GoTaq® Green Master Mix (Promega)

The reaction buffer (pH 8.5) contained bacterially derived *Taq* DNA polymerase, 400 μM of each dNTP and 3 mM $MgCl_2$ along with two dyes, blue and yellow, that allow monitoring of progress during electrophoresis. Moreover, the green dye confers DNA sufficient density for direct loading onto agarose gels. This solution is stored at -20 ^{o}C.

2.1.3.8 Gotaq® Colorless Master Mix (Promega)

The same as the GoTaq Green Master Mix but without the green dye.

2.1.4 Kits

All kits were stored at room temperature.

2.1.4.1 PowerSoil® DNA Isolation Kit (Mo Bio Laboratories inc.)

The contents of this kit consisted of:

1. PowerBead tubes, which contain a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.
2. Solution C1, contains sodium dodecyl sulphate (SDS) and other disruption agents.

3. Solution C2, is a patented Inhibitor Removal Technology® (IRT).
4. Solution C3, is a patented IRT®.

   Solutions C2 and C3 work to precipitate non-DNA organic and inorganic material including humic acid, cell debris, and proteins.
5. Solution C4, is a high concentration salt solution that allow binding of DNA to the spin filters.
6. Solution C5, is an ethanol solution used to wash bound DNA.
7. Solution C6, contains 10 mM Tris with no EDTA as an elution buffer.
8. Spin Filters (2 ml), DNA is selectively bound to the silica membrane in the Spin Filter in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.
9. Collection tubes (2 ml).

2.1.4.2 QIAEX II® Gel Extraction Kit (QIAGEN)

This kit contains the following reagents:

1. QIAEX II Suspension, used to solubilize agarose or polyacrylamide gel residues.
2. Buffer QX1 (with pH indicator), further solubilizes and remove gel residues and contains a high concentration salt solution to allow adsorption of DNA to QIAEX II silica particles.
3. Buffer PE (concentrate) with ethanol, used to wash DNA of agarose gel, proteins, and salt contaminants.

2.1.4.3 Wizard® SV Gel and PCR Clean-Up System (Promega)

1. Membrane Binding Solution, to allow binding of DNA to the minicolumns filter membrane.
2. Membrane Wash Solution (concentrated), to wash bound DNA.
3. Nuclease-Free Water, for final elution of the purified DNA.

4. Wizard® SV Minicolumns and (2 ml) collection tubes.

2.1.5 Primers

Primers from Invitrogen, US that were used in this study of methanotrophs in soil are shown in Table 2-1.

Table 2-1: Primers used for this study.

Primer	**Sequence[c] (5′ - 3′)**	**Target gene**	**Reference**
A189f[a,d]	GGNGACTGGGACTTCTGG	*pmoA/amoA* gene	Holmes *et al.*, 1995
A682r	GAASGCNGAGAAGAASGC		
mb661	CCGGMGCAACGTCYTTACC	*pmoA* gene	Costello and Lidstrom, 1999
A650r	ACGTCCTTACCGAAGGT		Bourne *et al.*, 2001
f326	TGGGGYTGGACCTAYTTCC		Fjellbirkeland *et al.*, 2001
r643	CCGGCRCRACGTCCTTACC		
Type IF	ATGCTTAACACATGCAAGTCGAACG	16S rRNA gene of type-I methanotrophs	Chen *et al.*, 2007
Type IR	CCACTGGTGTTCCTTCMGAT		
MethT1dF	CCTTCGGGMGCYGACGAGT		Wise *et al.*, 1999
MethT1bR	GATTCYMTGSATGTCAAGG		
Type IIF	GGGAMGATAATGACGGTACCWGGA	16S rRNA gene of type-II methanotrophs	Chen *et al.*, 2007
Type IIR	GTCAARAGCTGGTAAGGTTC		
533F	GTGCCAGCAGCCGCGGTAA		Wise *et al.*, 1999
MethT2R	CATCTCTGRCSAYCATACCGG		
341F_GC[b]	CCTACGGGAGGCAGCAG	DGGE (along with type-I R)	Muyzer *et al.*, 1993
518F_GC[b]	CCAGCAGCCGCGGTAAT	DGGE (along with type-II R)	

[a]The GC-clamp (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC) was attached to the 5′-end of this primer when used with DGGE (Rettedal *et al.*, 2010).

[b]The GC-clamp (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG) was attached to the 5′ end of these primers when used with DGGE (Chen *et al.*, 2007).

[c]S = G or C, R = A or G, W = A or T, M = A or C, Y = C or T, N = any (IUPAC).

[d]The WellRED fluorescent dye (D2) was attached to the 5′-end of this primer when used for T-RFLP analysis.

2.1.6 Restriction Enzymes

Restriction enzymes from Invitrogen that were used in this study of methanotrophs in soil are listed in Table 2-2.

Table 2-2: Restriction enzymes used for this study.

Enzyme	Cut Site	Incubation T.*	Inactivation T.
Hae III	5′-GG↓CC-3′ 3′-CC↑GG-5′	37°C	80°C for 20min
Hha I	5′-GCG↓C-3′ 3′-C↑GCG-5′	37°C	65°C for 20min
Mbo I	5′-↓GATC-3′ 3′-CTAG↑-5′	37°C	65°C for 20min
Taq I	5′- T↓CGA-3′ 3′- AGC↑T-5′	65°C	80°C for 20min

*Incubation time depends on the digested product and should be optimized for each experiment.

2.2 Methods

2.2.1 Soil Samples

Twenty-four samples of soil were collected from four ecologically different sites in Wooster, Ohio, United States; No-till corn fields, tilled corn fields, grasslands and forests. Samples were collected during the winter time in February, 2012. The samples were collected from three random locations at each site and at two depths; 0-5 cm and 5-20 cm, respectively. Soils were homogenized and sieved to remove large roots and rocks. The soil samples were bagged and labeled properly and stored at -20 ^{o}C for later DNA extraction. The labeling was as follows: Samples 1-6 for no-tillage, 7-12 for tillage, 13-18 for grasslands, and 19-24 for forest.

2.2.2 DNA Extraction from Soil Samples (Mo Bio PowerSoil® DNA isolation kit protocol)

Soil DNA was extracted using a PowerSoil DNA isolation kit in accordance with the manufacturer's instructions. This kit comes with a humic substance/brown color removal procedure, which is effective at removing PCR inhibitors from even the most difficult soil types. The principle of extraction is based on mechanical and chemical cell lyses followed by immobilization of the DNA on a silica spin column and subsequent washing and elution. Briefly:

- 0.25 g of soil was added to the PowerSoil bead beating tubes containing cell lysis solution, followed by the addition of 60 µl of C1 solution. The samples were homogenized using horizontal vortex at 10,000 x *g* for 10 min followed by centrifugation at 10,000 x *g* for 30 s.

- Solution C2 was added to the pellet, the samples were briefly vortexed, and then incubated at 4° C for 5 min, followed by 1 min centrifugation and the supernatant was discarded.
- This same step was repeated with all samples for after addition of solution C3.
- The next step involves adding the DNA binding solution (C4) to the cleared supernatant. The sample mixture was loaded onto the spin column.
- Washing of the spin column was performed with solution C5 according to the manufacturer's instructions with an additional centrifugation steps to remove any remaining ethanol from the column.
- The DNA was eluted with C6 solution (10 mM Tris). DNA was stored at -20 °C.

2.2.3 Selection and Testing of Primers

A literature survey was made to select the most appropriate primers for this study. Selected primers, given in Table 2-1, were tested for results comparison with their references. The primers was tested for specificity for both type-I and type-II methanotrophs sequences that were downloaded from GenBank (NCBI), using *in silico* PCR (http://insilico.ehu.es/PCR/). Analysis for potential dimers and secondary structures was carried out using the OLIGO 7 program (http://www.oligo.net/index.html). RDP II Probe Match program (https://rdp.cme.msu.edu/probematch/search.jsp) was utilized to test 16S rRNA genes-specific primers. Further testing with gel electrophoresis was performed for the amplified products.

2.2.4 PCR Amplification

The primers utilized in this study were shown in Table 2-2. Four sets of primers were used for the amplification of the *pmoA* gene; A189f-A682r targeting *pmoA/amoA* genes, A189f-A650r, f326-r643 and A189f-mb661 targeting *pmoA* gene. The latter primer pair was used in a semi-nested PCR strategy (Horz *et al.*, 2005). A189f-GC primer was used instead of A189f when the reaction was prepared for DGGE analysis.

A semi-nested PCR strategy was used for the amplification of the 16S rRNA of both type-I and type-II methanotrophs genes. The first round, type IF-type IR primer for type-I, and type IIF-type IIR for type-II methanotrophs were used, respectively. For the second round, the forward primers containing a GC-clamp sequence were used (341F_GC-type IR; 518F_GC-type IIR).

The reaction was carried out using the thermal cycler in a reaction mixture containing 25 µl of GoTaq Green Master Mix polymerase (1x), 1 µl (10 pMol) of each primer, and 0.5 µl (~5 ng) of template DNA. The volume was then completed to 50 µl using nuclease-free water (supplied). Thermocycling conditions for *pmoA* amplification were as follows: Initial denaturation at 94^{o} C for 4 min, followed by 35 cycles of denaturation at 94 ^{o}C for 35 s, annealing at 55 ^{o}C for 1 min, and elongation at 72 ^{o}C for 1 min, and a final elongation step at 72 ^{o}C for 10 min. The cycler was programmed to end by holding the tubes at 4 ^{o}C for approximately 2 h. In case of primer mb661, the annealing temperature of the second round was set to 60 ^{o}C and for 25 cycles. The same conditions were used for the 16S rRNA genes amplification, except for the annealing temperature; where it was set to 60 ^{o}C and the reaction was run for 30 cycles (Chen *et al.*, 2007). After PCR, 5 µl of each PCR product was resolved in 2% agarose gel to confirm product size and the negative control (i.e. the PCR mixture containing the primers but without DNA template).

2.2.5 Measuring DNA Concentration (Green and Sambrook, 2012)

DNA concentration and purity were measured, before amplification and following amplification, using 1 µl of each DNA sample according to the NanoDrop ND 1000 manual. For PCR products, the two dyes of the GoTaq Green Master Mix interfere with readings. Thus, the Colorless Master Mix was used instead. DNA purity was measured depending on the ratio of sample absorbance at wavelengths 260 and 280nm.

2.2.6 Agarose Gel Electrophoresis (Green and Sambrook, 2012)

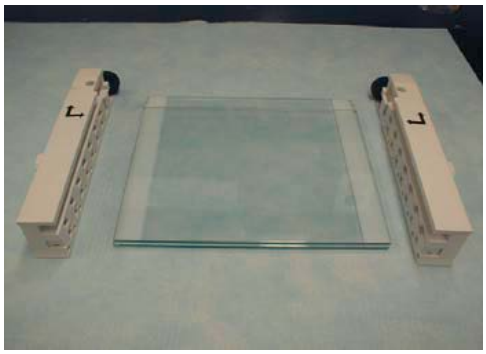
Agarose gel of 2% concentration was utilized to confirm product size and to conduct a negative control of the PCR products. The agarose gel consisted of 2.4 g agarose dissolved in 120 ml of 1x TAE buffer using a microwave. After the agarose solution cooled down to 55-60 ^{o}C, a 1 µl of 10 mg/ml ethidium bromide (EtBr) was added. Then, the solution was poured into the gel tank with the combs in place and let to cool for 30 min to set. The combs were removed carefully and the tank was placed in the electrophoresis system containing running buffer consisting of 1x TAE. The buffer was poured until it covered the gel for about 1-2 mm. Five µl of each PCR product along with the negative control and a 100 bp DNA ladder were loaded into the wells, the system cover was then put into place and the system was turned on. The gel is left to run for 2 h with a 100 volt/50 mAmp current. Following electrophoresis, visualization was conducted with a UV transilluminator, using Kodak's Gel Logic 200 Imaging System®. This system has the appropriate filter and a suitable program for illumination of EtBr-stained gels.

2.2.7 DGGE Analysis

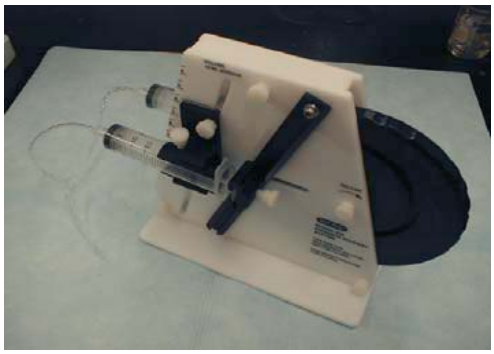
PCR-amplified products were subjected to DGGE analysis for resolution of bacterial communities based on DNA sequence differences. DGGE was performed using the Bio-Rad D-Code system (Figure 2-1) according to the manufacturer's directions using 1 mm thick, 6% (w/v) polyacrylamide gel (acrylamide/bis-acrylamide [37.5:1]) with a denaturing gradient of 30% to 70%. The gel was cast by preparing two falcon tubes; one for the high concentration and the other for the low concentration. In each tube, 17.5 ml of the denaturing reagent, 172 µl of ammonium persulfate and 17.2 µl TEMED were added. In addition, 350 µl of the DCode dye solution was added to the tube containing the high concentration reagent. Electrophoresis was carried out for 6 h at 60 ^{o}C and a constant voltage of 150 where the gel was submerged in 7 L of 1x TAE buffer.

Following electrophoresis, the gel was placed in a staining box (containing 300 ml of 1x TAE and 25 µl of 10 mg/ml EtBr) for 10 min and then in a de-staining box (containing buffer only) for approximately 15 min before imaging.

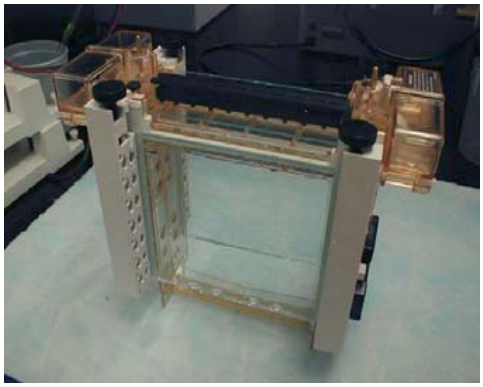
After DGGE, the gel was transferred on a tray onto a UV transmitter (the Chromato-Vue Transilluminator®) for band cutting. A sharp scalpel was used to cut around the shiny bands. Each band was transferred to a 2 ml tube containing 50 µl DDI H_2O; all tubes were stored at -4 ^{o}C overnight. A second amplification reaction for each band followed by electrophoresis was done to confirm band purity. The resulting bands were cut and purified from the gel and sent for sequencing.



A



B



C

D

Figure 2-1: DGGE assembly. (A) Casting the gel sandwich. Gel clamps and spacers between two glass plates. (B) The gradient wheel. The two syringes hold the high- and low-concentration solutions. (C) The gel, with the comb in place, is attached to the core assembly. (D) The DGGE apparatus in action.

2.2.8 DNA Extraction from Agarose Gel

DNA extraction and purification was carried out using QIAEX II gel extraction kit. Bands resulting from the second amplification reaction were cut using a clean, sharp scalpel, transferred to 2 ml tubes and the excess agarose was carefully removed. To each tube, 300 µl of buffer QX1 was added. QIAEX

II reagent was vortexed for 30 s for resuspension, and 30 µl of this reagent was added to each tube. Using a water bath, the tubes were incubated at 50 ^{o}C for 10 min to solubilize the agarose and bind the DNA. To keep QIAEX II in suspension, the tubes were vortexed every 2 min. Samples were centrifuged at 10000 x *g* for 30 s and the supernatant was carefully removed with a micropipette. The pellet was washed with 500 µl of buffer QX1 to remove residual agarose contaminants, followed by two washes with 500 µl of buffer PE for salt contaminants removal. All of these three washing steps were carried out by adding the buffer, resuspension by vortexing, centrifugation for 30 s and carefully removing the supernatant. Samples were left to air-dry for 30 min until the pellet became white.

For DNA elution, 20 µl of nuclease-free H_2O was added, the pellet was resuspended by vortexing and then the samples were incubated at room temperature for 5 min. The last step includes centrifugation for 30 s and carefully pipetting the supernatant, which now contains the purified DNA, to a clean 2 ml tubes. Samples were stored at -20 ^{o}C.

2.2.9 DNA Sequencing

DNA capillary sequencing was performed at the Molecular and Cellular Imaging Center (MCIC), Ohio Agricultural Research and Development Center (OARDC), the Ohio State University (OSU), using the ABI Prism 3100xl genetic analyzer (Applied Biosystems®). According to manufacturer's recommendations, two sets of 6 µl gel-purified DNA were transferred to PCR strips, the forward primer used in the PCR reaction was added to the first set and the reverse primer for the second. Following sequencing, the resultant sequences obtained were tested for length and quality using the CodonCode Aligner software (CodonCode Corporation®).

2.2.10 Data Analysis and Phylogenetics

Sequences obtained with good qualities were subjected to BLASTn searches using GenBank. Sequences that showed the highest identity and maximum coverage were downloaded. Alignment of the obtained and downloaded sequences was established using the ClustalX2 software (Larkin *et al.*, 2007). Phylogenetic trees were constructed using MEGA 5 software employing the neighborhood-joining method, based on the Poisson model with Nearest-Neighbor Interchange and a Bootstrap Test of Phylogeny. The Bootstrap was set to test 100 replicates in order to increase the reliability of the tree.

2.2.11 Terminal Restriction Fragment Length Polymorphism

The PCR reaction for the T-RFLP was performed using the GoTaq® Colorless Master Mix, to avoid dye interference during detection, and the resultant products were purified using the Wizard® SV Gel and PCR Clean-Up System. The PCR products were purified according to the kit's instructions without any modification. Digestion of PCR products was carried out following the supplier's recommendations. The enzyme (1 µl) was mixed with 2 µl of specific buffer and 4 µl (2000-2500 ng) of PCR product and volume was completed to 20 µl using nuclease-free H_2O. Samples were incubated for 5 h to insure complete digestion followed by enzyme inactivation. The enzymes used, along with their specific cleavage site, incubation and inactivation temperatures are listed in Table 2-2. For characterization, digested samples were sent to MCIC/OARDC, OSU.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Primer Testing

A computational analysis using *in silico* PCR was conducted to test the validity of the most commonly used primers that were used in this study to identify methanotrophs in soil samples. The analysis outcome mostly agreed with those done in previous studies, and as follows:

(a) *pmoA*-specific primers. The A682r primer pair gave products of ~531 bp, and covered mostly species of *Methylocystis, Methylomanas, Methylobacter,* and ammonia-oxidizing bacteria (Holmes *et al.*, 1995); the mb661 primer gave products of 518-526 bp, and covered the majority of known methanotrophs (Costello and Lidstrom, 1999); the primer A650r gave a product of ~500 bp and covered limited species of *Methylocystis*, *Methylococcus*, and *Methylobacter* (Bourne *et al.*, 2001); and the f326-r643 primer set which gave a product of ~750 bp and covered species of *Methylocystis*, *Methylococcus*, and *Methylobacter*, but missed *pmoA* of other methanotrophs (Fjellbirkeland *et al.*, 2001).

(b) 16S rRNA primers. The MethT1df-MethT1b for type-I (gave product of ~923 bp) and MethT2R for type-II (gave product of ~505 bp) methanotrophs failed to target the 16S rRNA genes of several methanotrophs, such as *Methylosphaera, Methylocaldum, Methylocella* and *Methylocapsa* (Wise *et al*., 1999); and the type IF-type IR for type-I (gave product size of ~669-673 bp) and type IIF-type IIR for type-II (gave product of ~525 bp) methanotrophs, these two sets were able to cover those species that were not covered previously (Chen *et al.*, 2007). PCR followed by gel electrophoresis were performed, and the resultant products match those obtained from *in silico* analysis. In case of

some primers however, it was difficult to obtain a proper, decisive amplification signal and thus were excluded from this study (results are not shown).

3.2 DNA Extraction from Soil Samples

Direct DNA extraction from soil is a basic technique in the study of microbial ecology. The DNA produced must be of high molecular weight and free of inhibitors of subsequent molecular techniques such as PCR and restriction digestion (Bakken and Frostegård, 2006). One of the major problems associated with studying genes and their expression in the environment is the difficulty to obtain adequate and pure nucleic acid samples. Some of the most difficult contaminants in soil DNA are humic acids, a large group of organic compounds associated with most soils that are high in organic content (Stach *et al.*, 2001). Conventional methods proved to be laborious and time consuming, i.e. from the preparation of a number of chemicals to the need for extra purification steps, all of which might produce inconsistent and inconvenient results. For this reason, commercial DNA extraction kits are now commonly used in the assessment of taxonomic and functional diversity, community composition, and population abundance (Lord *et al.*, 2002; O'Brien *et al.*, 2005; Wawrik *et al.*, 2005; Shanks *et al.*, 2006; Roesch *et al.*, 2007). The MoBio PowerSoil® DNA isolation kit, which was utilized in this study, offers a novel method for isolating genomic DNA from environmental samples utilizing a special patented Inhibitor Removal Technology®. This IRT removes enzymatic inhibitors including humic acids, polyphenols, polysaccharides, heme, or dyes and it is intended for use with environmental samples containing a high humic acid content including difficult soil types such as compost, sediment, and manure (Nagissa *et al.*, 2011; Subramanya *et al.*, 2013).

Genomic DNA was successfully extracted from all soil samples. In almost all cases, the extracted DNA concentration was about 10-18 ng/µl when measured using the Nanodrop ND 1000. The obtained DNA was of sufficient amount for downstream PCR. In fact, most of the used primers gave visible products even when less than 5 ng of DNA was used as a template (data not shown). The UV absorbance at the A260/A280 ratio was also measured for the extracted DNA and it ranged ~1.84-2.01. This ratio indicates sufficient removal of proteins or other contaminants for the majority of samples. The extracted DNA was stored at -20 $^{\circ}$C, to prevent degradation, in a solution containing Tris buffer, to maintain neutral pH and without EDTA. EDTA was excluded from this storage mixture because it has the ability to inhibit DNA synthesis by chelating the Mg^{2+} necessary for the activity of DNA polymerase during subsequent PCR reaction (Khosravinia and Ramesha, 2007; Huggett *et al*., 2008).

3.3 PCR Amplification

Several primer sets were tested for the successful amplification of the *pmoA* gene. These includes f326-r643 (Fjellbirkeland *et al.*, 2001), A189f-A650r (Bourne *et al.*, 2001), A189f-A682r (Holmes *et al.*, 1995), and A189f-mb661 (Costello and Lidstrom, 1999). The *pmoA* gene was detected in all soil samples. The first two sets (i.e. f326-643 and A189f-A650r) gave faint bands in some cases and non-specific amplification was also observed even after some optimization processes (results not shown). On the other hand, the A189f-A682r and A189f-mb661 primers gave visible, distinct bands with amplicons of the expected size, and they were both chosen for this study (Figure 3-1). The A189f-mb661 primer set was used to carry out a semi-nested PCR according to Horz *et al.* (2005). In this procedure, the primer pair A189f-A682r was used in

the first PCR round and A189f-mb661r was used in the second round. This gave consistently high yields of *pmoA* amplicons. The semi-nested PCR technique has several useful advantages. It increases PCR yield and generates more specific DNA fragments suitable for DGGE analysis by avoiding the possible detrimental effects of PCR amplification with primers that have a GC-clamp (Mühling *et al*., 2008).

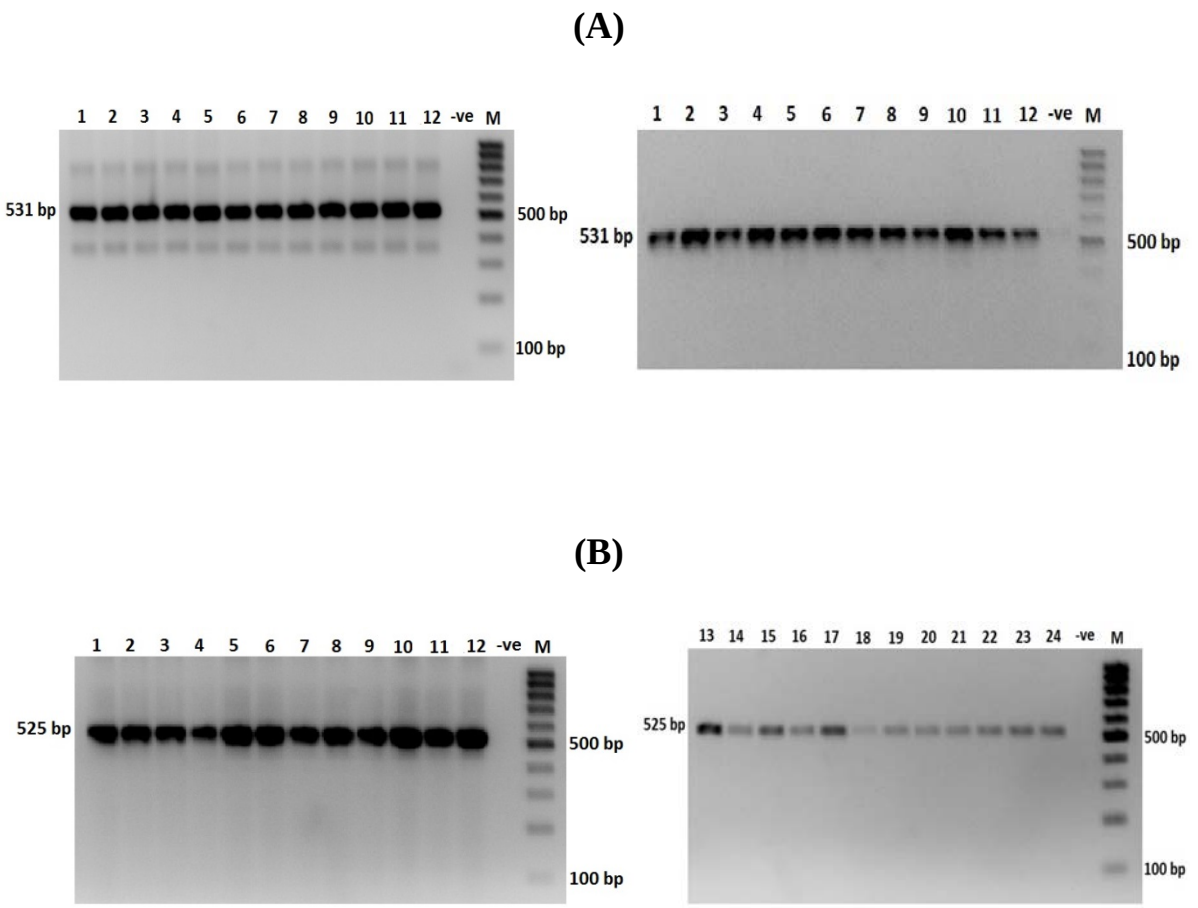


Figure 3-1: Agarose gel electrophoresis of the amplified *pmoA* gene. **(A)** Gene amplified with the A189-A682r primer set (531 bp). **(B)** Gene amplified with the A189-mb661 primer set (~525 bp). (A 2% agarose gel run at 100 V for 2 h).

The PCR conditions differ between the two rounds of this approach. In the first PCR round, increased number of cycles and a relatively lower annealing temperature was used to allow the most possible amplifications from the environmental DNA samples, which contain mixed populations of bacteria. The second round employed a higher annealing temperature, to increase the specificity of primers annealing, with less DNA template, less primers, and fewer number of cycles in an attempt to limit the number of non-specific amplifications that might occur (Shabir *et al.*, 2005).

The A189f-A682r primers have been used extensively in environmental studies to provide a molecular profile of the methane oxidizers (Holmes *et al.*, 1999; Bourne *et al*, 2001; Horz *et al*, 2001; Heyer *et al.*, 2002; Kalyuzhnaya *et al.*, 2002; and Radajewski *et al.*, 2002) and have proved useful in detecting novel sequences (Knief *et al.*, 2003). This primer set was designed to amplify internal fragments of the genes encoding pMMO (particulate methane monooxygenase) and AMO (ammonia monooxygenase) enzyme complexes (Holmes *et al.*, 1995). The phylogeny of *pmoA/amoA* is reasonably congruent with the 16S rRNA gene phylogeny of the organisms from which the gene sequences were retrieved (Kolb *et al.*, 2003). Therefore, retrieval of *pmoA* and *amoA* sequences provides information on the diversity of these organisms in the environment. The pMMO and AMO genes are evolutionarily related (Holmes *et al.*, 1995), and at the amino acid level they share a number of highly conserved residues (Ricke *et al.*, 2004). Based on alignments of the predicted peptide sequences of the α subunits of 112 particulate methane monooxygenases and 349 ammonia monooxygenases, Tukhvatullin *et al.* (2001) identified residues common to both proteins.

The A189f-A682r primer set is used in conjunction with another set, the *pmoA*-specific primer A189f-mb661r (Lin *et al.*, 2005) and demonstrated specificity in amplifying *pmoA* sequences but not *amoA* sequences (Shrestha *et*

al., 2008). The results indicated that the mb661r primer gave the best results in covering methanotroph diversity, however, while the primer A682r excludes *Methylocapsa*, as well as genes from other uncultivated bacteria, which are indicated to be methane oxidizers (Pacheco-Oliver *et al.*, 2002); it managed to detect novel groups of *pmoA* sequences where mb661r fails to detect. For this reason, studies suggest using both the A189f-A682r and the A189f-mb661r primer sets in order to obtain the best coverage of methanotroph diversity (Morris *et al.*, 2002; Hutchens *et al.*, 2004; Lin *et al.*, 2004; Knief *et al.*, 2005; Lin *et al.*, 2005; Chen *et al.*, 2007; and Ruo *et al.*, 2012). NanoDrop results showed very good amplification, where the DNA concentration ranged between 500-550 ng/µl, and the A260/A280 ratio readings was ~1.88-2.10 for all of the amplified samples.

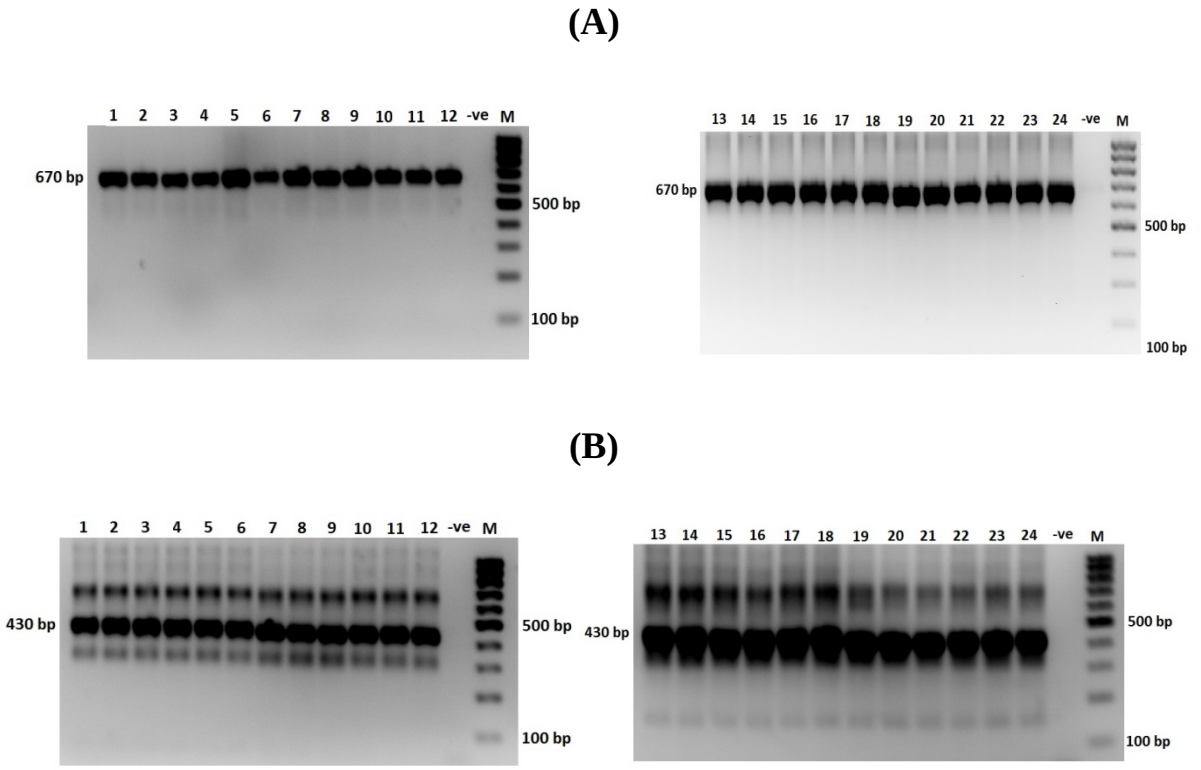


Figure 3-2: Agarose gel electrophoresis of 16S rRNA gene of type-I methanotrophs. **(A)** Gene amplified using type IF-IR primer set (670 bp). **(B)** Re-amplification using 318f-IR primer set (430 bp). (A 2% agarose gel run at 100 V for 2 h).

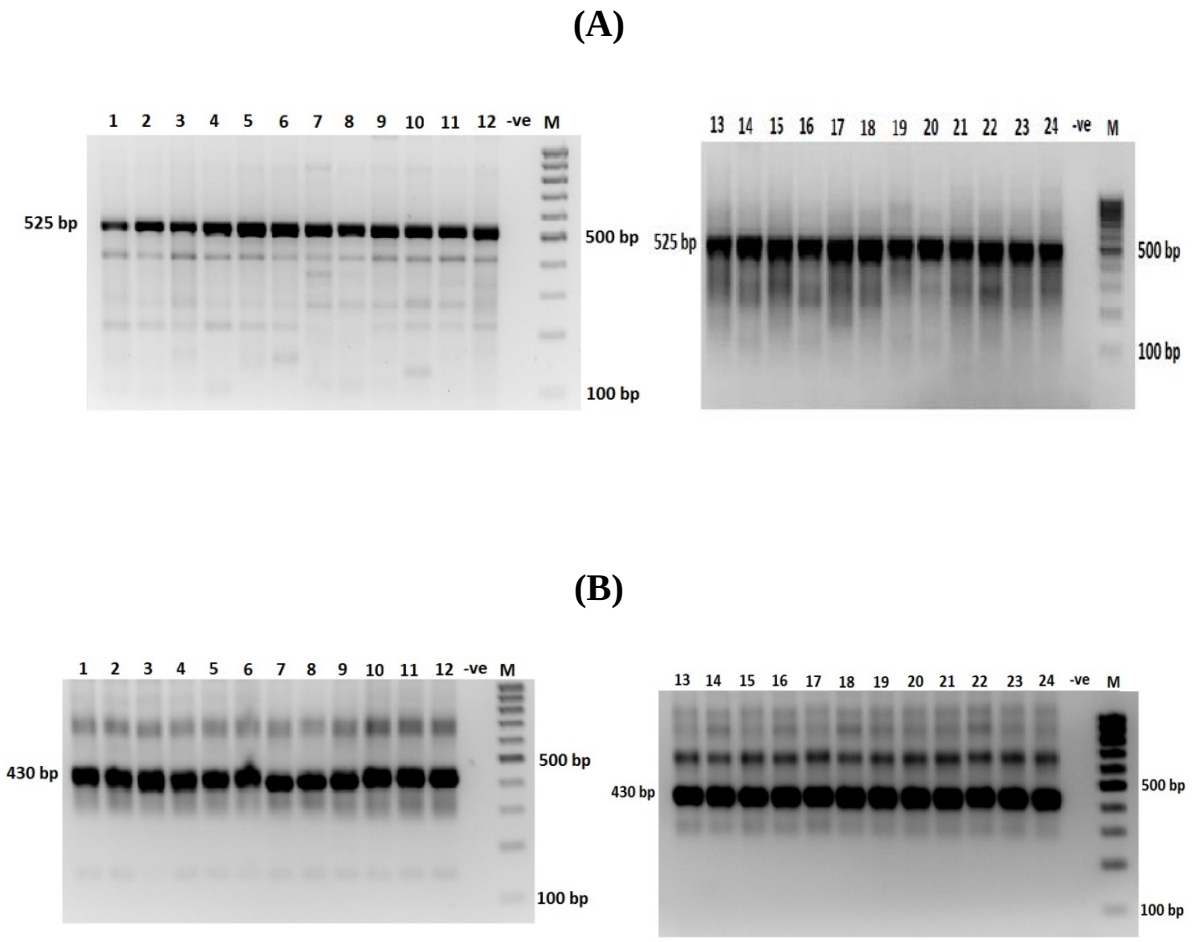


Figure 3-3: Agarose gel electrophoresis of 16S rRNA gene of type-II methanotrophs. **(A)** Gene amplified using type IIF-IIR primer set (525 bp). **(B)** Re-amplification using 518f-IIR primer set (430 bp). (A 2% agarose gel run at 100 V for 2 h).

The amplification of the 16S rRNA genes of methanotrophs was tested using two sets of primers for each type (i.e. type-I and type-II) of methanotrophs; MethT1dF-MethT1bR, for type-I, 533F-MethT2R, for type-II methanotrophs (Wise *et al.*, 1999). These two sets gave contradictory results and rather weak signals on agarose gel (results not shown). The other two pairs of type IF-type IR for type-I methanotrophs, and type IIF-type IIR for type-II methanotrophs, showed much better results and gave products of the expected size (Figure 3-2A and 3-3A). Thus, they were chosen for this study. A semi-

nested PCR approach was used with these primers. In the first round, they were used to selectively amplify methanotrophs 16S rRNA genes from soil. In the second round, the forward primers containing GC-clamps were used, i.e. 341F_GC-type IR for type-I methanotrophs, and 518F-type IIR for type-II methanotrophs, respectively (Muyzer *et al.*, 1993). The second round of amplification gave products suitable for DGGE profiling (Figure 3-4B and 3-5B).

3.4 DGGE and Sequencing

For DGGE profiling, amplification with the primer A189f with a GC-clamp attached to it was successful. The resultant gel showed several dominant bands that appeared at a different position in the gel, indicating the potential of the primers to detect different bacterial taxa. Other produced bands were neglected because the intensities of these bands were always much lower than the intensity of the dominant bands. Figure 3-4 gives an example of a DGGE profile picture.

A thing that should be noted is there are no established standards for DGGE, especially when studying mixed population where the use of a standard does not have much meaning. The best approach is to create set of standards by mixing PCR products of a number of differently migrating isolates; achieved by running the PCR of each isolate independently and then mixing the PCR yield, thus making a large stock. Such procedures are impractical for most general diversity studies where comparison is not something to be focused on (Shabir *et al.*, 2005). Adding a standard to each lane for technicality studies is another matter not concerned with this study.

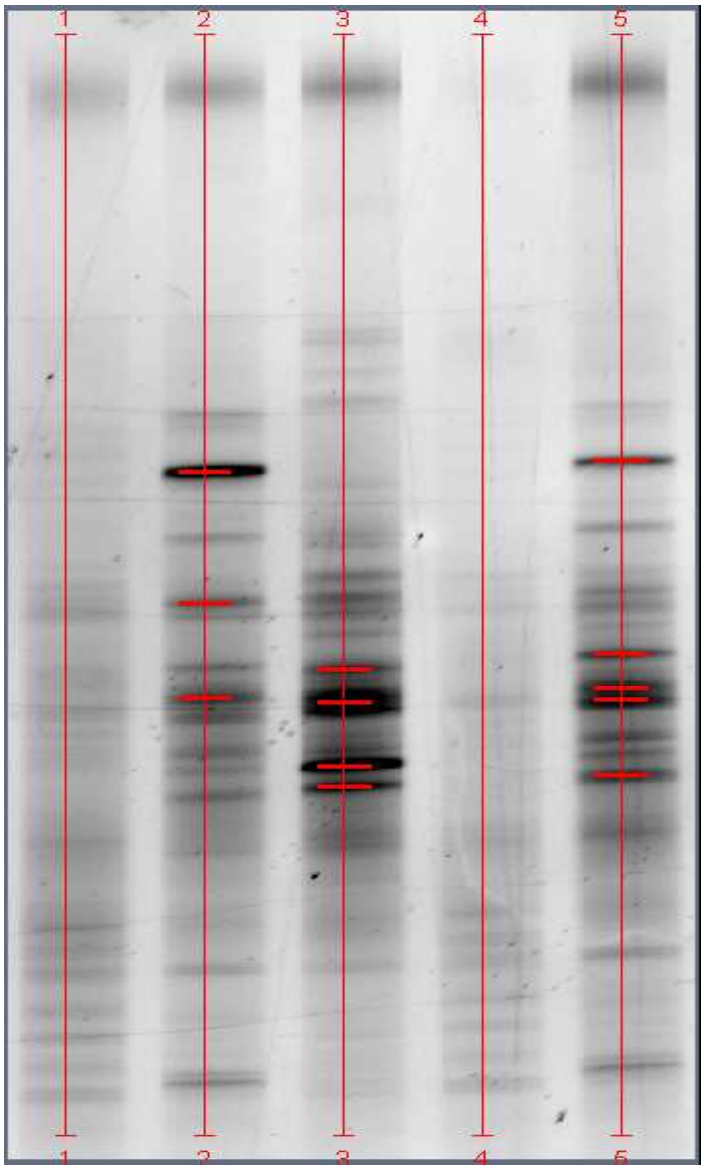


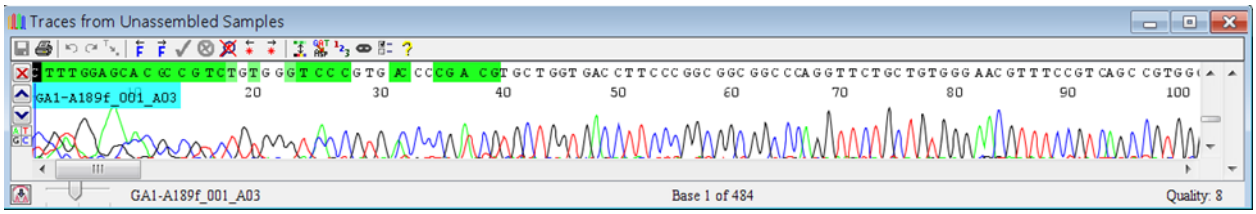
Figure 3-4: Denaturant gradient gel electrophoresis (DGGE) profile of PCR-amplified fragment of *pmoA* gene. The red markers indicate intense bands. (A 6% polyacrylamide gel with a 30% to 70% denaturant gradient, run at 60 °C and 150 V for 6 h).

Purity of selected bands was assessed by re-amplification of the cut bands, and the resultant products were resolved on agarose gel. Only the products that gave sharp and distinct bands were cut and purified from the gel, and then sent for sequencing. Assessment of sequence quality was conducted for the retrieved raw sequences using the CodonCode Aligner® software. All

sequences had to be trimmed to remove low-quality (represented by trashy-looking peaks) information, i.e. the first 20-60 nucleotides and, sometimes, the last 20 nucleotides. These "trashy-looking" peaks are formed due to some primer dimers or from small PCR products that cannot be seen on an agarose gel (Figure 3-5). Once the raw sequences were trimmed down, they were subjected to BLAST analysis.

Because most of the primers designed for functional genes are highly degenerate, a number of problems during PCR and DGGE may arise. Since the whole point of DGGE is to separate fragments that differ in sequence, identical PCR fragments that have different primer sequences can sometimes generate multiple bands on DGGE.

(A)



(B)

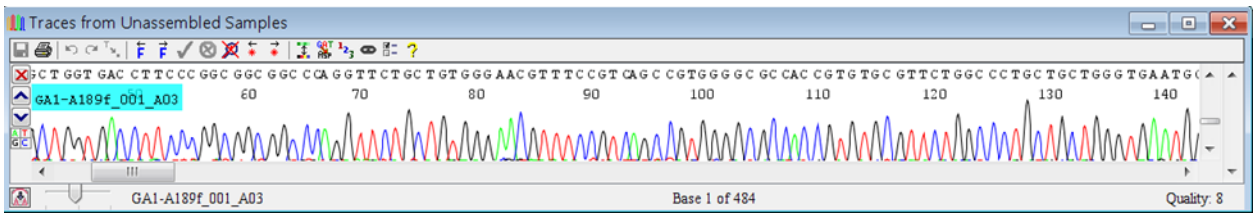


Figure 3-5: Sequence quality check using the CodonCode Aligner® software. (A) Retrieved raw sequence showing low-quality sequencing, represented by "trashy-looking" peaks. (B) The same sequence but with the first 40 nucleotides removed. Only proper nucleotide sequence remains.

The problem originates during the PCR reaction, when using degenerate primers. For these situations, a low annealing temperature must be used to accommodate all the possible primer combinations (that may occur when using degenerate primers). However, at the low annealing temperature, some of the primers can anneal non-stringently to DNA target, and thus the non-stringent primer becomes incorporated into the growing DNA fragment. So, multiple primer combinations with different primer sequences can anneal to the same template DNA and generate copies of the same fragment. If differences in these primer sequences are great enough, DGGE analysis will separate out the identical PCR fragments by the differences existing in the primer region. Sequencing DGGE analysis can help resolve this issue. However, there is not much that can be done to avoid this problem and it must be noted that this problem can complicate measurements of diversity when analysed using DGGE gels (Janse *et al.*, 2004).

3.5 Analysis of the Functional Gene *pmoA*

To characterize the methanotrophic community in soil samples, the functional gene encoding pMMO was investigated using two sets of primers. From DGGE profiling, a total of 30 sequences were obtained using both sets (Fig. 3-6).

With the *pmoA/amoA* phylogeny that was generated with the A189-A682 primer set, ten sequences were obtained. Six of these were grouped as *amoA* sequences and were closely related to *amoA* sequences of *Nitrospira* sp., *Nitrosovibrio* sp. RY3C and *Nitrosolobus multiformis* (96-98%). The remaining four *pmoA* sequences were of relative similarity with species of *Methylobacter* (98%), *Methylosoma* (91%) and *Methylmonas* (89%). For the *pmoA* phylogeny generated with the A189f-mb661 primer set, twenty sequences were obtained.

Of these, two sequences showed 99% identity to *Methylocystis* sp. SC2 and *Methylocystis parvus*, and another two showed 99% identity to *Methylobacter albus* and *Methylobacter* sp. LW14. One sequence was highly similar to *Methylomicrobium album* (95%) and another six sequences exhibited similarity to that of *Methylococcus capsulatus* sp. Bath (86-89%). The remaining nine sequences were mostly clustered with sequences of uncultured methanotrophs recovered from Genbank with similarities ranging from 92% to a relatively low 84%. As expected, *Methylocella* was not detected since it does not possess *pmoA* (Theisen *et al.*, 2005), but its presence was detected with the 16S rRNA gene profile.

Further detailed analysis revealed the exclusion of other species, like *Methylosarcina*, *Methylocapsa*, *Methylocaldum*, and *Methylosinus*, but their presence was also detected by 16S rRNA gene analysis. An explanation for this observation could be primers bias against some of these species; however, this might or might not be the case since it has been proven otherwise in a number of studies (Bourne *et al.*, 2001; Hutchens *et al*., 2004).

It is difficult to make assumptions based on the number of sequences in a profile without making a sufficient study regarding the abundance of methanotrophs genes in the soil. A possibility which must not be excluded is whether these bacteria were actually expressing pMMO in that soil samples or whether the genes were dormant. The *pmoA* data suggested that type-I methanotrophs were more active than type-II methanotrophs, at the time of sampling (February 2012). This may be related to the temperature at the time of sampling as found by Börjesson and colleagues (2004) who studied the effects of temperature on methanotrophs in three different landfill cover soils. They showed that the PLFA (phospholipids-derived fatty acids) marker for type-II methanotrophs (18:1w8c) that was highly elevated only at high temperatures (20 ˚C) and PLFA markers for type-I methanotrophs (16:1w5t, 16:1w6c,

16:1w8c) primarily increased at low temperatures (5–10 ˚C). A subsequent study showed that methanotrophs distribution is also affected by parameters such as O_2 and CH_4 concentrations (Wang *et al.*, 2008).

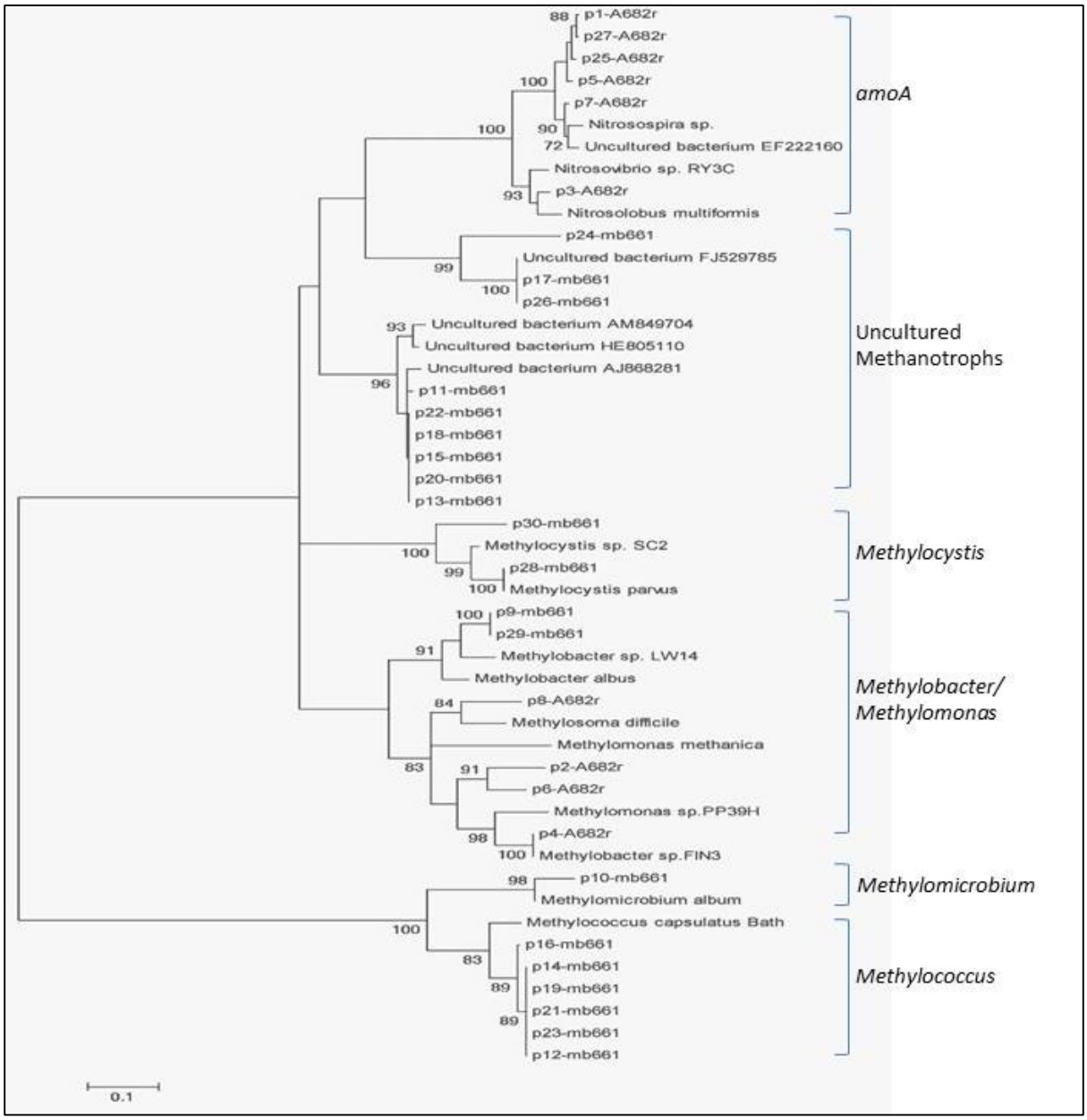


Figure 3-6: Neighbor-joining phylogenetic tree of deduced *pmoA* gene sequences. Bootstrap values greater than 70 are shown (100 replications). The scale bar represents 0.1 substitutions per nucleotide position.

3.6 Analysis of Methanotrophs by *16S rRNA*

The DGGE profile of type-I methanotrophs 16S rRNA gene, amplified with the primer pair type IF-type IR, showed quite diverse groups of methanotrophs that match the expected results obtained *in silico* from this primer set (Figure 3-7). Two sequences showed a high similarity of 96% and 98% respectively to *Methylomonas*, and another two were also very similar to *Methylocaldum* (95% and 98%, respectively). At least one sequence was obtained for species of *Methylomicrobium* (99%), *Methylosarcina lactus* (99%), *Methylobacter* (97%), and *Methylococcus* (99%).

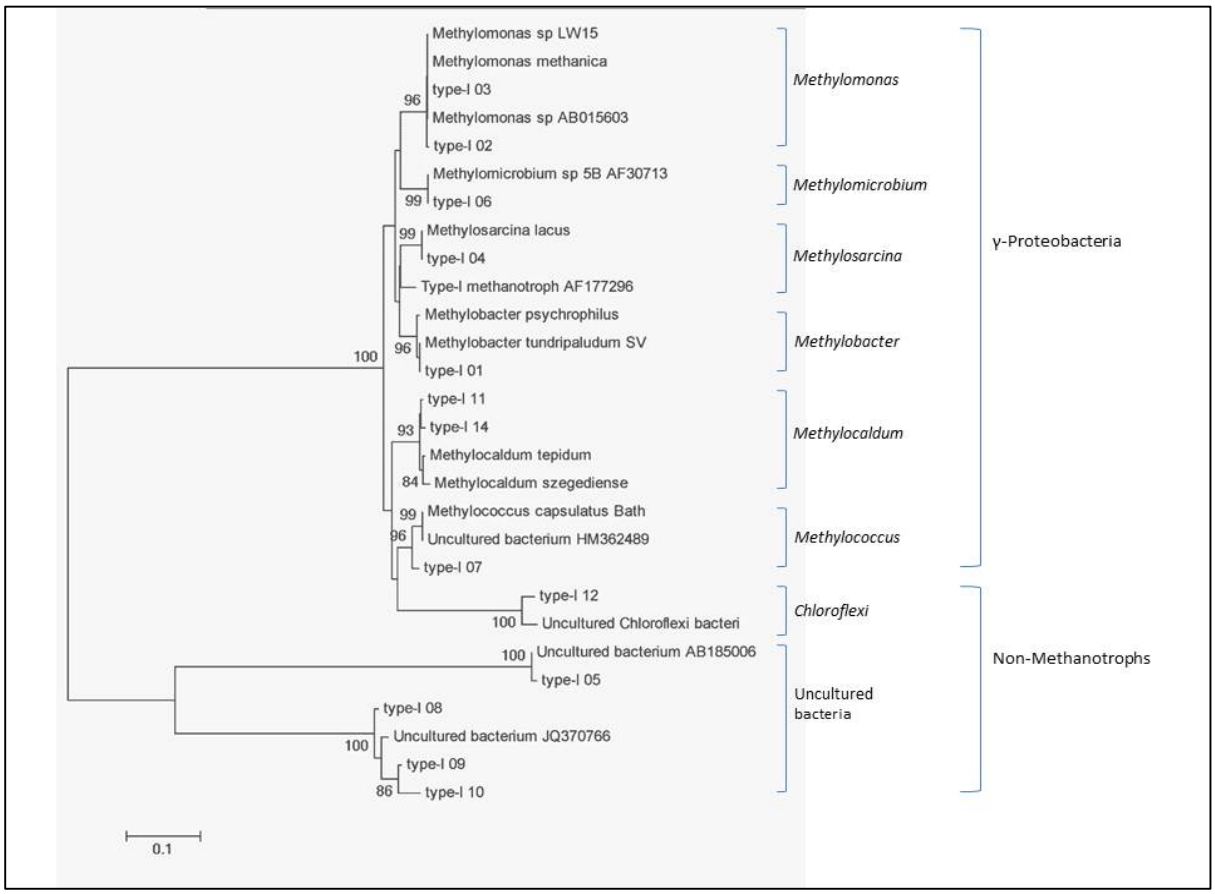


Figure 3-7: Neighbor-joining phylogenetic tree of deduced type-I methanotrophs 16S rRNA gene sequences. Bootstrap values greater than 70 are shown (100 replications). The scale bar represents 0.1 substitutions per nucleotide position.

Five obtained sequences corresponded to sequences of uncultured bacteria other than methanotrophs. One of these five sequences was classified as a species of *Chloroflexi*.

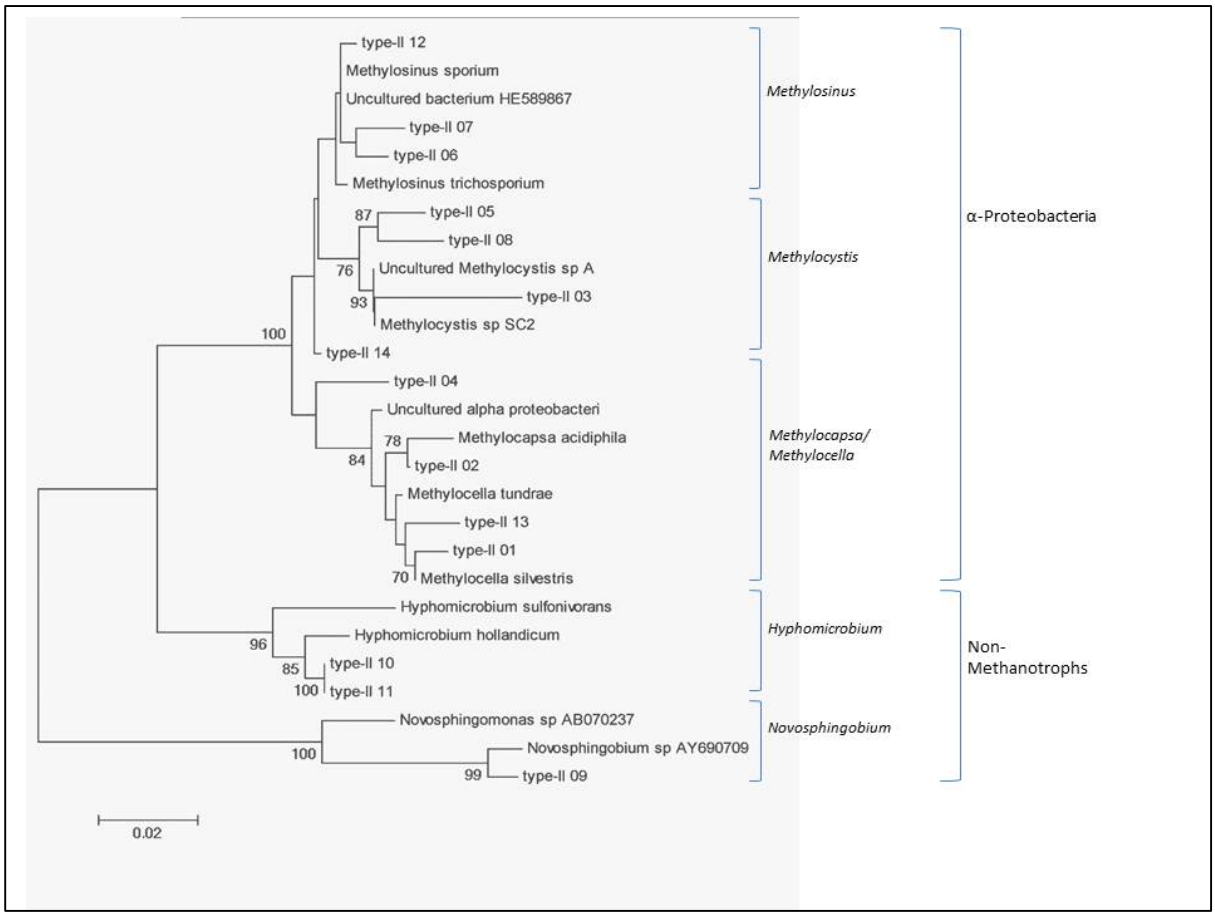


Figure 3-8: Neighbor-joining phylogenetic tree of deduced type-II methanotrophs 16S rRNA gene sequences. Bootstrap values greater than 70 are shown (100 replications). The scale bar represents 0.02 substitutions per nucleotide position.

The 16S rRNA gene of type-II methanotrophs amplified with the type IIF-type IIR primer pair was also analyzed (Figure 3-8). The sequences obtained were grouped to four major type-II methonotrophs. Three sequences belong to *Methyosinus sporium* and *M. trichosporium* (95-98%), four sequences matches those of *Methylocystis* spp. (97-99%), and another four sequences showed high

similarity (97-99%) to spp. of *Methylocella tundra*, *M. silvestris* and *Methylocapsa acidiphila*. Three sequences were found to be closely related to the 16S rRNA genes of non-methanotrophs, with two of these sequences identified as species of *Hyphomirobium* (97%) and the third as *Novosphingobium* (95%) bacteria. The DGGE profiling of both type-I and type-II methanotrophs revealed diversities that were consistent with other reports studying methanotrophs in soils (Börjesson *et al.*, 2004; Stralis-Pavese *et al.*, 2006). In this study, 16S rRNA DGGE analysis has reflected the major genera of type-I methanotrophs.

Although the resolution of DGGE fingerprints for type-II methanotrophs was not very high, reflecting high similarity of 16S rRNA sequences among these organisms, it was able to successfully identify the predominant methanotrophs within type-II category. Further optimization of DGGE conditions would obviously be required in future studies where these primer sets are applied to DNA extracted from such environment. The analysis of both of type-I and type-II methnotrophs 16S rRNA genes also revealed sequences of non-methanotrophs bacteria, indicating a lack of primers specificity in some cases. These primers, among others, such as MethT1dF-MethT1bR for type-I methanotrophs and 533F-MethT2R for type-II methanotrophs, amplified sequences for non-methanotrophs and they have been reported in a number of studies (Wise *et al.*, 1999; Wartiainen *et al.*, 2003; Newby *et al.*, 2004; Carini *et al.*, 2005; and Chen *et al.*, 2007).

3.7 T-RFLP Analysis

An attempt to identify and quantify methanotrophs using T-RFLP was carried out in this study via targeting the pMMO gene. Four four-base cutter restriction enzymes were used (Table 2-2). The forward primer was labeled with

the D2 dye for a more sensitive detection. D2 is WellRED dye-labeled oligos licensed by Beckman Coulter, inc. to be used with their CEQ Genetic Analysis System, the CEQ^{tm}8800, which was the system used for detection at MCIC/OARDC, OSU.

Results were obtained in the form of raw data files, but unfortunately, there was no database for the *pmoA* gene, for the time being, to be used as a reference and make any meaning out of these data!

Conclusions and Recommendations

Conclusions

- Molecular tools used in this study are important to study the diversity of methanotrophs because the majority of these bacteria are difficult to isolate on agar plates, which makes growth-based assessment of natural populations problematic.
- Despite the difficulty in extracting DNA directly from soil, the MoBio PowerSoil DNA isolation kit proved to be efficient in obtaining DNA of sufficient quantity and purity.
- Up to the point of this study, the utilized primers gave a better coverage of methanotrophs than any other published primers.
- Adapting a semi-nested PCR approach was favorable in diversity studies; where increased specificity while covering the maximum possible number of species is needed. This is especially important when using more than one primer or different PCR parameters (to maximize coverage), or in the case of DGGE where attaching a GC clamp to the primer is necessary.
- DGGE is a powerful fingerprinting technique that can be used in diversity studies, since it managed to retrieve diverse species of methanotrophs.

Recommendations

- Since all of the known methanotrophs 16S rDNA primers can amplify sequences from non-methanotrophs; Considerations should be taken in future studies if these primers are used in qPCR experiments that aim to quantify methanotrophs.
- There is still a need for designing new primers that have increased specificity to methanotrophs and are able recover a wider range of taxa.
- More genetic markers can be targeted in future studies such as the *mmox*, encoding the soluble methane monooxygenase enzyme (sMMO), and *mxaF*, encoding the methanol dehydrogenase enzyme, or other biomarkers, e.g. lipids.
- Since one of the molecular methods used in the study of methanotrophs is terminal restriction fragment length polymorphism (T-RFLP), and the vast majority of electropherogram databases are for 16S rRNA genes and not functional genes such as the *pmoA*; a database is necessary to be built in order to further utilize this technique in diversity studies.
- Finally, this study is a part of a larger study that involves (a) quantitatively differentiate between the four ecological sites, to prove that no-till management can actually help in maintain soil microbial reservoir and thus shift it to be a sink instead of a source to methane, therefore a quantitative study for methanotrophs should be considered; and (b) design a methanotrophs-specific FISH (fluorescent *in situ* hybridization) probes for rapid identification of methanotrophs; these probes will give an authentic profile of all the labeled methanotrophs in a soil sample simultaneously, thus continuous testing and optimizing experiments are needed.

References

Ali H., J. Scanlan, M. G. Dumont, and J. C. Murrell. 2006. Duplication of the mmoX gene in Methylosinus sporium: cloning, sequencing and mutational analysis. *Microbiol*. 152:2931–2942.

Altschul S. F.; T. L. Madden; A. A. Schäffer; J. Zhang; Z. Zhang; W. Miller and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.

Amann R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59:143–169.

Auman A. J., S. Stolyar, A. M. Costello, and M. E. Lidstrom. 2000. Molecular characterization of methanotrophic isolates from freshwater lake sediment. *Appl. Environ. Microbiol.* 66:5259–5266.

Ausubel F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 2002., Short Protocols in Molecular Biology. 2nd edi. John Wiley & Sons, Inc., New York, NY.

Azam F., and F. Malfatti. 2007. Microbial structuring of marine ecosystems. *Nat. Rev. Microbiol.* 5: 782–791.

Bakke, B., P. A. Stewart, and M. A. Waters. 2007. Uses of and exposure to trichloroethylene in US industry: a systematic literature review. *J. Occup. Environ. Hyg.* 4: 375-390.

Bakken L. R. and Å. Frostegård. 2006. Nucleic Acid Extraction from Soil. Soil Biology, Volume 8. Nucleic Acids and Proteins in Soil by Nannipieri P. and K. Smalla (Eds.) ©Springer-Verlag Berlin Heidelberg.

Baxevanis A. D. 2005. Assessing sequence similarity: BLAST and FASTA, p295-324. In A. D. Baxevanis and B. F. F. Ouellette (ed.), Bioinformatics: a Practical Guide to the Analysis of Genes and Proteins, 3rd ed. John Wiley & Sons, Inc., New York, NY.

Becker S., P. Böger, R. Oehlmann, and A. Ernst. 2000. PCR bias in ecological analysis: case study for quantitative Taq nuclease assay in analysis of microbial communities. *Appl. Environ. Microbiol.* 66:4945–4953.

Beja O., E. V. Koonin, L. Aravind, L. T. Taylor, H. Seitz, J. L. Stein, D.C. Bensen, R. A. Feldman, R. V. Swanson, and E. F. DeLong. 2002. Comparative genomic analysis of archaeal genotypic variants in a single population and in two different oceanic provinces. *Appl. Environ. Microbiol.* 68: 335–345.

Benson D. A.; I. Karsch-Mizrachi; D. J. Lipman; J. Ostell and E. W. Sayers. 2009. GenBank. *Nucleic Acids Res.* 37 (database issue): D26-D31.

Bodelier P.L., M. Meima-Franke, G. Zwart, and H. J. Laanbroek. 2005. New DGGE strategies for the analyses of methanotrophic microbial communities using different combinations of existing 16S rRNA-based primers. *FEMS Microbiol. Ecol.* 52: 163–174.

Bodrossy L., and A. Sessitsch. 2004. Oligonucleotide microarrays in microbial diagnostics. *Curr. Opin. Microbiol.* 7:245–254.

Bodrossy L., N. Stralis-Pavese, J. C. Murrell, S. Radajewski, A. Weilharter, and A. Sessitsch. 2003. Development and validation of a diagnostic microbial microarray for methanotrophs. *Environ. Microbiol.* 5:566–582.

Bodrossy L., N. Stralis-Pavese, M. Konrad-Ko¨szler, A. Weilharter, T. G. Reichenauer, D. Scho¨fer, and A. Sessitsch. 2006. mRNA-based parallel detection of active methanotroph populations by use of a diagnostic microarray. *Appl. Environ. Microbiol.* 72:1672–1676.

Bolt, H. M. 2005. Vinyl chloride - a classical industrial toxicant of new interest. *Crit. Rev. Toxicol.* 35: 307-323.

Börjesson G., I. Sundh, A. Tunlid, A. Frostegard, and B. H. Svensson. 1998. Microbial oxidation of CH_4 at high partial pressures in an organic landfill cover soil under different moisture regimes. *FEMS Microbiol. Ecol.* 26:207–217.

Börjesson G., I. Sundh, and B. Svensson. 2004. Microbial oxidation of CH_4 at different temperatures in landfill cover soils. *FEMS Microbial. Ecol.* 48: 305–312.

Boschker H. T. S., S. C. Nold, P. Wellsbury, D. Bos, W. de Graaf, R. Pel, R. J. Parkes, and T. E. Cappenberg. 1998. Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labeling of biomarkers. *Nature* 392:801–805.

Bourne D. G., I. R. McDonald, and J. C. Murrell. 2001. Comparison of *pmoA* PCR primer sets as tools for investigating methanotroph diversity in three Danish soils. *Appl. Environ. Microbiol.* 67:3802–3809.

Bowman J. P., J. H. Skerratt, P. D. Nichols, and L. I. Sly. 1991. Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. *FEMS Microbiol. Ecol.* 85:15–22.

Bowman J. P., L. Jimenez, I. Rosario, T. C. Hazen, and G. S. Sayler. 1993. Characterization of the methanotrophic bacterial community present in a

trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. *Microbiol.* 59:2380–2387.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997. Diversity and Association of Psychrophilic Bacteria in Antarctic Sea Ice. *Appl. Environ. Microbiol.* 63(8):3068–3078.

Brons J. K., and J. D. van Elsas. 2008. Analysis of bacterial communities in soil by use of denaturing gradient gel electrophoresis and clone libraries, as influenced by different reverse primers. *Appl. Environ. Microbiol.* 74: 2717–2727.

Bull I. D., N. R. Parekh, G. H. Hall, P. Ineson, and R. P. Evershed. 2000. Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature* 405:175–178.

Bussmann I., M. Pester, A. Brune, and B. Schink. 2004. Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance). *FEMS Microbiol. Ecol.* 47:179–189.

Carini S., N. Bano, G. LeCleir, and S. B. Joye, S. 2005. Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). *Environ. Microbiol.* 7: 1127–1138.

Cébron, A., L. Bodrossy, N. Stralis-Pavese, A. C. Singer, I. P. Thompson, J. I. Prosser, and J. C. Murrell. 2007. Nutrient amendments in soil DNA stable isotope probing experiments reduce observed methanotroph diversity. *Appl. Environ. Microbiol.* 73: 789–807.

Chao, Kun-Mao. 2005. Dynamic Programming – a Quick Review. csie.ntu, Taipei, Taiwan.

Chen Y., and J. C. Murrell. 2010. When metagenomics meets stable isotope probing: progress and perspectives. *Trends Microbiol.* 18:157–163.

Chen Y., Dumont, M.G., Cébron, A., and Murrell, J.C. 2007. Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. *Environ. Microbiol.* 9: 2855–2869.

Chen Y., M. G. Dumont, N. P. McNamara, P. M. Chamberlain, L. Bodrossy, N. Stralis-Pavese, and J. C. Murrell. 2008. Diversity of active methanotrophic community in acidic peatland as assessed by mRNA and SIP-PLFA analyses. *Environ. Microbiol.* 10:446–459.

Chenna R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson. 2003. "Multiple sequence alignment with the Clustal series of programs". *Nucleic Acids Res.* 31 (13):3497–3500.

Cole J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): Sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33:D294-D296.

Coleman, N. V., T. E. Mattes, J. M. Gossett, and J. C. Spain. 2002. Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. *Appl. Environ. Microbiol.* 68: 6162-6171.

Conrad, R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiol. Rev*. 60:609–640.

Conrad, R. 2007. Microbial ecology of methanogens and methanotrophs. *Adv. Agron.* 96:1–63.

Costello A.M., and Lidstrom, M.E. 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl. Environ. Microbiol.* 65: 5066–5074.

Crossman Z. M., F. Abraham, and R. P. Evershed. 2004. Stable isotope pulse-chasing and compound specific stable carbon isotope analysis of phospholipid fatty acids to assess methane oxidizing bacterial populations in landfill cover soils. *Environ. Sci. Technol.* 38:1359–1367.

Dai, Y., H. J. Di, K. C. Cameron, and J. Z. He. 2013. Effects of nitrogen application rate and a nitrification inhibitor dicyandiamide on methanotroph abundance and methane uptake in a grazed pasture soil. *Environ. Sci. Pollut. Res. Int.* 20(12):8680-8689.

Dedysh S. N., M. Derakshani, and W. Liesack. 2001. Detection and enumeration of methanotrophs in acidic Sphagnum peat by 16S rRNA fluorescence in situ hybridization, including the use of newly developed oligonucleotide probes for *Methylocella palustris*. *Appl. Environ. Microbiol*. 67:4850–4857.

Dedysh S. N., P. F. Dunfield, M. Derakshani, S. Stubner, J. Heyer, and W. Liesack. 2003. Differential detection of type II methanotrophic bacteria in acidic peatlands using newly developed 16S rRNA-targeted fluorescent oligonucleotide probes. *FEMS Microbiol. Ecol*. 43:299–308.

Derpsch, R. 2012. No-Tillage, Sustainable Agriculture in the New Millenium. http://www.rolf-derpsch.com.

Didelot, X. 2010. "Sequence-Based Analysis of Bacterial Population Structures". In Robinson, D. A., D. Falush, and E. J. Feil. Bacterial

Population Genetics in Infectious Disease. John Wiley & Sons, Inc., New York, NY. pp. 46–47.

Dumont M. G., and J. C. Murrell. 2005a. Community-level analysis: key genes of aerobic methane oxidation. *Environ. Microbiol*. 397:413–427.

Dumont M. G., and J. C. Murrell. 2005b. Stable isotope probing: linking microbial identity to function. *Nat. Rev. Microbiol.* 3:499–504.

Elias, Isaac. 2006. "Settling the intractability of multiple alignments". J. *Comput. Biol.* 13 (7): 1323–1339.

Eller G., S. Stubner, and P. Frenzel. 2001. Group-specific 16S rRNA targeted probes for the detection of type I and type II methanotrophs by fluorescence *in situ* hybridisation. *FEMS Microbiol. Lett.* 198:91–97.

Elshahed M. S., N. H. Youssef, A. M. Spain, C. Sheik , F. Z. Najar, L. O. Sukharnikov, B. A. Roe, J. P. Davis, P. D. Schloss, V. L. Bailey, and L. R. Krumholz. 2008. Novelty and uniqueness patterns of rare members of the soil biosphere. *Appl. Environ. Microbiol.* 74: 5422–5428.

Ercolini D. 2004. PCR-DGGE fingerprinting: Novel strategies for detection of microbes in food. *J. Microbiol. Meth.* 56:297-314.

Felsenstein J. 2004. Inferring Phylogenies. Sinauer Associates: Sunderland, MA.

Fierer N., C. L. Lauber, N. Zhou, D. McDonald, E. K. Costello, and R. Knight. 2010. Forensic identification using skin bacterial communities. P *Natl. Acad. Sci.* USA 107: 6477–6481.

Fjellbirkeland, A., V. Torsvik, and L. Øvreås. 2001. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel

electrophoresis profiles of *pmoA*, *mxaF* and 16S rDNA sequences. Antonie Leeuwenhoek 79:209–217.

Fliermans, C. B., T. J. Phelps, D. Ringelberg, A. T. Mikell, and D. C. White. 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. *Appl. Environ. Microbiol.* 54:1709-1714.

Forster P., V. Ramaswamy, P. Artaxo, T. Berntsen, R. Betts, D. W. Fahey, J. Haywood, J. Lean, D. C. Lowe, G. Myhre, J. Nganga, R. Prinn, G. Raga, M. Schultz, and R. Van Dorland. 2007. Changes in atmospheric constituents and in radiative forcing. Cambridge University Press, Cambridge, United Kingdom.

Fromin N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ. Microbiol.* 4: 634–643.

Futamata, H., S. Harayama, K. and Watanabe. 2001. Diversity in kinetics of trichloroethylene-degrading activities exhibited by phenol-degrading bacteria. *Appl. Microbiol. Biotechnol.* 55: 248-253.

Garrity G. M., J. A. Bell and T. G. Lilburn. 2004. Taxonomic outline of the prokaryotes. *Bergey's Manual of Systematic Bacteriology*, 2nd ed., release 5.0. Springer-Verlag, New York, NY.

Gascuel O., and M. Steel. 2006. "Neighbor-joining revealed". *Mol. Biol. Evol.* 23 (11): 1997–2000.

Gilbert B., I. R. McDonald, R. Finch, G. P. Stafford, A. K. Nielsen, and J. C. Murrell. 2000. Molecular analysis of the *pmo* (particulate methane monooxygenase) operons from two type II methanotrophs. *Appl. Environ. Microbiol.* 66:966–975.

Gilbert, B., B. Aßmus, A. Hartmann, and P. Frenzel. 1998. *In situ* localization of two methanotrophic strains in the rhizosphere of rice plants. *FEMS Microbiol. Ecol.* 25(2):117–128.

Gill S. R, M. Pop, R. T. DeBoy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. I. Gordon, D. A. Relman, C. M. Fraser-Liggett, and K. E. Nelson. 2006. Metagenomic analysis of the human distal gut microbiome. *Science* 312, 1355–1359.

Green R. M., and J. Sambrook. 2012. Molecular Cloning: A Laboratory Manual, Fourth Edition. CSHL Press.

Hakemian A. S., A. C. Rosenzweig. 2007. The biochemistry of methane oxidation. *Annu. Rev. Biochem.* 76:223-41.

Hall N. 2007. Advanced sequencing technologies and their wider impact in microbiology. *J. Exp. Biol.* 290:1518-1525.

Hall, B. G. 2008. Phylogetic trees made easy: a How-To Manual, 3rd ed. Sinauer Associates, Inc., Sunderland, MA.

Hanson R. S., and T. E. Hanson. 1996. Methanotrophic bacteria. *Microbiol. Rev.* 60:439–471.

He Z., T. J. Gentry, C. W. Schadt, L. Wu, J. Liebich, S. C. Chong, Z. Huang, W. Wu, B. Gu, P. Jardine, C. Criddle, and J. Zhou. 2007. GeoChip: a comprehensive microarray for investigating biogeochemical, ecological, and environmental processes. *ISME J.* 1:67–77.

Hermansson, A., and P.-E. Lindgren. 2001. Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Appl. Environ. Microbiol*. 67:972–976.

Heyer, J., V. F. Galchenko, and P. F. Dunfield. 2002. Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiol*. 148:2831–2846.

Higgs, P. G., and T. K. Attwood. 2005. Bioinformatics and molecular evolution. Malden, MA : Blackwell.

Holder, M., and P. O. Lewis. 2003. Phylogeny estimation: traditional and Bayesian approaches. *Nat. Rev. Genet.* 4:275-284.

Holmes, A. J., A. M. Costello, M. E. Lidstrom, and J. C. Murrell. 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol. Lett.* 132:203–208.

Holmes, A. J., P. Roslev, I. R. McDonald, N. Iversen, K. Henriksen, and J. C. Murrell. 1999, Characterization of methanotrophic bacterial population in soils showing atmospheric methane uptake. *Appl. Environ. Microbiol.* 65:3312-3318.

Horz, H. P., A. S. Raghubanshi, J. Heyer, C. Kammann, R. Conrad, and P. F. Dunfield. 2002. Activity and community structure of methane-oxidizing bacteria in a wet meadow soil. *FEMS Microbiol. Ecol.* 41:247–257.

Horz, H. P., M. T. Yimga, and W. Liesack. 2001. Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA* based terminal restriction fragment length polymorphism profiling. *Appl. Environ. Microbiol.* 67:4177–4185.

Horz, H. P., V. Rich, S. Avrahami, and B. J. M. Bohannan. 2005. Methaneoxidizing bacteria in a California upland grassland soil: diversity

and response to simulated global change. *Appl. Environ. Microbiol.* 71:2642–2652.

Hou, S., K. S. Makarova, J. H. Saw, P. Senin, B. V. Ly, Z. Zhou, Y. Ren, J. Wang, M. Y. Galperin, M. V. Omelchenko, Y. I. Wolf, N. Yutin, E. V. Koonin, M. B. Stott, B. W. Mountain, M. A. Crowe, A. V. Smirnova, P. F. Dunfield, L. Fen, L. Wang, and M. Alam. 2008. Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum *Verrucomicrobia*. *Biol. Direct* 3:26.

Huggett J. F., T. Novak, J. A. Garson, C. Green, S. D. Morris-Jones, R. F. Miller, and A. Zumla. 2008. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. *BMC Research Notes* 2008, 1:70.

Hutchens E., S. Radajewski, M. G. Dumont, I. R. McDonald, and J. C. Murrell. 2004. Analysis of methanotrophic bacteria in Movile Cave by stable-isotope probing. *Environ. Microbiol.* 6:111–120.

Hütsch, B. W. 2001, "Methane oxidation in non-flooded soils as affected by crop production", *European J. Agro.* 14:237-260.

International Union of Pure and Applied Chemistry, The (IUPAC).

IPCC (Intergovernmental Panel on Climate Change), 2001. The Scientific Basis. Cambridge Univ. Press, Cambridge UK and New York, NY USA.

IPCC (Intergovernmental Panel on Climate Change), 2007. The Physical Science Basis. Cambridge Univ. Press, Cambridge UK and New York, NY USA.

Janse I, J. Bok, and G. Zwart. 2004. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *J. Microbial. Methods.* 57:279-281.

Janssen, P. H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72:1719–1728.

Jaynes, E. T., and G.L. Bretthorst. 2003. Probability theory: the logic of science. Cambridge University Press, Cambridge, New York, NY.

Kalyuzhnaya M. G., V. A. Makutina, T. G. Rusakova, D. V. Nikitin, V. N. Khmelenina, V. V. Dmitriev, and Y. A. Trotsenko. 2002. Methanotrophic communities in the soils of the Russian Northern Taiga and Subarctic tundra. *Mikrobiologiya* 71:227–233.

Karsch-Mizrachi, I., Y. Nakamura, G. Cochrane. 2011. The International Nucleotide Sequence Database Collaboration. *Nucleic Acids Res.* 40 (database issue): D33–D37.

Ke, M., R. Conrad, and Y. Lu. 2013. Dry/wet cycles change the activity and population dynamics of methanotrophs in rice field soil. *Appl. Environ. Microbiol.* 79(16):4932-4939.

Khadem, A. F., A. Pol, M. S. Jetten, and H. J. M. Op den Camp. 2010. Nitrogen fixation by the verrucomicrobial methanotroph '*Methylacidiphilum fumariolicum*' SolV. *Microbiol.* 156:1052–1059.

Khmelenina, V. N., N. G. Starostina, M. G. Tsvetkova, A. P. Sokolov, N. E. Suzina, and Y. A. Trotsenko. 1996. Methanotrophic bacteria in saline reservoirs of Ukraine and Tuva. *Mikrobiologiya*. 65(5):696-703.

Khosravinia, H. and K. P. Ramesha. 2007. Influence of EDTA and magnesium on DNA extraction from blood samples and specificity of polymerase chain reaction. *African J. of Biotech.* 6(3):184-187.

Kip, N., W. Ouyang, J. van Winden, A. Raghoebarsing, L.van Niftrik, A. Pol, Y. Pan, L. Bodrossy, E. G. van Donselaar, G-J. Reichart, M. S. M. Jetten, J. S. S. Damstě, and H. J. M. Op den Camp. 2011. Detection, Isolation, and Characterization of Acidophilic Methanotrophs from *Sphagnum* Mosses. *Appl. Enviorn. Microbiol.* 16:5643–5654.

Knief, C., A. Lipski, and P. F. Dunfield. 2003. Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microbiol.* 69:6703–6714.

Knief, C., S. Vanitchung, N. W. Harvey, R. Conrad, P. F. Dunfield, and A. Chidthaisong. 2005. Diversity of methanotrophic bacteria in tropical upland soils under different land uses. *Appl. Environ. Microbiol.* 71:3826–3831.

Knittel, K., and A. Boetius. 2009. Anaerobic Oxidation of Methane: Progress with an Unknown Process. *Annu. Rev. Microbiol.* 63:311–334.

Kolb S., C. Knief, P. F. Dunfield, and R. Conrad. 2005. Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. Environ. Microbiol. 7:1150–1161.

Kolb, S., C. Knief, S. Stubner, and R. Conrad. 2003. Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl. Environ. Microbiol. 69:2423–2429.

Kolenbrander, P. E., R. J. Palmer Jr., S. Periasamy and N. S. Jakubovics. 2010. Oral multispecies biofilm development and the key role of cell-cell distance. Nat. Rev, Microbiol. 8:471–480.

Kong, J.-Y., Y. Su, Q.-Q. Zhang, Y. Bai, F.-F. Xia, C.-R. Fang, and R. He. 2013. Vertical profiles of community and activity of methanotrophs in landfill cover soils of different age. *J. Appl. Microbiol.* 115(3):756-765.

Kubota, K., A. Ohashi, H. Imachi, and H. Harada. 2006. Visualization of *mcr* mRNA in a methanogen by fluorescence in situ hybridization with an oligonucleotide probe and two-pass tyramide signal amplification (two-pass TSA-FISH). *J. Microbiol. Methods* 66:521–528.

Larkin M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. "ClustalW and ClustalX version 2". *Bioinformatics* 23(21):2947–2948.

Lee, S.-W., D. R. Keeney, D.-H. Lim, A. A. Dispirito, and J. D. Semrau. 2006. Mixed Pollutant Degradation by *Methylosinus trichosporium* OB3b Expressing either Soluble or Particulate Methane Monooxygenase: Can the Tortoise Beat the Hare? *Appl. Environ. Microbiol.* 72(12):7503–7509.

Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl. Environ. Microbiol.* 68:673–690.

Ley, R. E., D. A. Peterson, and J. I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837–848.

Li, M., B. Wang, M. Zhang, M. Rantalainen, S. Wang, H. Zhou, Y. Zhang, J. Shen, X. Pang, M. Zhang, H. Wei, Y. Chen, H. Lu, J. Zuo, M. Su, Y. Qiu, W. Jia, C. Xiao, L. M. Smith, S. Yang, E. Holmes, H. Tang, G. Zhao, J. K. Nicholson, L. Li, and L. Zhao. 2008. Symbiotic gut microbes modulate human metabolic phenotypes. *Proc. Natl. Acad. Sci. US* 105:2117–2122.

Lin J. L., S. B. Joye, J. C. M. Scholten, H. Schäfer, I. R. McDonald, and J. C. Murrell. 2005. Analysis of Methane Monooxygenase Genes in Mono Lake Suggests That Increased Methane Oxidation Activity May Correlate with a Change in Methanotroph Community Structure. *Appl. Environ. Mirobiol.* 10:6458–6462.

Lin J. L., S. Radajewski, B. T. Eshinimaev, Y. A. Trotsenko, I. R. McDonald, and J. C. Murrell. 2004. Molecular diversity of methanotrophs in Transbaikal soda lake sediments and identification of potential active populations by stable isotope probing. *Environ. Microbiol.* 6:1049–1060.

Lindahl, T. 1993. Instability and decay of the primary structure of DNA. *Nature* 362:709–715.

Lord, N. S., C. W. Kaplan, P. Shank, C. L. Kitts, and S. Elrod. 2002. Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. *FEMS Microbiol. Ecol.* 42:327–337.

Ma, B., J. Tromp, and M. Li. 2002. PatternHunter: faster and more sensitive homology search. *Bioinformatics* 18:440-445.

Mao, D., Q. Zhou, C. Chen and Z. Quan. 2012. Coverage evaluation of universal bacterial primers using the metagenomic datasets. *BMC Microbiol.* 12:66.

Marie, D., N. Simon, and D. Vaulot. 2005. Phytoplankton Cell Counting by Flow Cytometry. *Algal Culturing Techniques by R. Anderson*. Elsevier press 17:253-268.

Martin-Cuadrado, A. B., P. López-García, G. Gottschalk, F. Rodríguez-Valera. 2007. Metagenomics of the deep Mediterranean, a warm bathypelagic habitat. *PLoS ONE*. 2(9):e914.

Maxfield, P. J., E. R. C. Hornibrook, and R. P. Evershed. 2006. Estimating high-affinity methanotrophic bacterial biomass, growth, and turnover in soil by phospholipid fatty acid ^{13}C labeling. *Appl. Environ. Microbiol.* 72:3901–3907.

Maymo-Gatell, X., T. Anguish, and S. H. Zinder. 1999. Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by "Dehalococcoides ethenogenes" 195. *Appl. Environ. Microbiol.* 65:3108-3113.

McDonald, I. R., L. Bodrossy, Y. Chen, and J. C. Murrell. 2008. "Molecular ecology techniques for the study of aerobic methanotrophs", *Appl. Environ. Microbiol.* 74:1305-1315.

McDonald, I. R., S. Radajewski, and J. C. Murrell. 2005. Stable isotope probing of nucleic acids in methanotrophs and methylotrophs: a review. *Organic Geochem.* 36:779–787.

Medini, D., D, Serruto, J. Parkhill, D. A. Relman, C. Donati, R. Moxon, S. Falkow, and R. Rappouli. 2008. Microbiology in the post-genomic era. *Nat. Rev. Microbiol.* 6:419-430.

Mihaescu, R., D. Levy, L. Pachter. 2009. "Why neighbor-joining works". *Algorithmica* 54(1):1–24.

Morris S. A., S. Radajewski, T. W. Willison, and J. C. Murrell. 2002. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. *Appl. Environ. Microbiol.* 68: 1446–1453.

Mount, D. M. 2004. Bioinformatics: Sequence and Genome Analysis (2nd ed.). Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Mühling, M., J. Woolven-Allen, J. C. Murrell, and I. Joint. 2008. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME Journal* 2:379–392.

Murrell, J. C. 1994. Molecular genetics of methane oxidation. *Biodegradation* 5:145–160.

Murrell, J. C., and S. Radajewski. 2000. Cultivation-independent techniques for studying methanotroph ecology. *Res. Microbiol*. 151:807–814.

Murrell, J. C., B. Gilbert, and I. R. McDonald. 2000. Molecular biology and regulation of methane monooxygenase. *Arch. Microbiol.* 173:325–332.

Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* 73:127-141.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695–700.

Mygind, T., S. Birkelund, E. Falk, and G. Christiansen. 2001. Evaluation of real-time quantitative PCR for identification and quantification of

Chlamydia pneumoniae by comparison with immunohistochemistry. *J. Microbiol. Methods* 46:241.

Nagissa M., G. F. Slater, and R. R. Fulthorpe. 2011. Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soil. *J. Microbiol.* 57(8):623-8.

National Center for Biotechnology Information. 2002. The NCBI handbook. Bethesda MD. http://www.ncbi.nlm.nih.gov/books/NBK21101.

National Institute of Health, The. 2012. www.nih.gov

Neufeld, J. D., and W. W. Mohn. 2006. Assessment of microbial phylogenetic diversity based on environmental nucleic acids. In Molecular Identification, Systematics, and Population Structure of Prokaryotes. Stackebrandt, E. (ed.). Heidelberg, Germany: Springer-Verlag, 219–260.

Newby D. T., D. W. Reed, L. M. Petzke, A. L. Igoe, M. E. Delwiche, F. F. Roberto, J. P. McKinley, M. J. Whiticar, and F. S. Colwell. 2004. Diversity of methanotroph communities in a basalt aquifer. *FEMS Microbiol. Ecol.* 48:333–344.

Ng, P. C., and S. Henikoff. 2001 Predicting deleterious amino acid substitutions. *Genome Res.* 11(5):863-74.

Nielsen, A. K., K. Gerdes, and J. C. Murrell. 1997. Copper-dependent reciprocal transcriptional regulation of methane monooxygenase genes in *Methylococcus capsulatus* and *Methylosinus trichosporium*. *Mol. Microbiol.* 25:399–409.

NOAA Annual Greenhouse Gas Index (AGGI), The. 2012. US Department of Commerce, National Oceanic & Atmospheric Administration (NOAA). http://www.esrl.noaa.gov/gmd/aggi/.

O'Brien, H. E., J. L. Parrent, J. A. Jackson, J. M. Moncalvo, and R. Vilgalys. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Appl. Environ. Microbiol.* 71:5544–5550.

Op den Camp, H. J. M., T. Islam, M. B. Stott, H. R. Harhangi, A. Hynes, S. Schouten, M. S. M. Jetten1, N.-K. Birkeland, A. Pol, and P. F. Dunfield. 2009. Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environ. Microbiol. Rep.*1:293–306.

Pacheco-Oliver M., I. R. McDonald, D. Groleau, J. C. Murrell, and C. B. Miguez. 2002. Detection of methanotrophs with highly divergent *pmoA* genes from Arctic soils. *FEMS Microbiol. Lett.* 209:313–319.

Radajewski S., G. Webster, D. S. Reay, S. A. Morris, P. Ineson, D. B. Nedwell, J. I. Prosser, and J. C. Murrell. 2002. Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. *Microbiol*. 148:2331–2342.

Radajewski, S., I. R. McDonald, and J. C. Murrell. 2003. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Curr. Opin. Biotechnol.* 14:296–302.

Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature* 403:646–649.

Raeymaekers, L. 2000. Basic principles of quantitative PCR. *Mol. Biotechnol.* 15:115–122.

Rappe, M. S., and S. J. Giovannoni. 2003. The uncultured microbial majority. *Annu. Rev. Microbiol.* 57:369–394.

Reay, D. S., S. Radajewski, J. C. Murrell, N. McNamara, and D. B. Nedwell. 2001. Effects of land use on the activity and diversity of methane oxidizing bacteria in forest soils. *Soil Biol. Biochem.* 33:1613–1623.

Rettedal, E. A., S. Clay and V. S. Brözel. 2010. GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. *FEMS Microbiol. Lett.* 312:55–62.

Ricke P., M. Erkel, R. Kube, R. Reinhardt, and W. Liesack. 2004. Comparative analysis of the conventional and novel *pmo* (particulate methane monooxygenase) operons from *Methylocystis* strain SC2. *Appl. Environ. Microbiol.* 70:3055–3063.

Roesch, L. F. W., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie, and E. W. Triplett. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J.* 1:283–290.

Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Lilies, K. A. Loiacono, B. A. Lynch, A. Berkley, I. A. Macneil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osborne, J. Clardy, J. Handelman, and R. M. Goodman. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* 66(6):2541-2547.

Ruo H., M. J. Wooller, J. W. Pohlman, C. Catranis, J. Quensen, J. M. Tiedje, and M. B. Leigh. 2012. Identification of functionally active aerobic methanotrophs in sediments from an arctic lake using stable isotope probing. *Environ. Microbiol.* 1462-2920.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.

Sanders, E. R., and J. H. Miller. 2010. I, Microbiologist: A Discovery-based Undergraduate Research Course in Microbial Ecology and Molecular Evolution. ASM Press, Washington DC.

Schmeisser, C., H. Steele, and W. R. Streit. 2007. Metagenomics, biotechnology with nonculturable microbes. *Appl. Microbiol. Biotechnol.* 75:955–962.

Schuster, S. C. 2008. Next-generation sequencing transforms today's biology. *Nat. Methods* 5:16-18.

Scott, C. S., and W. A. Chiu. 2006. Trichloroethylene cancer epidemiology: a consideration of select issues. *Environ. Health Perspect.* 114:1471-1478.

Semrau, J. D., A. A. DiSpirito, and J. C. Murrell. 2008. Life in the extreme: thermoacidophilic methanotrophy. Trends Microbiol. 16:190–193.

Semrau, J. D., A. A. DiSpirito, and S. Yoon. 2010. Methanotrophs and copper. *FEMS Microbiol. Rev.* 34:496–531.

Semrau, J. D., A. Chistoserdov, J. Lebron, A. Costello, J. Davagnino, E. Kenna, A. J. Holmes, R. Finch, J. C. Murrell, and M. E. Lidstrom. 1995. Particulate methane monooxygenase genes in methanotrophs. *J. Bacteriol.* 177:3071–3079.

Shabir, A. D., J. G. Kuenen, and G. Muyzer. 2005. Nested PCR-Denaturing Gradient Gel Electrophoresis Approach To Determine the Diversity of Sulfate-Reducing Bacteria in Complex Microbial Communities. *Appl. Environ. Micrbiol.* 71(5):2325–2330.

Shanks, O. C., J. W. S. Domingo, R. Lamendella, C. A. Kelty, and J. E. Graham. 2006. Competitive metagenomic DNA hybridization identifies host-specific microbial genetic markers in cow fecal samples. *Appl. Environ. Microbiol.* 72:4054–4060.

Shao, Z., W. A. Dick, and R. M. Behki. 1995. An improved *Escherichia coli-Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* spp using electroporation. *Lettr. Appl. Microbiol.* 21:261-266.

Shrestha M., W. R. Abraham, P. M. Shrestha, M. Noll, and R. Conrad. 2008. Activity and composition of methanotrophic bacterial communities in planted rice soil studied by flux measurements, analyses of *pmoA* gene and stable isotope probing of phospholipid fatty acids. *Environ. Microbiol.* 10:400–412.

Sievers, F., A. Wilm, D. G. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson, and D. G. Higgins. 2011. "Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega". *Mol. Syst. Biol.* 7(539).

Smith, K. S., A. M. Costello, and M. E. Lidstrom. 1997. Methane and trichloroethylene oxidation by an estuarine methanotroph, *Methylobacter* sp. strain BB5.1. *Appl. Environ. Microbiol.* 63:4617–4620.

Stach J.E.M., S. Bathe, J. P. Clapp, and R. G. Burns. 2001. PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using

different isolation and purification methods. *FEMS Microbiol. Ecol.* 36:139–151.

Stolyar, S., A. M. Costello, T. L. Peeples, and M. E. Lidstrom. 1999. Role of multiple gene copies in particulate methane monooxygenase activity in the methane-oxidizing bacterium *Methylococcus capsulatus* Bath. *Microbiol*. 145:1235–1244.

Strait, D. S., and F. E. Grine. 2004. Inferring hominoid and early hominid phylogeny using craniodental characters: the role of fossil taxa. *J. Hum. Evol*. 47(6):399-452.

Stralis-Pavese N., L. Bodrossy, T. G. Reichenauer, A. Weilharter, and A. Sessitsch. 2006. 16S rRNA based T-RFLP analysis of methane oxidising bacteria – assessment, critical evaluation of methodology performance and application for landfill site cover soils. *Appl. Soil Ecol.* 31:251–266.

Stubner, S. 2002. Enumeration of 16S rDNA of *Desultomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen™ detection. *J. Microbiol. Methods* 50:155–164.

Studier, J. A. and K. J. Keppler. 1988. A note on the neighbor-joining algorithm of Saitou and Nei. *Mol. Biol. Evol.* 5:729-731.

Subramanya R., K. D. Hyde, and S. B. Pointing. 2013. Comparison of DNA and RNA, and Cultivation Approaches for the Recovery of Terrestrial and Aquatic Fungi from Environmental Samples. *Curr. Microbiol.* 66:185–191.

Suzuki, M. T., L. T. Taylor, and E. F. DeLong. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5-nuclease assays. *Appl. Environ. Microbiol.* 66:4605–4614.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28:2731-2739.

Tavormina, P. L., V. J. Orphan, M. G. Kaluzhnaya, M. S. M. Jetten, and M. G. Klotz. 2010. A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. *Environ. Microbiol. Rep.* 3:91–100.

Temmerman, R., I. Scheirlinck, G. Huys, and J. Swings. 2003. Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 69:220-226.

Theisen A. R., M. H. Ali, S. Radajewski, M. G. Dumont, P. E. Dunfield, I. R. McDonald, S. N. Dedysh, C. B. Miguez, J. C. Murrell. 2005. Regulation of methane oxidation in the facultative methanotroph *Methylocella silvestris* BL2. *Mol. Microbiol.* 58:682–692.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools". *Nucleic Acids Res.* 25(24):4876–4882.

Tiquia, S. M. 2010. Using Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis to Assess Microbial Community Structure in Compost Systems. S.P. Cummings (ed.), Bioremediation, *Methods in Molecular Biology*, 599.

Tringe, S. G., C. von Mering, A. Kobayashi, A. A. Salamov, K. Chen, H. W. Chang, M. Podar, J. M. Short, E. J. Mathur, J. C. Detter, P. Bork, P.

Hugenholtz, and E. M. Rubin. 2005. Comparative metagenomics of microbial communities. *Science* 308:554–557.

Tringe, S. G., T. Zhang, X. Liu, Y. Yu, W. H. Lee, J. Yap, F. Yao, S. T. Suan, S. K. Ing, M. Haynes, F. Rohwer, C. L. Wei, P. Tan, J. Bristow, E. M. Rubin, and Y. Ruan. 2008. The airborne metagenome in an indoor urban environment. *PLoS ONE* 3(4):e1862.

Triplett Jr. G. B. and W. A. Dick. 2008. No tillage crop production: A revolution in agriculture. *Agronomy J.* 100:S153-S165.

Trotsenko, Y. A., and V. N. Khmelenina. 2002. Biology of extremophilic and extremotolerant methanotrophs. *Arch. Microbiol.* 177:123–131.

Tukhvatullin I. A., R. I. Gvozdev, and K. K. Anderson. 2001. Structural and functional model of methane hydroxylase of membrane-bound methane monooxygenase from *Methylococcus capsulatus* (Bath). *Russ. Chem. Bull.* 50:1867–1876.

Uz, I., M. E. Rasche, T. Townsend, A. V. Ogram, and A. S. Lindner. 2003. Characterization of methanogenic and methanotrophic assemblages in landfill samples. *Proc. Biol. Sci.* 270:S202–S205.

Vanhoutte, L. T., G. Huys, and I. S. Cranenbrouck. 2005. Exploring microbial ecosystems with denaturing gradient gel electrophoresis (DGGE). *BCCM Newsl.* 17(2).

Verce, M. F., R. L. Ulrich, and D. J. Freedman. 2000. Characterization of an isolate that uses vinyl chloride as a growth substrate under aerobic conditions. *Appl. Environ. Microbiol.* 66:3535-3542.

Wang H., J. Einola, M. Heinone, M. Kulomaa, and J. Rintala. 2008. Group-specific quantification of methanotrophs in landfill gas-purged laboratory

biofilters by tyramide signal amplification-fluorescence *in situ* hybridization. *Bioresour*. *Technol.* 99:6426–6433.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73:5261-5267.

Wartiainen I., A. G. Hestnes, and M. M. Svenning. 2003. Methanotrophic diversity in high arctic wetlands on the islands of Svalbard (Norway) – denaturing gradient gel electrophoresis analysis of soil DNA and enrichment cultures. *Can. J. Microbiol.* 49:602–612.

Wawrik, B., L. Kerkhof, J. Kukor, and G. Zylstra. 2005. Effect of different carbon sources on community composition of bacterial enrichments from soil. *Appl. Environ. Microbiol.* 71:6776–6783.

West, T., and W. Post. 2002. Soil organic carbon sequestration rates by tillage and crop rotation: A global data analysis. *Soil Sci. Soc. Am. J.* 66:1930-1946.

Westrick, J. J., J. W. Mello, and R. F. Thomas. 1984. The groundwater supply survey. *J. Am. Water Works Assoc.* 76:52-59.

Whittenbury, R., K. C. Phillips, and J. F. Wilkinson. 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. *J. Gen. Microbiol.* 61:205–218.

Wild, C., and G. Seber. 2000. Chance Encounters: A First Course in Data Analysis and Inference. John Wiley & Sons, Inc., New York, NY.

Wilkinson, M., J. O. McInerney, R. P. Hirt, P. G. Foster, and T. M. Embley. 2007. Of clades and clans: terms for phylogenetic relationships in unrooted trees. *Trends. Ecol. Evol.* 3:114-115.

Wilson, J. F. 2002. The Rise of Biological Databases. *The Scientist*, 34.

Wise, M. G., J. V. McArthur, and L. J. Shimkets. 1999. Methanotroph diversity in landfill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Appl. Environ. Microbiol*. 65:4887–4897.

Wu, L., D. K. Thompson, G. Li, R. A. Hurt, J. M. Tiedje, and J. Zhou. 2001. Development and evaluation of functional gene arrays for detection of selected genes in the environment. *Appl. Environ. Microbiol.* 67:5780–5790.

Yu, Z. and M. Morrison. 2004. Comparisons of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70:4800–4806.

Zahn, J. A., and A. A. DiSpirito. 1996. Membrane-associated methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Bacteriol.* 178:1018–1029.

الخلاصة

تعتبر البكتريا المحللة للميثان في التربة من اهم مصادر تخفيض الميثان في الجو , و ان نظام معالجة التربة له تأثير مباشر على التنوع الحيوي في التربة. حضرت هذه الدراسة لتشخيص انواع البكتريا المحللة للميثان في التربة و تحت اربعة انظمة ادارة مختلفة. وقد أجريت هذه الدراسة في مركز اوهايو لبحوث وتطوير الزراعة/جامعة ولاية اوهايو/مدينة ووستر/ولاية اوهايو/الولايات المتحدة الامريكية. حيث تم جمع اربع و عشرين عينة خلال فصل الشتاء (شهر شباط , 2012) و من مواقع بيئية مختلفة: (1) اللاحرث و (2) الحرث و (3) الارض المعشوشبة و (4) الغابة. عملية استخلاص الدنا المباشرة قد مكنت من تضخيم الجين المشفر لوحيدة انزيم الاوكسيجيناز الاحادي الجسيمي *pmoA* , بالاضافة الى جينات ال 16S الرايبوسومية للبكتريا مما ادى الى تشخيص البكتريا بشكل مباشر.

تم استغلال عملية تفاعل انزيم البلمرة التسلسلي الحاسوبي (*in silico*) من اجل اختبار البوادئ المستهدفة لجين *pmoA* و جينات 16S الرايبوسومية و لكلا نوعي بكتريا المحللة للميثان (type-I و type-2). حيث تم اختيار البوادئ التي اعطت افضل النتائج و كانت كالاتي: زوجا بوادئ A189f-A682r و A189f-mb661 من اجل التضخيم المحدد لجين *pmoA* , و زوجا بوادئ IF-type IR و type IIF-type IIR من اجل تضخيم جينات ال 16S الرايبوسومية و لكلا نوعي البكتريا المحللة للميثان , وعلى التوالي. عملية تفاعل انزيم البلمرة التسلسلي كانت ناجحة في تضخيم جميع الجينات المستهدفة. و قد تم تحسين البوادئ المستخدمة مع ظروف التفاعل من اجل عملية التحليل باستخدام طريقة تغيير طبيعة التدرج الكهربائي للهلام (DGGE). ان استخدام طريقة شبه متداخلة في التضخيم ادى للحصول على نتائج افضل. تم تنقية الحزم الناتجة من عملية ال DGGE و تنقيتها من الهلام و اجراء تضخيم ثاني للدنا من الحزم و من ثم ترحيلها مرة اخرى على الهلام للتأكد من مدى نقاوة هذه الحزم. الحزم النقية الناتجة تم تنقيتها و ارسالها من اجل اجراء تحديد التتابع لها (sequencing).

تم صف التتابعات الناتجة مع تتابعات قاعدة البيانات المطابقة , و تم تجميع هذه التتابعات و بحسب طريقة التمهيد (Bootstraping) باستخدام مصادر المعلوماتية الحيوية (Bioinformatics) المتوفرة. حيث اظهر تصنيف الجين الوظيفي تنوع في مجاميع البكتريا المحللة للميثان , من ضمنها: *Methylobacter, Methylomonas, Methylocystis, Methylomicrobium, Methylococcus* بالاضافة الى عدد من محللات الميثان الغير قابلة للزرع. أما البوادئ المستخدمة في تضخيم جينات ال 16S الرايبوسومية فقد تمكنت من تحديد: *Methylomonas, Methylomicrobium, Methylosarcina, Methylobacter, Methylococcus, and Methylocaldum* ضمن فئة النوع الاول من البكتريا , و *Methylosinus, Methylocystis, Methylocapse, and Methylocella* ضمن فئة النوع الثاني من البكتريا. و قد تم تمثيل كل هذه النتائج بشكل رسم الشجرة التطورية.

تم اجراء محاولة تحليل اضافي لهذه البكتريا بتقنية تباين أطوال قطع الدنا المقيدة الطرفية (T-RFLP) و ذلك عن طريق أستهداف جين *pmoA*. الحزم الناتجة من الترحيل تم انتاجها عن طريق القطع بأربع أنزيمات قاطعة حيث ان هذه الانزيمات تستهدف تتابعات مكونة من اربع قواعد في كل مرة. ان عدم وجود قاعدة بيانات خاصة بالجينات الوظيفية قد أدى الى عدم التمكن من تفسير هذه النتائج , و لكن مجموع هذه النتائج بالاضافة الى اي نتائج ممكن الحصول عليها من دراسات اخرى ستكون مصادر مهمة من أجل انشاء قاعدة بيانات لهذا الجين.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانة الاحيائية

التحري عن مجتمع البكتريا المؤكسدة للميثان في التربة بأستخدام تقنية الترحيل الكهربائي على الهلام متدرج المسخ

رسالة مقدمة الى مجلس كلية العلوم , جامعة النهرين كجزء من متطلبات نيل درجة الماجستير في التقانة الاحيائية

من قبل:

سامر عماد الصفار

بكلوريوس تقانة احيائية / جامعة النهرين / 2009

بأشراف:

ألاستاذ الدكتور ماجد حسين الجيلاوي

الاستاذ المساعد الدكتور علي عبد الحافظ أبراهيم

صفر , 1435

كانون الاول , 2013