Understanding methanotroph ecology in a biofilter for efficiently mitigating methane emissions

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Abstract

In New Zealand, the majority of the greenhouse gas (GHG), methane (CH_4) emissions are from the agriculture sector (enteric fermentation, manure management) and the remainder from solid waste disposal, coal mining and natural gas leaks. A soil-based biofilter made from volcanic pumice soil (isolated from a landfill in Taupo, New Zealand) and perlite has been tested and promoted to mitigate high concentrations (3 300 ppm – 100 000 ppm) of CH_4 emissions from a dairy effluent storage pond. This soil-perlite mixture exhibited excellent physical (porosity, water holding capacity and bulk density) characteristics to support the growth and activity of an active methanotroph community. Methanotrophs comprise a diverse group of aerobic alpha and gamma proteobacteria (type I and type II methanotrophs, respectively) that are present naturally in soils where CH_4 is produced. However, there is little information on the methanotrophs community structure, population diversity and abundance in this soil-based biofilter. Understanding the activity of these diverse genera under varying soil conditions is essential for optimum use of biofiltration technology, and is the main aim of this thesis.

This thesis describes a study to use molecular techniques (PCR, quantitative PCR, T-RFLP and molecular cloning) (Chapter 3) to reveal the population dynamics of methanotrophs (type I, type II and various genera – *Methylobacter/Methylomonas/Methylosarcina*, *Methylococcus* and *Methylocapsa*), in order to build a more efficient CH_4 biofiltration system. Methanotroph population dynamics in two fundamentally different prototypes of volcanic pumice soil biofilters – a column and a floating/cover biofilter studied are presented in Chapters 4 and 5.

The column biofilter study (Chapter 4) examined the performance of a previously used acidic soil-biofilter medium that was further acidified from pH 5.20 ± 0.20 to 3.72 ± 0.02 by H_2S present in the biogas (from the dairy effluent pond).. The more acidic soil biofilter medium (volcanic pumice soil and perlite, 50:50 v/v) was reconstituted with optimal moisture content (110% gravimetric dry wt or ~ 60 % WHC) and achieved a maximum CH_4 removal rate of 30.3 g m^{-3} h^{-1}. In addition, the population of *Methylocapsa*-like methanotroph increased by 400 %, demonstrating

the ability of these soil microorganisms to adapt and grow under acidic pH conditions in the biofilter. The results from this study indicated that (i) when primed with CH_4, a soil biofilter can effectively regain efficiency if sufficient moisture levels are maintained, regardless of the soil acidity; (ii) changes in the methanotroph population did not compromise the overall capacity of the volcanic pumice soil to oxidise CH_4; and (iii) the more acidic environment (pH 3.72) tends to favour the growth and activity of acid-loving *Methylocapsa*-like methanotroph while being detrimental to the growth of the *Methylobacter* / *Methylococcus* / *Methylocystis* group of methanotroph.

In the floating biofilter (Chapter 5), original acidic soil biofilter medium (pH 5.20) as used in column study was assessed to remove CH_4 from the effluent pond surface for a period of one year (December 2013 to November 2014). Field evaluation was supported with a concurrent laboratory study to assess their CH_4-oxidising capacity, in addition to identifying and comparing the methanotroph community changes in the soil when exposed to field conditions. Results indicated that (i) irrespective of the season, the floating biofilters in the field were removing 67 ± 6% CH_4 throughout the study period with a yearly average rate of 48 ± 23 g CH_4 m^{-3} h^{-1}; however, the highest CH_4 removal rate achieved was 101.5 g m^{-3} h^{-1} CH_4, about 300 % higher than the highest CH_4 removal rate by the acidified column biofilter (Chapter 4); (ii) the acidity of the field floating biofilters increased from a pH value of 5.20 to 4.72, but didn't suppress the genera of methanotrophs (particularly *Methylobacter*/*Methylosinus*/*Methylocystis*); (iii) the laboratory-based floating biofilters experienced biological disturbances with low and high CH_4 removal phases during the study period, with an yearly average CH_4 oxidation removal of 58%; and (iv) both type I and type II methanotrophs in the field floating biofilters were more abundant, diverse and even compared with the methanotroph community in the laboratory biofilters. This study has demonstrated the ability of the floating biofilters to efficiently mitigate dairy effluent ponds emissions in the field, without requiring any addition of nutrients or water; however, during very dry conditions, occasional addition of water might be needed to keep the biofilter bed moist (≥ 23±4 % dry wt).

Earlier New Zealand studies and the current studies (Chapters 4 and 5) were based on the use of a particular volcanic pumice soil as biofilter medium. However, the limited availability of volcanic pumice soil and associated transportation costs limited the wider application of this technology within New Zealand and internationally. This necessitated the assessment of other farm soils and potentially suitable, economical, and locally available biofilter materials that could potentially be used by the farmers to mitigate CH_4 emissions (Chapter 6). The potential biofilter materials, viz. farm soil (isolated from a dairy farm effluent pond bank area), pine biochar, garden waste compost, and weathered pine bark mulch were assessed with and without inoculation with a small amount of volcanic pumice soil. All materials supported the growth and activity of methanotrophs. However, the CH_4 removal was high (> 80%) and consistent in the inoculated - farm soil and biochar, and was supported by the observed changes in the methanotroph community. The CH_4 removal was further enhanced (up to 99%) by the addition of nutrient solution. Field evaluations of these potential materials are now needed to confirm the viability of these materials for recommending them for use on farms.

Chapter 7 summarises the molecular results from all the above studies, and describes the future studies. Molecular techniques indicated that a very diverse (Shannon's diversity, $H' = 3.9$ to 4.4) group of type I and type II methanotrophs were present in the volcanic pumice soil, which assisted the biofilter materials to perform under varying abiotic conditions. Many novel species and strains of type I and type II methanotrophs were also identified in these soils. For long-term, low cost and efficient and stable CH_4 removal, the presence of an even and abundant population (of type I and type II methanotrophs) is however essential. Nevertheless, biofilters offer much promise for mitigating CH_4 emissions from dairy ponds, piggeries, and landfills, thereby contributing to the lowering of emissions of this potent greenhouse gas to mitigate the effects of climate change.

Dedication

This work is dedicated to my parents (Farzana, Mazheruddin), siblings (Imad, Fuad, Hafsa and Dr Iqra), wife (Abeer) and my mentors (Drs Surinder, Kevin and Bernd) for their encouragement and unending support throughout my PhD.

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Publications and Presentations

Peer reviewed International scientific journals

1. **Syed R**, Saggar S, Tate KR and Rehm BHA (2016). *Does acidification of soils compromise its methane-oxidizing capacity?* Biology & Fertility of Soils 52:573-583.
2. **Syed R**, Saggar S, Tate KR and Rehm BHA (2016). *Assessing farm soil, biochar, compost and weathered mulch to mitigate methane emissions*. Applied Microbiology & Biotechnology 100:9365-9379.
3. **Syed R**, Saggar S, Tate KR, Rehm BHA and Berben P (2017): *Assessing the performance of floating biofilters for oxidation of methane from dairy effluent ponds*. Journal of Environmental Quality 46:272-280.

Conference/Workshops/meetings

4. **Syed R**, Saggar S, Tate K and Rehm B (2014). *Characterizing a field methane oxidation biofilter – treating farm CH_4 emissions from Massey No. 4 dairy pond*. (Poster presentation) In: Nutrient Management for the Farm, Catchment and Community, (Eds LD Currie & CL Christensen). http://firc.massey.as.nz/publications.html Occasional Report No. 27, Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand, 11p.
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6. **Syed R**, Saggar S, Tate K and Rehm B (2015). *Assessment of Potential Biofilter Materials to Mitigate Methane Emissions*. (Poster presentation) In: Moving Farm Systems to Improved Attenuation, (Eds LD Currie and LL Burkitt). http://firc.massey.ac.nz/publications.html Occasional Report No.

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List of Abbreviations

µ	Micro
AN	Avogadro's number
ANOVA	Analysis of variance
B	Boron
bp	base pair
C	Carbon
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CH_3OH	Methanol
CH_4	Methane
CMM	Coal mine methane
CO_2–e	Carbon dioxide equivalent
$CoCl_2.6H_2O$	Cobalt chloride hexahydrate
COD	Chemical oxygen demand
Cp	Crossing point
$CuCl_2.2H_2O$	Copper Chloride dihydrate
DGGE	Denaturing gradient gel electrophoresis
EBRT	Empty bed residence time
EC	Elimination Capacity
EDTA	Ethylene diamine tetra acetic acid
EEA	European Environment Agency
E_H	Evenness
EPA	Environmental protection agency
EPS	Exopolymeric Substances
FADH	Formaldehyde dehydrogenase
FAM	6-carboxy fluorescein
FDH	Formate dehydrogenase
$FeSO_4.7H_20$	Iron sulphate heptahydrate
FID	Flame ionisation detector
FR	Francop
GC	Gas Chromatography
Gg	Giga gram
GHG	Greenhouse gas
GWP	Global warming potential
H`	Shannon's diversity
H_2S	Hydrogen sulphide
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
HCHO	Formaldehyde
HDPE	High density poly ethylene
IPCC	Intergovernmental panel for climate change
K_2SO_4	Potassium sulphate
KCl	Potassium chloride
KH_2PO_4	Potassium mono phosphate
KJ	Kilo Joule
KNO_3	Potassium nitrate
Kt	Kilo tonne
LFG	Landfill gas

MBC	Microbial biomass C
MBN	Microbial biomass N
MDH	Methanol dehydrogenase
MfE	Ministry for Environment
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MMO	Methane monooxygenase
MMT	Million metric tonne
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
MOB	Methane oxidising bacteria
MSW	Municipal sewage waste
MVA	Mine ventilation air
Na_2HPO_4	Disodium phosphate
Na_2Mo_4, $2H_2O$	Sodium molybdate dihydrate
NCBI	National Centre for Biotechnology Information
$NiCl_2.6H_2O$	Nickel chloride hexahydrate
NMVOC	Non-methane volatile organic compounds
NPK	Nitrogen phosphorous potassium
PADS	Passive air diffusion system
PBS	Phosphate buffer saline
PCA	Principal Component Analysis
PCR	Polymerase Chain reaction
PHA	Polyhydroxy alkanoates
PLFA	Phospholipid-derived fatty acids
pmoA	Particulate methane monooxygenase subunit A
PMOB	Passive methane oxidation biocover
PVC	Poly vinyl chloride
qPCR	Quantitative Polymerase Chain reaction
Rpm	Revolutions per minute
RSKB	Rashad Surinder Kevin Bernd
RuMP	Ribulose monophosphate
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TCD	Thermal conductivity detector
TCE	Trichloro ethylene
T-RFLP	Terminal-restriction fragment length polymorphism
T-RFs	Terminal-restriction fragments
UV	Ultra violet
VOC	Volatile organic compounds
VS	Volatile solids
WHC	Water holding capacity
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

Chapter 1

Introduction and Objectives

1.1 Background

After carbon dioxide (CO_2), the greenhouse gas methane (CH_4) is most prevalent in the atmosphere over a period of 100 years with a global warming potential (GWP) of 34 as indicated by IPCC (2013). Methane concentrations in the atmosphere have been increasing rapidly, mostly (60%) coming out from human activities (EPA, 2014) whereas the natural sources of CH_4 include wetlands and the deeper layers of the Oceans. In New Zealand, CH_4 emissions contributed 29,038.5 Gg CO_2 equivalents (38.2%) of total GHG emissions, mostly from the enteric fermentation of grazing animals (~84%). The remainder was from manure management (~2.5%), solid waste disposal (~11.2%), coal mining, and natural gas (~2.2%) (MfE, 2015).

Methane emissions from New Zealand dairy lagoons contribute more than 50% of CH_4 emissions from the manure management sector and these emissions are likely to increase in future as 80% of total dairy farms (~ 9660) use an open effluent pond system (or a similar system) to store the waste from milking sheds and feeding pads (and stand-off pads on some farms) for pasture irrigation purposes (Laubach *et al.*, 2015). Due to high organic C content and low oxygen/anaerobic environment in the effluent ponds, CH_4 is produced by a group of archaea, methanogens during a complex decomposition process of the organic matter. On the contrary, a diverse group of naturally inhabiting proteobacteria called methanotrophs or methane oxidising bacteria (MOB) are present in the soils where CH_4 is produced (Tate *et al.*, 2012; Tate, 2015). In the presence of oxygen (O_2), methanotrophs convert CH_4 to CO_2 and microbial biomass with a small amount of water produced from the oxidation reaction. For every mole of CH_4 consumed, 0.3–0.4 moles of CO_2 are produced. Even though, one GHG (CH_4) is converted to other GHG (CO_2), the net removal of CH_4 with GWP of 34 is reduced, thus having a negative effect on global warming.

Further investigation revealed that the volcanic pumice soil isolated from the Landfill in Taupo, North Island exhibited excellent physical and chemical characteristics to support CH_4 oxidation. Based on this research, prototype CH_4 oxidation biofilters (column and floating bed) packed with volcanic pumice soil have been developed that consume high CH_4 concentration in biogas from a Massey University No.4 dairy waste pond (Pratt *et al.*, 2012a, b). Even though lot of work was done on the CH_4 removal ability of volcanic pumice soil using biofilters, very limited work was done on identifying the microbes involved in the CH_4 oxidation process and the factors affecting them. Literature suggests that methanotrophs are majorly grouped as type I and type II, based on phylogenetical, morphological and biochemical differences. Type I methanotrophs are more diverse and include genera viz., *Methylobacter, Methylomonas, Methylosarcina, Methylococcus, Methylomicrobium, Methylosphaera, Methylocaldum* and unclassified *Methylococcales*. On the other hand, *Methylocystis, Methylosinus, Methylocapsa* and *Methylocella* forms the genera under type II methanotrophs. The growth and activity of these different genera of methanotrophs are optimal at different abiotic and biotic conditions (Knief *et al.*, 2003; Chang *et al.*, 2010; Henneberger *et al.*, 2011; Ruo *et al.*, 2012b). There is a need for knowledge of the characteristics of these genera and how they respond to different conditions so that these organisms can be efficiently utilised to mitigate CH_4 emissions by the soil.

The emphasis of our current research is to make the biofilters as cost-effective as possible, and to ensure they can operate in all weathers for prolonged periods (e.g., several years) with little or no maintenance. This PhD aims to study methanotrophs population dynamics in biofilters (column and floating bed), simultaneously understanding the effect of various biotic and abiotic factors controlling their growth and activity. Novel molecular techniques like quantitative polymerase chain reaction (qPCR), terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) were used to identify the active groups of methanotrophs involved in the CH_4 oxidation process. These techniques also helped us understand changing population dynamics of the methanotrophs under extreme environmental conditions. Several novel species of methanotrophs were also identified using cloning and Sanger sequencing. The other challenge of scaling up this biofilter technology, for use nationally to mitigate emissions was the limited availability of volcanic pumice soil and associated

transportation costs. Therefore, several cheaply and widely available materials were tested to assess their efficacy to remove CH_4 and support active growth of methanotrophs. This PhD project enhanced the understanding of the microorganisms involved in the efficient biofiltration of CH_4, and developed the best way to introduce and sustain active methanotroph populations in cost effective alternative biofilter media.

Passing biogas (CH_4, CO_2 and volatile organic compounds) through a column or filter bed packed with volcanic pumice soil (or any other material) seeded with methanotrophs offers an economical and cleaner approach to mitigate emissions from sources where energy capture and flaring (conventional mitigation options) is not possible such as from dairy farms, piggeries, barns or animal sheds, diluted coal mine CH_4 gases, wetlands, landfills, petroleum industries exhaust and solid manure storages.

1.2 Thesis objectives

The aim of this thesis is to

- Understand methanotroph ecology to efficiently develop cost-effective CH_4 removal technologies for livestock farming systems.

Specific objectives include

- To evaluate the CH_4 removal potential of two different biofilter systems (Column and floating cover)
- Studying the effect of important biotic and abiotic factors controlling CH_4 oxidation potential of the biofilter and population dynamics of methanotrophs
- Screening widely and cheaply available alternate potential biofilter materials that can support methanotrophs growth and activity
- Identifying active groups of methanotrophs involved in CH_4 oxidation using molecular techniques – qPCR, T-RFLP and molecular cloning.

1.3 Thesis structure

Structure of the thesis is presented in Figure 1.1. **Chapter 1** outlines the brief introduction on the CH_4 emissions from New Zealand dairy ponds and its mitigation by identifying the gaps to enhance our understanding of biofilter technology. **Chapter 2** reviews national and international literature available on CH_4 sources, emissions, underlying processes and mitigation options for CH_4 removal from agriculture and waste sectors. Several biotic and abiotic factors affecting the methanotrophs growth and activity is also discussed in addition to discussion on the novel molecular techniques used. **Chapter 3** describes the methodology used to study the biofiltration systems. Particularly in regards to soil/materials physical, chemical and microbial/molecular analysis to enhance understanding of CH_4 oxidation and methanotroph population dynamics in the biofilters. **Chapter 4** assesses the ability of a reconstituted acidic biofilter to remove high concentrations of CH_4 from effluent pond at Massey No.4 dairy farm. This study reported the presence of acidophilic *Methylocapsa* like methanotrophs in the biofilter, which were majorly contributing to CH_4 removal along with type I methanotrophs. This study also indicated that regardless of soil acidity, a biofilter can perform efficiently over a large period if sufficient moisture content levels are maintained. **Chapter 5** assesses the ability of the floating cover biofilter prototype to remove CH_4 over a period of one year under field conditions at Massey No.4 dairy farm (without controlling any environmental factors). Changes in the methanotroph community abundance and diversity was also studied in the floating biofilter during its transfer from laboratory to field conditions. **Chapter 6** refers to screening of alternate economical and widely available materials for use as a biofilter medium to partly or completely replace the experimental biofilter medium – the volcanic pumice soil. Promising alternate biofilter materials included soil and biochar which were more stable and resilient than the materials tested and supported active CH_4 removal and methanotrophs growth and activity. **Chapter 7** brings together the molecular results from all the experimental chapters and presents a general discussion on methanotroph abundance and diversity in relation to CH_4-oxidation potential, and **Chapter 8** highlights the main achievements of this study and recommends some aspects of future research.

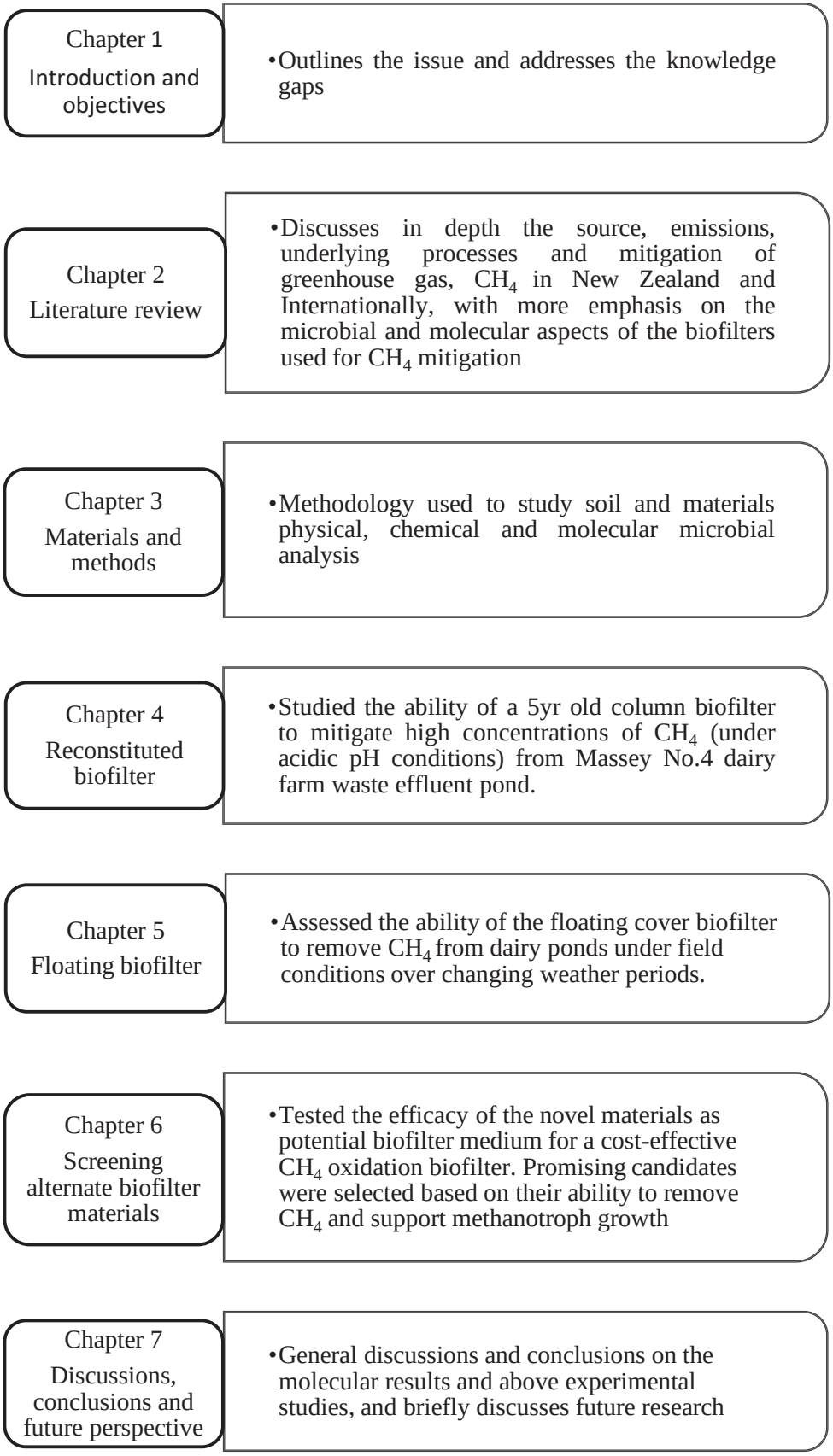


Figure 1.1 Thesis structure

Chapter 2

Review of Literature

To achieve the objectives of this PhD, it is important to identify the sources of methane & its production, understand the microbial aspects of methanotrophs and underlying factors affecting their growth and activity. The importance of using molecular techniques like quantitative PCR, T-RFLP, DGGE and cloning to identify the methanotrophs involved in active CH_4 oxidation is also emphasised to achieve the objectives of this research.

2.1 Methane emissions

Globally, livestock manure management is one of the largest contributors of the potent greenhouse gas, methane (CH_4), which accounts for 10% of total agriculture emissions (Owen and Silver, 2015). In the US, dairy cattle emit 41.9 and 32.2 MMT CO_2-equivalents of CH_4 by enteric fermentation and manure management, respectively (EPA 2016). Owen and Silver (2015) estimated more CH_4 emissions from the US anaerobic lagoons (368±193 kg herd^{-1} yr^{-1}) than is produced from enteric fermentation in grazing animals (120 kg herd^{-1} yr^{-1}) and highlighted liquid manure systems as promising areas for greenhouse gas mitigation.

New Zealand has a unique greenhouse gas (GHG) emission profile with a greater proportion of methane (CH_4) and nitrous oxide (N_2O) from agriculture. Majority of the CH_4 emissions are contributed from enteric fermentation and manure management and the remainder from solid waste disposal, coal mining and natural gas leaks. In the context of this PhD, only emissions from waste management and manure management is discussed.

2.1.1 Waste Management

In New Zealand, the CH_4 emissions from waste sector includes the solid waste disposal sites (4600.30 Kt CO_2-e) and waste water treatment plants (450.5 Kt CO_2-e). Solid waste disposal sites include managed landfills (municipal waste) and unmanaged landfills (farm and industrial waste). Worldwide, landfills are used to store solid wastes and municipal sewage waste for biological degradation which take several years to degrade. The organic fraction in the waste is degraded under anaerobic conditions to produce landfill gas (LFG) mainly containing CH_4 (55-60% v/v) and CO_2 (40-45% v/v) (Scheutz *et al.*, 2009) but water, hydrogen sulphide (H_2S), hydrogen (H_2) and non-methane organic compounds (NMOC) are also produced (Menard *et al.*, 2012).

Globally, CH_4 emissions from landfills have increased by about 12%, from 706 to 794 MtCO_2-e between 1990-2005 and are projected to increase by 21% from 2005 to 2030 (EPA, 2012). In United States, landfills are the third largest source of CH_4 production, accounting for about 18% of the total U.S. anthropogenic CH_4 emissions in 2013. U.S. landfills generated 114.6 Mt CO_2-e in 2013 (EPA, 2015). In New Zealand, emissions from solid waste disposal sites account for 13% of the total CH_4 emissions. However, emissions from solid waste disposal on land have decreased by 2.1% from 4698.6 to 4600.3 Kt CO_2-e between 1990 and 2013 level (MfE, 2015). This reduction in emissions is due to the decrease in the number of legally operating landfills and efficient management of the landfill sites. A total of 327 legally operating landfills were reported in 1995 but they were reduced by 85% to 49 in 2013 (MfE, 2015).

2.1.2 Manure management

Management of livestock manure produces CH_4 and N_2O. Methane is produced by anaerobic decomposition of manure when stored in anaerobic lagoons, liquid systems or pits, but when handled as solids or dry lots produce less CH_4. In 2013, US CH_4 emissions from manure management were 61.4 MT CO_2-e, 9.64% higher than in 1990. This increase is due to an increased use of liquid systems to store manure because of new regulations limiting the practice of daily manure spreading on the pasture or paddock lands (EPA, 2015). In Europe, liquid systems are used to store manure from dairy cattle, non-dairy

cattle and swine. About 42% of the manure coming from dairy cattle and swine is stored in anaerobic lagoons or liquid systems (EEA, 2012). However, no activity data is reported by US and European GHG inventory on measured CH_4 emissions from stored anaerobic ponds/liquid systems

In New Zealand, CH_4 emissions from manure management of dairy cattle's increased by 76% from 390.1 Kt CO_2-e to 896.9 Kt CO_2-e between 1990 and 2013 (MfE, 2015). According to Ledgard and Brier (2004), CH_4 emissions from manure stored in the New Zealand effluent ponds contribute to 6% of the total on-farm emissions based on the amount of time the livestock spend at the milking shed. However, Chung *et al.* (2013) have questioned the accuracy of this agricultural waste CH_4 emission estimate as reported by (MfE, 2007); MfE (2012), showing that the inventory could be estimating as much as 18% of the actual CH_4 emissions from this sector. Based on a review of CH_4 emissions from farm effluents (Saggar *et al.*, 2004), dung deposited in the milking shed and feeding pads is diluted with 90 L of water per kg of dry dung matter and then stored in the anaerobic dairy ponds. Methane emissions from the slurry in the pond are higher than those emitted from dung pats on pasture. Average CH_4 production from the slurry is 3 and 26 times more than the solid manure and pasture pats dropped on the field respectively (Holter, 1997). Dung dropped on the pasture by the grazing cattle stopped producing CH_4 after 1.5 – 2 months (Saggar *et al.*, 2004) as it dried out and conditions became aerobic. By contrast, the CH_4 produced from stored manure slurry in dairy ponds is an on-going process. When the stored slurry dries out during the non-milking season and pastures are dry, the pond is then emptied and the contents applied on the pastures as a fertilizer. As per the local council regulations, the effluent pond should be emptied every three months by farmers, but in practice they are emptied only one to three times per year.

Methane emissions from New Zealand dairy effluent ponds are not accurately known (Chung *et al.*, 2013). The amount of CH_4 produced depends on the herd size, the pond size and the amount and quality of effluent been stored in the pond and the time of year. Methane emissions from New Zealand dairy lagoons contribute more than 50% of CH_4 emissions from the manure management sector and these emissions are likely to increase in future as 80% of total dairy farms (~ 9660) use an open effluent pond system (or a similar system) to store the waste from milking sheds and feeding pads (and stand-off

pads on some farms) (Laubach *et al.*, 2015). Typical values for CH_4 emissions are reported by Craggs *et al.* (2008). Average methane emissions from a 1700 m^2 sized dairy effluent pond receiving the manure from ~ 700 dairy cattle were 45 m^3 d^{-1} or 6.4 Kg CH_4 /cow yr. This was much higher than CH_4 emissions of 5.2 m^3 d^{-1} reported by McGrath and Mason (2004) for a 900 m^2 No.4 Massey dairy effluent pond holding waste from 435 cows. In the recent study of Pratt *et al.* (2012c) they measured 180 m^3 methane d^{-1} from the current 928 m^2 Massey No.4 dairy effluent pond receiving waste from a herd of 450 cows.

New Zealand has about 11500 dairy farms with an average herd size of about 300 cows in North island and 500 cows in South Island (Bolan *et al.*, 2009). Capturing CH_4 for energy production is only considered economically possible if the herd size is large (~1000) (Pratt *et al.*, 2012c) and if the CH_4 flow rates are high, with concentrations of 30–40% v/v (Haubrichs and Widmann, 2006). Due to increased use of anaerobic ponds for deferred irrigation purposes, more waste from feed pads, stand-off pads, milk waste and feeding residues are being added in to the ponds. As a consequence, CH_4 emissions from farm waste are likely to be increasing. However, as CH_4 capture for energy or flaring is not currently feasible for an average sized dairy farm, CH_4 is left untreated from most of the anaerobic ponds, thus contributing to total agricultural CH_4 emissions.

2.2 Methane production process

Methane is produced by decomposition of organic matter under anaerobic conditions. This process is mediated by three groups of microorganisms as described in the figure 2.1. The first group - hydrolytic and fermentative microorganisms - convert polymers like carbohydrates, proteins and fats into short chain carboxylic acids, alcohols, carbon dioxide and hydrogen. Proton reducing acetogens (second group) oxidise the fermentation products to acetate, carbon dioxide and hydrogen. The final step is performed by the third group – CH_4 producing archaea, methanogens. They utilize various sources like hydrogen plus carbon dioxide, acetate and other substrates including formate, methanol and methylamine to produce CH_4 and water. Hydrogenotrophic methanogens produce CH_4 from hydrogen plus carbon dioxide only and the acetotrophic

group of methanogens produce CH_4 from acetate. The former reaction yields 135.6 KJ of energy per mole of CH_4 produced and the latter reaction mediated by acetophilic methanogens yield 30.6 KJ/molCH_4 energy (Barlaz *et al.*, 1990).

Methanogens play an important role in maintaining the balance of the ecosystem by controlling the pH by consumption of acetate, regulating favourable catabolic conditions by consuming hydrogen and excreting organic growth factors that are used by other bacteria in the ecosystem (Barlaz *et al.*, 1990). High chemical oxygen demand (COD) indicates the onset of methanogenesis and the higher the COD the more CH_4 is produced (Saggar *et al.*, 2004). Methane production under anaerobic conditions is influenced by different factors- organic C content, methanogen population, moisture content and leachate flow, pH and temperature. The optimum environmental conditions for CH_4 production are pH 6.8-7.4, moisture content (35-55%) and temperature (30-45^oC) (Barlaz *et al.*, 1990).

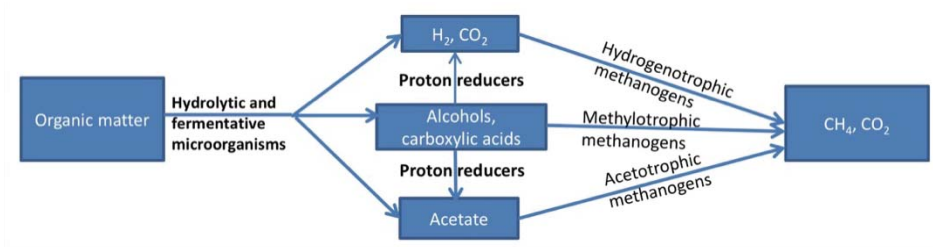


Figure 2.1 Organic matter decomposition and methanogenesis involving non-methanogenic and methanogenic microorganisms. Methanogens convert partially reduced carbon compounds to CH_4 and CO_2.

2.3 Methane mitigation options

2.3.1 Gas capture for flaring and power generation

Methane can be captured for heating and energy use. The calorific value of biogas is about half that of natural gas. Biogas from landfills is collected through a network of gas wells

and piping material and supplied to a boiler or biogas generator for combustion. Power is generated based upon the principle of electromagnetic induction, where the electrical energy is generated from the mechanical energy of the motor, powered by the biogas. Methane capture for energy production can be economically viable only if high rates and concentrations of CH_4 are produced. At standard temperature and pressure conditions, CH_4 concentrations above to 30-40% (v/v) with a minimum flow rate of 30-50 $m^3 h^{-1}$ are typically needed for biogas valorisation (Haubrichs and Widmann, 2006; Menard *et al.*, 2012).

On industrial scale, CH_4 is captured from large anaerobic digesters and used for power generation. Maciel and Jucá (2013) designed a pilot biogas recovery plant capable of capturing biogas coming from about 35,000 tonne municipal sewage waste (MSW) experimental landfill cell with 5880m^2 surface area. Biogas was collected from the landfills via vertical wells and then transferred to the pilot plant through the network of high density poly ethylene (HDPE) pipes. The pilot plant consisted of a complex system of HDPE pipes, a 20kW generator, radial compressor, biogas filter (to remove water vapour and H_2S) and a heat exchange system (to maintain an operational temperature of 29^oC). Heubeck and Craggs (2013) have designed a similar system in New Zealand to capture biogas from 7000m^3 swine effluent ponds to generate on-site heat and electricity with the use of a 48kW electrical generator. This technology is promising but has got its own complications. PVC tubes which are used to collect biogas are permeable to gases and reactive to CH_4, thus decreasing the capture efficiency. On the other hand, HDPE tubes are susceptible to thermal expansion when used to transport hot biogas coming from landfills (Maciel and Jucá, 2013). Trace components like H_2S, Siloxanes and other volatile organic compounds can severely impair plant operation and longevity. Hydrogen sulphide (H_2S) present in the biogas during combustion form corrosive and oxidative sulphuric acid which damages iron and zinc fractions in the gas engine. Siloxanes produced during the combustion form abrasive fine sands (silicon oxides) damage the gas engine and interfere with the combustion performance. This can result in increased nitrous oxide and carbon monoxide emissions (Coffey, 2009). Experiments conducted by Maciel and Jucá (2013) showed the purification efficiency of the trace components was only 50% which had resulted in the poor performance of the plant. The total costs for CH_4 capture for energy production include capital investment for biogas capture, electrical energy

generation and biogas combustion systems, operating and maintenance costs. In New Zealand the costs for setting up a biogas capture unit on a waste effluent pond for energy generation is estimated to be 20000-25000 NZD which excludes operational/maintenance costs. However, this technology is economically feasible only for large industrial anaerobic digesters, landfills and effluent dairy ponds systems with high methane flow rates.

Biogas for flaring is generally used to limit CH_4 gas emissions contributing to global warming. According to Haubrichs and Widmann (2006); Menard *et al.* (2012), flaring is only possible when the CH_4 concentrations are 20-25% (v/v) and with flow rates of 10-15 $m^3 h^{-1}$. However, this should be carried out only at a combustion temperature of 1200 $^{\circ}C$, otherwise toxic by-products like dioxins are formed. Furthermore, flaring poses a fire hazard to its surroundings, if not carried out under controlled conditions.

2.3.2 Biotechnology using methanotrophs

Use of microorganisms or living systems to develop a technological application is defined as biotechnology. Bio-filtration is one of the oldest biotechnologies and is used for treating contaminated air, odours and several organic and inorganic volatile compounds (Nikiema *et al.*, 2007). Biofilter seeded with CH_4 eating bacteria (methanotrophs) have been used to mitigate CH_4 emissions from various CH_4 emitting sources viz. landfills, coal mines, anaerobic waste, animal herd homes/winter barns.

Unlike conventional CH_4 removal technologies, CH_4 gas biofiltration is a clean and green process, which does not generate hazardous products like carbon monoxide (CO), nitric oxides (NO_x), particulate matter and sulphur dioxide (SO_2) (Limbri *et al.*, 2013). Methane gas is typically passed through the biofilter, where the methanotrophs in the biofilter in the presence of O_2 converts CH_4 to CO_2, water and biomass. Apart from treating CH_4 emissions, biofilter is also capable of removing odours (H_2S) and ammonia gas emissions (Scheutz *et al.*, 2009). There are typically two variants of biofilter technology - active and passive biofilters. In the actively aerated biofilters, a mixture of CH_4 and air is passed

through the biofilters from the bottom, whereas in the passive system, CH_4 passively diffuses from the bottom of the biofilter and the air from the top into the biofilter.

Biofilters / biocovers seeded with methanotrophs are being developed worldwide to mitigate CH_4 emissions from landfills, waste systems, coal mines and various low CH_4 emitting sources with concentrations less than 20% (v/v) in the air (Menard *et al.*, 2012), but concentrations more than 60%(v/v) with high CH_4 loading rates (~700g CH_4 m^{-3} day^{-1}), could also be mitigated using large biocovers (Capanema and Cabral, 2012)

2.4 Methanotrophs

Methanotrophs are gram negative, rod - cocci shaped aerobic proteobacteria that convert CH_4 into CO_2 in the presence of oxygen. As they rely on single carbon atoms for their energy, they are also called methylotrophs. They are naturally found in soils where they act as CH_4 sink (Czepiel *et al.*, 1995; Tate *et al.*, 2007; Meijide *et al.*, 2010; Tate, 2015a), and have the ability to form resting stages - cysts or exospores under unfavourable conditions or when CH_4 is not available (Whittenbury *et al.*, 1970)

2.4.1 Characteristics

Methanotrophs are mainly classified as type I and type II based on physiological, biochemical, and morphological properties. Type I methanotroph are more diverse and include genera, viz., *Methylobacter, Methylomonas, Methylosarcina, Methylococcus, Methylomicrobium, Methylosphaera, Methylocaldum*, and unclassified *Methylococcales*. *Methylocystis, Methylosinus, Methylocapsa*, and *Methylocella* form the genera under type II methanotroph (Hanson and Hanson, 1996; Dedysh *et al.*, 2003; Ho *et al.*, 2011).

Two forms of methane monooxygenase enzyme are involved in the CH_4 oxidation reaction- soluble cytoplasmic form methane monooxygenase (sMMO) and membrane-bound particulate methane monooxygenase (pMMO). Soluble MMO is present in few species of type I and II methanotrophs and pMMO is present in most of the type I and II methanotrophs (Hanson and Hanson, 1996). Methanotrophs have also been identified

from various diverse environments including polluted environment (McDonald *et al.*, 2006). Characteristics of methanotrophs in wetland, rice soil and biocover soil are discussed below.

Gupta *et al.* (2012) investigated the diversity of methanotrophs in two contrasting North American peatlands (wetland) systems- a nutrient rich sedge fen with a neutral pH and a nutrient poor Sphagnum bog with a low pH (4.8). Analysis of 16S rRNA, *pmoA* and *mmoX genes* revealed that bog systems were dominated by less diverse methanotrophs mainly belonging to *Methylosinus* and *Methylocystis* (Type II) group. Fen systems were inhabited by more diverse type I methanotrophs. Despite the neutral conditions in the fen peatlands which can accommodate a more diverse community, CH_4 oxidation rates were higher in low pH bog peatlands. The authors consider that higher CH_4 oxidation rates in wetland systems are due to the dominance or evenness of a community rather than the phylogenetic diversity. Basiliko *et al.* (2004) tested the effect on CH_4 oxidation rates by different species of *Sphagnum* (moss) with varying physiologies. The results indicate that the CH_4 oxidation rate is mainly dependent on the availability of CH_4 and O_2 rather on other soil or plant attributes in wetland systems. Yun *et al.* (2012) studied the methanotrophic community structure and activity in three different water logged marshes of the Zoige tundra wetlands, the largest CH_4 emission sources in China. The study zone was 10 cm below the water table and at an average temperature of 1.2–5 $^{\circ}$C. Molecular analysis showed that two genera of methanotrophs – *Methylobacter* and *Methylocystis* belonging to type I and type II methanotrophs respectively, were dominant and actively involved in CH_4 oxidation. In a similar study of methanotrophs diversity in the Xianghai wetland of northeast China, Yun *et al.* (2013) suggested that type I methanotrophs and more specifically methanotrophs belonging to *Methylobacter* genera were dominant at colder temperatures and actively involved in CH_4 oxidation. Samples from 0–30 cm deep layers revealed the presence of a large number of *Methylobacter* methanotrophs with marginal presence of *Methylococcus*, *Methylomonas* and *Methylocystis* genera.

Macalady *et al.* (2002) studied the methanotrophs (type I and II) population dynamics in Californian rice soils using phospholipid fatty acids analysis-16:1 w8 (type I) and 18:1 w8 (type II). They found that both type I and II were abundant throughout the year and the type II population were influenced by rice growth and CH_4 concentrations. Group-

specific quantitative analysis of methanotrophs in the rice soils were studied by Kolb *et al.* (2003). They developed quantitative PCR (polymerase chain reaction) assays for five groups (*Methylococcus* group, *Methylobacter/Methylosarcina* group, the *Methylosinus* group, the *Methylocapsa* group and the forest clones group) by targeting *pmoA* gene sequences. Their results suggest that the methanotrophs belonging to the *Methylosinus group* (classical type II; *Methylocystaceae*) and the *Methylobacter/Methylosarcina* group (*Methylosarcina*, *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*; classical type I methanotrophs) were predominant in the rice fields. The above results concur with the findings of Henckel *et al.* (1999) in rice field soils demonstrating the presence of both type I and type II methanotroph communities belonging to *Methylobacter*, *Methylococcus* and *Methylocystis* based on 16S rRNA, *pmoA* and DGGE analysis.

Chi *et al.* (2011) studied the diversity of methanotrophs in a biocover made up of materials prepared from compost (leaves plus chicken manure) with a volume of $0.021m^3$. The type II methanotrophs didn't exhibit spatial variability but the spatial distribution within the reactor varied with type I methanotrophs. Type I were also influenced by the air distribution in the reactor. PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) analyses indicated that methanotrophs belonging to *Methylobacter* and *Methylocystaceae* groups were found in the biocover. Diagnostic microarray targeting of different genera of methanotrophs developed by Gebert *et al.* (2008), revealed the diversity and abundance of methanotrophs in bioreactors operating at two different landfill sites in Germany – Francop (FR) and Muggenburger Strabe (MU). The two bacterial communities in the biofilters operating at an average temperature of 22 $^{\circ}C$ differed significantly with the presence of type I methanotrophs only in the biofilter FR. On the other hand, type II methanotroph communities dominated by *Methylocystis* group and *Methylosinus* species were present in biofilter FR and MU respectively. The authors speculate that the additional presence of NMVOC's (non-methane volatile organic compounds) in MU landfill gas might have resulted in the selection of type II methanotroph community, which has the ability to co-degrade NMVOC's along with the CH_4. Understanding methanotrophs abundance and activity is therefore critical for efficient development of bio mitigation technologies.

2.4.2 Methane oxidation process

Methanotrophs possess the unique ability to utilise CH_4 as a sole carbon and energy source. The first enzyme involved in the CH_4 oxidation process is MMO (Figure 2.3) Depending upon the presence of the soluble or particulate form of MMO enzyme, sMMO or pMMO converts CH_4 to CH_3OH (methanol). This is then further converted to HCHO (formaldehyde) by methanol dehydrogenase (MDH) enzyme, which is present in all known methanotrophs. Carbon assimilation or biomass formation from HCHO is carried out by two characteristic pathways- serine and RuMP.

Most of the type I methanotrophs assimilate the carbon from HCHO by the RuMP pathway and type II methanotrophs assimilate by serine pathway. Formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FDH) enzymes further convert the HCOOH to CO_2.

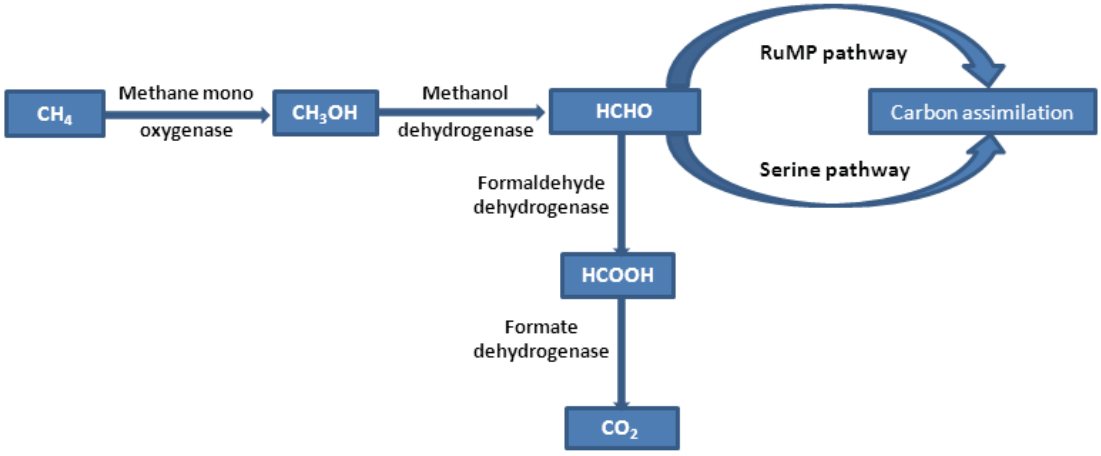


Figure 2.2 Methane oxidation process involving RuMP and serine pathway of carbon assimilation in methanotrophs. (Hanson and Hanson, 1996)

According to Whittenbury *et al.* (1970) enrichment culture studies, 1 mole of CH_4 plus 1.0-1.1 mole of O_2 is converted to 0.2-0.3 moles of CO_2 and the remaining 0.7-0.8 moles of carbon is assimilated as biomass. The stoichiometric equation for methane oxidation process is as follows

1 **CH_4** + *1.0-1.1* **O_2** ---------> *0.2-0.3* **CO_2** + **2 H_2O** + **biomass**

Methanotrophs can co-metabolise different sources of carbon viz., methanol, methylated amines, halomethanes, trichloro ethylene and various short chain and long chain

chloroalkanes. Few of the methanotrophs can also oxidise ammonia and various NOx forms. Type II methanotrophs also possess the capability of fixing N_2 when needed (Hanson and Hanson, 1996).

2.4.3 Factors effecting methanotroph activity

Methanotrophs activity and the CH_4 oxidation process are affected by various abiotic and biotic sources, which are discussed in the below sections.

2.4.3.1 Methane and O_2 ratio

Oxygen and CH_4 concentrations are the most important abiotic parameters controlling CH_4 oxidation rates. The literature suggests that type I methanotrophs are more dominant at lower CH_4 and higher O_2 concentrations, while type II methanotrophs are dominant in higher CH_4 and lower O_2 concentration (Ho and Frenzel, 2012; Duan, 2012; Ruo *et al.*, 2012a; Tate *et al.*, 2012; Zheng *et al.*, 2012). According to the review of Nikiema *et al.* (2007), CH_4 concentrations <1000 ppmv and O_2 concentration of 21% (v/v) favour type I methanotrophs growth. By contrast, CH_4 concentration of greater than 1% (v/v) and lower concentration of O_2 (1% v/v) appears to support type II methanotrophs, but the distinction between these two scenarios is not very clear.

The effect of CH_4 and O_2 concentrations on CH_4 oxidation dynamics was studied by Chi *et al.* (2012). Biofilter material prepared from topsoil of MSW (municipal sewage waste) and leaves plus chicken manure compost were homogeneously mixed and used to study CH_4 oxidation rates based on the Michaelis-Menten model. Oxygen to CH_4 molar ratio of 3:1 favours full aerobic CH_4 oxidation and the ratio lower than that limits the CH_4 oxidation rate, thus confirming that oxygen is the limiting factor for CH_4 oxidation and the CH_4 oxidation rate is influenced more by CH_4 concentration rather than O_2. The effect of aeration on the CH_4 removal efficiency of a lab-scale biofilter column was investigated by Haubrichs and Widmann (2006). An actively aerated biofilter made up of finely grained compost material exhibited a 5.5 times higher CH_4 oxidation rate than the passively aerated one. They also found that the removal efficiency of the biofilters decreased from 92% to 88% when the O_2/CH_4 inlet ratio was lowered from 2.5 to 2. All

the above studies confirm that O_2/CH_4 ratio is one of the most important factors affecting CH_4 oxidation rates and an O_2/CH_4 ratio greater than 2.5 should be maintained for effective methane oxidation in the biofilter. A recent study (Pratt *et al.*, 2013) also showed that CH_4/CO_2 ratios were a useful index of efficiency of CH_4 oxidation in a landfill cover soil.

Empty bed residence time (EBRT) is an important factor contributing to CH_4 oxidation rate in the biofilters. Residence time is defined as the amount of time a substrate spends in a particular system. EBRT for CH_4 biofilters is calculated as follows

$$\tau = (V_f \times \Theta) / Q$$

Where, τ = residence time (s); V_f = filter bed volume (m^3); Q= air flow rate ($m^3\ s^{-1}$) and Θ = porosity = (volume of void space/ filter material volume).

Nikiema and Michele (2009b) reported CH_4 removal efficiencies of 90-100% and 30-90% for an EBRT of 8.7 min and 5.8 min respectively, when the CH_4 inlet load of $\leq$ 55 g $m^{-3}h^{-1}$ was passed through inorganic biofilter material made up of gravel with a void space of 40%. Higher CH_4 removal efficiencies can be achieved at longer EBRT, which is primarily due to increased contact time between the methanotrophs and the CH_4 gas molecules.

2.4.3.2 Temperature

Visvanathan *et al.* (1999) studied the effect of temperature and moisture content on CH_4 oxidation rates in biofilter columns made up of 70 % sand, 5-15 % silt and 15-25 % clay landfill cover soils. Methane oxidation rate batch experiments targeting temperatures from 5 – 45 $^{\circ}$C was studied; biofilter columns operating at temperatures below 20 $^{\circ}$C and above 45 $^{\circ}$C were not affective at removing CH_4, while the highest CH_4 oxidation rate was measured for temperatures of 30-35 $^{\circ}$C. However, temperature dependency factor depends upon the presence of an active type or group of methanotrophs in the biofilter. The CH_4 oxidation process in landfill cover soils operating at low temperatures (1-19 ^{o}C) was studied by Einola *et al.* (2007). Though the rate of CH_4 oxidation was lower at 1 ^{o}C (0.06 µmol CH_4 g $dw^{-1}\ h^{-1}$) compared to that at 12 ^{o}C (0.70 µmol CH_4 g $dw^{-1}\ h^{-1}$), this

experiment demonstrated that methanotrophs can actively consume CH_4 even at lower temperatures.

Careful identification and selection of these temperature-specific microorganisms and their incorporation in biofilters will allow effective removal of CH_4 over a range of temperatures. The importance of temperature in the selection of methanotrophs population dynamics was demonstrated by Borjesson *et al.* (2004). Swedish landfill cover soils from three different sites were placed in a 1.1 L gas tight flask and were incubated at different temperatures (3–5 °C, 10 °C, 15 °C and 20 °C) to monitor CH_4 consumption over time. PLFA analysis was used to identify type I and type II methanotrophs to provide the composition of the methanotroph population at a given temperature. At lower temperature (5-10 °C), PLFA markers related to type I methanotrophs were abundant and at 20 °C temperature, type II methanotrophs were abundant. This experiment suggests that temperature is one of the major factors determining the selection of active populations of methanotrophs.

2.4.3.3 pH

The pH is another abiotic factor controlling the CH_4 oxidation process. Optimal pH for methanotroph growth and activity is about 6.8–7 (Whittenbury *et al.*, 1970; Hanson and Hanson, 1996) but they can survive in alkaline and acidic conditions. Alkaline or acidic condition can be a determining factor for choosing a specific group of methanotroph community. For example, methanotroph community structure in an alkaline upland landfill soil was studied by Chang *et al.* (2010). Terminal-RFLP and qPCR analyses revealed the abundance of type I (*Methylobacter*, *Methylomicrobium* and *Methylomonas*) and type II populations in alkaline soils. Molecular biology studies on low pH peatland systems by Gupta *et al.* (2012) revealed the presence of type II methanotrophs, establishing the fact that type II methanotrophs are also active at low pH. Pratt *et al.* (2012b) studied the CH_4 oxidation in a biofilter column made up of a volcanic pumice soil. In their study, pH had dropped to 4.2 at the end of 16 months but no change in the overall CH_4 removal efficiency of the biofilter was observed. The above studies indicate that methanotrophs are capable of oxidising CH_4 in acidic, neutral and alkaline conditions.

2.4.3.4 Moisture content

An optimal moisture condition allows effective gas diffusion, maintains osmotic balance and provides a medium for nutrient availability to the methanotrophs. Moisture content of the soil depends upon the porous nature of the soil and its water holding capacity (Tate *et al.*, 2007). High moisture contents are likely to decrease methanotroph activity due to gas diffusion limitations and low moisture content tends to reduce methanotroph activity, due to osmotic stress or unavailability of nutrients (Semrau *et al.*, 2010). As moisture content is linked to other factors viz. substrate, physical characteristics, O_2, and CH_4 diffusion and type of methanotroph population and temperature (Tate *et al.*, 2007); it is very difficult to indicate the optimal moisture content range. Pratt *et al.* (2012b) suggested the strong influence of moisture content on biofilter performance. Biofilters oxidised CH_4 twice as efficiently at 40% moisture content (wt/dry wt) than at 85% moisture content (wt/dry wt). Moon *et al.* (2010) found the optimal moisture content range to be 15-40 % when he performed experiments using a paddy soil and earthworm casts as a biofilter material. From the above studies, it is evident that optimal moisture content depends largely on the physical characteristic of the biofilter material. For biofilters made up of volcanic pumice soil, an optimal moisture content of 60% water holding capacity (WHC) was indicated by experiments carried out by Pratt *et al.* (2012a, b, c)

2.4.3.5 Macro and micro nutrients

With regard to the availability of macro nutrients, Whittenbury *et al.* (1970) reports that for a phosphorus concentration of above 0.2% (w/v) and ammonium chloride concentrations greater than 0.05% (w/v) growth of methanotrophs is inhibited in liquid culture. Nitrogen is generally available inorganically to microorganisms as nitrate (NO_3^-), ammonium (NH_4^+) or nitrite (NO_2^-). Typically, the sources of N used for laboratory studies include ammonium chloride, ammonium sulphate and urea for NH_4^+; sodium nitrate and potassium nitrate for NO_3^-. Whittenbury *et al.* (1970) used potassium nitrate as a source of NO_3^- in his culture studies. According to Wilshusen *et al.* (2004) hypothesis, addition of nitrogen (N) elevates the activity and abundance of type I methanotrophs as they are not capable of nitrogen fixation and depend on soil inorganic nitrogen for meeting their demand for N. Type II methanotrophs on the other hand, can

biologically fix nitrogen, and the absence of inorganic nitrogen in the soil does not seem to affect their activity or abundance. The effect of nitrogen salts and NPK fertilisers on CH_4 oxidation potential was studied by Jugnia *et al.* (2012). A landfill cover soil (10g) was mixed with 10ml of de-ionised water and used as a microcosm. This was incubated under different nitrogen and NPK conditions in gas tight serum bottles. Methane oxidation potential was higher for microcosms amended with NPK fertiliser compared to the soil amended with nitrogen salts, thus concurring with the fact that phosphorus also plays an important role in CH_4 oxidation. Experiments performed by Zheng *et al.* (2008) demonstrated that NPK application on the paddy soils had an elevated effect on the abundance of methanotrophs. A significantly higher ratio of type I methanotrophs was found in all the N, NPK and NPK+C treatments, suggesting that nitrogen, phosphorus and potassium fertilizers could be important factors controlling methanotrophs ecology.

Generally, compost has been widely used as a biofilter material (Melse and Vanderwerf, 2005; Scheutz *et al.*, 2009) as it serves as a complex nutrient media containing almost all nutrients. Composts also contain inhibitor compounds in unknown quantities that can adversely affect the CH_4 oxidation process. The use of chemically defined media in an inorganic biofilter was demonstrated by Nikiema *et al.* (2009a, 2010). The relationship between N concentration and CH_4 inlet load was studied by Nikiema *et al.* (2009a). For CH_4 inlet loads of 20–55 g m^{-3} h^{-1} and 55–95 g m^{-3} h^{-1}, N concentrations of 0.50 g L^{-1} and 0.75 g L^{-1} respectively were required for optimal biofilter performance. For a CH_4 inlet load of 55 g m^{-3} h^{-1} and N concentration of 0.50 g L^{-1}, the maximum CH_4 oxidation achieved was 26 g m^{-3} h^{-1} which is 80% and 135 % higher than the oxidation rates achieved at N concentrations of 0.25 and 0.14 g L^{-1} respectively. For a higher N concentration of 1 g L^{-1}, CH_4 oxidation was inhibited in the biofilters. In another experiment, the influence of phosphorus (P), potassium (K) and copper (Cu) on CH_4 biofilter performance was studied. Phosphorous concentrations ranging from 0.1 to 6.2 g L^{-1} were added to an inorganic biofilter bed and the biofilters performance was assessed. Methane oxidation by the biofilter increased with the increase in P concentration. Maximum oxidation or elimination capacity (EC) of 44.7 g m^{-3} h^{-1} was obtained at an optimal P concentration of 3.1 g L^{-1}, this EC value is 35% and 175% higher than the EC obtained at 0.3 g L^{-1} and 6.2 g L^{-1} of P respectively. However, the EC of 44.7 g m^{-3} h^{-1} obtained at a P concentration of 3.1 g L^{-1} was maintained only for a short period of 30

days. At the end of the period, the biofilter clogged up due to excessive accumulation of biomass thus disturbing the gas diffusion properties of the biofilter, resulting in a decrease in its performance. Thus lower P concentrations (1.5-3.1 g L^{-1}) and higher N/P mass ratios (0.5-2.5) are preferable for long term operation of biofilter. Despite the formation of excess biomass at higher concentration, P concentration also had an effect on the start-up time period. A biofilter with a P concentration of 3.1 g L^{-1} took 7-10 days for start-up/waking up period, whereas a minimum of 2 – 3 week start-up time was needed typically for a P concentration of 0.3 g L^{-1}.

Copper, acts as a cofactor for the pMMO enzyme and plays a key role in regulating methanotroph activity (Hanson and Hanson, 1996; Duan, 2012). A concentration of 1-5 µ mol L^{-1} Cu supports the increased activity of pMMO enzyme, while concentrations below 1 µ mol L^{-1} inhibited pMMO enzyme activity (Hanson and Hanson, 1996). Little was known about the effect of micro nutrients on the performance of the biofilter until the experiments carried out by Nikiema *et al.* (2010). According to their results, potassium (K), magnesium (Mg) and calcium (Ca) do not seem to have a major effect on biofilter performance compared to the influence of N and P. However, K, Mg and Ca concentrations of 0.076 g L^{-1}, 0.004 g L^{-1} and 0.002 g L^{-1} respectively favours the optimal performance of the inorganic biofilter. Among the micronutrients studied have been - copper (Cu), zinc (Zn), manganese (Mn), boron (B), molybdenum (Mo), cobalt (Co) and iron (Fe); Cu is one of most studied trace elements but didn't have any significant impact on biofilter performance. Nevertheless, a Cu concentration of 0.003 g L^{-1} is preferable for optimal biofilter performance. This behaviour of methanotrophs to Cu addition (Nikiema *et al.*, 2010) could be explained by the presence of type II methanotroph - *Methylocystis parvus* in their biofilter, which has the ability to grow in low Cu conditions.

2.5 Molecular methods to study methanotrophs ecology

Methanotrophs are very difficult to grow in the laboratory (Whittenbury *et al.*, 1970; Hanson and Hanson, 1996; Singh and Tate, 2007). Several culture independent techniques like Guanine plus cytosine (G+C) content analysis, DNA microarrays and PCR-based approaches including denaturing gradient gel electrophoresis (DGGE),

restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP) and real time or quantitative PCR (qPCR) techniques have been used to study methanotroph ecology (Kirk *et al.*, 2004). Most of the studies (Gebert *et al.*, 2008; Gulledge *et al.*, 2001; Henneberger *et al.*, 2011; Henckel *et al.*, 1999) extracted RNA instead of DNA to study active methanotrophs in the sample because DNA will indicate the presence of both living and dead methanotrophs. On the other hand, RNA indicates only live or active methanotrophs in the population. However, the shorter life of RNA in the bacterial cell and presence of inhibitors in the soils makes the RNA extraction procedure difficult.

Group and genera-specific phylogenetic and functional primers have been designed by many researchers. Phylogenetic markers like 16S rRNA primers are available to target *Methylobacter*, *Methylococcus*, *Methylomonas*, *Methylosinus*, *Methylocapsa*, *Methylosphaera* and *Methylocystis* genera, thus covering the majority of methanotrophic diversity (Kolb *et al.*, 2003). Primers targeting sMMO and pMMO genes were also used by many authors to study type I and type II methanotroph diversity by molecular cloning and sequencing techniques (McDonald *et al.*, 2008; Ho *et al.*, 2011)

Microbial community diversity or community fingerprinting can be analysed using DGGE. Agarose gel electrophoresis is typically used to separate oligonucleotides of variable length; whereas DGGE is applied to analyse sequence variations in PCR-amplified identical DNA fragments. DGGE separates the DNA fragments based on their mobility in an increasing denaturing polyacrylamide gel gradient. The separated bands can be directly visualised, excised and sequenced to provide community diversity, without the use of molecular cloning procedures (Murrell *et al.*, 1998). Wise *et al.* (1999) used DGGE to study methanotroph community diversity. They targeted variable region 3 of 16S rRNA using 40bp GC clamped primer 517R (5'-ATTACCGCGGCTGCTGG-3'). DGGE gels were made up of 6.5% polyacrylamide gel with 7M Urea and 40% deionised formamide as denaturant. A denaturing gradient from 20-70% was used for type I sequences and 30-60% for type II DNA fragments. They were then visualised by staining with ethidium bromide. Horz *et al.* (2001) used a 35-80% denaturing gradient gel made up of 6.5% (w/v) polyacrylamide, 5.6M Urea and 32% deionized formamide to

separate amplified pmoA fragments. The amount of denaturant and the range of gradients usage varied among the authors.

Quantitative or real time PCR is a novel molecular biological approach to quantify methanotroph abundances by determining the concentration of target DNA in the sample. In the qPCR technique, the fluorescence signal during the amplification of labelled primers is measured against the diluted standard DNA concentrations. Both phylogenetic (16S rRNA) and functional markers (*pmoA*, *mmoX*) have been used to quantify methanotroph abundance in soils (Kolb *et al.*, 2003; Chen *et al.*, 2007; Ho *et al.*, 2011). Both sets of pmoA primers A189F/A682R and A189F/Mb661R have been used to quantify all known methanotroph population, but the former set of primers amplified a small proportion of non-specific products as revealed by DGGE (Knief *et al.*, 2003). A189F/Mb661R primers are more specific to methanotrophs and generally do not target other ammonia oxidising bacteria as A682R does (Bourne *et al.*, 2001; Kolb *et al.*, 2003). MethT1dF, MethT1bR and MethT1cR are the other set of 16S rRNA primers which are used to target 16S rRNA genes of type I methanotrophs, but they lack the specificity to target *Methylocaldum* (type I) and *Methylocella* (type II) species, whereas Type IF/IR and Type IIF/IIR primers can target all the methanotroph diversity. Sequencing of the molecular clone libraries of type I and type II methanotrophs generated from the 16S rRNA primers (Type IF/IR, type IIF/IIR, MethT1dF and MethT1bR) revealed the presence of a small proportion of non-methanotroph sequences (Chen *et al.*, 2007) which indicates the characteristic of 16S rRNA probes can target some non-methanotroph population. On the other hand, pmoA based primers are more specific to target the methanotroph community and are widely used to study methanotroph community diversity (Kolb *et al.*, 2003). Nevertheless, the use of phylogenetic or functional based primers depends upon the sample source and the presence of type of methanotroph community diversity in the soil. Commonly used phylogenetic (16S rRNA) and functional (pmoA) primers are tabulated in table 2.1 and 2.2.

Table 2.1 Functional probes (*pmoA*) targeting methanotrophs

***PmoA* primers**	**Sequence (5` – 3`)**	**Target Group**	**Reference**
A189 F/Mb601 R	GGN GAC TGG GAC TTC TGG / ACR TAG TGG TAA CCT TGY AA	*Methylobacter/Methylosarcina* group	(Holmes et al., 1995; Kolb *et al.*, 2003; Yun *et al.*, 2012)
A189 F/Mc468 R	GGN GAC TGG GAC TTC TGG / GCS GTG AAC AGG TAG CTG CC	*Methylococcus* group	(Kolb *et al.*, 2003; Yun *et al.*, 2012)
A189 F/Mcap630	GGN GAC TGG GAC TTC TGG / CTC GAC GAT GCG GAG ATA TT	*Methylocapsa*	(Kolb *et al.*, 2003; Yun *et al.*, 2012)
A189 F/Mb661 R	GGN GAC TGG GAC TTC TGG / GGT AAR GAC GTT GCN CCG G	*Methylobacter/Methylosarcina, Methylococcus, Methylosinus* group, *Methylocapsa and Nitrosococcus*	(Costello et al., 1999; Kolb *et al.*, 2003; Yun *et al.*, 2012)
II223 F/II646 R	CGT CGT ATG TGG CCG AC / CGT GCC GCG CTC GAC CAT GYG	*Methylosinus* group	(Kolb *et al.*, 2003; Yun *et al.*, 2012)
A189F/A682R	GGN GAC TGG GAC TTC TGG / GAA SGC NGA GAA GAA SGC	*pmoA* methanotrophs	(Murrell *et al.*, 1998; Horz *et al.*, 2001; McDonald *et al.*, 2008)

Table 2.2 Phylogenetic probes (16S rRNA) targeting methanotrophs

16S rRNA Primer	**Sequence (5` – 3`)**	**Target Group**	**Reference**
MethT1dF/ MethT1bR/ MethT1cR	CCT TCG GGM GCY GAC GAG T / GAT TCY MTG SAT GTC AAG G / ATC CAA TCG AGT TCC CAG GTT AAG CCC	*Methylomonas* *Methylobacter* *Methylomicrobium* *Methylococcus*	(Wise *et al.*, 1999)
MethT2R	CATCTCTGRCSAYCATAC CGG	*Methylocystis* *Methylosinus*	(Wise *et al.*, 1999)
Type IF	ATG CTT AAC ACA TGC AAG TCG AAC G	Type I methanotrophs	(Chen *et al.*, 2007)
Type IR	CCA CTG GTG TTC CTT CMG AT	Type I methanotrophs	(Chen *et al.*, 2007)
Type IIF	GGG AMG ATA ATG ACG GTA CCW GGA	Type II methanotrophs	(Chen *et al.*, 2007)
Type IIR	GTC AAR AGC TGG TAA GGT TC	Type II methanotrophs	(Chen *et al.*, 2007)
Am445	CTTATCCAGGTACCGTCA TTATCGTCCC	Type II methanotrophs	(Gulledge *et al.*, 2001)
Gm633	AGTTACCCAGTATCAAA TGC	Methylobacter and Methylomicrobium	(Gulledge *et al.*, 2001)
Gm705	CTGGTGTTCCTTCAGATC	Type I methanotrophs except *Methylocaldum*	(Gulledge *et al.*, 2001)
Mlc1436	CCCTCCTTGCGGTTAGAC TACCTA	*Methylococcus*	(Gulledge *et al.*, 2001)
Mcd77	GCCACCCACCGGTTACC CGGC	*Methylocaldum*	(Gulledge *et al.*, 2001)

T-RFLP is a novel technique used for profiling of microorganisms based on restriction site close to a florescent labelled end of an amplified conserved sequence. Mostly *pmoA* based primers were used for methanotroph diversity analysis and in addition the

construction of clone libraries helped to interpret the T-RFLP profile by in silico digestion of cloned sequences thus providing diversity data of the sample (Ho *et al.*, 2011; Henneberger *et al.*, 2011). Terminal-RFLP is considered advantageous over DGGE technique.

Both functional (pmoA) and phylogenetic (16SrRNA) primers will be used in this study. Type I and Type II 16SrRNA probes will be used to identify all the major groups belonging to type I and type II family. Functional primers will be used mainly to identify genera within the type I and type II family. For instance, MCAP assay will be used to identify all the methanotrophs belonging to *Methylocapsa* group (type II)); MBAC for *Methylobacter*/*Methylomonas/Methylosarcina* group (type I); MCOC for *Methylococcus* group (type I); II 223F/646R for *Methylosinus* group (type II)

2.6 Biofilters

A biofilter is an engineered ecosystem where the methanotrophs seeded on the carriermaterial biologically convert the CH_4 to biomass and CO_2 in the presence of O_2. Biofilters have been extensively used and studied to mitigate CH_4 gas emissions from landfills. Recently, CH_4 emissions from anaerobic dairy ponds have also been successfully treated using biofilters. The biofilter material is the main engine that drives the CH_4 oxidation process. Different types of biofilters used for mitigating CH_4 emissions from landfills, anaerobic dairy ponds, wastes and coal mines are discussed below.

2.6.1 Biofilter matєrial

In Scheutz *et al.* (2009) review, different biofilter bed materials were identified viz. glass beads, perlite, compost made up of leaves, peat, woodchips and other green waste. Use of earthworm casts as a biofilter bed material was studied by Moon *et al.* (2010). At optimal moisture content of 40 % and 25 $^{\circ}$C temperature; a biofilter comprised of earthworm casts plus soil mixture (3:7) achieved 80% more CH_4 removal (highest CH_4 oxidation rate 17.9 g m^{-3} h^{-1}) compared to the paddy soil without earthworm cast. The 16S

rRNA gene T-RFLP analysis was used to show that both groups of methanotrophs-type I (majorly Methylocaldum) and type II (majorly Methylocystis) were present in both the soils. These results give an indication that addition of earthworm cast elevates methanotroph activity by releasing nutrients during compost decomposition and by enhancing diffusion of gases (CH_4 and CO_2). Pratt *et al.* (2012c) has reported a much higher CH_4 oxidation rate (27.3 g CH_4 m^{-3} h^{-1}) during the floating biofilter cover laboratory experiments with volcanic pumice soil as a filter material. Volcanic pumice soils have high porosity (77%) and low bulk density (545 kg m^{-3}), which appear to provide favourable biofilter physical properties for effective CH_4 oxidation.

Though many authors have demonstrated high CH_4 oxidation rates from compost, N_2O emissions were not monitored during these experiments. It is worth noting that while additions of compost might elevate CH_4 removal rates, they can also produce N_2O, which has 14 times greater global warming potential than CH_4. During the floating biofilter laboratory studies with compost as biofilter material, Pratt *et al.* (2012c) found that one of the biofilter made up of compost produced about 19.9 mg N_2O $m^{-3}h^{-1}$. This is very little compared to the amount of CH_4 oxidised by that filter. In another experiment using columns of pasture soils as a biofilter material, about 1.94 mg N_2O m^{-3} h^{-1} was produced when the CH_4 influx was increased to 24 g m^{-3} h^{-1} (Pratt *et al.*, 2012a). This indicates that surplus availability of CH_4 could have encouraged ammonia oxidising and denitrifying bacteria present in the compost and pasture soils to co-metabolise NO_3^- / NH_4^+ along with CH_4 to produce N_2O gas. This characteristic of ammonia oxidising and denitrifying bacteria has been reported by Hanson and Hanson (1996). No N_2O emissions were reported from biofilters made up of volcanic pumice soil by Pratt *et al.* (2012a, b, c), suggesting that N_2O production is related mainly to the presence of other bacterial community members and the nutrient composition of the biofilter bed material.

Ideal biofilter characteristics for effective CH_4 oxidation are:

- High porosity
- High water holding capacity
- Large surface area
- Low bulk density
- Supports growth and activity of methanotrophs

- Cost effective
- Long life with no/little maintenance

2.6.2 Landfill methane gas mitigation

Ruo *et al.* (2012a) developed a waste biocover soil (WBS) made up of soil and municipal sewage waste (MSW), packed in a 40 cm high biofilter column weighing 180 kg. Oxygen was passively diffused from the top and CH_4 was passed through the biofilter with a moisture content set at 45 wt. %. They tested the activity of the waste cover soil for 100 days and the highest removal efficiency achieved was 94-96 % and the bottom layers were doing most of the oxidation. Biofilter soil made up of yard waste compost and MSW landfill topsoil, when fed with 40 mL/min of landfill gas (CH_4 and CO_2 1:1 v/v) oxidised 100% of the CH_4 and the highest CH_4 oxidation capacity reached was 31.34 mol m^{-3} d^{-1}, when O_2 was supplied via passive air diffusion system (PADS) (Zifang *et al.*, 2012). Pratt *et al.* (2012b) tested the activity of methanotrophs in top and sub soil layers of a volcanic pumice soil from the Taupo landfill, New Zealand. The top soil exhibited highest CH_4 oxidation rate up to 24 g CH_4 m^{-3} h^{-1}. Their experiment demonstrated that volcanic pumice soil has a high surface area and excellent gas diffusion properties which are essential parameters for high CH_4 oxidation rates.

Methane oxidation efficiencies of the passively aerated biocovers constructed at the middle of capped area of St-Nicephore landfill in Quebec, Canada were evaluated by Capanema and Cabral (2012). Landfill gas collected from the wells was fed to a passive CH_4 oxidation biocovers (PMOB) of 2.75 m (W) x 9.75 m (L) x 1.2 m (D) made up of coarse sand and compost (1:5 ratio). High CH_4 removal efficiencies of about 92% were obtained at high CH_4 inlet loads of 818 g CH_4 m^{-2} d^{-1}. During cold temperatures removal efficiency of the PMOB dropped to 45.5% (371.3 gCH_4 m^{-2} d^{-1}). This demonstrates that biofilters can offer mitigation solutions for treating not only low loads but also high loads of CH_4 coming from large and young landfills.

Laboratory biofilter studies by Haubrichs and Widmann (2006) in a stainless steel column (1.5 m height and 0.5 m diameter) filled with 167 L of compost materials demonstrated 96% removal efficiency when fed at a CH_4 loading rate of 28 g m^{-3} h^{-1}. They had noticed

the formation of exopolymeric substances (EPS) in the compost biofilter at the end of 100 day of the experiment, but EPS formation didn't have any significant effect on biofilter performance by the end of the trial (222 days). Long term effects of EPS on biofilter performance needs to be assessed as EPS formation hinders the gas transport properties of the biofilter by creating micro anaerobic conditions for the methanotrophs around the biofilm (Scheutz *et al.*, 2009).

The influence of inlet load on CH_4 removal efficiency of the inorganic biofilter was studied by Nikiema *et al.* (2009a). An Inorganic biofilter was made up of gravel material with an average cylindrical length of 5-6 mm and a void fraction of 40%. The biofilter had a height of 1m and a volume of 0.018 m^3. A methane elimination efficiency of 38% was achieved, when an inlet load of 95 g m^{-3} h^{-1} of CH_4 was passed through the biofilter. In another experimental study, Nikiema and Michele (2009b) studied the influence of different gas flow rates (1-5.5 L/min) on the CH_4 removal efficiencies of the biofilter. Greater than 90% efficiencies were achieved at gas flow rates of <2 L/min and CH_4 loading rates of < 55 g m^{-3} h^{-1}. When the gas flow rate was set to more than 3 L/min, a decrease in biofilter performance was noted. This might have been caused by a shorter contact time between the CH_4 gas molecules and the methanotrophs in the biofilter. Based on their results, the maximum volumetric CH_4 load for optimal biofilters CH_4 removal capacity achieved by their biofilter was 0.075 m^3 (CH_4) m^{-3} (biofilter) h^{-1}.

2.6.3 Biofilters for waste treatment

Methanotrophs are unique proteobacteria which can co-degrade pollutants along with the CH_4 (Hanson and Hanson, 1996; Duan, 2012; Menard *et al.*, 2012). Both forms of methane monooxygenase enzyme- pMMO and sMMO are capable of co-metabolising a range of substrates, but sMMO has the broader substrate range compared to pMMO. The latter is known to have lower substrate specificity.

Enzyme sMMO can oxidise alkanes up to C-8, ethers, cyclic alkanes and aromatic hydrocarbons; whereas pMMO can oxidise only up to C-5 alkanes. This characteristic of methanotrophs has been used to degrade pollutants like halogenated alkenes e.g. trichloroethylene (TCE). However, oxidation of trichloroethylene negatively affects the

CH_4 oxidation process, due to substrate binding competition to MMO and formation of toxic products like epoxide (Semrau *et al.*, 2010). Kuo *et al.* (2012) investigated the bioremediation characteristics of methanotrophs to degrade trichloroethylene pollutant in contaminated ground water during a 140-day pilot scale study. TCE concentrations were reduced from 210 μ g L^{-1} to 18 μ g L^{-1}, when the contaminated water was passed through the inoculum of methanotrophs immobilised on the bio sparger. Molecular analysis revealed the involvement of both types (I & II) of methanotrophs in TCE degradation. This study shows that methanotrophs can be used for bioremediation of pollutants like TCE from contaminated ground water. Lee *et al.* (2006) studied the ability of *Methylosinus trichosporium* OB3b to degrade mixed pollutants (TCE, Trans dichloroethylene (t-DCE) and vinyl chloride (VC)) along with the CH_4. The hypothesis that sMMO could efficiently degrade TCE due to its broader specificity was proved wrong, when it was found that cells expressing pMMO can actually degrade more of these compounds at higher concentrations. This phenomenon is explained by the characteristic of pMMO, which has greater specificity for CH_4 compared to other substrates; pMMO expressing methanotrophs can degrade these compounds rather slowly but over a longer time frame without producing harmful products. This differs from sMMO-expressing methanotrophs, which are capable of rapid degradation of TCE but with the formation of toxic products.

As the degradation of hazardous compounds is carried out by the same enzyme which catalyses CH_4, important factor like competitive inhibition that controls the kinetics of growth and co-metabolism need to be understood in detail to help design an efficient co-metabolism process of biodegradation using methanotrophs (Limbri *et al.*, 2013).

2.6.4 Anaerobic dairy effluent methane mitigation

Though CH_4 emissions from manure management accounts for a considerable proportion of agriculture GHG emissions globally, only few studies have been carried out of CH_4 mitigation using biofilter technology on anaerobic dairy ponds. Girard *et al.* (2011) studied the efficiency of an inorganic biofilter by loading different low concentrations of CH_4 (0.16-2.8 g m^{-3}). For an inlet load of 38 g m^{-3} h^{-1}, maximum elimination capacity

obtained was 14.5 g m^{-3} h^{-1} (38% removal efficiency). Biogas emissions from New Zealand waste dairy ponds was first treated using a column biofilter by Pratt *et al.* (2012b). CH_4 emissions from a 4 m^2 section of dairy effluent pond were passed through 70L biofilter made up of the volcanic pumice top soil and perlite in 1:1 ratio for 16 months; CH_4 removal rates up to 16 g m^{-3} h^{-1} were obtained; this biofilter is still operating nearly after 5 years. A negligible effect from biogas H_2S on biofilter performance was found despite a low pH and H_2SO_4 accumulation at the base of the biofilter at the trial's conclusion. Interestingly they also found an increase in N content during the trial period, suggesting ammonia capture and/or N_2 fixation characteristic of the methanotrophs from the atmosphere and the biogas. Although biofilters offer an effective mitigation option for treating CH_4 emissions from average sized dairy ponds and landfills, the scale up cost and the process of collecting the CH_4 from the pond using piping's and other materials makes this approach costly. To offset 720 g h^{-1} CH_4 emissions from a typical 1000 m^2 dairy effluent pond, a 50 m^3 biofilter column would be needed as reported by Pratt *et al.* (2012b)

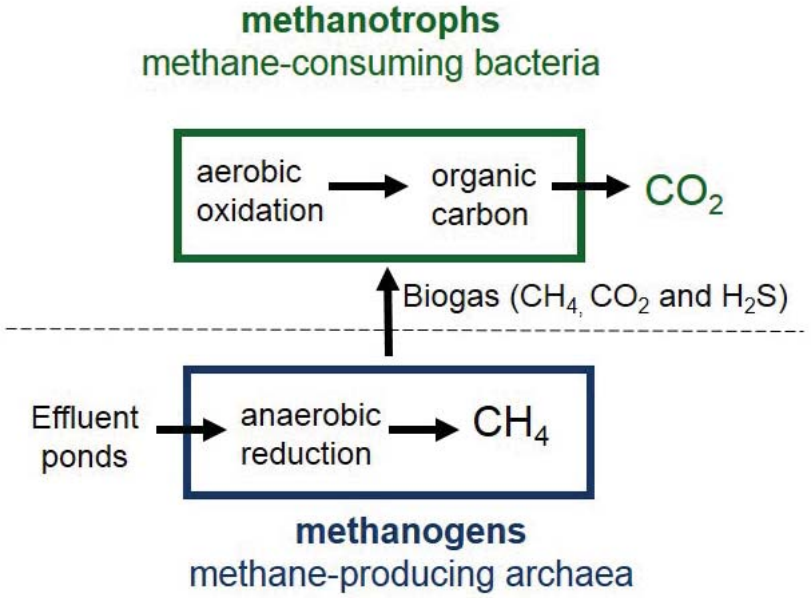


Figure 2.3 Methane production from the pond, and methane mitigation from the above (using biofilters)

Accordingly, to develop a robust, low cost biofilter, Pratt *et al.* (2012c) have designed a floating biofilter that can sit on top of the effluent pond. In a laboratory study effluent

pond conditions were simulated in a 50 L container and 95% methane oxidation was achieved when CH_4 was passed through a biofilter (5 cm thick with 8 L volume) (Pratt *et al.*, 2012c). During the third month of the experiment, the CH_4 inlet load was doubled to 27 g m^{-3} h^{-1}, interestingly methanotrophs sustained these rates and oxidised more than 94% of the CH_4; establishing the fact that methanotrophs can adapt quickly to high CH_4 fluxes. Long term performance of this prototype in the laboratory and on-field is now being tested. However, in the Pratt *et al.* (2012b, c) studies knowledge of the methanotrophic population dynamics in the biofilter was limited. These studies have established the high CH_4 removal properties of the biofilter. Understanding the engine part, mainly looking at the active methanotrophs responsible for high CH_4 removal activity is essential for developing an efficient biofiltration system.

2.7 Conclusions

As methanotrophic proteobacteria are highly diverse, their population dynamics and ecology are poorly understood. Several culture independent techniques have led to the identification of novel uncultured methanotrophs. Identification or selection of dominant groups or species of methanotrophs is needed to use them as inoculum in the biofilter.

The volcanic pumice soil isolated from Taupo Landfill has been found to mitigate CH_4 emissions from New Zealand dairy effluent ponds. However, the knowledge about the methanotrophs involved in oxidising CH_4 is limited. After reviewing the literature (Chapter 2) the knowledge gaps which might limit the development of an efficient biofilter identified were: (i) understanding the effect of acidification on column biofilter performance and methanotroph population dynamics; (ii) Can floating biofilter prototype be resilient to changing weather conditions and efficiently mitigate CH_4 from a dairy effluent pond?; (iii) Exploring the relationship between CH_4 removal and other physico-chemical and biological factors like moisture, pH, C and N, and methanotroph abundance and diversity; (iv) Could other cheaply and widely available materials be used as a biofilter media, by introducing active methanotrophs population and priming them with CH_4?; (v) Could addition of nutrients enhance the CH_4 oxidation and methanotrophs abundance

Chapter 3

Materials and Methods

In this study, the biofilter materials were assessed by analyzing the physical, chemical and biological parameters controlling CH_4 removal. The moisture content, pH, total C and N, nitrate and ammonium – N, microbial biomass C and N were among the parameters measured. The ability to remove CH_4 was analyzed by collecting gas samples for gas chromatography to quantify CH_4 concentrations. Ammonia and hydrogen sulphide were not measured as they were not the objective of this thesis, however increase in total N was measured and reported. The DNA was extracted from the biofilter media and molecular techniques like PCR, qPCR and T-RFLP were used to study abundance and diversity of methanotrophs (type I, type II and various genera within) involved in aerobic CH_4 oxidation. Molecular cloning was used to prepare clone library of conserved *pmoA* sequences and was helpful in identifying novel species or strains of methanotrophs. The methodology used in details is described in below sub-headings

3.1 Gas chromatography

Gas samples were collected in gas tight vials and were analysed in the laboratory for CH_4, N_2O, and CO_2 concentrations by gas chromatography (GC) (Varian CP-3800) using flame ionisation (FID), thermal conductivity (TCD), and electron capture (ECD) detectors, respectively. A method file was built using the GC software for incorporating CH_4, CO_2 and N_2O standards concentrations ranging from 0 to 6 000 000 ppmv, 0 to 200 000 ppmv, and 0 to 2000 ppbv, respectively.

3.2 Moisture content

Soil moisture plays an important role in controlling CH_4 oxidation and activity of methanotrophs. Even though methanotrophs produces water vapour during CH_4

oxidation, it is important to keep the soil moist for effective nutrient and gas transfer. Moisture content was determined by oven drying at 105 °C for 15–18 h. Moisture content (% dry wt) was calculated as the percentage of water before and after drying using the below formula

$$Gravimetric\ water\ content\ (\%\ dry\ wt) = weight\ of\ moisture \times 100\ \div weight\ of\ oven\ dry\ soil$$

3.3 pH

The pH plays an important role in influencing the chemical and biological process. Previous studies indicated that the methanotrophs live comfortably at slightly acidic pH (~ 5.5). Soil pH was measured by suspending the air dried soil in the water at a ratio of 1:10 (Blakemore *et al.*, 1987). The beaker with the suspension was then stirred vigorously with a mechanical stirrer, covered overnight with a loose plastic (to minimise evaporation losses) and measured next day at the interphase of soil and water using a pH meter after fresh calibration with buffers (4, 7 and 10).

3.4 Microbial biomass carbon and nitrogen

Procedures described by Cabrera and Beare (1993) and Vance et al. (1987) were followed for microbial biomass C and N analysis. Briefly, chloroform fumigated and non-fumigated portions (equivalent to 5g dry wt) of the soil were extracted using 0.5M K_2SO_4 and analysed using a TOC (total organic carbon) analyser. The difference between the C content of fumigated and non-fumigated is measured as extractable C flush. Microbial biomass C was then estimated by multiplying the C flush by a factor k_{EC} of 0.41, which represents the extraction efficiency of microbial biomass C (MBC). For microbial biomass N (MBN), the 0.5M K_2SO_4 extract (from MBC) was further treated with alkaline persulphate solution, autoclaved, and the oxidisable N flush measured using a flow injection analyser (FIA) and prepared standards. A k_N factor of 0.45 was used to calculate microbial N. Both MBC and N Values are expressed in mg C kg^{-1} or N kg^{-1} of soil.

3.5 Total Carbon (C) and Total Nitrogen (N)

Total C and N were measured (%) by sieving the air dried soils to <2 mm and combusting in a FF-2000 CNS analyser (LECO Corporation, St Joseph, MI, USA).

3.6 Nitrate and Ammonium N

Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 2 M KCl using a 1:10 material: extractant ratio and a 1 hour end-over-end shaker followed by filtration, as described by Blakemore *et al.* (1987)

3.7 DNA extraction and PCR

Soil samples were extracted in duplicate using a Mobio™ Powersoil DNA extraction kit (Mobio Laboratories, USA) according to the manufacturer's instructions. About, 0.25 gm of soil was added to the bead tube containing proprietary buffer (supplied by the manufacturer) and vigorously vortexed for 10–12 minutes to break the bacterial cells. Cleaning reagents were then added according to the protocol, to extract and purify DNA. Extracted DNA was later quantified (OD at 260/280 nm) using a UV Spectrophotometer (Nanodrop™).

Functional primers, *pmoA* (particulate methane monooxygenase gene sub-unit A) designed by Kolb et al. (2003) MBAC (A189F/Mb601R) – targeting *Methylobacter* and *Methylosarcina*, MCOC (A189F/Mc468R) – *Methylococcus*, MCAP (A189F/Mcap630R) – *Methylocapsa*, type II *pmo*A (II646F/II223R) – *Methylosinus and Methylocystis*; and Chen et al. (2007) 16S rRNA primers – Type IF/IR – targeting type I methanotroph (*Methylobacter*, *Methylosarcina*, *Methylococcus*, *Methylocaldum*, *Methylomicrobium*, *Methylomonas*, *Methylosphaera*, and unclassified *Methylococcales*) and Type IIF/IIR – type II methanotroph (*Methylocystis*, *Methylosinus*, *Methylocella*, *Methylocapsa*, and unclassified *Methylocystaceae)* were used to amplify conserved sequences of methanotroph community (Table 3.1).

Table 3.1 Primers used in this study to target different groups and genera of methanotrophs

Primers	**Assay**	**Target group/genera**	**Reference**
A189F (GGNGACTGGGACTTCTGG) Mb661R (GGTAARGACGTTGCNCCGG)	MTOT pmoA	Type I & II methanotrophs (except *Methylomonas* and *Methylocaldum*) (along with few *Nitrosococcus* Species)	Kolb *et al.* (2003)
II223F (CGTCGTATGTGGCCGAC) II646R (CGTGCCGCGCTCGACCATGYG)	Type II pmoA	*Methylosinus* group	Kolb et al. (2003)
A189F (GGNGACTGGGACTTCTGG) Mb601R (ACRTAGTGGTAACCT TGYAA)	MBAC pmoA	*Methylobacter* and *Methylosarcina* group	Kolb et al. (2003)
A189F (GGNGACTGGGACTTCTGG) Mc468R (GCSGTGAACAGGTAGCTGCC)	MCOC pmoA	*Methylococcus* group	Kolb et al. (2003)
A189F (GGNGACTGGGACTTCTGG) Mcap630R (CTCGACGATGCGGAGATATT)	MCAP pmoA	*Methylocapsa*	Kolb et al. (2003)
Type IF (ATGCTTAACACATGCAAGTCGAACG) Type IR (CCACTGGTGTTCCTTCMGAT)	Type I 16SrRNA	Type I methanotroph (*Methylobacter*, *Methylosarcina*, *Methylococcus*, *Methylocaldum*, *Methylomicrobium*, *Methylomonas*, *Methylosphaera* and unclassified *Methylococcales*)	Chen *et al.* (2007)
Type IIF (GGGAMGATAATGACGGTACCWGGA) Type IIR (GTCAARAGCTGGTAAGGTTC)	Type II 16SrRNA	Type II methanotroph (*Methylocystis*, *Methylosinus*, *Methylocella*, *Methylocapsa* and unclassified *Methylocystaceae*)	Chen *et al.* (2007)

Reaction conditions were modified and standardised to suit this study. PCR was carried out in 40 µL reaction volumes using a thermocycler (MaxyGene™). Reaction mixtures were prepared as follows: 20 µL of 100 nM one taq™ master mix (New England Biolabs, UK), 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 2 µL of target DNA (diluted 1:25 with sterile PCR-grade water), and sterile water were added to adjust the volume to 40 µL. The thermal profile consisted of 40 cycles of denaturation at 95 °C for 25s, annealing at assay-specific temperature (MBAC, MCOC, MCAP, type II *pmoA*, type I 16SrRNA, and type II 16SrRNA at 54, 58, 50, 63, 60, and 60 °C, respectively) for 30s and elongation at 72 °C for 45s.

3.8 Quantitative PCR

Quantitative PCR standards were prepared by cloning purified, assay-specific amplified genes (from the volcanic pumice soil) into the *E. coli* host using TOPO® TA cloning® kit (Invitrogen Life Technologies), following manufacturer's instructions.

Table 3.2 Thermal reaction conditions of qPCR assays used in this study.

Primer Assay	**Annealing temperature (°C)**	**Data acquisition temperature (°C)**	**Product size (bp)**
MTOT	69	69	530
MBAC	58	82	432
MCOC	58	82	299
MCAP	55	82	461
Type II pmoA	69	82	444
Type I 16SrRNA	65	65	673
Type II 16SrRNA	65	65	525

Assay-specific amplified portions of plasmid DNA were sequenced and were BLAST searched to validate against the publicly available nucleotide databases of methanotrophs at NCBI. Plasmids containing gene of interest were quantified and serially diluted from 1/100 to 1/1000000 using sterile PCR grade water and were then used as standards (Figure 3.1). Each qPCR reaction volume (10 µL) consisted of 5 µL ssofast™ qPCR master mix, 0.6 µL each of 10 nM forward and reverse primers, 1 µL of sample DNA, and sterile PCR grade water made up to final volume of 10 µL. Sample DNA were diluted 1/25 times to reduce the effect of inhibitors in the sample. Assay-specific standards (for calibration curves) and negative controls were run along with the samples. Reactions in duplicates were carried out in a Roche Light cycler 480™ machine with the following thermal profile: initial denaturation at 94 °C for 15s; 40 cycles of denaturation at 95°C for 5s; annealing at assay-specific temperature (MBAC, MCOC, MCAP, type II *pmoA*, type I 16SrRNA and type II 16SrRNA –58, 58, 55, 69, 65, and 65 °C, respectively) for 25 s; and data acquisition at 82 °C (16S rRNA type I & II at 65 °C) for 4s (Table 3.2).

Melt curve analysis was done post-qPCR by acquiring fluorescence data by continuous melting of samples from 65 °C to 95 °C for 30s. Formation of assay specific product size was verified by gel electrophoresis on a 2% w/v agarose gel in 1×TBE buffer and stained with SyberSafe gel stain and visualized under UV light. Gene copy numbers of the samples were calculated by plotting linear regressions of crossing point (C_p) values and logarithmic gene copy number values of the standards (calculations adapted from Lee *et al.* (2008)). The r^2 values of the plots ranged from 0.9743 to 0.9999. Melting curve analysis was done at the end of the qPCR cycle to confirm gene specific amplification. One peak indicates amplification of one product (Figure 3.2). Melt curve analysis temperature ranged from 65 °C to 95 °C with a holding time of 1s at each 1 °C increment of temperature. A negative control (sterile water instead of DNA) was also run along with the standards on every plate. Post qPCR run, gene specific qPCR amplification was also reconfirmed by running the samples on 2% TBE gel. An example post-run gel is shown in the Figure 3.2b.

A

Results

Inc	Pos	Name	Type	CP	Concentration	Standard	Status
☑	A1	P1_IFR	Standard	5.00	1.01E-2	1.00E-2	<
☑	A2	P1_IFR	Standard	5.00	1.01E-2	1.00E-2	<
☑	A3	P2_IFR	Standard	12.21	9.94E-4	1.00E-3	
☑	A4	P2_IFR	Standard	12.24	9.87E-4	1.00E-3	
☑	A5	P3_IFR	Standard	19.41	9.88E-5	1.00E-4	
☑	A6	P3_IFR	Standard	19.33	1.01E-4	1.00E-4	
☑	A7	P4_IFR	Standard	26.41	1.05E-5	1.00E-5	
☑	A8	P4_IFR	Standard	26.66	9.62E-6	1.00E-5	
☑	A9	P5_IFR	Standard	35.00	6.63E-7	1.00E-6	
☑	A10	P5_IFR	Standard	32.74	1.37E-6	1.00E-6	
☑	A11	Water	Negative Control	36.64	3.92E-7		
☑	A12	Water	Negative Control	40.00	1.33E-7		>
☑	B1	Sample_IFR	Unknown	20.32	7.37E-5		
☑	B2	Sample_IFR	Unknown	20.41	7.17E-5		

< - Early Cp call (first five cycles) has higher uncertainty, > - Late Cp call (last five cycles) has higher uncertainty

B

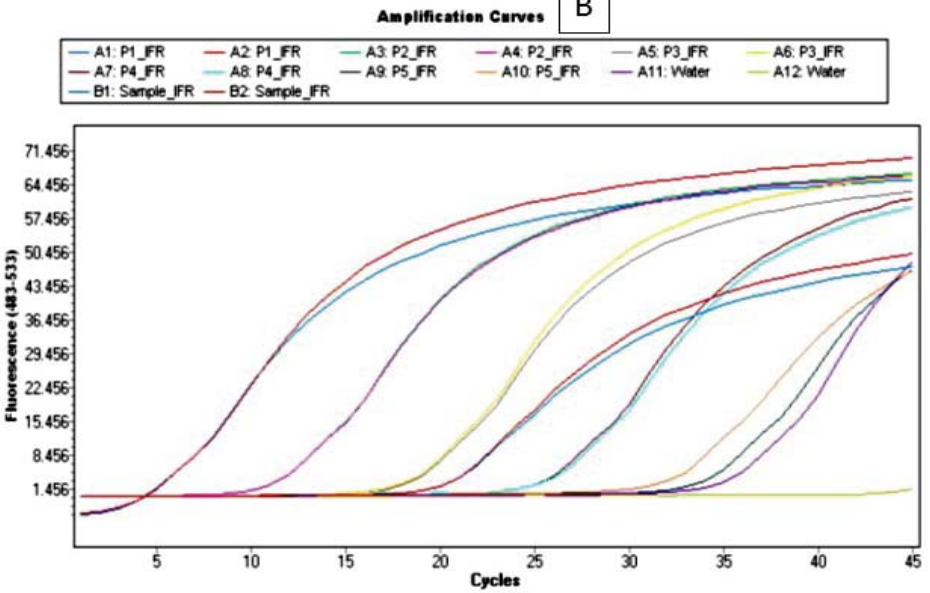


Figure 3.1 Example showing the crossing point values and the amplification curves for Type I 16S rRNA standards (ranging from 10^{-2} to 10^{-6} dilutions)

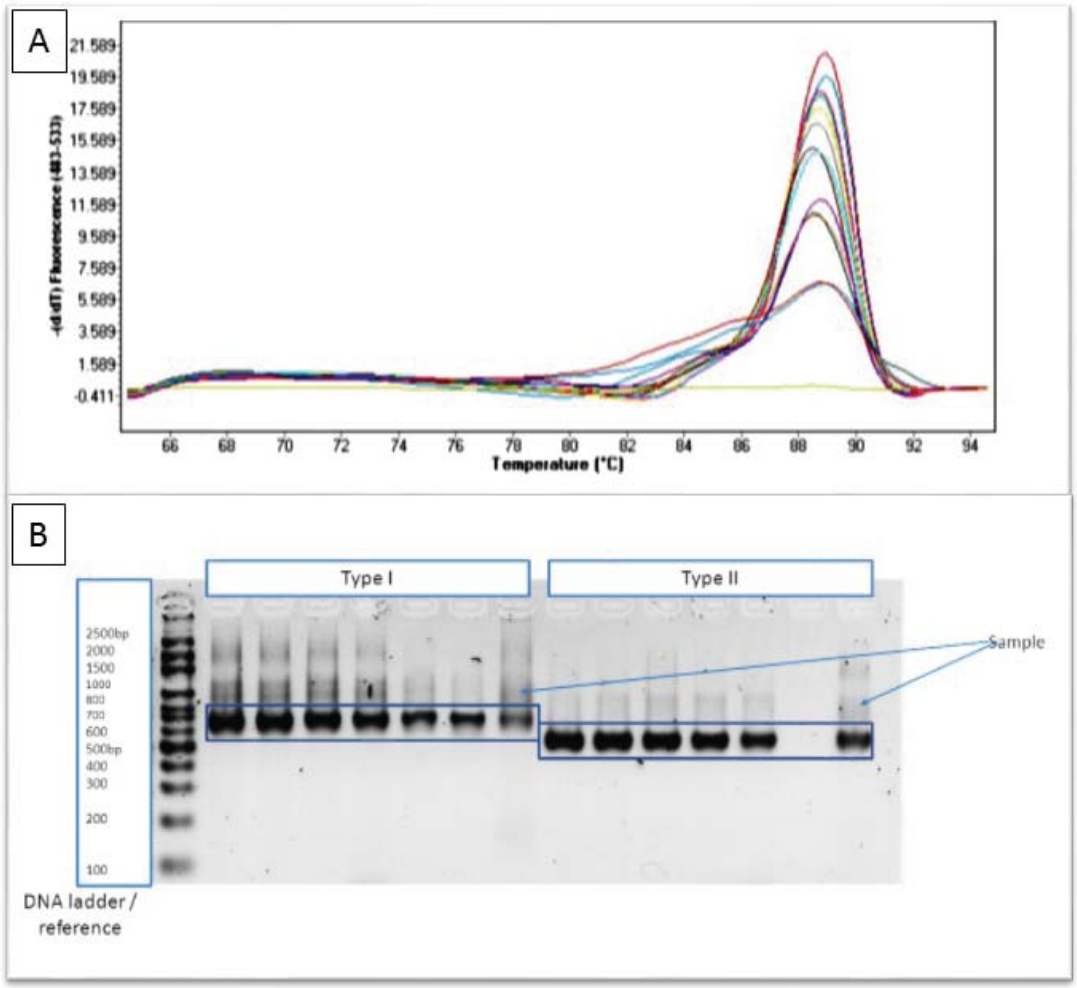


Figure 3.2 Post-qPCR run analysis: (a) Melting curves and (b**)** 2% TBE gel. This was done to confirm specific amplification of target genes during the qPCR run

Gene copy number is measured based on the "crossing point (C_p)" values (Figure 3.1). Crossing point is the point where the fluorescence signal rises above the background fluorescence during the amplification of the target gene. The lower the $C_{p,}$ the higher the target DNA amount in the sample. Gene copy number of the sample DNA is analysed by plotting crossing point values against log gene copy numbers of the standards (Figure 3.3). Gene copy number for the standards is calculated based on the below formula.

$$Gene\ copy\ number = \frac{6.023\times10^{23}\ (copies\ mol^{-1})\times amount\ of\ DNA\ (ng)}{DNA\ length\ (bp)\times(1\times10^{9})\times650\ (ng\ mol^{-1}bp^{-1})}$$

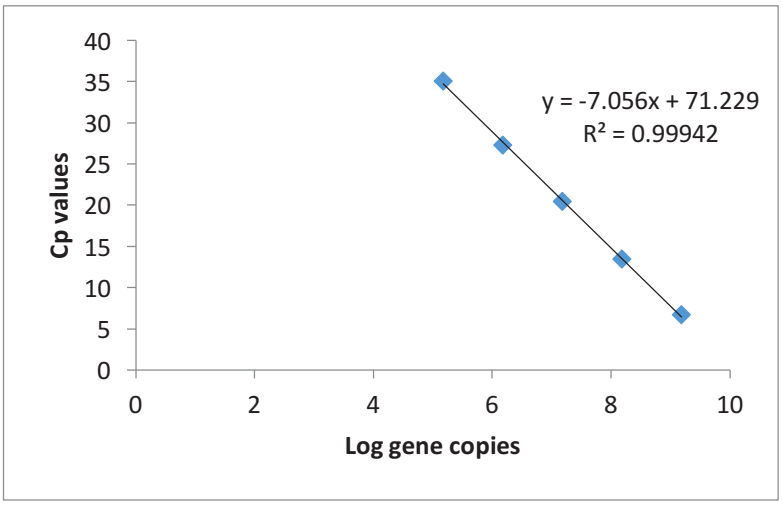


Figure 3.3 Generating standard curves by plotting log gene copy numbers and crossing point values.

Gene copy number for a given sample DNA is calculated using the linear regression plot of Cp values and log gene copy numbers of the standards.

Number of gene copies per "sample" is calculated from the above slope as $= \mathbf{10^{(Cp - b)/m,}}$ where C_p= crossing point of the DNA sample; b and m = y-intercept and slope of the line, respectively.

3.9 Terminal-RFLP

FAM (6-carboxy-flourescein) labelled primer A189F and unlabelled primer Mb661R were used to generate PCR products targeting both type I and type II methanotroph for T-RFLP analysis. Reaction conditions were similar to those described above except with annealing at 50 °C. PCR products were digested for 4 h at 37 °C with 2.5U of *MspI* restriction enzyme in 40-ul reaction volumes. A PCR purification kit was used to purify the digested products and 1–2 µL of the products were analysed by ABI genetic analyser (Genotyping performed by Massey Genome Service, New Zealand). Length and abundance of the terminal restriction fragments (T-RF's) were calculated using Peak

Scanner and the data were standardised according to Dunbar et al. (2000). Shannon's diversity index (H`) and evenness (E_H) were calculated using the formulae below (Equations 1 and 2). As the H values indicate changes in the diversity. Evenness assumes a value between 0 and 1, with 1 being complete evenness:

$$H' = -\sum_{i=1}^{n} \text{Pi Ln(Pi)} \qquad \text{(Eq 1)}$$

$$E_H = \text{H/H}_{max} = {}^{\text{H}}/_{\text{LnS}} \qquad \text{(Eq 2)}$$

where Pi = Relative abundance of each phylotype with respect to total number of phylotypes in a sample, and S = Phylotype richness, i.e. sum number of phylotypes in a sample.

3.10 Cloning, sequencing and phylogenetic analysis

PmoA genes amplified using A189F and Mb661R were purified by gel extraction and cloned using TOPO® TA cloning® kit (Invitrogen Life Technologies) following manufacturer's instructions. The procedure involved ligation of amplified gene into a TOPO™ TA vector and the recombinant vector was then transformed into *E. coli* cells by heat shock. The cells were then plated on media with antibiotics and incubated at 37 °C overnight to allow formation of colonies. Few colonies are randomly selected and colony PCR was done using M13 primers to identify the presence of target gene in the clones. Positive clones were then inoculated into sterile LB media flasks and grown overnight to produce several copies of the cells containing gene of interest.

Cells were then harvested and the plasmid was extracted using a commercial Kit (DNA and plasmid purification kit, MN™). Plasmids containing gene of interest were quantified using a UV- Spectrophotometer (Nanodrop™). Extracted plasmids were subjected to sequencing of the gene of interest using vector specific M13 primers (sequencing was performed by Massey Genome Service, Massey University, New Zealand). The identities of the *pmo*A gene sequences were confirmed by BLAST searching the publicly available

nucleotide databases with NCBI. Phylogenetic neighbour joining tree analysis of the clones was performed using the MEGA 7 software package. Sequences obtained from the studies were deposited at the NCBI nucleotide sequence database (accession numbers: KT424049 – KT424060; KU840813 – KU84082; KU215855 – KU215865). Details of the cloned gene sequences are attached in Appendix iv of this thesis.

Chapter 4

Does acidification of a soil biofilter compromises its methane oxidising capacity?

4.1 Introduction

To mitigate CH_4 emissions from dairy-farm effluent produced on an average-sized New Zealand dairy farm (300–500 cows), a soil-based biofilter containing a very active methanotroph community has been developed (Tate *et al.*, 2012; Pratt *et al.*, 2012b, 2013). Methane oxidising bacteria (MOB) or methanotrophs are the "engine" of this technology, and comprise a diverse group of aerobic gamma (type I) and alpha (type II) proteobacteria that are present naturally in soils where CH_4 is produced (Tate 2015). In the presence of O_2, CH_4 is converted to CO_2, water and biomass (Whittenbury *et al.*, 1970). Excess C from CH_4 consumption in some groups of methanotrophs is assimilated as polyhydroxyalkanoates (PHA) storage bodies inside the cell (Asenjo and Suk, 1986).

As CH_4 oxidation is mediated by methanotrophs, soil biofilter performance can be optimised through a better understanding of factors influencing the activity of these diverse groups of microorganisms. During restrained growth conditions, some methanotroph can form resting stages (spores or cysts), making them very resilient and able to become active again when conditions become more favourable. Based on physiological, biochemical, and morphological properties, methanotroph are mainly classified as type I and type II. Type I methanotroph are more diverse and include genera, viz., *Methylobacter*, *Methylomonas*, *Methylosarcina*, *Methylococcus*, *Methylomicrobium*, *Methylosphaera*, *Methylocaldum*, *Methylomarinum*, *Methylosoma*, and unclassified *Methylococcales*. *Methylocystis*, *Methylosinus*, *Methylocapsa*, *Methylopila*, and *Methylocella* form the genera under type II methanotroph (Hanson and

Hanson, 1996; Dedysh *et al.*, 2003; Ho *et al.*, 2011). The growth and activity of these different genera of methanotroph are optimal under different abiotic and biotic conditions (Knief *et al.*, 2003; Chang *et al.*, 2010; Henneberger *et al.*, 2011; Ruo *et al.*, 2012b). For this reason, there is a need for knowledge of the characteristics of these genera and how they respond to different conditions so they can be efficiently utilised to mitigate CH_4 emissions by soil.

Before the start of the experiment, very low CH_4 removal rates were evident in the soil biofilter previously studied by Pratt *et al.* (2012b) (data not shown). The low removal rates were probably either due to the drying out of the biofilter material (soil moisture 15 % gravimetric dry wt) or to the temporary disconnection of the CH_4 feed line 2–3 months before the experiment began. In addition, due to the presence of H_2S in the biogas, the soil had been further acidified to a pH of 3.72. In this study, the biofilter material was remoistened by moistening to 110 % gravimetric dry wt and gently mixing the soil before repacking to the same density in the column (Table 4.1). The focus of our current research was to determine how methanotroph abundance and diversity have influenced biofilter performance since it began operating about 5 years ago. The objectives were: (1) to assess the performance of the soil biofilter under acidic conditions, and whether reconstitution of biofilter soil material (with 110 % gravimetric dry wt) could allow the biofilter to regain its efficiency; and (2) to characterise methanotroph abundance and diversity using novel molecular techniques like quantitative PCR and T-RFLP.

4.2 Materials and Methods

4.2.1 Preparation of the biofilter

The biofilter was reconstructed by using the soil medium from the biofilter established by Pratt *et al.* (2012b) containing a 50/50 (v/v) mixture of volcanic pumice soil (Andisol) and perlite enriched with methanotroph, and acidified to pH 3.72 by the oxidation of H_2S produced from the dairy effluent pond over its 5-year use. This was done by gentle mixing and wetting the soil to about 110 % gravimetric dry wt (~ 60% water-holding capacity

(WHC)) (previously suggested by Pratt *et al.* (2012b)) and refilling the biofilter column (1 m high and 0.35 m in diameter) with 58 L of soil medium up to a height of 54 cm. The biofilter column had inlet port at the bottom, ten sample ports – spaced 5 cm apart down the side of the biofilter and an outlet port to facilitate gas sampling at various depths in the biofilter (Figure 4.1). The physico-chemical properties of the biofilter are from Pratt *et al.* (2012b) and are as follows – porosity (80%), bulk density (310 kg m^{-3}), total C(5 %) and total N (0.38 %). Moisture content (110 (% dry wt) was calculated in this study.

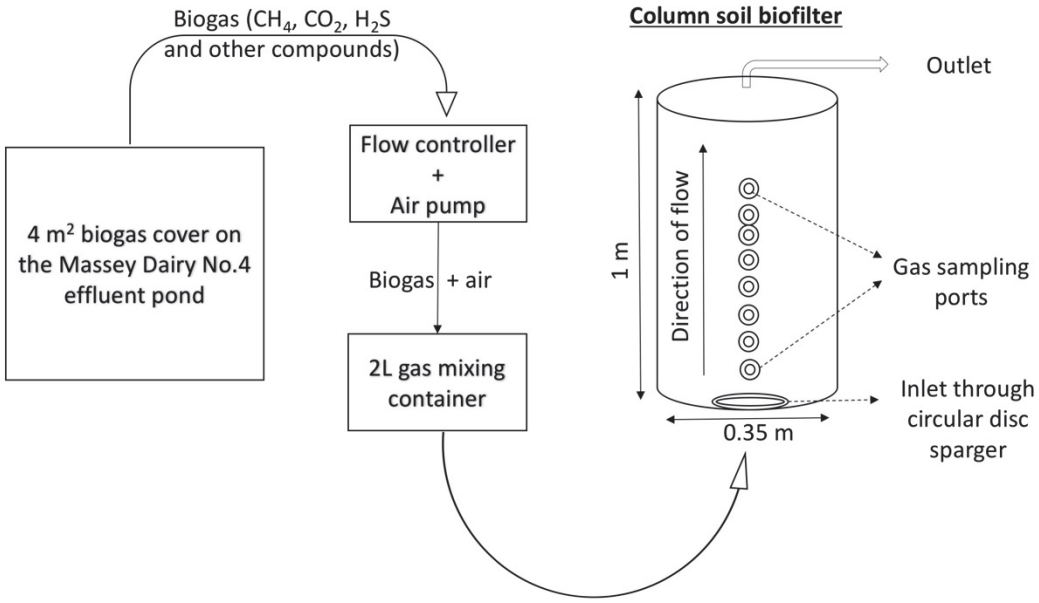


Figure 4.1 Schematic diagram showing biogas capture from the Massey Dairy No.4 effluent pond, and feeding it through a column biofilter filled with soil seeded with methanotrophs. Methane gets converted to CO_2 during the process.

4.2.2 Biofilter setup and experiment

The soil biofilter was sited beside Massey University No. 4 dairy farm effluent pond in Palmerston North, New Zealand. Air samples containing biogas (65% CH_4 (v/v), 25% CO_2 (v/v), and traces of H_2S and other unknown volatile compounds) were collected from a 4-m^2 section of the effluent pond. Considering the relatively small volume of the biofilter, about 10% of the biogas was fed to the biofilter via a flow controller that monitors the biogas flow rate and the temperature of the pond and biofilter was measured half hourly. During the 90-day study period, air was fed to the biofilter (flow rate of 1000

ml/min) to keep the CH_4/O_2 ratio at more than 1:3, to maximise CH_4 oxidation (Nikiema *et al.*, 2009a). Biofilter temperature (average 21 ^oC) was monitored every half hour throughout the study period using an automatic data logger. Methane oxidation measurements were conducted on 4 occasions (days 0, 10, 29, and 90). Soil samples were collected from the biofilter and analysed for pH, moisture content, and microbial biomass C and N, and soil DNA was extracted to determine gene copy numbers by qPCR. Sigmaplot (Version 12) was used for statistical analysis.

4.2.3 Gas samples

Gas samples were taken from the biofilter during this experimental study on days 0, 10, 29, and 90. Samples were collected in gas tight vials from the inlet, 10 ports along the height of the biofilter, and the outlet. The gas samples were analysed in the laboratory for CH_4, CO_2, and N_2O concentrations by gas chromatography (GC) as described in Chapter 3.1. A method file was built using the GC software for incorporating CH_4, CO_2 and N_2O standards concentrations ranging from 0 to 600 000 ppmv, 0 to 200 000 ppmv, and 0 to 2000 ppbv, respectively. For CH_4 – 500, 250 00, 500 00, 200 000 and 600 000 ppmv standards were used, with an r^2 value of 0.9994.

The CH_4 oxidation rate (g m^{-3} h^{-1}) was calculated from the difference in input and output CH_4 flow rate (g m^{-3} h^{-1}). The CH_4 flow rate at the outlet was calculated by considering the concentration of CH_4, the volume of the biofilter material (0.057 m^3), the density of CH_4 at 20 °C (0.667 g/ L), and the flow rate of air and CH_4, according to the following relationship:

$$CH_4\ (gm^{-3}h^{-1}) = \frac{CH_4\ concentration\ (ppm)}{1000000\ (ppm)} \times \frac{(air\ flow + CH_4\ inflow)\ L}{min} \times \frac{0.667\ g}{L} \times \frac{60\ min}{hr} \times \frac{1}{0.057\ m^3}$$

4.2.4 Soil samples

Using a 3 cm diameter soil auger, representative biofilter soil samples were taken aseptically (after the gas sampling) on day-0, 10, 29, and 90 down the full vertical length of the biofilter soil column. The auger was sterilised with 70% ethanol between samplings. Soil samples were stored at 4 $^{\circ}$C for further analysis but soil samples for DNA analysis were extracted immediately. The sample slots were refilled with volcanic pumice soil-perlite mixture of the same composition and labelled to avoid resampling. Soil moisture content (gravimetric), pH, microbial biomass C and N were determined as described in sections 3.2, 3.3 and 3.4 of Chapter 3 (Materials and Methods).

4.2.5 Molecular analysis

DNA was extracted from the soil samples as described in Chapter 3.7. Quantitative PCR analysis was done as described in Chapter 3.8. The r^2 values of the linear regressions plots of crossing point (C_p) values and logarithmic gene copy number values of the standards ranged from 0.9743 to 0.9999. Terminal-RFLP analysis was done as described in Chapter 3.9. Cloning, sequencing and phylogenetic analysis was done as described in Chapter 3.10. Sequences obtained in this study were deposited at the NCBI nucleotide sequence database (accession numbers: KT424049 – KT424060).

4.3 Results

4.3.1 Methane removal by the soil-biofilter

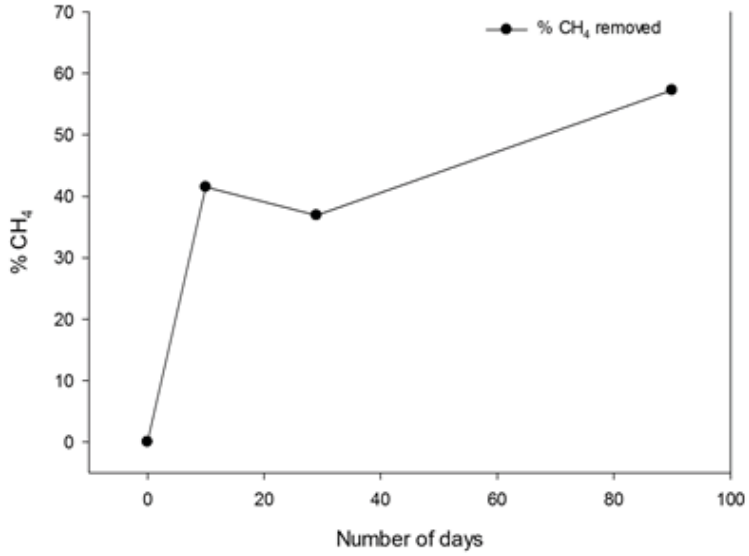
Methane removal by the soil-based biofilter (after reconstitution) is shown as a function of time (days of operation) (Figure 4.2a). During the 90-day study period, a constant supply of CH_4 was provided, i.e. at 52 ± 5 g m^{-3} h^{-1}. The CH_4 removal efficiency of the biofilter reached 40% in just 10 days of operation, and then it slowly increased to 57% on day-90. By the end of 90-day study period, the highest CH_4 removal achieved by the biofilter was 30.3 g m^{-3} h^{-1}. Figure 4.2b shows the CH_4 flux down the vertical length of the biofilter–depth, 0 and 54 cm representing the top/outlet and the bottom/inlet of the

biofilter respectively. Day-0 shows no difference in the CH_4 flux between the bottom and top ends of the biofilter. Subsequently, the difference increased over time. The lower and middle regions of the biofilter were sites of greatest oxidation compared with the top region. Evolved CO_2 and N_2O were also measured from the biofilter. As well as providing evidence of general microbial activity, CO_2 was produced during the CH_4 oxidation process (Figure 4.2c). Carbon dioxide increased in concentration from bottom to top of the soil column, suggesting that there were no gas leaks from the biofilter. Although the N_2O emissions increased slightly over time, concentrations remained close to ambient air concentrations ~ 280 ppbv. Nitrous oxide concentrations on day-0, 10, 29, and 90 were 253 ± 0.0, 177 ± 2.0, 203.5 ± 3.5, and 273.15 ± 17.5 ppbv, respectively.

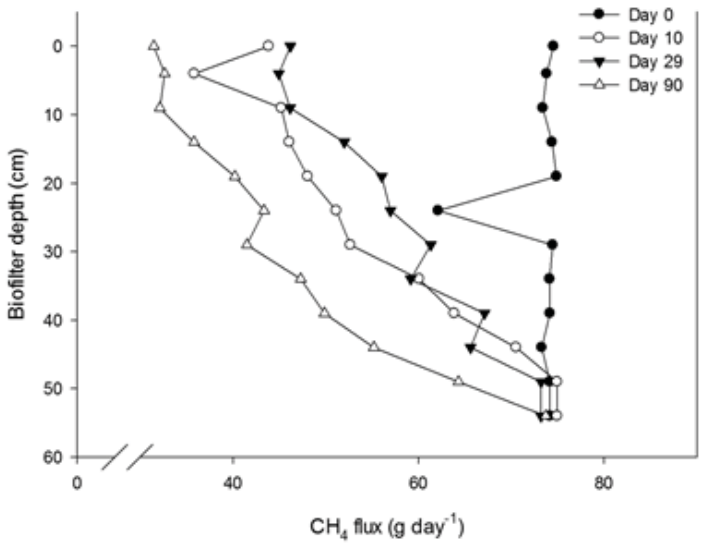
4.3.2 Factors affecting soil methane oxidation

On day-0, the moisture content was adjusted to 110 % gravimetric dry wt near the optimal range (72–108 % gravimetric dry wt) (Figure 4.3a), as suggested from earlier studies (unpublished data, C. Pratt). The soil was moist enough to encourage gas transport and nutrient distribution. Moisture data on day-10 and day-29 varied across the top, middle, and bottom portions of the biofilter, but these moisture values had overall decreased by the end of the study period (day-90). However, throughout the study period the moisture content was around the optimal range. Another key abiotic parameter, pH, remained around 3.8 during the study period (Figure 4.3b). The pH of soil did not change much along the length of the biofilter during the 90-day supply of the biogas, but the soil pH at the base of the biofilter declined from 3.72 to 3.25.

4.2a


% CH4 removed
% CH4
Number of days
0
10
20
30
40
50
60
70
0
20
40
60
80
100

4.2b


Day 0
Day 10
Day 29
Day 90
Biofilter depth (cm)
CH4 flux (g day-1)
0
10
20
30
40
50
60
0
40
60
80

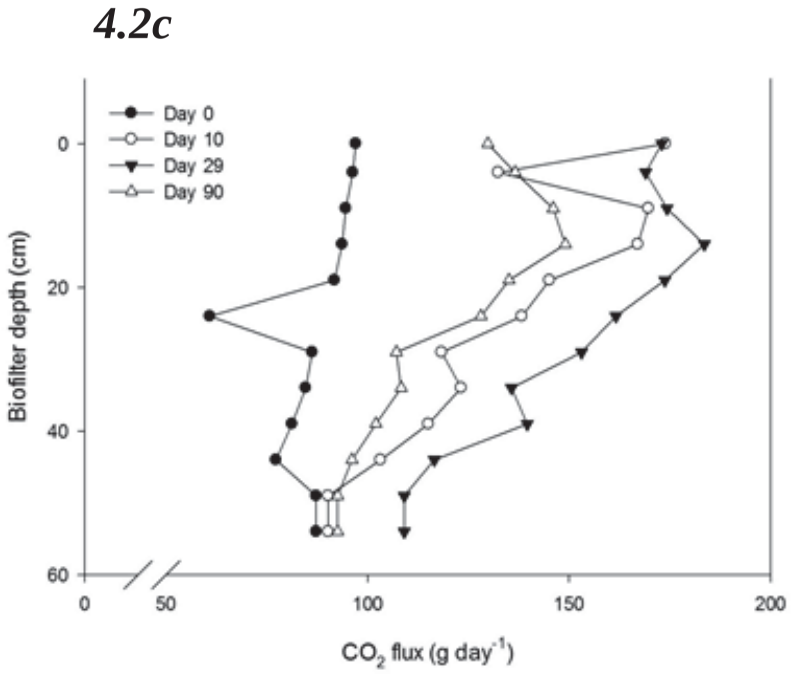


Figure 4.2 (a) Methane (%) removed by the biofilter on day-0, day-10, day-29 and day-90. **(b)** Methane flux across various depths of the biofilter – 0 cm and 54 cm indicating the top/outlet and bottom/inlet of the biofilter, respectively. **(c)** CO_2 concentration in g day^{-1} across various depths of the biofilter – 0 cm and 54 cm indicating the top/outlet and bottom/inlet of the biofilter, respectively.

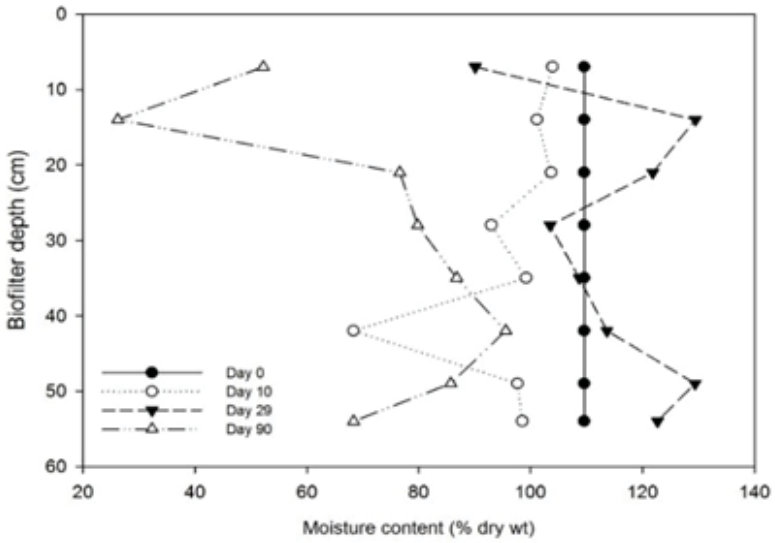
Table 4.1 Average gene copy number with standard deviations ($\times 10^8$) per gm of dry soil over the study period (90 days); n=8.

Days	**Type I methanotrophs**	**Type II methanotrophs**	***Methylocystis* and *Methylosinus***	***Methylocapsa***	***Methylobacter* and *Methylomonas***	***Methylococcus***
0	3.96 ± 0.16	1.91±0.05	1.06 ± 0.09	4.18 ± 0.21	0.39 ± 0.02	0.34 ± 0.02
10	3.26 ±0.24	0.91±0.03	0.58 ± 0.02	6.34 ± 0.63	0.20 ± 0.0	0.43 ± 0.13
29	4.10 ± 0.27	1.23±0.07	0.68 ± 0.04	7.25 ± 0.68	0.23 ± 0.0	0.33 ± 0.03
90	4.58 ± 0.20	0.93±0.05	0.62 ± 0.05	17.1 ± 1.84	0.33 ± 0.02	0.25 ± 0.02

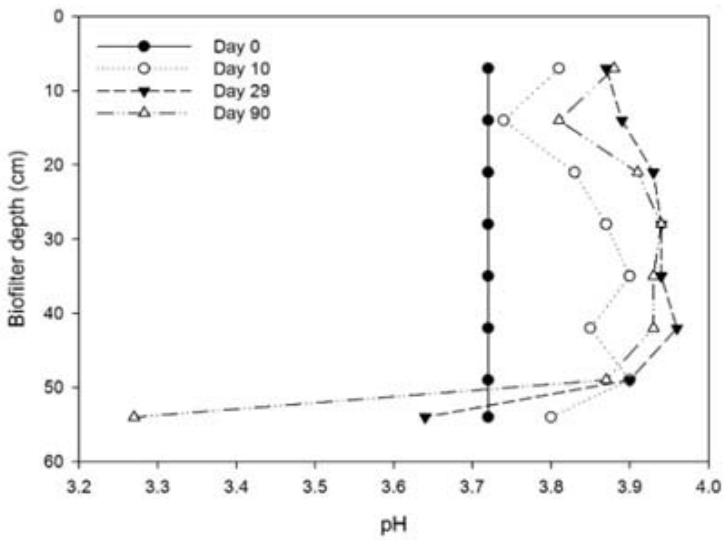
Table 4.2 Diversity and abundance based on T-RFLP analysis of pmoA genes of methanotroph. Phylotypes is indicative of the number of species present. Higher the H` value, more the diversity. Evenness assumes a value between 0 and 1, with 1 being complete evenness, n=2.

Reconstituted Biofilter	**Number of Phylotypes (richness)**	**T-RF's Abundance**	**Shannon's Diversity (H`)**	**Evenness (E)**
Day 0	55±7	2.0±0.3 x10^5	4.0	0.9
Day 90_7cm	74±1	2.1±0.0 x10^5	4.3	1.0
Day 90_14cm	70±2	1.9±0.1 x10^5	4.2	1.0
Day 90_21cm	76±1	1.7±0.1 x10^5	4.3	1.0
Day 90_28cm	71±3	1.9±0.2 x10^5	4.3	1.0
Day 90_35cm	78±1	2.9±0.1 x10^5	4.4	1.0
Day 90_42cm	79.5±0.5	2.8±0.0 x10^5	4.4	1.0
Day 90_49cm	56.5±4.5	2.0±0.0 x10^5	4.0	0.9
Day 90_55cm	59±1	2.1±0.0 x10^5	4.1	0.9

4.3a


Biofilter depth (cm)
Moisture content (% dry wt)
Day 0
Day 10
Day 29
Day 90

4.3b


Biofilter depth (cm)
pH
Day 0
Day 10
Day 29
Day 90

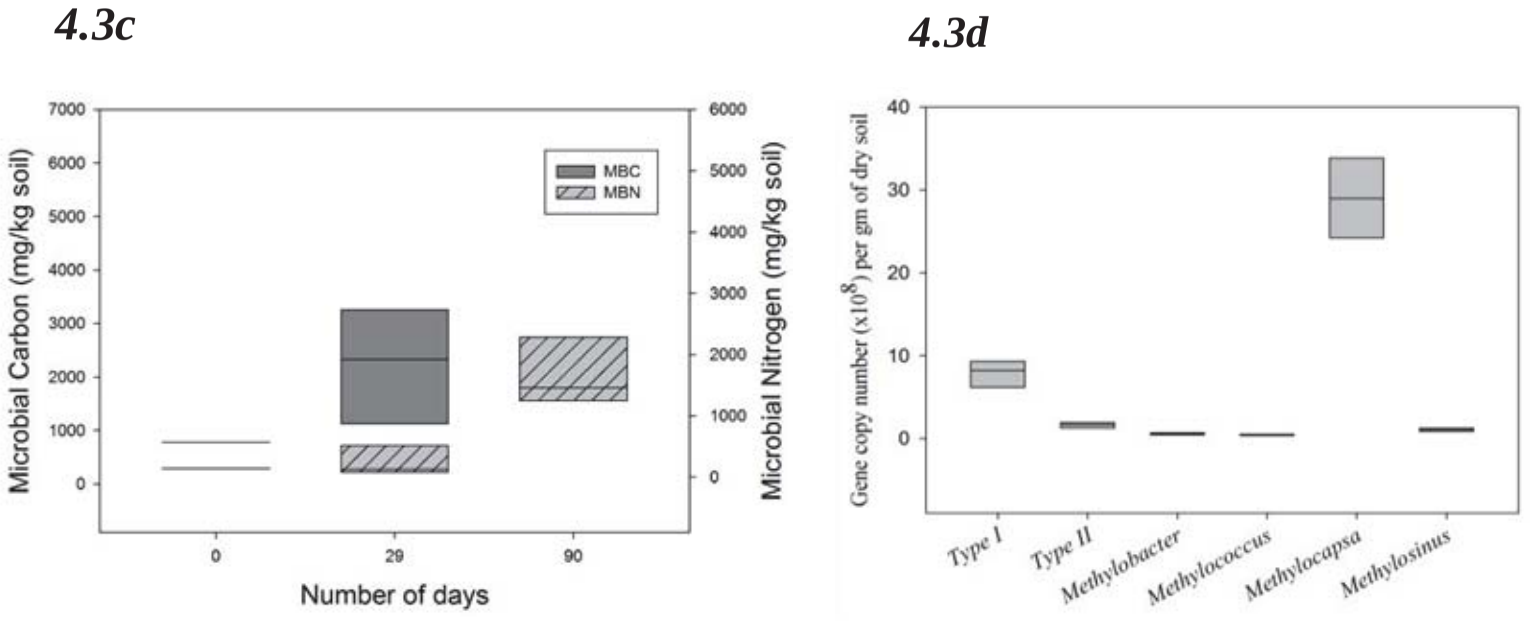


Figure 4.3 Scatterplot showing the moisture along the length of the Biofilter on day-0, day-10, day-29 and day-90. Values are represented based on % dry weight (oven drying). Fig.4.2b Scatterplot showing the pH change in the Biofilter across the various depths and study period. At the end of the study, base of the biofilter becomes more acidic due to the oxidation of H_2S to H_2SO_4. Fig.4.2c Microbial Biomass C and N in the Biofilter on day-0, day-29 and day-90 of the study period. Variability within the box plot indicates the variability along the length of the biofilter. Fig.4.2d Box plot showing methanotroph population on day-90. Variability within the box plot indicates the variability along the length of the biofilter. *Methylocapsa* like methanotroph is significantly different with $P < 0.005$ than other populations.

4.3.3 Microbial biomass C and N

Both MBC and MBN indicated an increase in total microbial biomass (Figure 4.3c). MBC increased from 800 mg kg^{-1} (on day-0) to about 2200 mg kg^{-1} (day-29), this was complemented by the increase in MBN from 200 mg kg^{-1} (day-0) to 500 mg kg^{-1} (day-29), and to 1800 mg kg^{-1} by the end of the study. Day-90 data for MBC are not available, but the increase in MBN on day-90 suggests there would also have been a proportionate increase in the C content. The microbial C/N ratio was about 6:1 during the study period.

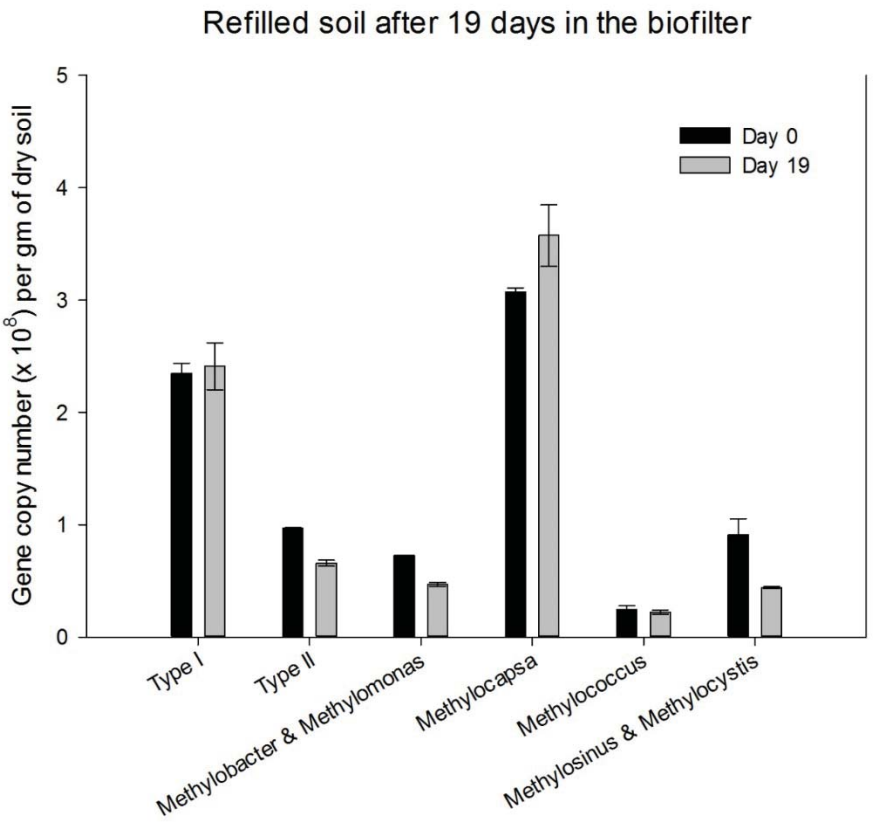


Figure 4.4 Methanotroph community change in refilled or backfilled soil: Day-0 represents-the first day of spare soil (pH-5.20) filled into the day-10 sample slot; Day-19 data represents, the spare soil analysed for a change in the community on day-29 sampling day. Except for type I and *Methylocapsa*, all other groups show a decreasing trend. Data = avg±SD (n=8).

4.3.4 Abundance and diversity of Methanotroph

According to the group-specific amplification results from qPCR, the total average gene copy number of the methanotroph (type I and *Methylocapsa*-like methanotroph) on day-90 ($21.68\pm2.04 \times10^8$) was higher than that present at day-0 ($8.14\pm0.37 \times10^8$), suggesting an increase in the population (Table 4.1). This increase in abundance concurs with the increase in abundance of the Terminal restriction fragments (T-RF's) from day-0 ($2.0 \pm0.3 \times10^5$) to day-90 ($2.2 \pm0.4 \times10^5$) (Table 4.2).

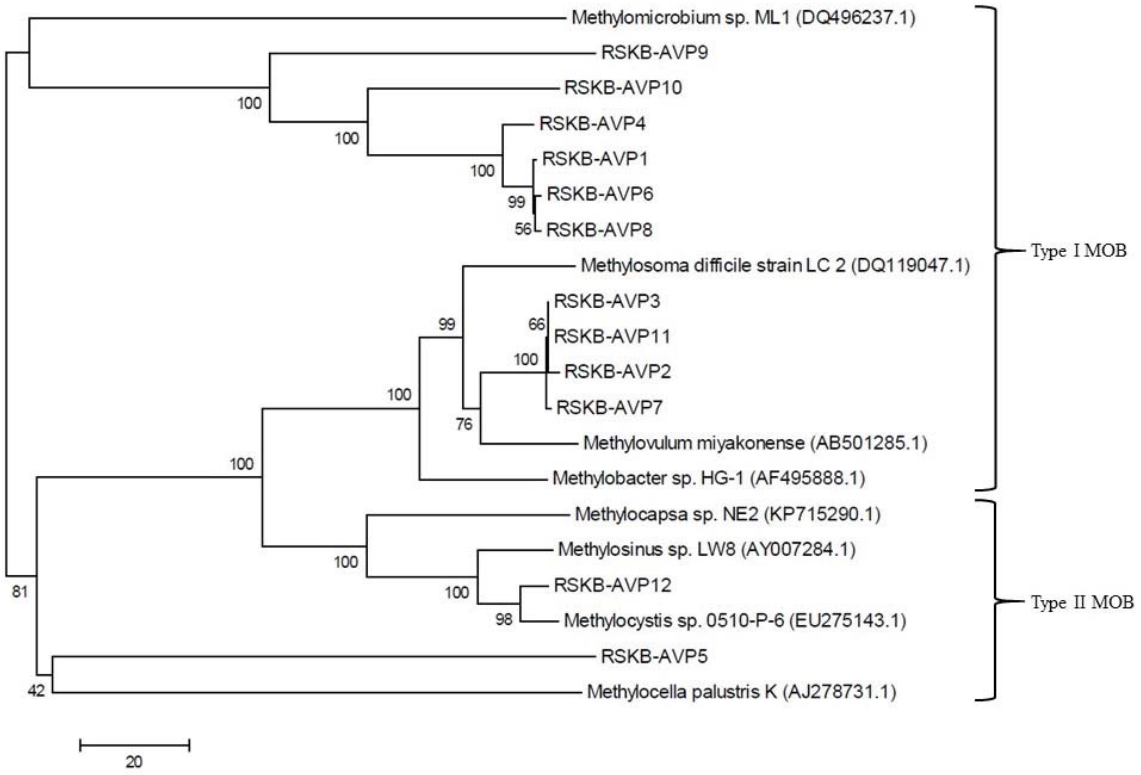


Figure 4.5 The phylogenetic tree was inferred using the Neighbour-Joining method based on the multiple alignment (clustal W) of nucleotides coding for pmoA gene. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. Evolutionary analyses were conducted in MEGA7 software package.

As indicated by the T-RFLP results, the diversity of the biofilter has increased during the study period (see richness, table 4.2). Methanotroph diversity and abundance did not vary much across the length of the biofilter, except the base of the biofilter had less diverse and minimally abundant population. By day-90, methanotroph belonging to the acidic group, *Methylocapsa*-like, was dominant, as shown by the gene copy numbers (Figure 4.2d).

The gene copy number for *Methylocapsa*-like methanotroph significantly ($P = 0.019$) increased 4-fold from $4.18\pm0.21 \times 10^8$ on day-0 to $17.1\pm1.84 \times 10^8$ on day-90 (Table 4.1). At the same time, the abundance of the type II methanotroph population decreased considerably from day-0 ($1.91\pm0.05 \times 10^8$) to day-90 ($0.93\pm0.05 \times 10^8$). In contrast, there was not much change observed in the abundance of *type I* methanotroph population from day-0 ($3.96\pm0.16 \times 10^8$) to day-90 ($4.58\pm0.20 \times 10^8$). Other genera within *type I* community – *Methylococcus*, *Methylobacter*, and *Methylomonas* – did not show an increase in their gene copy number as seen from Table 4.1.

Quantitative PCR analysis of the laboratory soil (less acidic soil) refilled or plugged in to the biofilter sampling slot showed the increasing trend of gene copy numbers of *type I* and *Methylocapsa* like methanotroph, whereas other methanotrophs gene copy numbers were declining during incubation in the biofilter for 19 days (between experimental period day 10 and day 29) (Figure 4.4). The pH of the refilled soil when measured after 19 days, dropped to 4.0 from the initial pH of 5.1. Figure 4.5 depicts the phylogenetic tree built from the randomly made cloned sequences library (12 novel sequences coding *pmo*A gene). Clearly four clusters were seen, with one cluster (RSKB-AVP 4, 1, 6 and 8) distantly related to *Methylomicrobium sp. ML1* (78- 88% sequence similarity); the second cluster (RSKB-AVP 3,11, 2 and 7) had 88–89% and 87–88% similarity to *Methylosoma difficile strain LC2* and *Methylovulum miyakonense*, respectively*;* the third cluster (RSKB-AVP12) closely related to *Methylocystis sp.* (97%) and distantly related to *Methylocapsa sp.* (73%); whereas the fourth cluster was distantly related to *Methylocella palustris* (89% sequence similarity).

4.4 Discussion

Adjustment of the soil moisture to 110 % gravimetric dry wt was apparently responsible for and increase to 40% of CH_4 removal within 10 days of operation of the reconstituted soil biofilter. This is attributed to the role soil moisture plays in regulating CH_4 oxidation and in reactivating the methanotroph population. This result also indicates that the methanotroph community is very resilient and is able to be revived quickly from a dormant state when sufficient substrates (CH_4 and O_2) and optimal moisture conditions are available. In this case the moisture appeared to be an important abiotic factor that had limited methanotroph activity. Several studies indicated the importance of moisture and the requirement of an optimum range for effective biofiltration, but the optimum range varies with different soil types (Tate *et al.*, 2007; Scheutz *et al.*, 2009; Moon *et al.*, 2010). The small dip on day-29 (~37% CH_4 removal) (Figure 4.2a), can be accounted for by the proportion of active soil (4.5 L) that was removed from the biofilter for sampling on day-10. While the sample slot was back-filled with fresh soil of the same volume, a few days would have been required for the back-filled soil to become acclimatised and fully active in consuming CH_4. Quantitative PCR data show no significant difference in the gene copy numbers of the methanotrophs on day-10 and day-29, suggesting that methanotroph (including *Methylocapsa*) were not stabilised but were in an adaptation phase during that period. At the end of the 90-day study period, the soil biofilter was removing 30.3 g m^{-3} h^{-1} of CH_4, much higher (47%) than was earlier reported by Pratt *et al.* (2012b). The average gene copy number of *Methylocapsa* on day-90 was significantly higher (P <0.005) than that on day-0, indicating *Methylocapsa* had increased, positively correlating (R^2 = 80.36%) with the increase in CH_4 removal by the soil. This indicates that *Methylocapsa*-like methanotroph was mainly contributing to the CH_4 removal. *Methylocapsa* abundance is also reflected in the values of MBC and MBN.

Based on the typical CH_4 emission value of 45 m^3 d^{-1} (Craggs *et al.*, 2008), a biofilter of about 50 m^3 volume would be required to treat all CH_4 emissions, as previously reported by Pratt *et al.* (2012b). However, a biofilter of about 41 m^3 (about 20% less in size) would be required to mitigate CH_4 emissions from a typical, average-sized New Zealand dairy effluent pond, based on the present CH_4 removal rate (30.3 g m^{-3} h^{-1}).

Earlier research indicated that N_2O (~ 480 ppbv) can be produced as a by-product during the CH_4 oxidation process, especially when compost biofilter materials are used (Pratt *et al.*, 2013). This is probably because the organic matter in the compost can be a rich source of N. However, the volcanic soil–perlite mixture in the biofilter produced no or very low levels of N_2O, with concentrations ranging between 270 and 290 ppbv. This highlights the importance of choosing the most appropriate soil material for constructing a biofilter.

Moisture is a major factor controlling CH_4 oxidation in soil (Tate 2015), including soil-based biofilters or biocovers. Optimal moisture enables the transport of nutrients, and gaseous exchange to the methanotrophs. Although moisture decreased overall from about 110% (dry wt) at the start of the study period to 72 (% dry wt) by day-90, values remained near optimal during the 90-day period (Figure 4.3a). The upper region in the biofilter appeared to become drier than the middle or lower regions of the biofilter during the 90-day period. This probably resulted from the CH_4 removal occurring in the middle and lower regions of the soil column. In addition, the presence of the high flow rate of air (1000 ml/min) tends to dry the biofilter material more than is compensated for by the water produced by methanotrophs during CH_4 oxidation.

Despite the pH being low (3.72±0.02) in the biofilter soil, CH_4 removal has considerably increased over several years, suggesting that the methanotroph population in the biofilter had also increased over the 5- years operation. The decrease in pH has negatively affected the growth of most of the methanotroph except the acidic *Methylocapsa*-like methanotroph. Dedysh *et al.* (2003) found a similar group of methanotroph belonging to *Methylocapsa* in acidic peat soils, confirming the ability of *Methylocapsa* to grow under acidic conditions. Our results suggest pH was an important factor in selecting for the active populations of *Methylocapsa*-like methanotroph, while negatively affecting the growth of other genera, viz. *Methylobacter*, *Methylococcus*, *Methylomonas* belonging to type I, and *Methylocystis* and *Methylosinus* belonging to type II. Despite the genera of type I (*Methylobacter*, *Methylomonas*, and *Methylococcus*) being less dominant or constant during the study period, type I methanotroph (Type I 16S rRNA primer) did seem to be slightly increasing (by ~13%) in gene copy numbers. This suggests that novel species (Figure 4.5) belonging to type I present in the soil were not picked up by genera-specific MBAC primers, but were identified by the Type I primers. On the other hand,

type II (16S rRNA) primers were designed to detect only a few species of the *Methylocapsa* genera, as they mainly target *Methylosinus* and *Methylocystis* genera (Chen *et al.*, 2007), which explains why the increase in *Methylocapsa*-like methanotroph was not reflected in the overall type II gene copy numbers. The cloned sequences library indicates that there are several novel species of Type I methanotroph present in the soil biofilter. Isolation and characterisation of these novel species will help us optimise the biofilter's performance.

The effect of pH on methanotroph growth was also observed when the laboratory soil of the same composition that was used by Pratt *et al.* (2012b), backfilled on day-10, was analysed for bacterial gene copy number on day-29 (i.e. after 19 days of incubation in the biofilter column). Initially the laboratory soil (control) at pH 5.14 ± 0.11 had balanced composition of type I and type II methanotroph (type I/type II ratio = 2.42). However, due to the effect of pH (dropped down to 4.0 ± 0.33), the population dynamics in the backfilled soil changed during the 19 days incubation period in the biofilter column. Results indicated a decreasing trend of gene copy numbers of the type II community (including *Methylosinus* and *Methylocystis*) and specific genera within the type I community, viz. *Methylomonas* & *Methylobacter*, and *Methylococcus* genera (Figure 4.4). On the other hand, there was a 16% increase in the gene copy number for *Methylocapsa* genera and the number remained almost constant for the overall type I population. This increase was not, however, statistically significant ($P > 0.005$). This shift in population indicates that these acid – loving methanotroph in the soil biofilter were better adapted to the increased acidity. This finding concurs with the study published by Delmont *et al.* (2014) indicating the important role played by the environment in selecting the composition of microbial communities.

Although the pH selectively enhanced the growth of one genera of methanotroph while limiting the growth of other sub groups, the total CH_4 removal (30.3 g m^{-3} h^{-1}) by the end of the 90-day study period was higher than had earlier been reported by Pratt *et al.* (2012b). It is important to note here that this soil-based biofilter had been in operation for about 5 years, and the total methanotroph population would have increased over time as the biofilter was almost always supplied with CH_4 and O_2, until it reached a point where the acidic conditions were favouring the growth of only specific groups of methanotroph

(*Methylocapsa*). If the pH was maintained at about 5.20, the methanotroph population of all groups could have increased and the total CH_4 removal by the biofilter would have been much higher. Pratt *et al.* (2012b) suggested that pH could be raised in the biofilter by passing biogas through lime or iron chips before feeding it in to the biofilter. This will allow the growth and activity of most of the genera within the methanotroph community, thus increasing the overall CH_4 removal rate.

Methanotrophs (including *Methylocapsa* and type I methanotroph) abundance did not exhibit any significant spatial variability (P = 1) in the biofilter, indicating these organisms are not much affected by the changing concentrations of CH_4 and O_2 with depth in the biofilter, although most CH_4 removal was taking place in the middle and lower regions of the biofilter. This concurs with the T-RFLP results (Table 4.2), where there was no significant difference in the Shannon's Diversity (H`) and Evenness (E) along the depth of the biofilter. In this study, type I methanotroph were better able to adapt to the unfavourable pH conditions than the type II community (except *Methylocapsa*-like methanotroph) in general. In addition, the fresh soil (pH – 5.14) that was backfilled into a day-10 sample slot also showed a decrease in the dominance of type II community over 19 days, which means *Methylocystis* and *Methylosinus* cannot grow under acidic conditions. Even though the study is based on a single biofilter column, the constant pH at different biofilter depths during the 90-day period indicated that the biofilter conditions provided enough time for the sub groups of methanotroph to react to changing conditions.

4.5 Conclusions

This study has demonstrated that CH_4-oxidising capacity of the diverse native population of methanotroph (types I and II) had the ability to adapt to changing acidic conditions. Reconstitution of biofilter material by mixing the soil with water to 110 % gravimetric dry wt enabled the CH_4 oxidation process to continue, suggesting that the biofilter could regain efficiency and operate with very little maintenance, with no media or chemical additions made except for the occasional addition of water to keep the soil material moist

enough for effective nutrient or gas transport. Along with other type I methanotroph, the presence of active *Methylocapsa*-like methanotroph supported the biofilters capacity to oxidise CH_4 at low pH (3.72). Other groups like *Methylobacter/Methylosinus/Methylococcus* were present at low abundances, suggesting that acidic pH was suppressing their growth and contributing little to the CH_4 oxidation. This study has demonstrated the adaptability of methanotroph population in the biofilter by revealing the capacity to oxidise CH_4 was not compromised by the acidification. For optimum performance, a biofilter should support the growth and activity of all communities of methanotroph, which can be achieved by manipulating the various physical and chemical parameters that control CH_4 oxidation in the soil. Our future research will focus on the effects of CH_4 oxidation of encouraging the growth of both types I and II methanotroph by adjusting the soil pH back to its original levels (at pH 5.20).

DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: RASHAD SYED

Name/Title of Principal Supervisor: Prof. Surinder Saggar

Name of Published Research Output and full reference:
Syed R, Saggar S, Tate KR, Rehm BHA (2016). Does acidification of soils compromise its methane-oxidising capacity? Biology & Fertility of Soils 52: 573-583.

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:
  and / or
- Describe the contribution that the candidate has made to the Published Work:
  First Author

Candidate's Signature — 4 OCT 2016 — Date

Principal Supervisor's signature — 4/10/2016 — Date

GRS Version 3– 16 September 2011

Chapter 5

Assessing the performance of floating biofilters for oxidation of methane from dairy effluent ponds

5.1 Introduction

Very few studies have been carried out on the application of biofilters enriched with methanotrophs for treating dairy effluent pond CH_4 emissions (Pratt et al. 2012b; Syed et al. 2016b). Engineered biofilter studies with volcanic pumice soil have demonstrated their ability to oxidise large concentrations (3 300– 100 000 ppm) of CH_4 under varying abiotic conditions (Tate *et al.*, 2012; Pratt *et al.*, 2012b; Syed *et al.*, 2016a, b). Molecular techniques (Syed *et al.*, 2016a, b) revealed that this soil was naturally enriched with genera of type I and type II methanotrophs, along with the presence of novel species and strains of methanotrophs. However, when this was used in a biofilter column to treat CH_4 emissions from a section of a dairy effluent pond, the columns effectiveness was limited by the surface area. The biofilter surface area is one of the most important limiting abiotic factors (Nikiema and Heitz 2010), as too were factors like the costs involved for collecting gas from the ponds, a pump (for feeding air), piping, and other material costs. Therefore, in this study a floating biofilter designed and briefly tested previously in the laboratory by Pratt *et al.* (2012c) is here assessed for its performance under field conditions for a period of one year. The objectives of the study are to (1) assess the performance of the floating biofilters in the field exposed to high CH_4–rich concentrated biogas, and (2) compare and contrast the methanotroph abundance and diversity under laboratory and field conditions using molecular techniques like quantitative PCR, T-RFLP and cloning to study population dynamics.

5.2 Materials and Methods

5.2.1 Preparation of the floating biofilter

The biofilter and control chambers (4 m^2) were sited at the centre of a 928-m^2 (29 m × 32 m) open effluent pond receiving waste from 450 cows at Massey University's research Dairy Farm No. 4, Palmerston North, New Zealand. The biofilter material (21 L) comprised volcanic pumice soil and perlite (light-weight alumino-silicate material) (1:1) mixture (subsequently called "volcanic pumice soil") and was filled to a depth of 6 cm in the chamber with 1 m × 0.5 m × 0.2 m dimensions. Porous supporting material (made from recycled plastic) (6–7 cm thick) at the base of the biofilter material prevented soil from falling off. The base of the chambers had holes to allow the passive influx of biogas into the chamber. The chambers were placed on a structure to allow floatation on the surface of the pond (thus avoiding penetration of effluent) as shown in Figure 5.1. No water and nutrients were added to the biofilters during the study period, however during extremely dry conditions (once in February and October), a little water was added to keep the biofilter bed moist, as our previous study (Syed *et al.*, 2016b) suggested dry soils (~ 15 % dry wt) do not support CH_4 oxidation. The control chambers had no biofilter materials and the same measurements were made as for the biofilter chambers. Before deployment of the floating biofilters onto the dairy ponds (and use for the laboratory experiments), the volcanic pumice soil (stored at 4 °C) was primed by feeding 800 000 ppm CH_4 (6 g m^{-3} h^{-1}) for almost a month in the laboratory chambers (at room temperature) to activate the methanotrophs in the soil. Priming of the soil was done only to eliminate the lag phase that usually occurs during its first few weeks of operation (Pratt *et al.*, 2012a, b).

Design of the laboratory-based biofilter chambers (50 L volume) is as described in Pratt *et al.* (2012c). In brief, about 8 L of the biofilter material (composition described above) was fed with a mixture of 80% CH_4 in CO_2, bubbled through the water present in the bottom 5 cm to simulate field conditions. Air (88 ml/min) was swept over the surface of the biofilter to allow passive diffusion into the biofilter from the top. An outlet port on the top of the biofilter facilitated gas sampling.

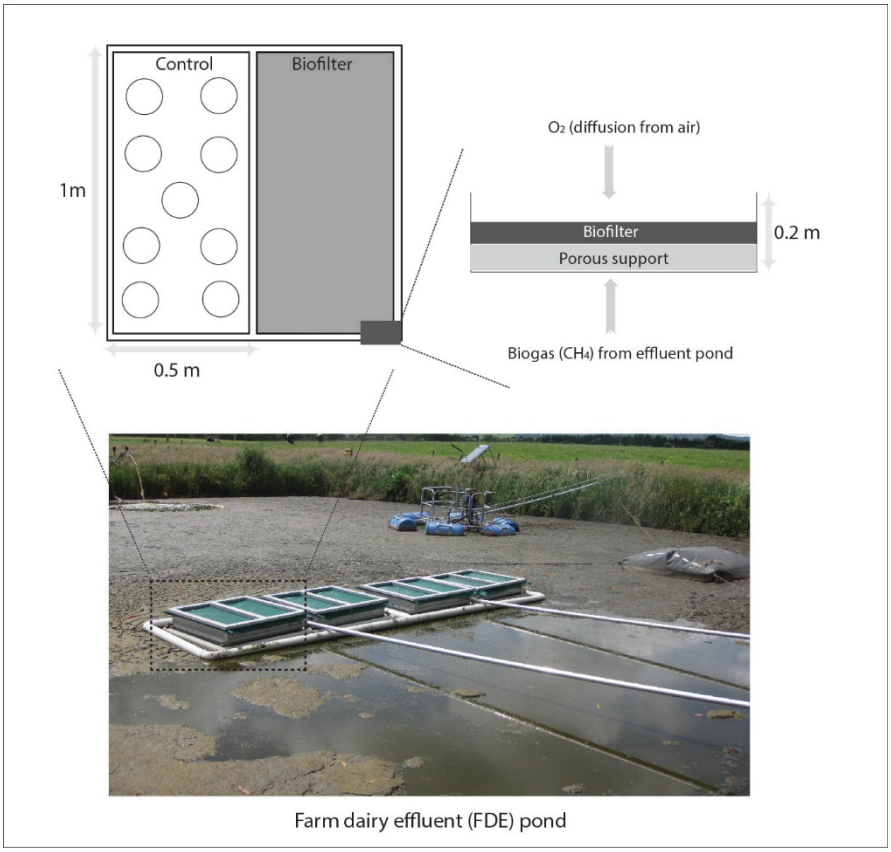

Figure 5.1 Floating biofilters used in this experimental study (11 months). Chambers (both control (without medium) and biofilter (with medium)) were placed on a floating structure to allow buoyancy and avoid penetration of effluent into the chambers. The holes present in the control and biofilter chambers allowed the methane to diffuse passively into the chambers from the pond. Biofilter and control chambers were kept open during the study period and were not covered during changing weathered conditions.

5.2.2 Gas samples

Gas emissions from the farm dairy effluent (FDE) were derived from concentration measurements of air samples from the control chambers. The emission reduction by the biofilters was determined from the concentration differences between air from the headspace of the biofilter chamber and from the control chamber. The effluent in the pond was not analysed for volatile solids (VS) or fresh inputs. For the pond biofilters, gas samples were taken at 0 min, 30 min and 60 min after closing the chamber lids (the photo in Figure 5.1 shows the lids in open position). The lids were internally fitted with battery-powered fans to allow gentle mixing of the accumulated gas in the headspaces of both

biofilters and controls. The samples were taken fortnightly throughout the study period, but during the winter (non-milking) season (May/July) only two measurements were made. Gas samples from the outlet ports of the biofilter and control chambers were analysed in the laboratory for CH_4, N_2O, and CO_2 concentrations by gas chromatography (GC) (CP-3800, Varian Inc, CA, USA) using flame ionisation (FID), thermal conductivity (TCD), and electron capture (ECD) detectors, respectively as described in Chapter 3.1.

5.2.3 Physical and chemical analysis of soil

Soil moisture content (gravimetric), pH, Ammonium (NH_4^+) and nitrate (NO_3^-), Total C and N were determined by following the protocols as described in sections 3.2, 3.3, 3.5 and 3.6 of Chapter 3 (Materials and Methods). The bulk density and porosity of the soil was calculated following techniques described by Gradwell (1972).

5.2.4 DNA extraction, PCR and T-RFLP

DNA extraction and PCR was performed as described in Chapter 3.7. Terminal-RFLP analysis was done using ABI 3730 genetic analyser (Genotyping performed by Massey Genome Service, New Zealand) and as described in Chapter 3.9.

5.2.5 Quantitative PCR

Three functional primers targeting *pmoA* used by Kolb *et al.* (2003) were used to amplify conserved sequences within type I and type II aerobic methanotroph. A189F/Mb601R was used to target *Methylobacter* and *Methylosarcina*, A189F/Mc468R – *Methylococcus*, A189F/Mcap630R – acidophilic *Methylocapsa*–like methanotrophs. Phylogenetic primers (16S rRNA) Type IF/IR and Type IIF/IIR, designed by Chen *et al.* (2007), were used to target all known type I and type II aerobic methanotrophs, respectively. Analysis was performed as described in Chapter 3.8. The r^2 values of the plots between Cp values and log gene copy numbers of the standards ranged from 0.9885 to 0.9999.

5.2.6 Cloning, sequencing, and phylogenetic analysis

PmoA genes amplified using unlabelled A189F and Mb661R were purified by gel extraction and cloned as described in Chapter 3.10. Phylogenetic neighbour joining tree-analysis of the clones (8) was performed using the MEGA 7 software package (Kumar *et al.*, 2016). Sequences obtained in this study were deposited at the NCBI nucleotide sequence database under accession numbers (KU840813 – KU840821).

5.2.7 Bio statistical analysis

Physical component (Multivariate) analysis was performed by relating the T-RFs pattern among the biofilter materials using Minitab® (version 16) software. Dendograms were produced based on similarity using Euclidean distances with single linkage method. A heat map was prepared using Genesis™ software (version 1.7.7). Relationships between CH_4 removal and other physical, chemical and biological characteristics were determined using multiple regression analysis and *Pearson correlation* using Minitab® (version 16) software. The Holm-Sidak test with $\alpha = 0.05$ significance level was used for the *post hoc* analysis to reveal the significant differences in the means of the replicates.

5.3 Results

5.3.1 Physical and chemical changes in the floating biofilters

Moisture (% dry wt) was initially 37.8±0.88 for both pond and laboratory-based biofilters. By the end of study period, the moisture levels had decreased in the pond biofilters to 23.50±4.33, whereas they decreased in the laboratory biofilters to 26.20±4.20. However, drying in the top layers of the soil biofilters (both lab and pond) was noted, whereas the bottom layers remained moist. Acidity in the pond biofilters

Table 5.1 Physico-chemical properties of the laboratory and pond biofilters during the 11-month study period. [a] indicates significant difference ($P < 0.05$) between the initial and final day of biofilter samples

Samples	Porosity (%)	Dry bulk density (g cm^{-3})	Moisture content (% dry wt)	pH	C (%)	N (%)	NO_3^- - N (mg kg^{-1})	NH_4^+ - N (mg kg^{-1})
Time 0 (floating biofilter)	75	0.42	37.8	5.29	4.17	0.36	34	27
Lab floating biofilter 1	75	0.42	22.0 [a]	5.47	5.17	0.36	1.14 [a]	28.7
Lab floating biofilter 2	75	0.42	30.4	5.37	6.09 [a]	0.39	0.22 [a]	34.3
Field floating biofilter 1	75	0.42	21.5 [a]	4.57 [a]	5.87	0.47	0.05 [a]	15.9 [a]
Field floating biofilter 2	75	0.42	30.7	4.69 [a]	6.77 [a]	0.52 [a]	0.00 [a]	12.9 [a]
Field floating biofilter 3	75	0.42	22.6 [a]	4.86 [a]	6.65 [a]	0.50 [a]	0.29 [a]	15.8 [a]
Field floating biofilter 4	75	0.42	19.2 [a]	4.76 [a]	5.21	0.41	0.15 [a]	9.4 [a]

increased significantly ($P < 0.05$) from pH 5.29±0.04 (day 0) to 4.72±0.11 (final day). The lab biofilters showed a slight decrease in the acidity – 5.42±0.05 (final day) from 5.29±0.04 (day 0). Total C and N increased significantly ($P < 0.05$) in the pond biofilters, whereas in the laboratory-based biofilters they increased over the study period (Table 5.1). Ammonia and hydrogen sulphide were not measured as they were not the objective of this thesis, however increase in total N was reported suggesting that the methanotrophs can metabolise NH_3 or it gets absorbed by the soil.

Both NO_3^- - N and NH_4^+ - N significantly decreased ($P < 0.005$) from 34 and 27 mg/kg (day 0) respectively, to 0.12±0.11 and 13.50± 2.66 mg/kg (day final) respectively, in the pond biofilters, whereas, NO_3^- - N decreased and NH_4^+ - N increased insignificantly in the lab biofilters. The temperature was not controlled in the laboratory, and was approximately 18±3 °C during the study period. In the field, temperature in the warmer months (Oct-Mar) ranged between 14 and 18 °C, whereas in the colder months (Apr–Aug) the temperature varied between 8 and 11 °C. Highest and lowest temperatures were recorded in February (22 °C) and August (8 °C), respectively (Figure 5.2).

5.3.2 Pond biofilter

Methane fluxes from the outlet ports of the control and pond biofilter chambers are shown in Figure 5.2. The CH_4 production from the pond (as depicted from the control chambers) was 0.12 m^3 day^{-1} at the beginning of the experiment (summer, January–March), later dropping during April–August (autumn–winter, 0.02 – 0.05 m^3 day^{-1}). The highest CH_4 fluxes were seen during September–October (spring, up to 0.18 m^3 day^{-1}). Methane flux from the biofilters followed a similar trend, but with a lower outlet flux, indicating the ability of the biofilters to oxidise CH_4.

The average CH_4 removal of the floating biofilters (n=4) during the study period was 48.2±23.1 g CH_4 m^{-3} h^{-1}; highest removals were seen during September–October (66–101 g m^{-3} h^{-1}) and lowest during April–July (4.96 – 17.91 g m^{-3} h^{-1}). Methane oxidation by the pond biofilters was consistent during the study period, with an average % CH_4

oxidation of 66.7±5.7 (Fig. 5.3), even during the cold temperature period (April–August). Overall, very little variation in the CH_4 oxidising (%) capability of the pond biofilters was noticed irrespective of the changing weather conditions in the field. The average N_2O concentrations from pond biofilter and control chambers were 370±53 ppbv and 447±140 ppbv, respectively. The pond biofilters removed about 243×10^{-9} m^3 day^{-1} of N_2O (~85%) during the study period (influx from the controls = 283×10^{-9} m^3 day^{-1}) (Fig. 5.4).

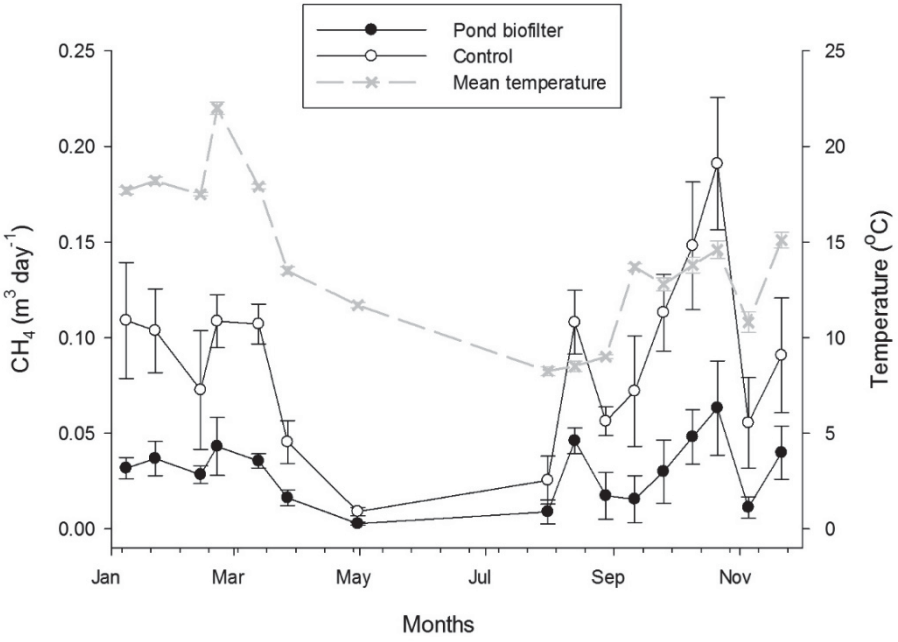

Figure 5.2 Methane flux (m^3 day^{-1}) from the outlet of pond biofilters (n=4) and control chambers (n=4) during the study period (January–November). Standard deviations indicate the deviation from the mean of the replicates. Dashed line indicates mean temperature (°C) during the study period. Year 2014 particularly had highest temperature records in February.

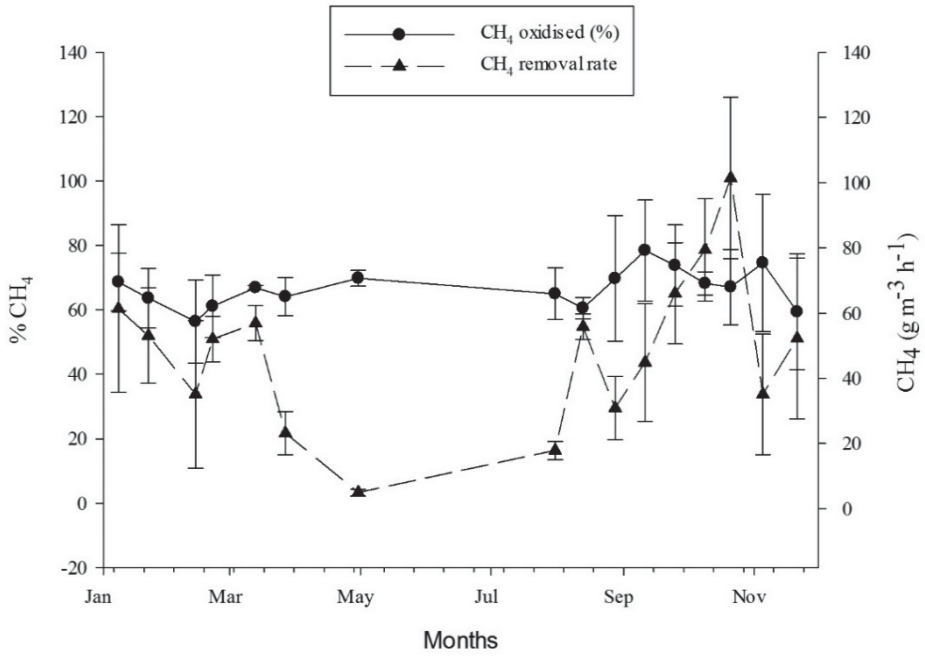

Figure 5.3 Methane removed (g m^{-3} h^{-1}) and fraction oxidised (%) by the pond biofilters during the study period. Error bars represent the standard deviation from the mean of replicates (n=4).

However, this is very negligible amount equivalent to 0.17 g N_2O-N yr^{-1} During the January–March period, about 80% of the N_2O produced (425 × 10^{-9} m^3 day^{-1}) was removed by the biofilters. In April–August about 77% (of 114 × 10^{-9} m^3 day^{-1}) was removed, whereas higher removals were observed in September–November, about 96% (of 255 × 10^{-9} m^3 day^{-1}).

5.3.3 Laboratory-based biofilter

Methane influx was varied during the study period in the lab biofilters (Figure 5.5). Initially, about 10 g m^{-3} h^{-1} of CH_4 was fed to the biofilters; later in the months of February and March the CH_4 influx was doubled and for the rest of the period (August–November) CH_4 feed was kept constant at 6.8 g m^{-3} h^{-1}. The CH_4 removed varied during the study period in relation to the amount of CH_4 fed (Figure 5.5). Initially, about 95% of the CH_4 was oxidised when 9–11 g m^{-3} h^{-1} of CH_4 was fed; however, when the influx was more than doubled, a gradual decline in the fraction of CH_4 oxidised (from 77 to 34%) was observed during the months of February–April. Despite the CH_4 flux being

brought back to a low influx rate (6.8 g m^{-3} h^{-1}) during the later stages of the study period, the average CH_4 oxidation fraction never reached more than 64%.

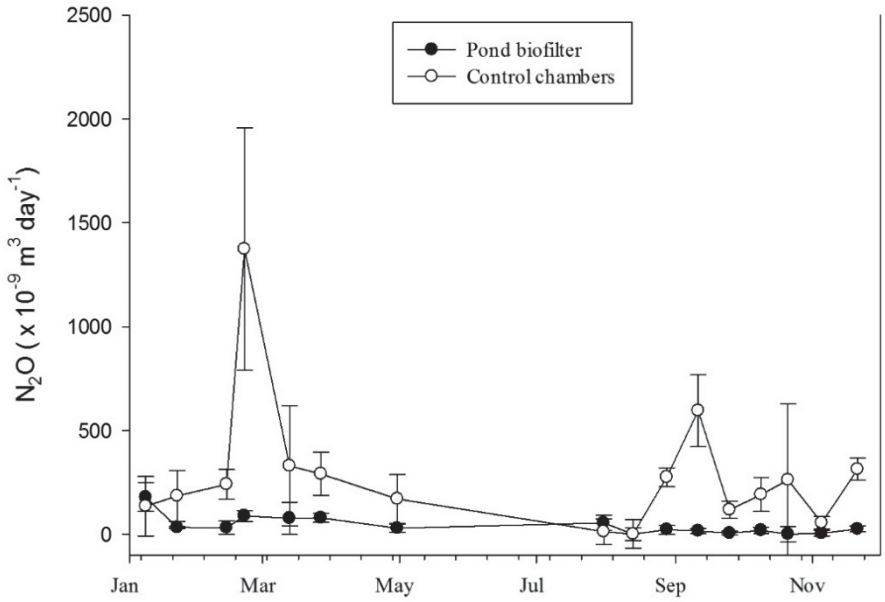

Figure 5.4 Net nitrous oxide (N_2O) emissions (× 10^{-9} m^3 day^{-1}) from the outlets of control (n= 4) and pond biofilters (n= 4). Error bars represents the standard deviation from the mean of the replicates. Higher the difference between the two series, higher the N_2O removed by the pond biofilters.

During the 11-month study period, the average fraction of CH_4 oxidised was 58.6%, with a minimum and maximum oxidation of 32.1% and 96.9%, respectively (Fig. 5.6). The N_2O concentrations were always in the range of 269–347 ppbv, indicating little N_2O removal and production from the lab biofilters.

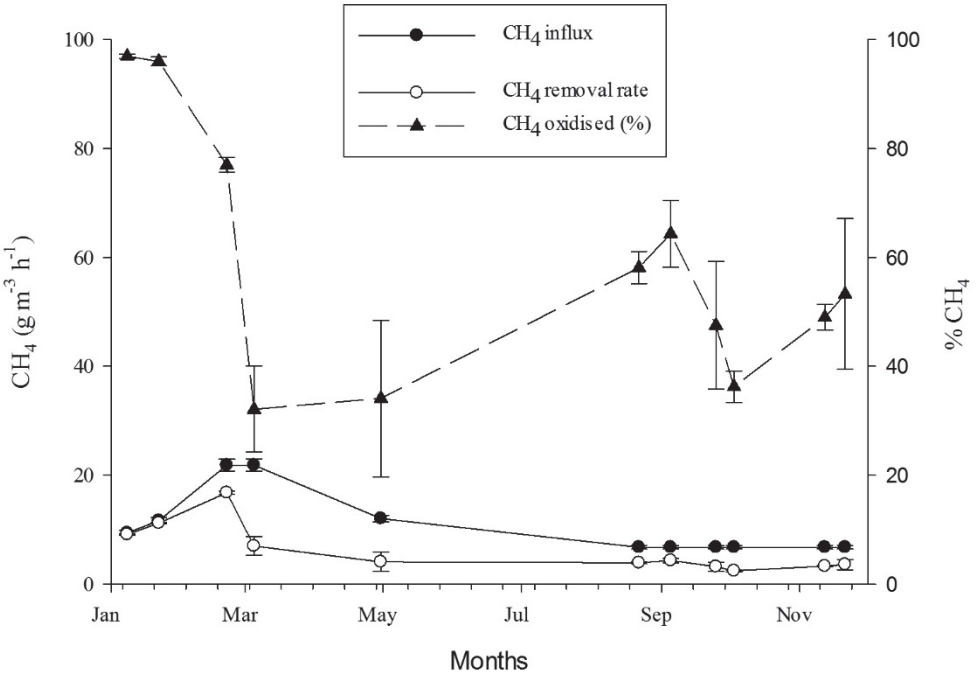

Figure 5.5 Methane influx (g m^{-3} h^{-1}), removal rate (g m^{-3} h^{-1}) and % CH_4 oxidised by the laboratory-based biofilters (n = 2) over the study period. Methane influx was increased after a month and then was decreased over the rest of the study period. Error bars represent the standard deviation of the mean from the triplicate gas measurements.

5.3.4 Methanotroph population dynamics in the biofilter

Quantitative PCR results indicate that by the end of 11 months study period in the pond biofilters, the type I methanotroph abundance increased (170%), whereas the type II methanotrophs had significantly (P < 0.005) increased by 340%. In the laboratory biofilters, the abundance of both type I and type II methanotroph decreased by 50% and 30%, respectively. No significant differences in gene copy numbers within the replicates of both field and

Table 5.2 Quantitative PCR results showing the abundance of gene copy number (× 10^8 per gm of dry soil) belonging to type I and type II methanotrophs and different genera within. [a] indicates significant difference ($P < 0.05$) between the initial and final day of biofilter samples. Data= avg±SD (n=2).

Samples	**Type I**	**Type II**	***Methylobacter***	***Methylococcus***	***Methylocapsa***	**Type I/Type II ratio**
Time 0 (floating biofilter)	5.08±0.64	2.24±0.08	1.12±0.02	0.82±0.04	4.73±0.06	2.27
Lab floating biofilter 1	2.25±0.02[a]	1.22±0.00[a]	0.56±0.03	0.09±0.01 [a]	1.35±0.07[a]	1.85
Lab floating biofilter 2	3.10±0.24[a]	1.89±0.04	0.81±0.00	0.12±0.01 [a]	1.87±0.04[a]	1.64
Pond floating biofilter 1	8.69±0.38[a]	6.44±0.03[a]	1.98±0.01	0.53±0.00	4.98±0.22	1.35[a]
Pond floating biofilter 2	8.62±0.01[a]	7.72±0.23[a]	1.48±0.03	0.63±0.03	6.17±0.22[a]	1.12[a]
Pond floating biofilter 3	8.90±0.11[a]	7.28±0.05[a]	1.58±0.08	0.61±0.06	6.74±0.12[a]	1.22[a]
Pond floating biofilter 4	8.57±0.25[a]	7.21±0.14[a]	1.37±0.02	0.58±0.02	5.93±0.16	1.19[a]

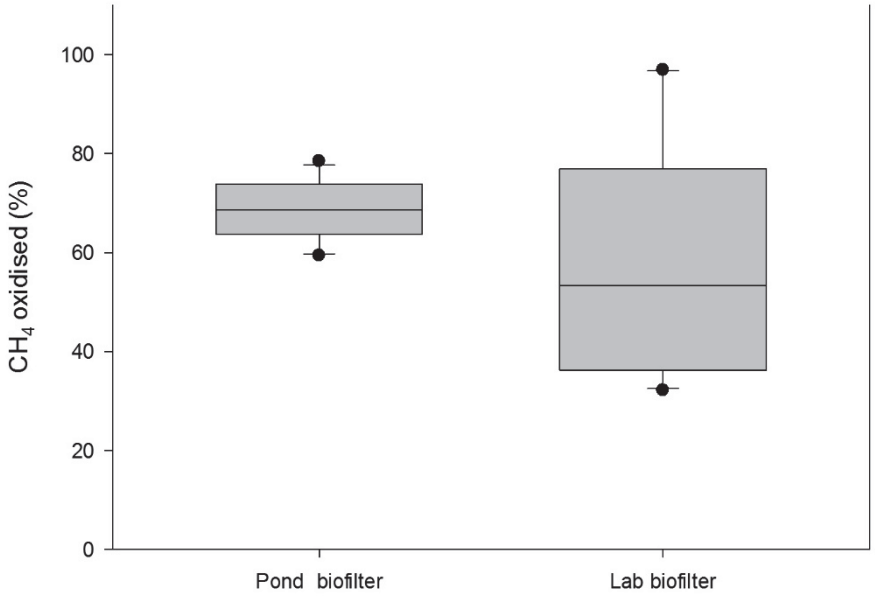

Figure 5.6 Comparing CH_4 oxidised (%) of laboratory and pond – floating biofilters. Outliers represent the minimum and maximum CH_4 oxidised, and the median represents the average CH_4 oxidised during the study period (n=4 and 2 for pond and laboratory biofilters, respectively).

Table 5.3 Methanotrophs diversity in the pond and lab – floating biofilters as indicated by T-RFLP. Data = avg±SD (n=2).

	Average richness	**Shannon's diversity**	**Evenness**
Time 0	67.0±4.0	4.20	0.96
Lab biofilter 1	59.5±5.5	4.08	0.93
Lab biofilter 2	58.5±3.5	4.07	0.93
Pond biofilter 1	65.5±4.5	4.18	0.96
Pond biofilter 2	67.5±0.5	4.21	0.96
Pond biofilter 3	70.1±1.0	4.25	0.97
Pond biofilter 4	67.6±6.0	4.20	0.96

lab biofilters were seen, except for pond biofilter 1, which had a significantly ($P < 0.05$) lower type II population in comparison with pond biofilter 3. By the end of the study period, *Methylocapsa*-like methanotrophs had decreased significantly ($P < 0.005$) by 194% in the lab biofilters, while in the pond biofilters the abundance increased significantly ($P < 0.05$) by 21% (Table 5.2). Gene copy numbers of the *Methylobacter*

group decreased by 20% in the lab biofilters, whereas the abundance increased significantly ($P < 0.05$) in the pond biofilters by 140% fold. Interestingly, methanotrophs belonging to the *Methylococcus* group decreased significantly by 688 % ($P < 0.005$) and 40% ($P < 0.05$) in both laboratory and pond biofilters, respectively.

Overall, the methanotroph population was more abundant in the pond biofilters than in the lab biofilters. At the start of the study (day 0), the type I and type II methanotrophs ratio was 2.27; however, by the end of the study period, the ratio decreased to 1.74±0.10 ($P > 0.05$) and 1.22±0.08 ($P < 0.05$) in lab and pond biofilters, respectively. Diversity analysis (using T-RFLP) indicated that the pond biofilters had a more diverse methanotroph population than the lab biofilter (See richness in table 5.3). Evenness remained constant during the study period for the pond biofilters (0.96), but had decreased to 0.93 in the lab biofilters. Principal component analysis of the samples based on T-RFs indicated differences between the pond and lab based biofilters (Figure 5.7). Except for pond biofilter 1, all the pond biofilters shared similarity of 80% within the replicates, whereas lab biofilters shared similarity of 75% and were more closely related to the Day 0 population in the biofilter. Heat map analysis with relative abundance of 1% supported the PCA data and indicated less abundance of particular T-RFs (81 bp, 213 bp and 248 bp) in laboratory-based biofilters when compared with pond biofilters (Figure 5.8).

A phylogenetic tree constructed from the *pmoA* sequences of pond and lab biofilter depicts three clusters representing novel strains belonging to type I methanotroph (Figure 5.9). The first cluster – RSKB-FBF1, 8, and 9 clones – were 93%, 90%, and 90%, respectively, similar to the *Methylomicrobium album* strain. In contrast, among the second cluster the RSKB-FBF6 clone was 95% similar to *Methylobacter albus*, the RSKB-FBF4 clone was 87% similar to *Methylosoma difficile strain*, and the RSKB-FBF5 and 10 were 87% and 88% similar to *Methylovulum miyakonense*. The third cluster had only one clone (RSKB-FBF11) distantly related (90%) to *Methylobacter albus*.

Regression analysis indicated that CH_4 removal was significantly correlated to the abundance of methanotrophs (R^2 (adj) = 91%, $P = 0.01$) whereas it was correlated (R^2 (adj) = 75%, $P = 0.32$) to other factors (moisture content, pH, C and N).

5.4 Discussion

5.4.1 Performance of the floating biofilters

After deployment, the pond biofilters were removing >68% of CH_4 within 3 weeks, indicating the ability of these biofilters once primed to adapt quickly to field conditions. The CH_4 oxidation never decreased below <60% throughout the study period, irrespective of whether the weather was warm or cold. During the colder season (Apr–Jul) the CH_4 flux from the biofilters and the corresponding flux from the ponds (control chambers) were at their lowest; however, the % CH_4 oxidised by the biofilters was not affected, indicating that the methanotrophs in the biofilters can perform well even at lower temperatures. A similar trend was reported previously by Pratt *et al.* (2012b), where both CH_4 production and oxidation was suppressed during the colder periods. However, it is important to note that the colder period also concurs with the non-milking season (April to July), which means a much lower amount of effluent is added to the pond that could have also slowed down CH_4 production by the methanogens. Even though the highest CH_4 removal rate achieved by the floating biofilters was 101.5 g m^{-3} h^{-1}, which is 31 % higher than has been reported previously (Menard et al. 2012); floating biofilters were able to remove only 70 % of the CH_4 produced from the effluent ponds. Considering the typical emission value (14 g m^{-3} h^{-1}) reported by Craggs *et al.* (2008), a floating biofilter (when covering the surface of the pond) has the potential to remove nearly 100% of the CH_4 produced, at an average (yearly) CH_4 removal rate of 48 g m^{-3} h^{-1}. Regardless, the average CH_4 oxidation was about 59% for the 11-month study period. During the highest CH_4 feeding period (21 g m^{-3} h^{-1}), the maximum CH_4 removed by the biofilters was 17 g m^{-3} h^{-1}. The inability of the laboratory biofilters to adapt to varying CH_4 conditions might depend upon competition between the methanotroph community and other soil bacterial communities for the available nutrients. Interestingly, the methanotrophic community make-up of the laboratory-based biofilters was less abundant and less diverse when compared with the pond biofilters (Figures 5.7 and 5.8), possibly contributing to the higher variability of the laboratory-based biofilters. Previous research (Syed *et al.*, 2016a) indicated that the low CH_4 removal phases could be avoided by adding minute quantities of nutrients (Nitrate mineral salts), which should be tested in future studies.

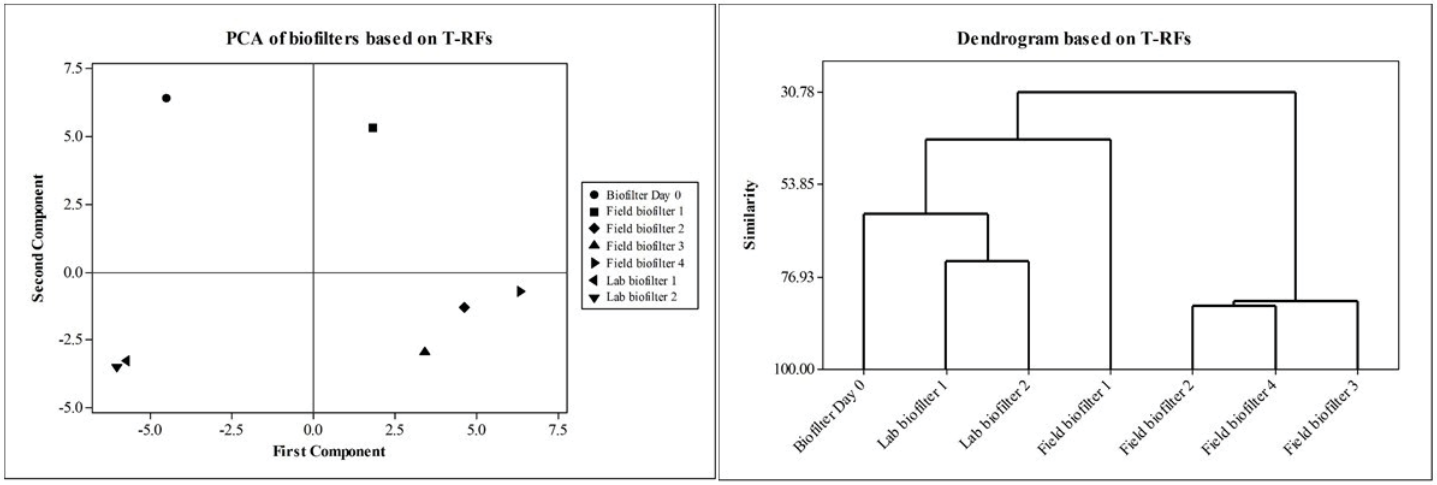

Figure 5.7 Principal component analysis of biofilter materials on initial and final day of study period based on terminal-restriction fragments (T-RFs). Dendogram was produced by considering the similarities between samples using Euclidean distance with single linkage method.

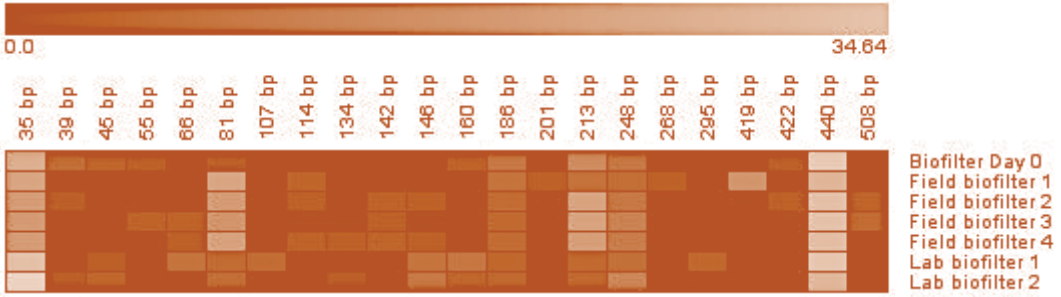

Figure 5.8 Heat map analysis of terminal-restriction fragments (T-RFs) profile of the biofilter materials. Data segregated based on the relative abundance of more than 1%.

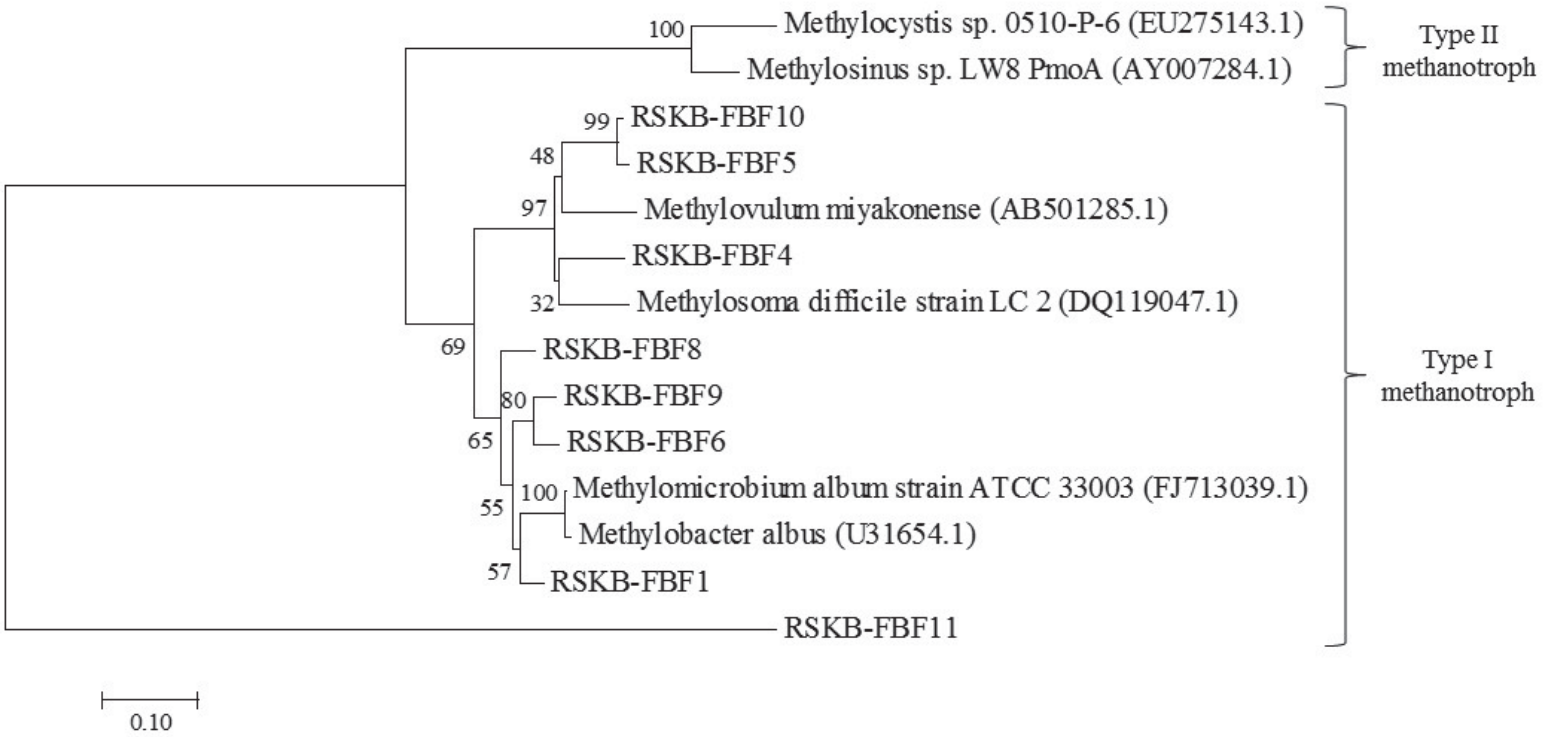

Figure 5.9 Unrooted phylogenetic tree constructed using Neighbour Joining method by considering the differences in nucleotides using a bootstrap value of 1000. All the cloned *pmoA* sequences (500 bp) showed similarities to different genera within type I methanotrophs (*Methylovulum*, *Methylosoma*, *Methylobacter* and *Methylomicrobium*), indicating presence of novel species or strains.

The CH_4 removal rate from these biofilters could be a function of many factors, including the importance of the surface area. In the previous study (Syed *et al.*, 2016b), the maximum CH_4 removal rate of 30.3 g m^{-3} h^{-1} was achieved with a 58-L biofilter column (54 cm height and 0.35-m diameter/surface area), whereas the floating biofilter with less than half of this volume (21 L, 6-cm depth and 0.5 m × 1 m surface area) removed up to 101.5 g m^{-3} h^{-1} of CH_4. This indicates that the CH_4 oxidising capacity of the biofilters can be limited by the surface area (Cohen, 2001), rather than the height/depth of the biofilter. However, it is also important to note here that the biofiltration systems are fundamentally different; the column biofilter was actively fed with CH_4 and air from the bottom, whereas the floating biofilter received passively-fed CH_4 from the bottom and air from the top.

5.4.2 Methanotroph population dynamics

Quantitative PCR results indicate that the field conditions encouraged the growth of both types of methanotrophs evenly, thus suggesting the importance of maintaining an abundant and even composition of these methanotrophs (Syed *et al.*, 2016a). Type II methanotrophs are known to co-metabolise various other organic compounds present in the biogas along with the CH_4 (Gebert *et al.*, 2008). This might explain the significant ($P < 0.005$) increase in type II methanotrophs over the study period in the field biofilters. The abundance of type I and type II methanotrophs in the field biofilters was higher than in the laboratory biofilters, indicating that the CH_4-oxidising capacity of the biofilters can be improved by exposing them to higher CH_4 loading rates (Dever *et al.*, 2013), until conditions are favourable for the growth and activity of methanotrophs. This is supported by the strong correlation found in this study between the CH_4 removal and abundance of methanotrophs (R^2 *(adj)* = 91%, $P = 0.01$). The increase in type I methanotroph abundance was supported by the increase in the *Methylobacter* group of methanotrophs, but the *Methylococcus* abundance was very low. The suppressed growth of the *Methylococcus* population at higher CH_4 concentrations was also evident in our previous studies (Syed *et al.*, 2016a, b). Interestingly, an increasing trend in the abundance of the *Methylocapsa*-like methanotrophs was noticed in the pond biofilters, and this could be related to the

ability of these methanotrophs to grow under acidic conditions. This concurs with our previous research finding (Syed *et al.*, 2016b). Even although the diversity (Shannon's) increased in the field biofilters over the study period, the population remained even, which suggested the presence of a stable methanotroph community in the pond biofilters. Data from PCA (Figure 5.7) and the heat map (Figure 5.8) analysis based on T-RF's confirms the significant differences between the laboratory and pond biofilters.

The conditions in the laboratory biofilters did not favour the growth of methanotrophs, especially the type II methanotrophs (including *Methylocapsa*). The reason for this decreased abundance is unknown, but could be related to the inability of these methanotrophs to adapt to varying CH_4 feeding conditions. In addition, the lab biofilters were fed with a mixture of CH_4 in CO_2 in the absence of other compounds (NH_3, H_2S and volatile organic compounds). Some of these other compounds might have had a stimulating effect on the growth and activity of type II methanotrophs. Shannon's diversity and evenness had also decreased over the study period, indicating a more unstable methanotroph population in the lab biofilter. Moisture levels of the lab biofilters (by the end of study period) were higher than those in the pond biofilters, so the effect of moisture on low abundance may not be evident.

5.4.3 Effect of H_2S on methanotroph population dynamics

Acidification of the biofilters was noticed in the floating field biofilters during the 11-month period. This is due to the oxidation of H_2S to H_2SO_4 in the biofilters. The abundance of the *Methylocapsa*-like methanotrophs showed an increasing trend in the floating biofilter. However, the acidity in the floating field biofilters did not influence the abundance of *Methylobacter* and type II methanotrophs, unlike in our previous study (Syed *et al.* 2016b). This earlier study indicated that the decrease in pH from between 4.72 and 3.8 was detrimental to these microorganisms but did not affect the overall CH_4 removal capacity of the soil; whereas a pH drop from 5.20 to 4.72 did not appear to suppress this group of methanotrophs. This finding is supported by other research (Manuel *et al.*, 2014), where higher H_2S (0.05% v/v) concentrations suppressed the growth of *Methylobacter* and *Methylocystis* methanotrophs. However, the process of acidification will be slower in a typical New Zealand dairy effluent pond due to its lower

organic content. This contrasts with our current experimental dairy effluent pond, where high milk inputs were occasionally dumped into the storage pond to increase the total volatile solids and subsequent production of H_2S and CH_4 by the methanogens. Acidification of the soil material may occur after a few years of operation, and the overall activity of the genera of methanotrophs could be affected when pH drops to 3.72 (Syed *et al.* 2016b). However, the pH could be restored to original levels by liming the soil, or by placing iron chips beneath the biofilter to capture H_2S before entering the biofilter bed as previously suggested by Pratt *et al* (2012a).

5.4.4 Practical considerations

For landfills, a cover biofilter could be easily installed, but constructing a floating structure on the effluent pond would incur some installation costs (up to 20 000 NZD) (Pratt *et al.*, 2012c). The alternative option would be to trap and collect biogas by covering the pond and passing the biogas through a network of pipes laid in soil under the surrounding effluent pond area. Nevertheless, our results confirm that the floating biofilter concept can offer an effective green mitigation technology for treating dairy effluent and other CH_4 emissions without requiring much maintenance.

5.5 Conclusions

Floating biofilters (containing primed volcanic pumice soil isolated from a Taupo landfill, New Zealand, and perlite) effectively removed high rates of CH_4-enriched biogas (up to 101.5 g CH_4 m^{-3} h^{-1}). The presence of a diverse and abundant methanotroph community facilitated CH_4 removal by the pond biofilter under varying field conditions. Methane removal was influenced more by the abundance and diversity of methanotrophs than by the other factors studied (moisture content, pH, total C and N). This study indicated that floating biofilters are potentially sustainable and offer the potential for mitigating high concentrations of CH_4 emissions from dairy effluent ponds, thereby contributing to the lowering of these greenhouse gas emissions to lessen their effects on climate change.

DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Rashad Syed

Name/Title of Principal Supervisor: Prof. Surinder Saggar

Name of Published Research Output and full reference:
Syed R, Saggar S, Tate KR and Rehm BHA (2016). Assessing the performance of floating biofilters for oxidation of CH_4 from dairy effluent ponds. Journal of Environmental Quality. (Manuscript under review).

In which Chapter is the Published Work: 5 – chapter

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:
  and / or
- Describe the contribution that the candidate has made to the Published Work:

First Author

Candidate's Signature — 4 OCT 2016 — Date

Principal Supervisor's signature — 4/10/16 — Date

GRS Version 3– 16 September 2011

Chapter 6

Assessment of potential biofilter materials to mitigate methane emissions

6.1 Introduction

Aerobic methanotrophs are present naturally in many New Zealand soils (pasture, forest and landfill) (Tate 2015; Tate *et al.* 2012). Biogas waste (containing CH_4) can be treated by passing it through a biofilter, where methanotrophs are immobilised on a carrier material. Previous New Zealand research on CH_4 mitigation focused only on using volcanic pumice soil - perlite mixture (subsequently called "volcanic pumice soil") (carrier material) as it had demonstrated excellent physical and chemical characteristics to support CH_4 oxidation (Pratt *et al.* 2012a, b, c; Syed *et al.* 2016b; Tate *et al.* 2007, 2012). Previous studies using molecular techniques indicated that the volcanic pumice soil had a healthy community of most of the genera of type I and type II aerobic methanotrophs favouring the removal of CH_4 under varied field conditions (Syed *et al.* 2016b).

Scaling up this volcanic pumice soil biofilter technology for national use to mitigate emissions is limited by the availability of volcanic pumice soil and has associated transportation costs. This study was therefore initiated to test alternative materials such as: farm soil, biochar, compost and pine bark that are cheaper and widely available locally, with excellent physical characteristics (porosity, bulk density) to support effective gas transfer. Even though the methanotrophs rely on CH_4 for C, these microorganisms might still require minute quantities of nutrients (N, P, K, Cu, Fe, Zn *etc.*) to enhance CH_4 oxidation (Asenjo and Suk, 1986; Anette Boiesen, 1993; Albanna *et al.*, 2007; Nikiema *et al.*, 2010; Semrau *et al.*, 2010). Another short experimental study (objective 2) was therefore conducted using inoculated farm soil and inoculated biochar to determine the

effect of nutrients addition on CH_4 oxidation and methanotrophs. The objectives of this study were (1) to compare the efficacy of cheaply and widely available materials (in New Zealand) with volcanic pumice soil, and to determine the ability of these biofilter materials to support growth and activity of methanotrophs, and (2) to assess the effect of nutrient addition in order to enhance the CH_4 oxidation process.

6.2 Materials and Methods

The potential biofilter materials tested were (i) farm soil (isolated from the area adjacent to an animal effluent storage pond), (ii) garden waste compost (3–5 months old), (iii) biochar from pine bark (prepared by pyrolysis at 450 °C), and (iv) sterilised weathered pine bark mulch (autoclaved at 121 °C for 15 min). These materials were inoculated with 20% of the active volcanic pumice soil (see next section for details) and CH_4 oxidation was measured in batch conditions for a period of about 6 months at constant temperature (25 °C). Moisture loss during the study period (1–1.5 g of water for every 5 weeks – data not shown) was compensated for by periodical spraying of about 1–1.5 mL distilled water onto the material. Physico-chemical properties of the materials tested are listed in Table 6.1.

6.2.1 Laboratory fed-batch experiments

Preliminary experiments were performed to select the best way to inoculate the alternative materials, whether by direct mixing or by suspending in buffers. Direct mixing of the inoculum (20%) with other alternative biofilter materials (80%) was established as the most effective approach (See Chapter 3.11). The total volume of the materials tested was kept constant at 100 mL; 20 mL of inoculum (volcanic pumice) was mixed with 80 mL of the material tested separately (in triplicates) in different air-tight 1800 mL AGEE^{TM} glass jars.

Table 6.1 Physico-chemical properties of the materials tested. [a] indicates significantly ($P < 0.05$) different value than the volcanic pumice soil.

Biofilter material	Dry bulk density (g cm^{-3})	Porosity (%)	Total C (%)	Total N (%)	NO_3^- - N (mg/kg)	NH_4^+ - N (mg/kg)
Volcanic pumice soil (inoculum)	0.42	75	4.17	0.36	27	34
Farm soil	0.63	75	4.67	0.48	683 [a]	224
Compost	0.44	80	14	1.35	1060 [a]	201
Biochar	0.19	85	86 [a]	0.19	1.44	20
Weathered Pine mulch	0.13	89	50 [a]	0.26	5.73	65

The CH_4 removal ability of the materials (without inoculum) was also studied. Volcanic pumice soil represents the positive control and is equivalent to 5X the inoculum compared to the other inoculated materials. For moisture content regulation, a 40-mL container half-filled with water was kept in the jars. Ports were fitted on the AGEE^{TM} jars for feeding CH_4 and for gas sampling purposes. All the materials were mixed with known amounts of water to adjust moisture content (Table 6.2) to suitable levels i.e., 40-60 % WHC suggested by Pratt *et al.* (2012b) and Moon *et al.* (2010)) to support CH_4 oxidation. Higher or lower moisture content could limit the gas transfer or nutrient transfer in the material. The pH of these materials varied between 2.8 to 7.0. The pH was not adjusted to optimum levels (5.5–6.5) as our intention was to simulate natural working conditions of these materials (see Table 6.2 for pH values).

Initially 10 mL of 60% CH_4 (in CO_2) was injected to supply CH_4 at 3 300 ppm, and then increased to 10 000 ppm and 20 000 ppm over the study period. Methane and O_2 were regularly fed at the start of each batch period, which lasted for 24 hours. Oxygen was supplied by opening the lid of the jar and letting fresh air passively diffuse into the jar for about 20 minutes, as previously reported by Pratt *et al.* (2012a). Gas samples containing CH_4, CO_2 and N_2O were analysed by gas chromatography (GC) (Schimadzu auto GC-2010) using flame ionisation (FID), thermal conductivity (TCD) and electron capture (ECD) detectors, respectively. GC was calibrated over the following gas standard ranges

CH_4 (0–25 000 ppmv), CO_2 (0–50 000 ppmv) and N_2O standards (0–2000 ppbv). Stabilisation period of the material was calculated by estimating the number of days a material took to consistently remove ≥80% CH_4, after an initial few weeks of the acclimatisation.

Methane removal (%) was calculated using the equation, where, Ct is concentration of CH_4 (ppm) at time t; and C_0 is concentration (ppm) at time 0.

$$(C_0–C_t)/C_0 \times 100$$

6.2.2 Addition of nutrients to soil and biochar

Another batch experiment as described above was set up for 3 months to assess the impact of nutrient supply on CH_4 oxidation, and methanotroph abundance and diversity. As farm soil and biochar biofilter materials performed best, they were used with the addition of 12 mL of NMS (nitrate mineral salts) media and were fed with two different CH_4 concentrations (3 300 ppm and 20 000 ppm) throughout the study period. For 100 mL of the material, amounts of N, P and K added to the materials were 1.66 mg, 0.26 mg and 4.73 mg respectively; whereas the amounts for micronutrients (Zn, Mn, B, Co, Cu, Ni, Mo, Fe, Mg and Ca) were very small (< 0.5 µg). The final composition of the NMS media in the materials was as follows KNO_3 (12 mg), Na_2HPO_4 (864 µg), KH_2PO_4 (336 µg), Tetra sodium EDTA (12 µg), $ZnSO_4.7H_2O$ (0.84 µg), $MnCl_2.4H_2O$ (0.36 µg), H_3BO_3 (3.6 µg), $CoCl_2.6H_2O$ (2.4 µg), $CuCl_2.2H_2O$ (0.12 µg), $NiCl_2.6H_2O$ (0.24 µg), Na_2Mo_4, $2H_2O$ (0.36 µg), $FeSO_4.7H_20$ (0.06 µg), $MgSO_4.7H_20$ (2.4 µg) & $CaCl_2.2H_2O$ (0.24 µg).

6.2.3 Physico-chemical analysis

Moisture content, pH, total C and N, nitrate and ammonium–N were determined as per the protocols described in Chapters 3.2, 3.3, 3.5 and 3.6. The particle density, dry and wet bulk density and porosity of the materials were calculated following the techniques described by Gradwell (1972).

6.2.4 Molecular analysis

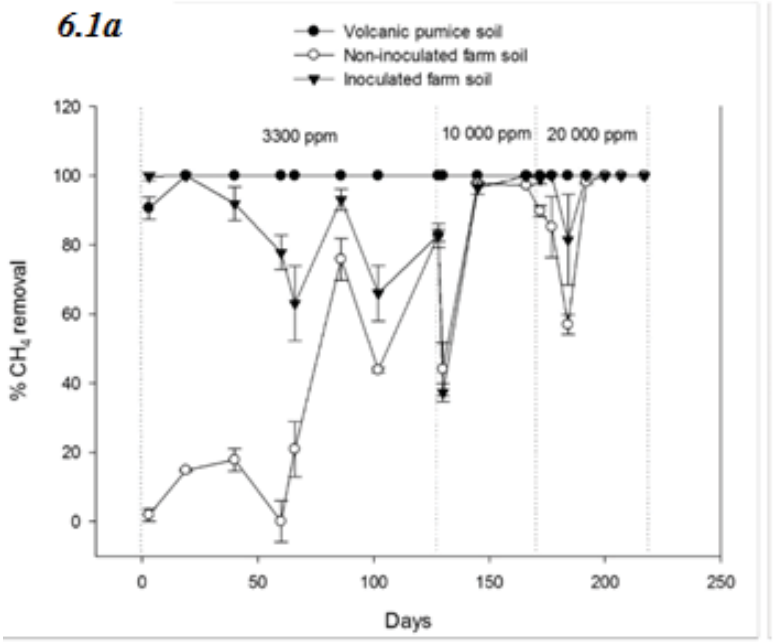
DNA was extracted from the soil samples by following the procedures described in Chapter 3.7. Quantitative PCR analysis was done as described in Chapter 3.8. The r^2 values of the linear regressions plots of crossing point (C_p) values and logarithmic gene copy number values of the standards ranged from 0.9925 to 1. Terminal-RFLP analysis was done as described in Chapter 3.9.

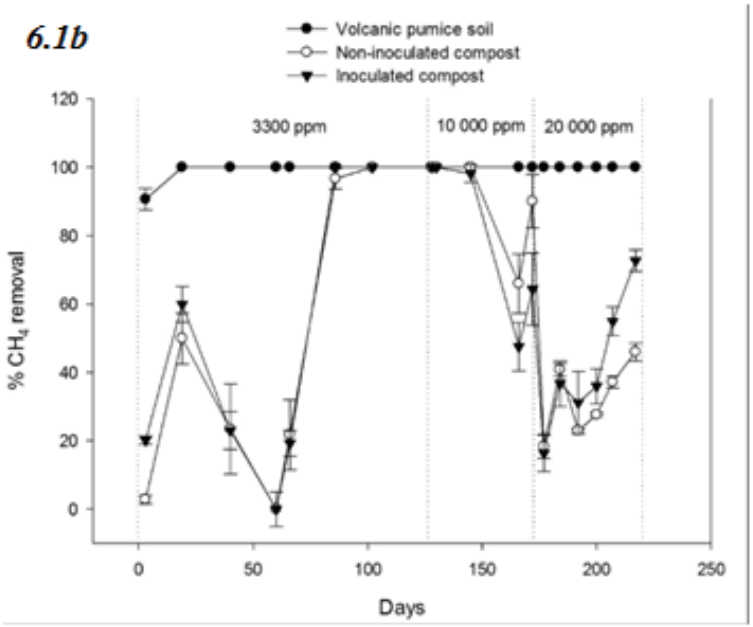
6.2.5 Phylogenetic tree construction

Soil DNA samples from the best performing biofilter candidates, i.e. farm soil, biochar and volcanic pumice soil were combined. *Pmo*A genes were amplified using A189F and Mb661R (Kolb *et al.*, 2013) and purified by gel extraction and cloned using a pGEM®-T vector system (Promega, In Vitro Technologies, New Zealand) following the manufacturer's instructions. Eleven randomly selected clones containing genes of insert (*pmo*A) were sub-cultured in Luria Bertani (LB) medium overnight and the plasmids were extracted using a commercial Kit (DNA and plasmid purification kit, MN™) as described in Chapter 3.10. Phylogenetic neighbour-joining tree analysis of the eleven clones was performed using the MEGA 7 software package (Kumar *et al.* 2016). Sequences obtained in this study were deposited at the NCBI nucleotide sequence database under accession numbers (KU215855 – KU215865). *In silico* digestion of clone sequences was performed using Snap Gene™ Version 1.1.3 and were related to experimental T-RF's.

6.2.6 Statistical analysis

Statistical analysis of the data was performed using Minitab ® Version 16 software. One-way analysis of variance was used to assess the differences in the means in gene copy numbers and T-RFs among different materials. The Holm-Sidak test with $\alpha = 0.05$ significance level was used for the *post hoc* analysis to reveal the significant differences in the means.


6.1a
Volcanic pumice soil
Non-inoculated farm soil
Inoculated farm soil
3300 ppm
10 000 ppm
20 000 ppm
% CH4 removal
Days
0
20
40
60
80
100
120
0
50
100
150
200
250


6.1b
Volcanic pumice soil
Non-inoculated compost
Inoculated compost
3300 ppm
10 000 ppm
20 000 ppm
% CH4 removal
Days
0
20
40
60
80
100
120
0
50
100
150
200
250

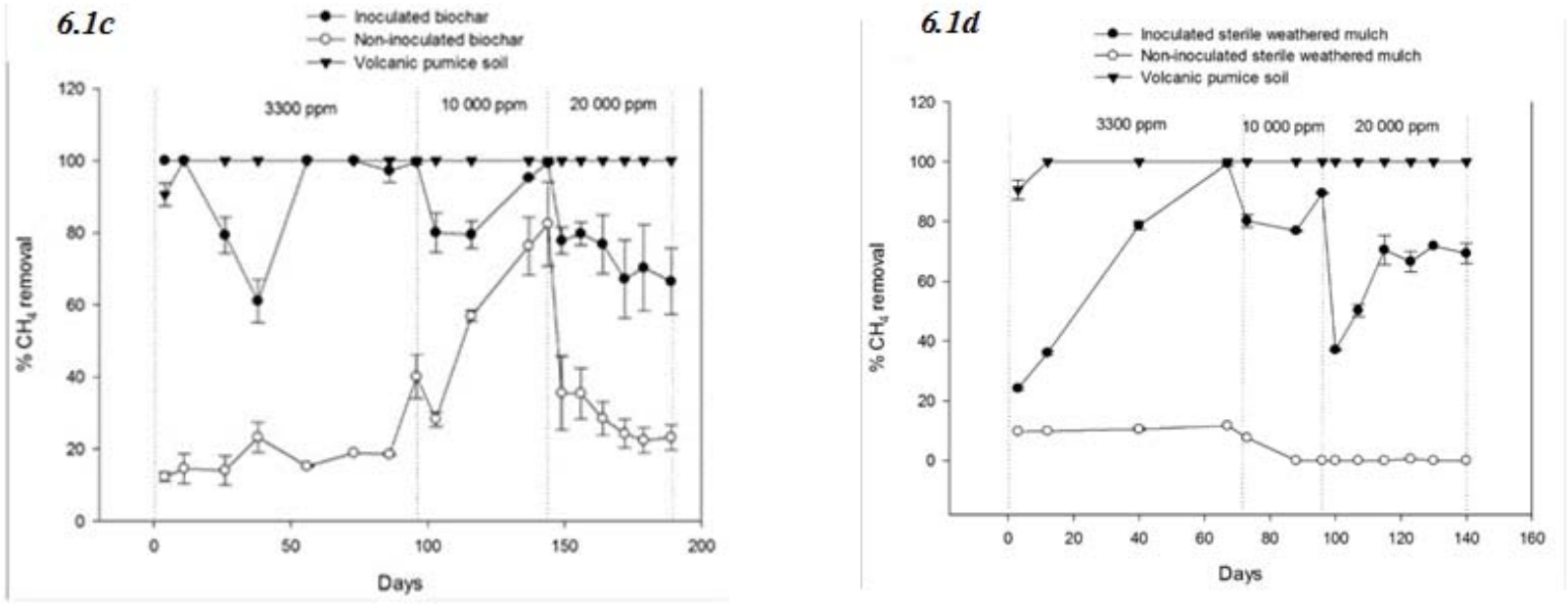


Figure 6.1 CH_4 removal of the inoculated and non-inoculated materials – (a) farm soil,(b) compost, (c) biochar and (d) sterile weathered mulch. Volcanic pumice soil (the positive control) had 5X inoculum compared to other materials tested (farm soil, compost, biochar and sterile weathered mulch). Error bars represent the standard deviation from the mean of triplicates. Each data point represents the % CH_4 calculated at the end of a 24 hour fed-batch period. Varying CH_4 feeding phases (3 300 ppm, 10 000 ppm and 20 000 ppm) are indicated using the dashed lines.

The relationship between CH_4 oxidation and different soil characteristics was determined using regression analysis and *Pearson correlation*. Principal component analysis (PCA) was used to evaluate the effect of community structure (T-RFs) on different materials. Cluster analysis was performed based on Euclidean distances using single linkage method to produce dendograms by revealing groupings of materials based on similar T-RFs profile.

6.3 Results

6.3.1 Methane removal of materials (with and without inoculum)

Methane removals (%) by the test materials (farm soil, compost, biochar and weathered mulch) inoculated with 20% volcanic pumice soil are shown in Figure 6.1a, b, c and d. Methane removal in all the inoculated materials fluctuated between 0 and 100% at different stages of the study. Among the inoculated materials, biochar and farm soil removed ≥80% CH_4 until day 23; then this decreased gradually and fluctuated during the later stages of the experiment (Figure 6.1a and c). During the last quarter stages of the study (from day 145), inoculated farm soil and inoculated biochar were removing ~100% and ~60–75% CH_4, respectively, although the high doses of CH_4 (20 000 ppm) during the later stages of the study period negatively affected the CH_4 removal ability of the inoculated biochar. Overall more than 80% of CH_4 was removed during the study period. Methane removal in the inoculated sterile weathered mulch and inoculated compost was initially low (up to day 60) but this was followed by higher CH_4 removal periods during the later stages of the study (Figure 6.1b and d). Of all the materials tested, inoculated farm soil and inoculated biochar performed best (Figure 6.2).

Among the non-inoculated or pure biofilter materials (farm soil, biochar, compost and weathered mulch), very low or no CH_4 removal was evident during the initial study period. However, during the later stages, farm soil and compost were removing up to 100% of CH_4. Nevertheless, low and high CH_4 removal phases were also noticed for non-

inoculated materials (farm soil, compost, and biochar) (Figure 6.1a, b, c and d). Throughout the study period, sterile weathered mulch was removing either no or <20% of CH_4 (Figure 6.1d). Average CH_4 removal (Figure 6.2) in the volcanic pumice inoculated farm soil, biochar, sterile weathered mulch, and compost was 89, 86, 67, and 55% respectively. In the inoculated farm soil CH_4 removal fluctuated between 37% and 100%. In the farm soil (without inoculum) CH_4 removal fluctuations were between 2% and 100%.

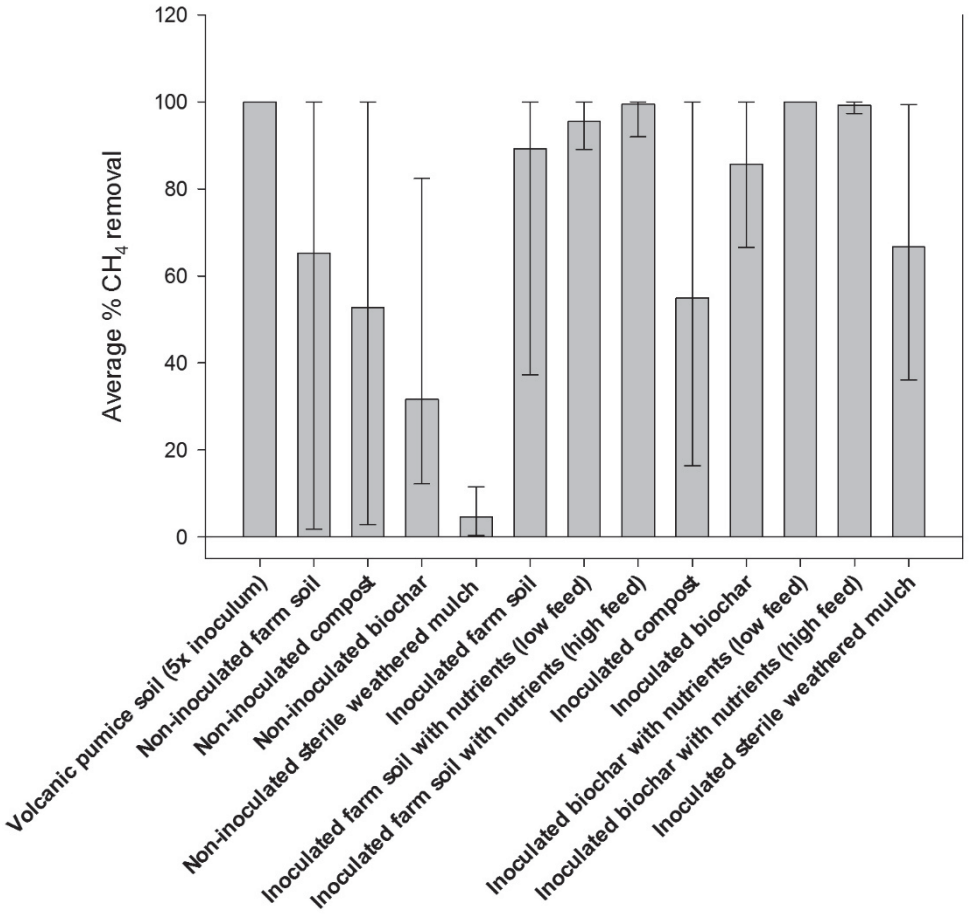


Figure 6.2 Average CH_4 removal (%) by all the biofilter materials tested over the study period. Error bars represents the maximum and minimum CH_4 removed during the study period (n=3). Volcanic pumice soil (the positive control) had 5x inoculum compared to other materials tested (farm soil, compost, biochar and sterile weathered mulch). Low feed-CH_4 concentration supplied at 3 300 ppm, and high feed-CH_4 concentration supplied at 20 000 ppm.

In a comparative study involving inoculated farm soil and inoculated biochar – with and without nutrients – the time taken (stabilisation period) for the materials to remove more than 80% CH_4 is shown in Figure 6.3. Inoculated farm soil and inoculated biochar amended with nutrients took ~20 days and <3 days respectively to stabilise, whereas materials without added nutrients took a much longer time (inoculated farm soil – 145 days; inoculated biochar – 58 days).

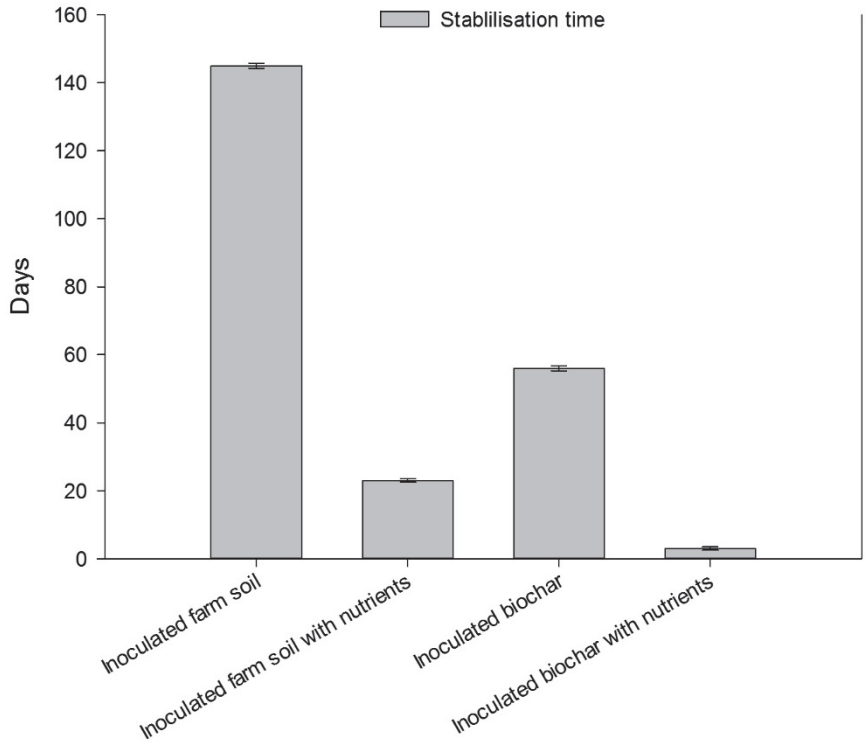


Figure 6.3 Comparing inoculated soil and biochar – with and without amended nutrients. Stabilisation time indicates the number of days required for a material to reach a stable CH_4 removal efficiency of more than 80% (n=3).

Ambient levels of N_2O concentrations were measured in the gas samples taken from all the materials and ranged between 330 and 370 ppbv (data not shown). Moisture content of all the materials remained c. 40–80% (dry wt), except for biochar and mulch where the moisture content levels were a little higher. No drying or clogging of the material was evident, suggesting the materials tested were moist enough to support CH_4 oxidation. In addition, no significant changes in pH (initial and final) were evident in the materials over the study period (see Table 6.2).

Table 6.2 Initial and final moisture content and pH measurements of the materials tested. n=3.

Biofilter materials	**Moisture (% dry wt)**		**pH**	
	Day 0	Final	Day 0	Final
Volcanic pumice	40.3	57.3	5.99	6.11
Farm soil	29.6	46.2	5.68	5.54
Compost	51.2	73.4	7.36	7.28
Biochar	84.7	175.0	6.84	6.7
Sterile weathered mulch	111.4	120.9	2.74	2.57
Inoculated farm soil	31.5	44.1	5.62	5.56
Inoculated farm soil amended with nutrients	31.3	40.6	5.14	5.66
Inoculated compost	48.4	76.2	7.36	7.13
Inoculated biochar	56.9	138.1	6.7	6.48
Inoculated biochar amended with nutrients	77.4	61.7	6.37	6.61
Inoculated sterile weathered mulch	110.3	69.7	3.38	3.32

6.3.2 Methanotroph abundance

Quantitative PCR results indicate the presence of both type I and type II methanotrophs in all the materials, but their ratios varied (Table 6.3). Among the inoculated materials, except for the compost all the materials had abundant methanotroph population (ratio between type I and II was between 1.70 and 3.05); whereas compost had significantly ($P < 0.05$) higher type I to type II methanotroph ratio (13.03) (Table 6.3). Over the study period, gene copy numbers of the type I methanotrophs increased significantly ($P < 0.05$) in all the materials except in the farm soil (inoculated and non-inoculated) and non-inoculated biochar – where the increase was not significant (Table 6.3). The type II methanotrophs increased significantly in all the materials, except in the inoculated compost. Gene copy numbers in non-inoculated biochar and non-inoculated weathered mulch were significantly ($P < 0.05$) lower than in the other two materials (non-inoculated soil and non-inoculated compost). By the end of the study, the type I methanotrophs of both inoculated and non-inoculated compost were significantly ($P < 0.005$) higher than the volcanic pumice soil (positive control); however, the type II methanotrophs gene copy numbers were significantly ($P < 0.005$) lower. Other non-inoculated materials (biochar and weathered mulch) had significantly ($P < 0.05$) lower type I and type II methanotrophs, when compared to volcanic pumice soil by the end of the study. In inoculated farm soil and inoculated biochar amended with nutrients – fed with high concentrations of CH_4 (20 000 ppm) both type I and type II methanotrophs increased significantly ($P < 0.05$) over the study period; however, in low CH_4 concentrations fed (3 000 ppm) materials, the increase was insignificant, thus indicating the effect of feeding concentrations on methanotrophs abundance. In inoculated farm soil amended with nutrients (high feed) – the type I methanotroph gene copy numbers increased within 52 days of incubation by 117% and were significantly ($P > 0.05$) higher when compared with inoculated farm soil (without nutrients) after 217 days. By contrast, the type II methanotroph gene copy numbers increased by 53% in less than 52 days. Similarly, within 52 days of incubation, the type I

Table 6.3 Gene copy number ($\times 10^8$) per gram of dry material. Table includes the data of initial and final days of the study period. Values are reported as Avg±SD. Volcanic pumice soil (the positive control) had 5x inoculum compared to other materials tested (farm soil, compost, biochar and sterile weathered mulch); # = indicates inoculated farm soil/biochar amended with nutrients supplied at a CH_4 concentration of 3 300 ppm (qPCR numbers are after 52 days of incubation) and * = indicates inoculated farm soil/biochar amended with nutrients supplied at CH_4 concentration of 20 000 ppm during the 24-hr fed-batch period (qPCR numbers are after 52 days of incubation). [a] indicates the materials are statistically significant ($P < 0.05$) from volcanic pumice soil, whereas [b] indicates the significant difference ($P < 0.05$) between respective inoculated and non-inoculated material.

Biofilter materials	***Gamma-proteobacterial* methanotrophs**		***Alpha-proteobacterial* methanotrophs**		***Methylobacter***		***Methylococcus***		***Methylocapsa***		***Type I / Type II ratio***	
	Day 0	*Day final*	*Day 0*	*Day final*	*Day 0*	*Day final*	*Day 0*	*Day final*	*Day 0*	*Day final*	*Day 0*	*Day final*
Volcanic pumice soil	6.78±0.00	5.36 ±0.05	1.39±0.05	2.32±0.03	1.13±0.06	1.24±0.02	0.38±0.00	0.15±0.01	2.98±0.21	2.32±0.04	4.88±0.18	2.31±0.01
Farm soil	4.43±0.09	4.73 ±0.00	0.46±0.00	1.39±0.01 [a b]	0.35±0.0	0.52±0.00	0.33±0.01	0.08±0.00	3.16±0.11	1.19±0.14	9.63±0.20	3.40±0.02
Compost	4.25±0.42	12.68±0.20[a]	0.39±0.01	0.56 ±0.02[a]	0.29±0.01	7.43±0.03[a]	0.76±0.00	0.32±0.02	2.59±0.08	-	10.88±0.88	22.66±0.45[a]
Biochar	0.14±0.00	0.63±0.05 [a b]	0.03±0.00	0.79 ±0.03[a]	0.01±0.00	0.17±0.01	0.14±0.02	0.02±0.00	0.05±0.00	0.82±0.02	4.67±0.00	0.80±0.03

Sterile weathered mulch	0.02±0.00	0.04±0[a b]	0.03±0.0	0.06±0[a]	0.29±0.01	0.51±0.02	0.07±0.02	0.03±0.01	0.81±0.00	1.63±0.02	0.67±0.00	0.68±0.02
Inoculated farm soil	4.87±0.09	5.24±0.05	0.75±0.01	2.02±0.06[b]	0.52±0	1.21±0.08	0.31±0.02	0.14±0.00	2.81±0.11	2.49±0.08	6.49±0.03	2.60±0.05
Inoculated farm soil with nutrients (low feed) [#]	2.95±0.20	4.32±0.07	0.84±0.04	0.73±0.01	0.52±0.00	1.27±0.04	0.28±0.01	0.07±0.01	1.28±0.02	1.05±0.11	3.51±0.07	5.92±0.18
Inoculated farm soil with nutrients (high feed) [*]	2.95±0.20	6.41±0.17[a b]	0.84±0.04	1.29±0.01[a]	0.52±0.00	3.11±0.11	0.28±0.01	0.12±0.00	1.28±0.02	1.63±0.01	3.51±0.07	4.97±0.09
Inoculated compost	4.80±0.14	10.68±0.37[a]	0.79±0.02	0.82±0.03[a b]	0.38±0.02	7.09±0.17[a]	0.45±0.01	0.26±0.00	2.07±0.08	-	6.08±0.02	13.03±0.03[a]
Inoculated biochar	3.88±0.06	5.46±0.03[b]	1.21±0.05	3.22±0.15[a b]	0.51±0.01	1.21±0.00	0.33±0.03	0.21±0.02	2.2±0.03	3.48±0.11	3.21±0.08	1.70±0.07
Inoculated biochar with nutrients_low feed [#]	2.34±0.06	3.59±0.03[a b]	1.04±0.01	2.03±0.12[a]	0.52±0.01	0.88±0.06	0.22±0.00	0.16±0.03	1.37±0.05	1.88±0.01	2.25±0.04	1.78±0.12
Inoculated biochar with nutrients_high feed [*]	2.34±0.06	3.07±0.05[a b]	1.04±0.01	2.16±0.06[a]	0.52±0.01	0.66±0.04	0.22±0.00	0.11±0.02	1.37±0.05	1.62±0.05	2.25±0.04	1.42±0.02
Inoculated sterile weathered mulch	1.17±0.03	5.19±0.07[a b]	0.52±0.01	1.7±0[a b]	0.46±0.01	1.08±0.12	0.24±0.01	0.06±0.01	1.22±0.09	1.44±0.03	2.25±0.01	3.05±0.04

and type II methanotrophs increased by 31% and 107%, respectively in inoculated biochar amended with nutrients (high feed) but were significantly ($P < 0.05$) lower than the methanotroph populations of inoculated biochar (Day 190). However, the ratio between type I and type II methanotrophs were similar to that of volcanic pumice soil (Table 6.3). In all the materials (both with and without inoculum), gene copy numbers belonging to type I methanotrophs group were comparatively higher than type II methanotrophs. Increases in overall type I methanotrophs were reflected in the increase in gene copy numbers belonging to the *Methylobacter* group (Table 6.3). In addition, gene copy numbers belonging to the *Methylococcus* genera decreased and those belonging to the *Methylocapsa* genera remained almost constant in all the materials over the study period.

6.3.3 Methanotroph diversity

Shannon's diversity and the evenness of all the materials are listed in Table 6.4. The number of phylotypes increased significantly ($P < 0.05$) in the non-inoculated biochar, whereas in other materials it slightly increased or remained more or less unchanged over the study period (Table 6.4). The evenness of the inoculated materials ranged from 0.90 to 0.95, except for inoculated sterile weathered mulch (0.88).

Based on the physical component analysis (Figure 6.4) of terminal-restriction fragments (T-RFs), in contrast to Day 0 the methanotroph diversity (T-RF's pattern) of inoculated and non-inoculated farm soil were similar up to 80%, by the end of study. The diversity of inoculated and non-inoculated compost was also similar up to 75%. However, the methanotroph diversity among both inoculated and non-inoculated biochar and sterile weathered mulch were different. Interestingly, the diversity of inoculated biochar and volcanic pumice soil were 50% similar by the end of the study, by contrast with other materials where they were less similar. Heat map analysis of T-RFs (with more than 0.05 relative abundance) is shown in Figure 6.5. Both inoculated farm soil and inoculated biochar had almost similar T-RF profile, however inoculated compost and inoculated weathered mulch had slightly different profile. Inoculated farm soil with nutrients (high feed) had slightly different profile than the inoculated farm soil (without nutrients).

Table 6.4 Richness, shannon's diversity and evenness of the biofilter materials analysed on initial and final days of the study period. The higher the shannon's diversity, the higher the diversity of the methanotrophs; evenness is measured between a value of 0 and 1 (I indicates the even distribution of the population). Volcanic pumice soil (the positive control) had 5X inoculum compared to other materials tested (farm soil, compost, biochar and sterile weathered mulch); # = indicates inoculated farm soil/biochar amended with nutrients supplied at a CH_4 feeding concentration of 3 300 ppm and * = indicates inoculated farm soil/biochar amended with nutrients supplied at CH_4 concentration of 20 000 ppm during the 24-hr fed-batch period. [a] indicates statistically signficant increase during the study period.

Biofilter materials	**Richness (S)**	**Shannon's Diversity (H′)**	**Evenness ($E_{H)}$**
Volcanic pumice_Day 0	51 ±8	3.91 ±0.15	0.85 ±0.03
Volcanic pumice_Day final	75 ±4	4.32 ±0.05	0.93 ±0.01
Farm soil_Day 0	77 ±4	4.35 ±0.05	0.94 ±0.01
Farm soil_Day final	75 ±2	4.32 ±0.03	0.93 ±0.01
Compost_Day 0	68 ±4	4.23 ±0.05	0.91 ±0.01
Compost_Day final	82 ±7	4.41 ±0.08	0.95 ±0.02
Biochar_Day 0[a]	21 ±1	3.07 ±0.02	0.66 ±0.01
Biochar_Day final[a]	62 ±2	4.13 ±0.03	0.89 ±0.01
Sterile weathered mulch_Day 0	37 ±10	3.46 ±0.12	0.76 ±0.02
Sterile weathered mulch_Day final	38 ±7	3.47 ±0.08	0.77 ±0.02
Inoculated farm soil_Day 0	62 ±2	4.12 ± 0.03	0.89 ±0.01
Inoculated farm soil_Day final	72 ±5	4.27 ±0.07	0.92 ±0.02
Inoculated farm soil with nutrients_Day 0	68 ±1	4.22 ±0.01	0.91 ±0.00
Inoculated farm soil with nutrients (low feed)_Day final[#]	34 ±1	3.54 ±0.01	0.76 ±0.00
Inoculated farm soil with nutrients (high feed)_Day final[*]	51 ±9	3.93 ±0.17	0.85 ±0.04
Inoculated compost_Day 0	61 ±5	4.1 ± 0.08	0.89 ±0.02

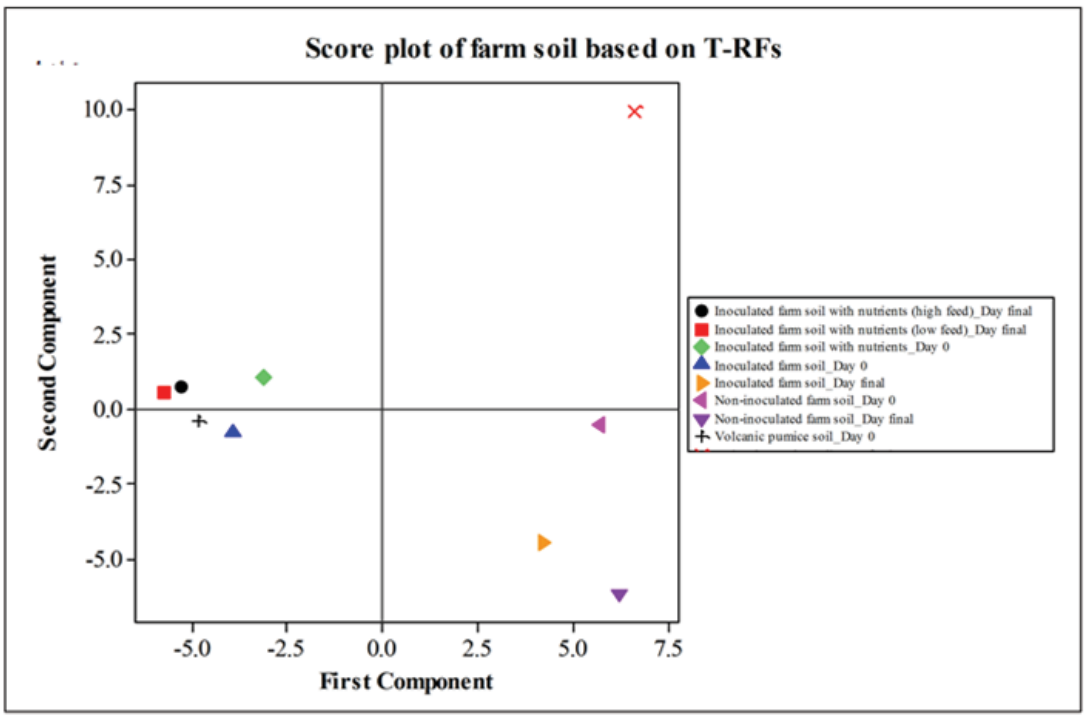
Inoculated compost_Day final	73 ±3	4.29 ±0.04	0.93 ±0.01
Inoculated biochar_Day 0	46 ±5	3.82 ±0.11	0.82 ±0.02
Inoculated biochar_Day final	71 ±11	4.25 ±0.16	0.92 ±0.03
Inoculated biochar with nutrients_Day 0	38 ±6	3.64 ±0.14	0.79 ±0.03
Inoculated biochar with nutrients (low feed)_Day final[#]	52 ± 2	3.96 ±0.03	0.85 ±0.01
Inoculated biochar with nutrients (high feed)_Day final[*]	45 ±2	3.81 ±0.04	0.82 ±0.01
Inoculated sterile weathered mulch_Day 0	70 ±1	4.25 ±0.01	0.92 ±0.00
Inoculated sterile weathered mulch_Day final	60 ±5	4.10 ±0.07	0.88 ±0.02

It is important to note that in the inoculated materials amended with nutrients (farm soil and biochar), both Shannon's diversity and evenness (Table 6.4) were less compared with their counterparts without nutrients, indicating that nutrient addition had an effect on the diversity of methanotrophs (Figure 6.4b and f).

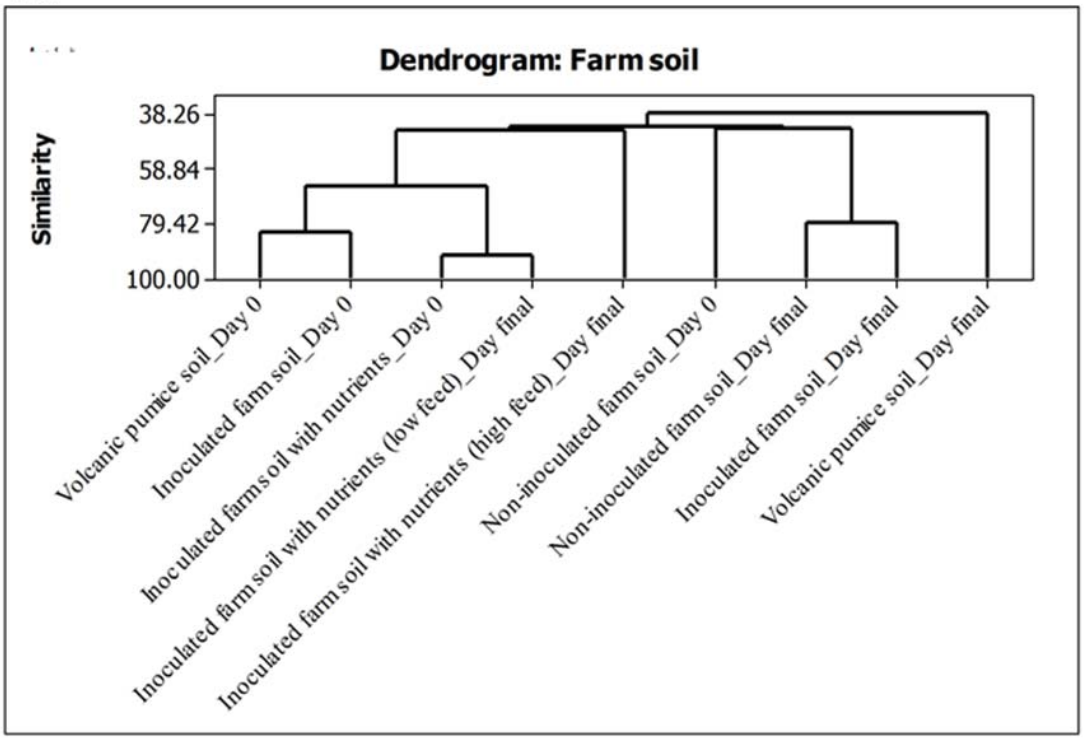
6.3.4 Factors affecting CH_4 removal

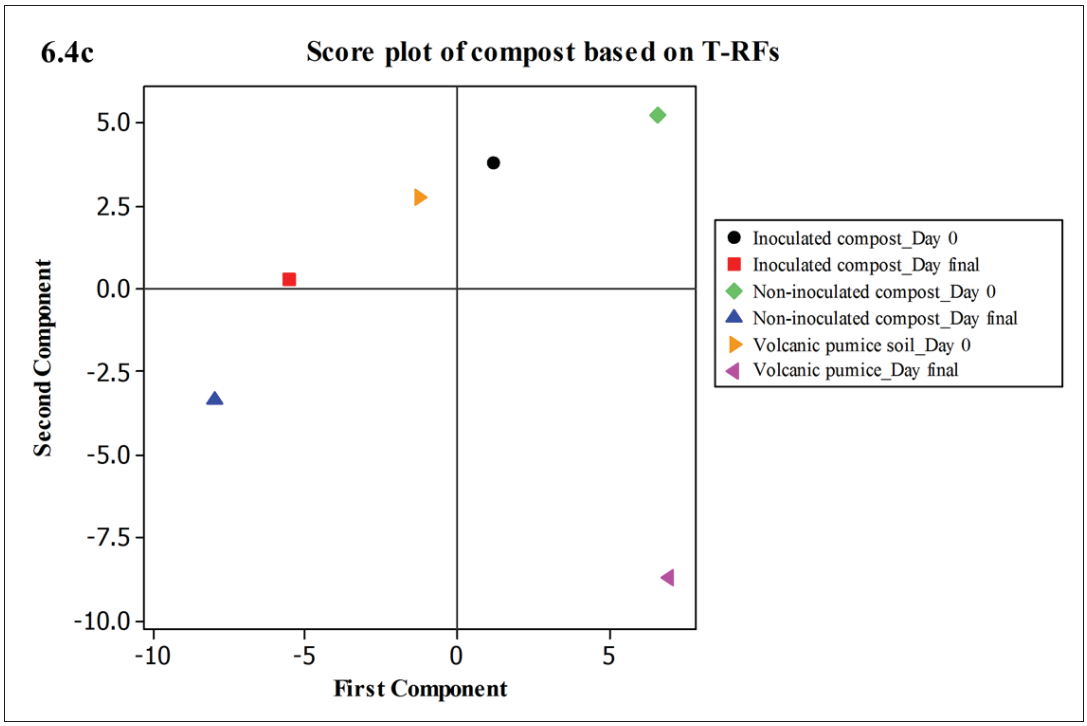
Factors affecting the performance of materials were assessed by preparing biplots using factoral analysis of physical, chemical and biological factors controlling the CH_4 oxidation (Figure 6.6). Methanotroph abundance and diversity had significant influence (R^2 = 68%, P = 0.001) on CH_4 oxidation, when compared with physical and chemical characteristics *viz.* porosity, bulk density, moisture content, pH, total C, nitrate and ammonium N (R^2 = 39%, P = 0.139). Based on the *Pearson correlation* analysis, CH_4 oxidation was positively correlated with type II methanotrophs gene copy numbers (r = 0.70). Interestingly NO_3-N had a positive effect on type I methanotrophs population (r = 0.60), whereas negatively but non-significantly affected type II population (r = –0.26).

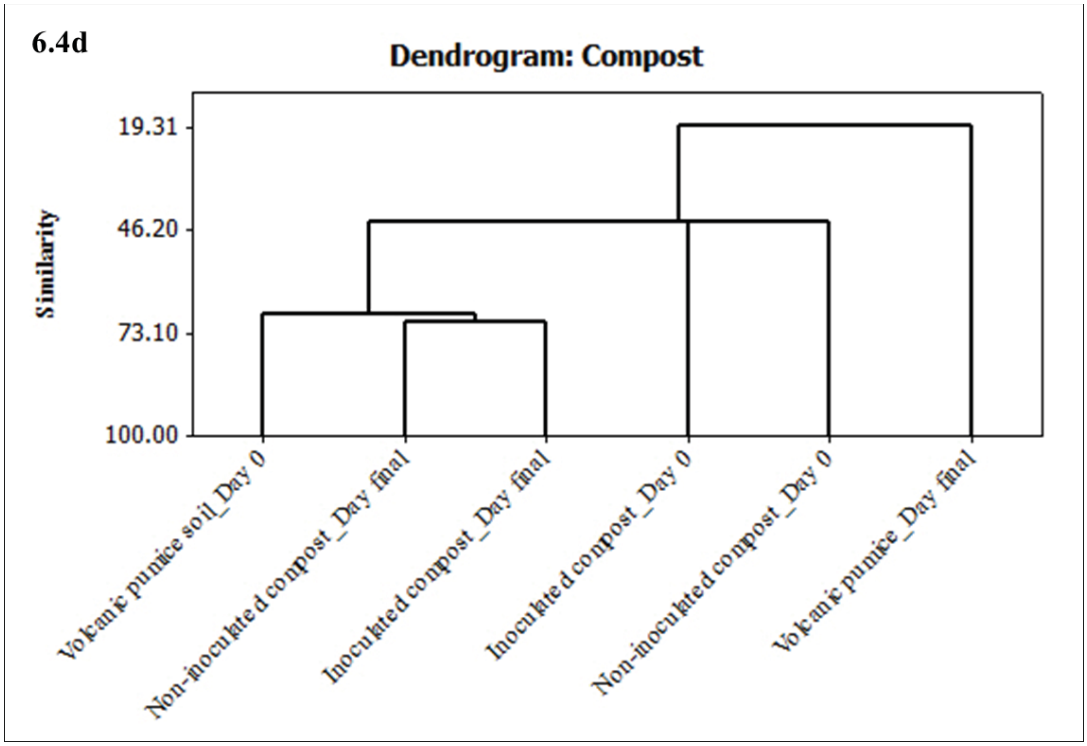
6.4a

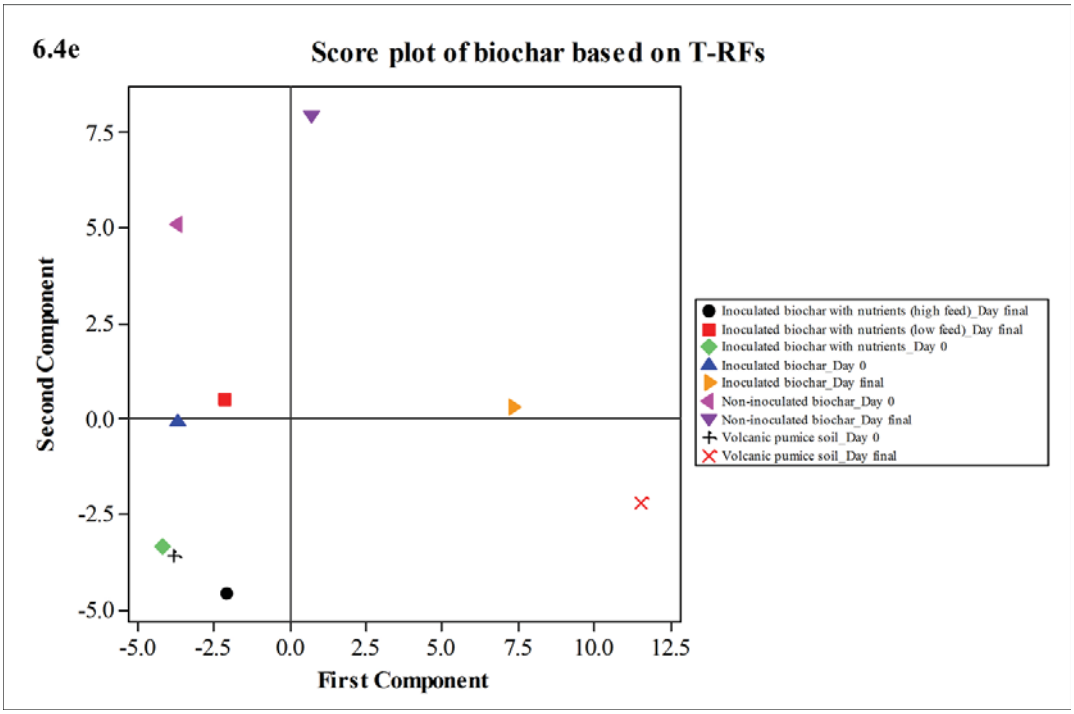

Score plot of farm soil based on T-RFs
Second Component
First Component
10.0
7.5
5.0
2.5
0.0
-2.5
-5.0
-5.0
-2.5
0.0
2.5
5.0
7.5
Inoculated farm soil with nutrients (high feed)_Day final
Inoculated farm soil with nutrients (low feed)_Day final
Inoculated farm soil with nutrients_Day 0
Inoculated farm soil_Day 0
Inoculated farm soil_Day final
Non-inoculated farm soil_Day 0
Non-inoculated farm soil_Day final
Volcanic pumice soil_Day 0

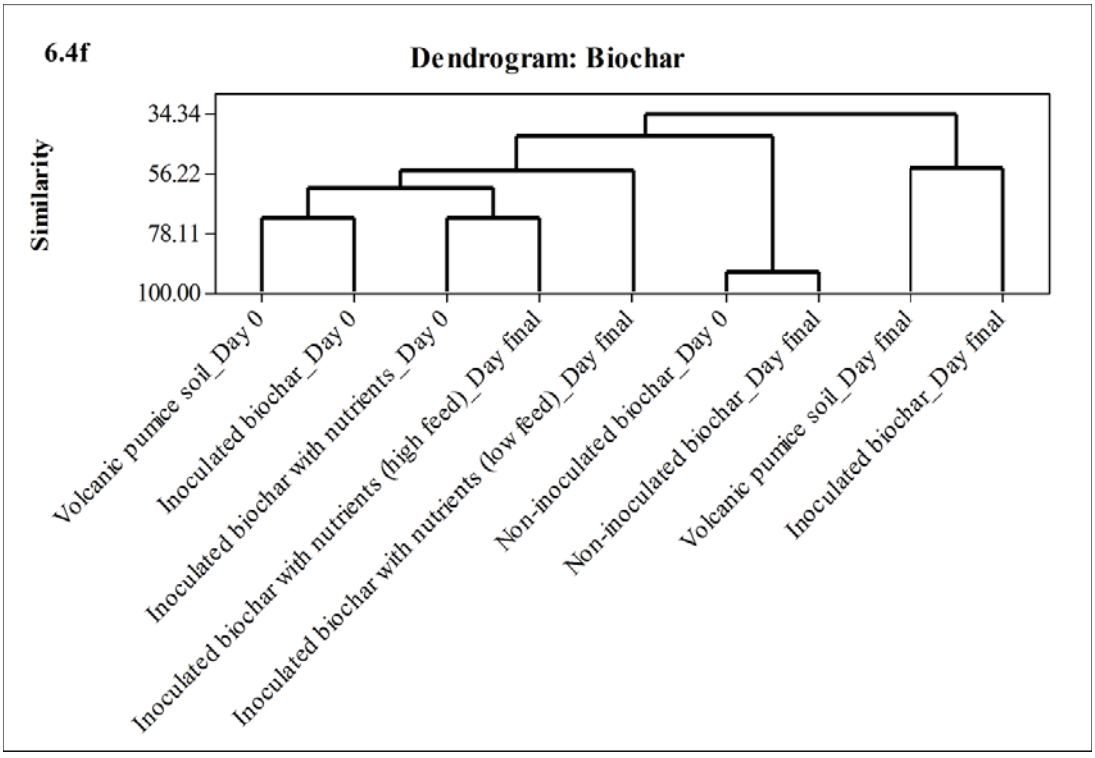
6.4b


Dendrogram: Farm soil
Similarity
38.26
58.84
79.42
100.00
Volcanic pumice soil_Day 0
Inoculated farm soil_Day 0
Inoculated farm soil with nutrients_Day 0
Inoculated farm soil with nutrients (low feed)_Day final
Inoculated farm soil with nutrients (high feed)_Day final
Non-inoculated farm soil_Day 0
Non-inoculated farm soil_Day final
Inoculated farm soil_Day final
Volcanic pumice soil_Day final


6.4c
Score plot of compost based on T-RFs
Second Component
First Component
5.0
2.5
0.0
-2.5
-5.0
-7.5
-10.0
-10
-5
0
5
Inoculated compost_Day 0
Inoculated compost_Day final
Non-inoculated compost_Day 0
Non-inoculated compost_Day final
Volcanic pumice soil_Day 0
Volcanic pumice_Day final


6.4d
Dendrogram: Compost
Similarity
19.31
46.20
73.10
100.00
Volcanic pumice soil_Day 0
Non-inoculated compost_Day final
Inoculated compost_Day final
Inoculated compost_Day 0
Non-inoculated compost_Day 0
Volcanic pumice_Day final


6.4e
Score plot of biochar based on T-RFs
Second Component
First Component
7.5
5.0
2.5
0.0
-2.5
-5.0
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
12.5
Inoculated biochar with nutrients (high feed)_Day final
Inoculated biochar with nutrients (low feed)_Day final
Inoculated biochar with nutrients_Day 0
Inoculated biochar_Day 0
Inoculated biochar_Day final
Non-inoculated biochar_Day 0
Non-inoculated biochar_Day final
Volcanic pumice soil_Day 0
Volcanic pumice soil_Day final


6.4f
Dendrogram: Biochar
Similarity
34.34
56.22
78.11
100.00
Volcanic pumice soil_Day 0
Inoculated biochar_Day 0
Inoculated biochar with nutrients_Day 0
Inoculated biochar with nutrients (high feed)_Day final
Inoculated biochar with nutrients (low feed)_Day final
Non-inoculated biochar_Day 0
Non-inoculated biochar_Day final
Volcanic pumice soil_Day final
Inoculated biochar_Day final

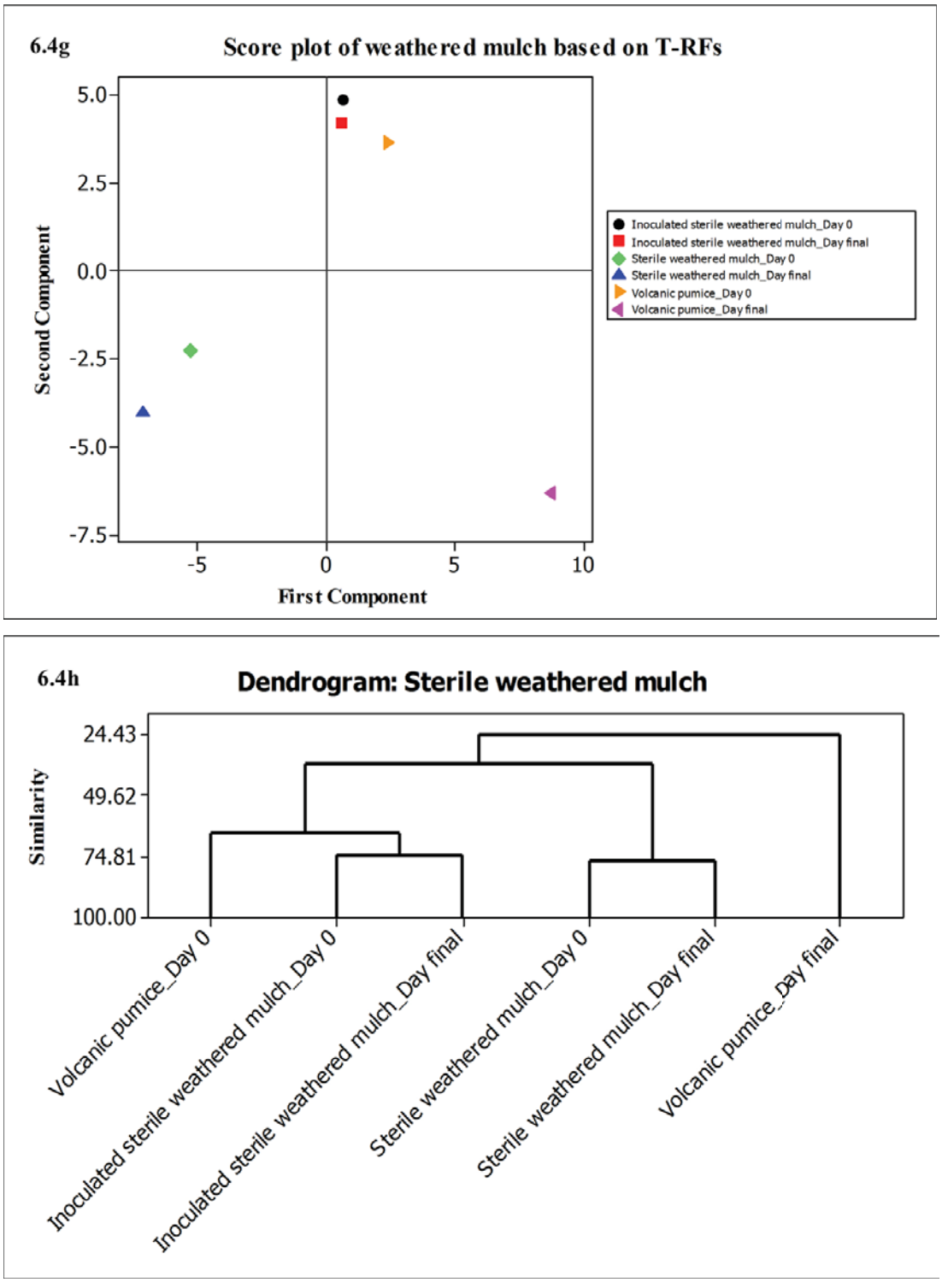


Figure 6.4 Principal component analysis (PCA) based upon the T-RFs profile at the initial and final study period of the materials tested. Dendograms were revealed by grouping the materials based on single linkage Euclidean distance method.

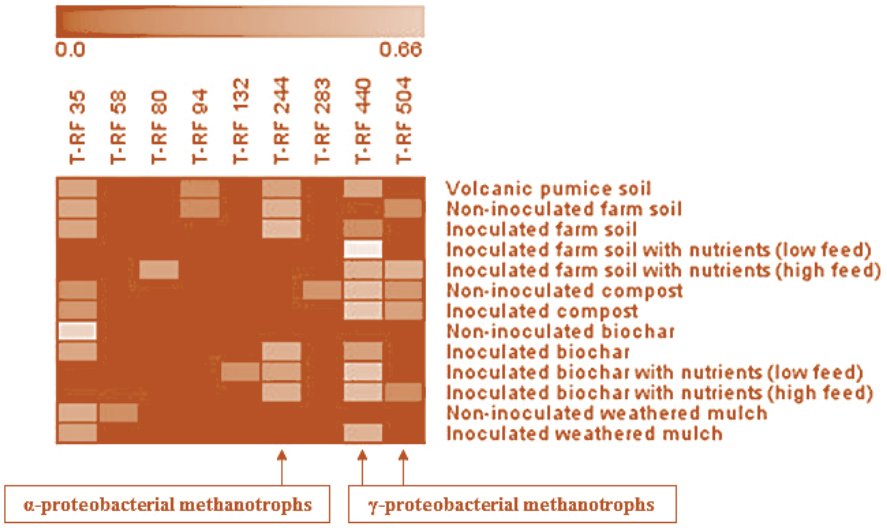


Figure 6.5 Heat map prepared using Genesis™ software Version 1.7.7. It is based on T-RF profile of all the materials on day final of the study period. Only the data with a relative abundance of >0.05 is included in this analysis. *Gamma-proteobacterial* (Type I) methanotrophs (*Methylobacter*, *Methylomicrobium*, *Methylosoma* and *Methylovulum*) were indicated by 440 and 504 bp, whereas 244 bp represents *α-proteobacterial* (type II) methanotrophs (*Methylocystis* and *Methylosinus* like)

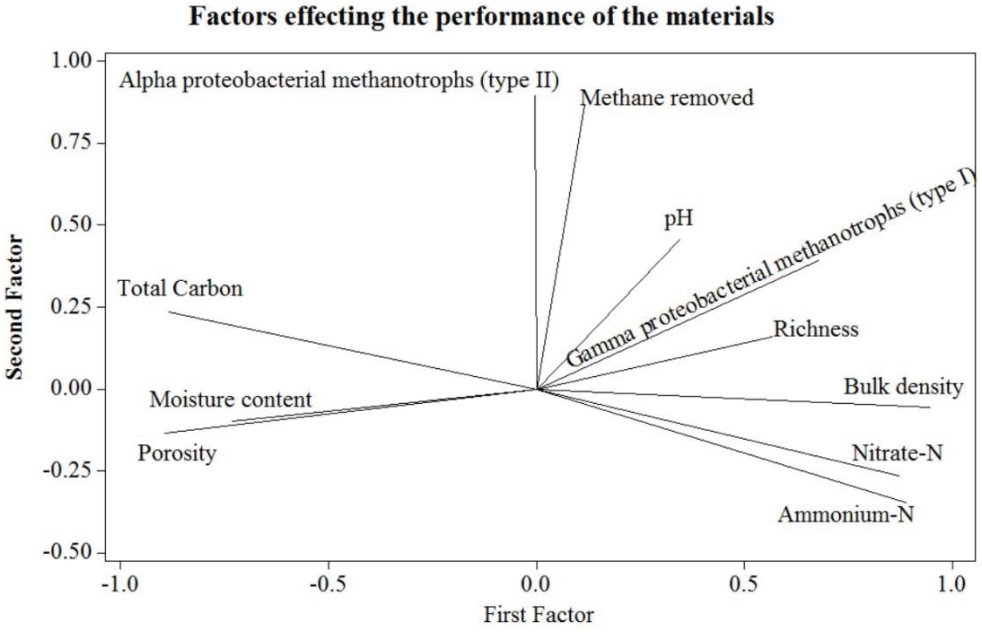


Figure 6.6 Factoral analysis indicating the correlation between CH_4 removal and various physical, chemical and biological factors studied.

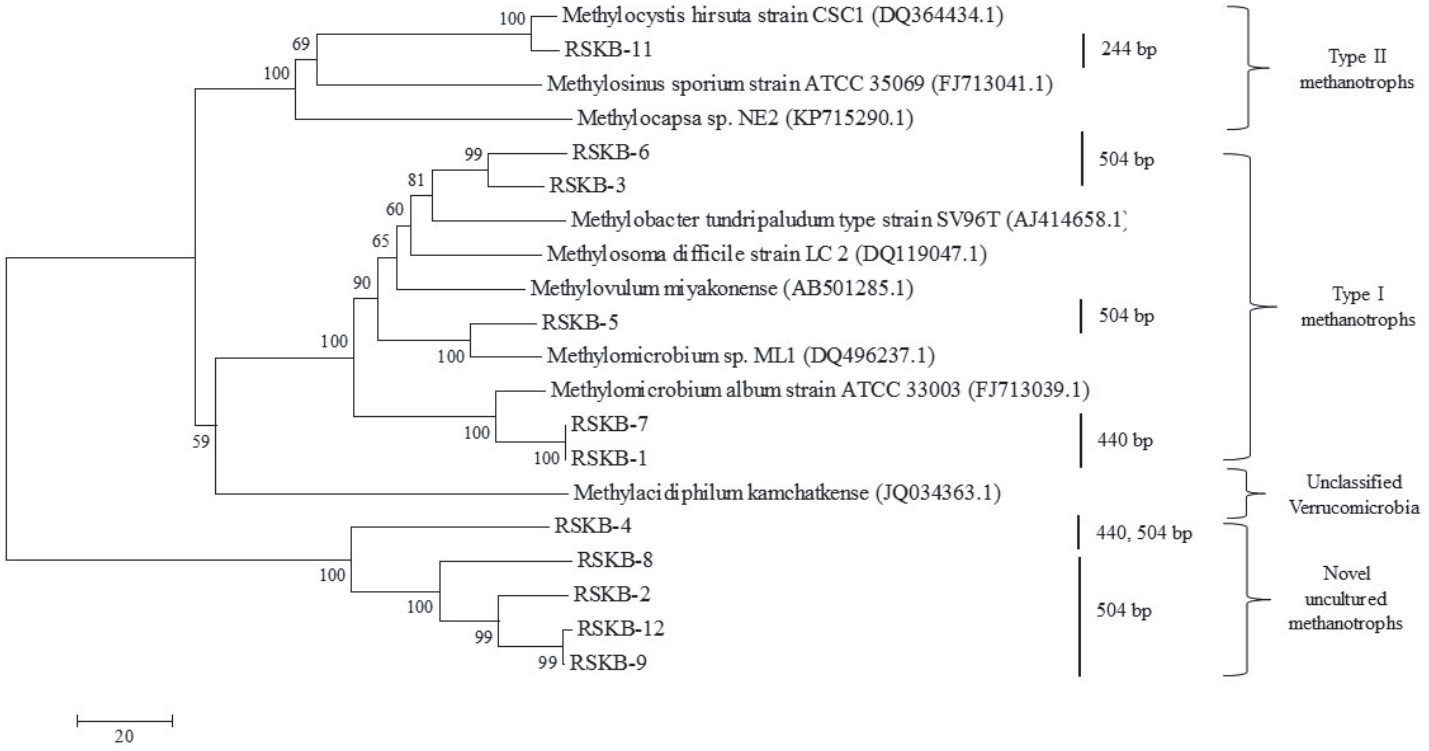


Figure 6.7 Unrooted phylogenetic neighbour-joining tree based upon nucleotides coding for partial methane monooxygenase gene subunit A (pmoA) of the cloned sequences (500 bp). Cloned sequences are compared to type strains or species of methanotrophs (accession numbers in the brackets). In silico digestion of the sequences (bp length) are presented. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

6.3.5 Phylogenetic analysis

Cloned library sequences were generated from the analysis of inoculated farm soil, inoculated biochar and volcanic pumice soil (Figure 6.7). Three clusters were identified, the first (RSKB-1, 3, 5, 6 and 7) being distantly related to known type strains or species of type I methanotrophs, the second cluster (RSKB-11) was closely related to type II methanotrophs, and the third (RSKB-2, 4, 8, 9 and 12) was very distantly related to known type I methanotrophs. Among the first cluster, both RSKB-3 and 6 clones were 86% and 85% related to *Methylosoma difficile* strain LC2 and *Methylovulum miyakonense* respectively; RSKB-5 was 93% similar to *Methylomicrobium* sp. ML1; whereas RSKB-1 and 7 were both 93% similar to *Methylomicrobium album* strain ATCC 35069. RSKB-11 clone in the second cluster was very closely related to *Methylocystis hirsuta* strain CSC1 and *Methylosinus sporium* strain ATCC 35069 with a % similarity of 97 and 88, respectively. The third cluster (RSKB- 2, 4, 8, 9 and 12) was very distantly related to type I methanotrophs (*Methylosoma difficile* and *Methylovulum miyakonense*) with a similarity of 85–87%. In addition, the cloned sequences were very distantly related (67% similarity) to unclassified *Verrucomicrobia* (*Methylacidiphilum kamchatkense*). Terminal-RF 244 bp was related to type II methanotrophs (*Methylocystis* and *Methylosinus* like); whereas 440 bp and 504 bp were related to type I methanotrophs (*Methylobacter*, *Methylomicrobium*, *Methylosoma* and *Methylovulum* like)

6.4 Discussion

6.4.1 Performance of the materials

All the materials (both with and without inoculum) went through a no or low CH_4 removal phase and high CH_4 removal phase during the study period. These biological disturbances (lower limits) were extreme in the materials without inoculum. Interestingly, inoculated soil and non-inoculated soil (without inoculum) followed a similar CH_4

removal trend (with the latter removing lower amounts of CH_4). Similarly, inoculated compost and compost (without inoculum) followed a similar trend line as evident in Figure 6.1. These biological disturbances could be attributed to the competition for CH_4 between microbial communities or other biological changes, rather than being the result of differences in physical and chemical properties of farm soil and compost. These phase changes were also noticed in previous published studies (Pratt *et al.* 2012a, c) with volcanic pumice soil. The low and high CH_4 removal phases could also either be attributed to the growth cycle of methanotrophs or to competition for sourcing of micro-nutrients from the dead bacterial cells. These biological disturbances were however not evident in inoculated farm soil and inoculated biochar amended with the nutrients (Figure 6.2). This indicates that with the addition of nutrients, the low CH_4 removal phases in the materials could be avoided. In inoculated biochar, unlike in farm soil and compost, very few disturbances/fluctuations were evident, probably because biochar had a lower resident methanotroph population (and microbial community) with which to compete (Table 6.3). Sterile weathered mulch removed no or very low levels of CH_4, probably because very much smaller microbial populations were present, given the sterilisation of the material at the start of the experiment.

Inoculated farm soil and inoculated biochar amended with nutrients removed all the CH_4 supplied (98 and 99%, respectively) with only small fluctuations reflected in CH_4 removal. This indicates that the CH_4 removal potential of farm soil and biochar can be accelerated with the addition of nutrients (Figure 6.3). It is important to note here that the inoculated farm soil and inoculated biochar amended with nutrients also took less incubation time to reach a higher CH_4 removal efficiency, than did inoculated farm soil and inoculated biochar without added nutrients. For instance, inoculated farm soil amended with nutrients took about 23 days, whereas the inoculated farm soil without nutrients took about 145 days. Even though sufficient N levels were present in the farm soil, other micronutrients stimulated the higher methanotroph gene copy numbers in the nutrient-amended materials. This indicates that faster acclimatisation can be expected by adding micro quantities of nutrients. The qPCR results also indicated that the presence of nutrients enhanced the growth of methanotrophs (Table 6.3).

6.4.2 Aerobic methanotroph community – abundance and diversity

Methanotroph gene copy numbers (with the exception of *Methylococcus*) increased during the study period for all the materials (Table 6.3), indicating that the conditions were favourable for their growth and activity. Results from qPCR (see Table 6.3) indicate all the materials (particularly farm soil and compost) had a resident type I and type II methanotroph population. The fluctuations or variability were less evident in the inoculated materials in comparison with their counterparts (without inoculum). This could be because the methanotrophs with higher starting population numbers might have competed more successfully for available nutrients (other than C) with the other bacteria present in the materials.

Amendment of inoculated farm soil and inoculated biochar influenced faster stabilisation of the material supporting active methanotroph growth and activity. Within just 52 days of incubation, the methanotroph population in inoculated farm soil and inoculated biochar amended with nutrients reached a stable and active population removing more than >90% of the CH_4. This is reflected in the increase of gene copy numbers in the materials (Table 6.3). In inoculated farm soil (with nutrients) the diversity or richness decreased over time, whereas in inoculated biochar (with nutrients) the diversity slightly increased. Amendment with nutrients has also reduced the evenness in both materials, indicating that micro- and macro-nutrients had positive effects on the growth and activity of selected species/strains of methanotrophs to effectively involve in CH_4 removal. In contrast, the diversity in all the inoculated materials increased over the study period, indicating more species or strains of methanotrophs were involved in CH_4 oxidation. In addition, the regression analysis indicated a week correlation between CH_4 removal and diversity (R^2 = 0.05%). This shows that the diversity is not directly related to the performance of the materials, rather the abundance and ratio between type I and type II methanotrophs are indicative of the stability of the materials.

The performance of the materials could have been affected by the difference in nutrient (particularly N) composition, which in turn influenced the population dynamics of type I and type II methanotrophs (Figure 6.6). Inoculated farm soil had similar porosity and C

and N composition to the volcanic pumice, but had a higher NO_3^- - N and NH_4^+- N content. Compost had much more NO_3^- - N and NH_4^+- N than in the volcanic pumice soil. In contrast, biochar and mulch were more porous and had lower N contents, especially the NO_3^- N. High N content favours type I methanotroph population, while suppressing type II methanotroph population (Hanson and Hanson, 1996; Mohanty SR *et al.*, 2006; Noll and Frenzel, 2008; Scheutz *et al.*, 2009). This concurs with the positive correlation shown between NO_3^--N and type I methanotroph (r = 0.60) in our study. Higher N content in both inoculated and non-inoculated compost could have adversely affected the growth of type II methanotroph population. Type II methanotroph are known to be stress tolerable (Ho *et al.*, 2013; Tate, 2015a) and its lower abundance in inoculated and non-inoculated compost could have made it less stable. In contrast, other inoculated materials (farm soil, biochar and sterile weathered mulch) had significantly ($P < 0.05$) higher abundance of type II *methanotrophs* thus contributing towards stable CH_4 removal. Inoculated biochar with nutrients (high feed) had even composition (1.42:1) of both type I and II methanotroph populations, with type II gene copies similar to that in the volcanic pumice soil. All the stable and higher CH_4 removing materials had abundant populations of type II methanotrophs, except for the inoculated farm soil amended with nutrients (low feed) which had gene copy numbers similar to that in inoculated compost. However, it is important to note that the inoculated farm soil amended with nutrients (low feed) was fed with significantly lower concentrations of CH_4 (3 000 ppm) compared to the other materials (20 000 ppm) throughout the study period and this population was enough to support the material remove >95% of the 3 300 ppm of CH_4 supplied. Nonetheless, the type I and II methanotroph ratio was significantly ($P < 0.05$) smaller in inoculated farm soil with nutrients (low feed) (5.92±0.08) in comparison to inoculated compost (13.03± 0.03). Regardless of the material type, gene copies of *Methylococcus* (from the type I methanotroph community) did not increase in number during the study period.

In both inoculated farm soil and inoculated biochar amended with nutrients, type II methanotrophs were lower compared to their counterparts (without amended nutrients). This could be due to the adverse effect of Cu (present in the nutrient solution) on their activity (Graham *et al.*, 1993; Scheutz *et al.*, 2009; Semrau *et al.*, 2010). In addition, the gene copy numbers of type II methanotrophs were significantly lower in low CH_4 concentrations (3 000 ppm) fed inoculated farm soil (with nutrients) in comparison to its

counterpart supplied at high concentrations of CH_4 (20 000 ppm) probably because of their affinity to grow at higher CH_4 concentrations (Hanson and Hanson, 1996; Dunfield *et al.*, 1999). On the other hand, inoculated biochar amended with nutrients had significantly ($P < 0.05$) higher type II methanotroph population when compared to those present in inoculated farm soil with nutrients. This might be due to the presence of significantly ($P < 0.005$) higher N content in farm soil comparatively. Interestingly, there was no significant difference in the gene copy numbers of type II methanotrophs in low and high CH_4 concentration fed inoculated biochar with nutrients; indicating that the effect of N content is much more profound than the effect of higher CH_4 concentrations on the activity of type II methanotrophs. However, it is important to note that the gene copy numbers for nutrient amended materials were analysed only after 52 days of incubation in comparison to their counterparts where the gene copy numbers were analysed on the final day of the study period (inoculated – farm soil and biochar – 215 and 190 days, respectively).

The performance of materials was supported by an abundant population of type I and type II methanotrophs, with the exception of sterile weathered mulch. Even though type I and type II gene copy numbers in inoculated weathered mulch have increased over the study period to a population number similar to volcanic pumice, the average % CH_4 removal was only 67. This indicates that either the methanotrophs were multiplying but not consuming CH_4 due to the presence of inhibitors or they were actively feeding on other substrates present in the weathered mulch (e.g. terpenes, *etc.*) along with the CH_4. There is much information on the co-metabolisation of various products by methanotrophs (Lee *et al.*, 2006; Jiang *et al.*, 2010; Kuo *et al.*, 2012) however, nothing has been reported on the consumption of terpenes. The reason for the lower activity of methanotrophs in weathered pine mulch is therefore still unknown.

Principal component analysis revealed that by the end of the study period, unlike biochar the T-RFs profile of inoculated farm soil was very similar to its counterpart (without inoculum) indicating its ability to build up stable methanotroph population over time (Figure 6.4). Heat map analysis (Figure 6.5) of T-RFs also complemented the data from qPCR analysis, which indicated the presence of both type I and II methanotrophs in inoculated farm soil and inoculated biochar. Interestingly, inoculated farm soil with

nutrients (high feed) has T-RF 80 bp (unidentified) which wasn't present in the volcanic pumice and inoculated farm soil suggesting its importance in contributing towards stable and higher CH_4 removal.

6.5 Conclusions

Understanding the factors affecting the ability of a biofilter to remove CH_4 is essential to develop efficient CH_4-mitigation technologies. This study showed strong correlation between CH_4 removal and type II methanotrophs, and the ratio between type I and II methanotrophs could be used as an indicative of stability of the biofilter materials. Other factors including moisture content and pH were less influential. Inoculated – farm soil and biochar materials were best in removing CH_4 among all the materials tested and could be considered as a potential biofilter material. It is important to note that other biochar's may or may not respond similarly, and in this study the results are drawn upon the biochar prepared from pine wood by pyrolysis at 450 °C. Feasibility of these materials to perform under varying flow rates of CH_4 needs further evaluation for use under field conditions.

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Rashad Syed

Name/Title of Principal Supervisor: Prof. Surinder Saggar

Name of Published Research Output and full reference:
Syed R, Saggar S, Tate KR and Rehm BHA (2016). Assessing farm soil, biochar, compost and weathered mulch to mitigate methane emissions. Applied Microbiology & Biotechnology (Published online; DOI 10.1007/s00253-016-7794-z)

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

First author

Candidate's Signature — 4 OCT 2016 — Date

Principal Supervisor's signature — 4/10/16 — Date

GRS Version 3– 16 September 2011

Chapter 7

General discussions and future perspective

In this chapter the importance of using molecular techniques to enhance an understanding of the conditions in biofilter media for efficiently mitigating CH_4 emissions from dairy effluent ponds is summarised. The discussion is ordered using the following sub-headings – (i) relationship between methanotrophs abundance and CH_4 removal rate, (ii) adaptability of the methanotroph community during unfavourable conditions, (iii) effect of acidity on community structure, (iv) type I/type II methanotroph ratio as an indicator of stable CH_4 removal, (v) effect of nutrient addition on CH_4 removal, (vi) the design considerations that should be used in choosing a biofilter material based on available molecular data and (vii) the limitations and challenges of molecular studies.

7.1 Effect of CH_4 flux on methanotroph abundance and activity

My results stress the need to determine factors that influence the abundance of the methanotrophs in achieving high CH_4 removal rates. The abundance of methanotrophs was strongly and positively correlated with the CH_4 removal rate. The pond floating biofilter had the highest CH_4 removal rate, whereas the laboratory-based floating biofilter had the least. Similarly, the pond biofilter had the highest methanotroph abundance and the laboratory biofilter had the least. Regression analysis indicated that the CH_4 removal rate was more strongly correlated with the abundance of methanotrophs (R^2 (adj)= 99%, $P < 0.05$) than with other factors (moisture content, pH and % C) tested. Interestingly % N also was correlated significantly (R^2 (adj)= 83%, $P < 0.05$) with the CH_4 removal rate. Methane removal had a negative *Pearson correlation*, r = - 0.37 and - 0.22, with the moisture content and pH, respectively.

Table 7.1 Physico-chemical properties (moisture, pH, total C and N) and methanotroph abundance (qPCR) of the volcanic pumice soil + perlite biofilter medium in different experimental systems, and associated maximum CH_4 removal rates. Abundance is represented as gene copy number $\times 10^8$ per gm of dry material.

	Max CH_4 removed ($g\ m^{-3}\ h^{-1}$)	**Methanotroph abundance**					**Moisture content** (% dry wt)	**pH**	**% C**	**% N**
		Type I	Type II	*Methylocapsa*	*Methylobacter*	*Methylococcus*				
Reconstituted biofilter	30.3	4.77×10^8	9.76×10^7	1.89×10^9	3.50×10^7	2.63×10^7	71.4	3.72	5	0.38
Volcanic pumice in Jars	36.2	5.41×10^8	2.35E+08	2.36×10^8	1.26×10^8	1.60×10^7	57.3	6.11	4.17	0.36
Pond floating biofilter	101.5	8.82×10^8	7.62×10^8	6.59×10^8	1.83×10^8	6.25×10^7	23.5	4.82	7	0.52
Laboratory floating biofilter	17.0	3.10×10^8	1.89×10^8	1.87×10^8	8.14×10^7	1.22×10^7	30.4	5.47	6.09	0.39

In addition, methanotrophs are present almost in all the media as seen in Chapter 6. All the non-inoculated materials when analysed for qPCR indicated the presence of a native or resident populations of methanotrophs. Interestingly, when the CH_4 was supplied, the abundances of these methanotrophs significantly increased over time (particularly in farm soil and compost). This indicates that priming with CH_4 is essential to increase methanotroph abundance and enable materials to act as a suitable biofilter material to oxidise CH_4, as long as it supports the growth and activity of both type I and type II methanotrophs.

7.2 Methanotrophs during unfavourable conditions

Before the start of the reconstituted biofilter experiment (Chapter 4), very low or no CH_4 removal rates were evident in the soil column biofilter probably either from the drying out of the biofilter material (soil moisture 15±2 % dry wt) or from the temporary disconnection of the CH_4 feed line 2–3 months before the experiment began. Even though the methanotrophs were present in the acidic volcanic pumice soil-perlite mixture, the populations were not active. However, when the soil was remoistened, the populations of type I and *Methylocapsa* like – methanotrophs increased over the 90-day study period, correlating with the CH_4 removal rate of 30.3 g m^{-1} h^{-1} (~ 58%). Terminal-restriction fragment length polymorphism (T-RFLP) analysis indicated that the profiles of the acidic volcanic pumice was quite different (Figures 7.1 and 7.2) from the volcanic pumice soils used in the floating biofilters (Chapter 5) and Laboratory Jar experiments (Chapter 6). This indicates that the volcanic pumice soil had a diverse group of methanotrophs and the changing environmental conditions triggered the activation or deactivation of particular genera of methanotrophs. The methanotrophic population in the volcanic pumice soil might have changed over a period of 5 years with the increasing acidity, as evidenced by the presence of abundant 45bp, 61 bp, 96bp, 116 bp 134bp and 150 bp T-RFs in the acidic soil (Fig 7.2).

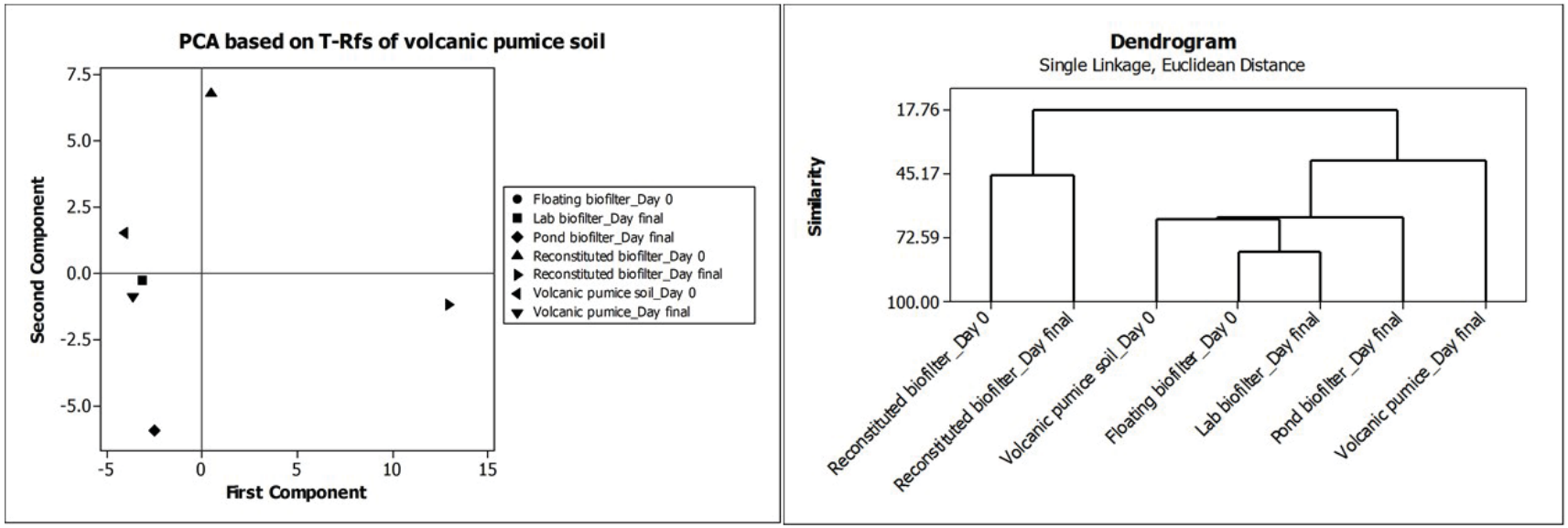


Figure 7.1 Pricipal compoent analysis of terminal-restriction fragment (T-RFs) of the volcanic pumice soil in the acidic biofilter (Chapter 4), floating biofilters (Chapter 5) and laboratory jar experiements (Chapter 6). Samples from the initial and final period of study are shown here for a comparison.

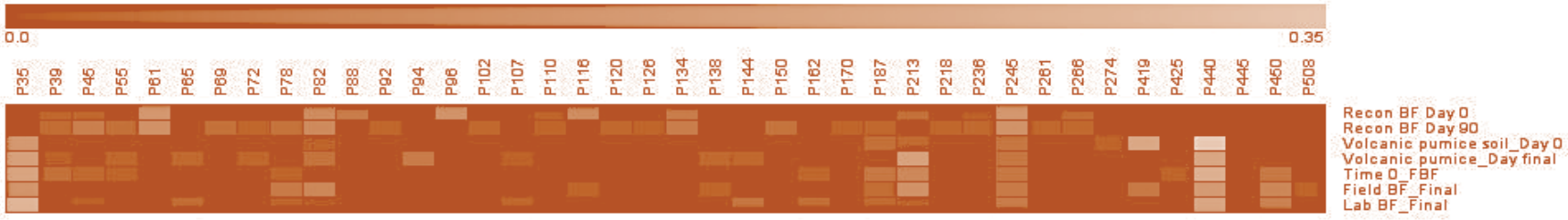


Figure 7.2 Heatmap analysis (based on T-RF's >1% relative abundance) of the volcanic pumice soil in the acidic reconstituted biofilter (Chapter 4), floating biofilters (chapter 5) and laboratory Jar experiments (Chapter 6).

The quantitative PCR results indicated that the population of *Methylocapsa* – like methanotrophs quadrupled during the 90-day study period (Figure 4.2), when adequate moisture conditions were available. This underlines the importance of having sufficient moisture in the soils to facilitate methanotroph growth and activity, and CH_4 oxidation. In another study with the floating biofilters, although a moisture content of 23 ± 3 % dry wt didn't suppress the growth and activity of methanotrophs, levels below this could potentially limit the methanotroph activity from oxidising CH_4. This was seen on the initial day of the reconstituted biofilter which had a moisture content of 15 ± 2 % dry wt, and indicates that although low pH suppresses the activity of genera of methanotrophs, moisture content is the most important factor controlling the CH_4 oxidation process.

7.3 Effect of pH on methanotroph community structure and biofilters performance

Controlling the acidity of the biofilter is the second most important parameter that needs to be taken into consideration for enhancing the biofilters performance. The acidity in the reconstituted biofilter did have an effect on changing the population dynamics of the methanotrophs. At a pH of 3.72 the abundances of type II methanotrophs (*Methylocystis* and *Methylosinus*) and *Methylobacter* (belonging to type I) were suppressed, whereas pH had little effect on the overall type I methanotroph population. Statistical analysis indicated that *Methylocapsa* – like methanotroph abundance was positively correlated to increasing acidity. In the floating biofilter study, after a year of operation, its pH had dropped to 4.7, but this did not suppress *Methylobacter* (type I) and *Methylocystis/Methylosinus* (type II). This indicates that a pH of 4.7 is not limiting the performance of most of the methanotrophs genera. In fact, the highest CH_4 removal rate was evident in the volcanic pumice soils present in the floating biofilters. These results indicate that for higher and efficient CH_4 removal, the biofilter materials should support the growth of most of the genera of methanotrophs (*Methylobacter*/ *Methylomonas*/*Methylosinus*/*Methylocystis*/*Methylocapsa*) as seen in the floating biofilters.

7.4 Ratio between type I and type II methanotrophs as an indicator of stable CH_4 removal

The ratio of type I/type II methanotrophs (based on qPCR) is useful indicator of the biofilter materials to stably remove CH_4. In the potential biofilter materials experiments (Chapter 6), all the stable and more efficient inoculated materials (*viz*, farm soil, biochar, weathered mulch) had a ratio between 1.70–3.05, whereas less stable material – Inoculated compost) had a ratio of 13.03. This indicated that the abundance of type II methanotrophs contributed to the stable and efficient CH_4 removal. Of all the materials tested, compost had a lower abundance of type II methanotrophs that are known to be stress-tolerant and their lower abundance contributed to its lower stability.

As evident from table 7.2, the higher the abundance of the methanotrophs (type I and type II), the higher was the CH_4 removal of the biofilter material. Interestingly, the compost never removed >80% CH_4 during the study period regardless of having an abundant type I methanotroph population, stressing the importance of the presence of abundant type II methanotroph for higher and stable CH_4 removal.

7.5 Effect of nutrients on stable CH_4 removal

Maintaining a balanced N content in the material to support methanotroph activity is important for effective CH_4 oxidation. Results from chapter 6 clearly indicated that the addition of micronutrients enhanced methanotroph growth and activity, and contributed towards higher and stable CH_4 removal. The materials amended with nutrients (farm soil and biochar) showed higher and stable CH_4 removal. Even though methanotrophs depend upon CH_4 for C, they still require minute quantities of micro nutrients for their metabolism and/or growth. Therefore, addition of nutrients might have suppressed the competition for sourcing micro-nutrients from dead bacterial cells. In addition, nutrients like NO_3-N and NH_4-N can suppress the growth of type II methanotrophs, as evidenced in both inoculated and non-inoculated compost (Chapter 6).

Table 7.2. Methanotroph (Type I and II) abundance (× 10^8 gene copy numbers per gm of dry material), richness (number of phylotypes based on T-RFLP), maximum CH_4 removed and time the materials took to remove >80% of CH_4 over the study period (stability) of volcanic pumice soil, farm soil, compost, biochar and sterile weathered mulch. * materials never removed >80% CH_4 during the study period.

Characteristics	**Volcanic pumice soil**	**Farm soil**	**Compost**	**Biochar**	**Sterile weathered mulch**
Type I abundance @ Day 0	6.78±0.00	4.43±0.09	4.25±0.42	0.14±0.00	0.02±0.00
Type I abundance @ Day final	5.36±0.05	4.73±0	12.68±0.20	0.63±0.05	0.04±0
Type II abundance @ Day 0	1.39±0.05	0.46±0.00	0.39±0.01	0.03±0	0.03±0.0
Type II abundance @ Day final	2.32±0.03	1.39±0.01	0.56±0.02	0.79±0.03	0.06±0
Richness @ Day 0	51±8	77±4	68±4	21±1	37±10
Richness @ Day final	75±4	75±2	82±7	62±2	38±7
Stability (number of days)	< 3	145	> 217	> 217*	> 217*
Maximum CH_4 removed ($g\ m^{-3}\ h^{-1}$)	36.18±0.27	14.65±0.27	4.93±1.40	2.75±0.09	0.45±0.50

7.6 Design considerations

In order to build an efficient CH_4 biofiltration system, the molecular results indicated that the following factors need to be considered

- Selecting biofilter materials that support the growth and activity of most of the genera of methanotrophs (both type I and type II).
- Avoiding the moisture content falling below 23±3 % dry wt.
- Avoiding the acidification of the materials below a pH of 4.8±0.1.
- Addition of minute quantities of micronutrients (nitrate mineral salts) to enhance methanotroph growth and activity.
- Maintaining N content (0.36 – 0.52 %) of the biofilter materials to support CH_4 removal by methanotrophs, as higher N contents of 1.35 % in compost suppressed type II methanotroph activity.

7.7 Limitations and challenges of molecular tools

The molecular techniques used in this study were based on the DNA extracted from the soil. Microbial DNA extracted from the soils could be retrieved from both dead and live microbes. In this study, the DNA was quantified and compared in a before–and–after scenario in a similar system, which gave an indication of the total increase in abundance (from both live and dead microbes). However, a one-off measurement of the abundance of methanotrophs based on DNA has limited usefulness due to its inability to differentiate between dead and live microbes and effectively assess the changes in live microbes. Therefore, RNA-based approaches measuring the abundances of only live microbes should be used in future for comparing and quantifying methanotroph efficiency to oxide CH_4 before and after priming, and isolating efficient biofilter media.

Relating CH_4 removal to the activity of a specific species/genera of Methanotroph is quite challenging because all the studies demonstrated the participation of most of the groups

of methanotrophs in CH_4 oxidation. Methanotrophs are very diverse, having 20 or more genera and thousands of species in the family. It is therefore challenging with the available molecular tools, and with limited resources, to quantify the abundance of a target species/genera and relate this to CH_4 removal activity, especially under different abiotic conditions. It might have been possible, if a specific pure species/strain had been used in the biofilter for mitigating CH_4, and might have made it scientifically possible to relate to CH_4 oxidation. Regardless, my studies have shown that the abundant presence of both type I and type II methanotroph populations is essential for the biofilter to operate effectively under different abiotic conditions. This study has also emphasised the main problem facing microbial ecology namely reconciling the large number of unknown species in complex environments like soil, and our inability to identify the active species and connect them to a specific process.

7.8 Future directions

Methane removal using biofilters offers a great potential for mitigating CH_4 emissions from the agricultural and waste sectors. Potential future research directions are briefly discussed below.

7.8.1 Mitigating high concentrated CH_4 emissions

This PhD has successfully extended the potential use of soil biofilters for mitigating high concentrations (3 300 – 100 000 ppmv) of CH_4 from New Zealand Dairy effluent ponds, by enhancing our understanding of the microbial processes involved, and showing that readily available inexpensive alternative biofilter materials can also perform well. Now, future studies should utilise this knowledge and focus on mitigating high concentrated CH_4 emissions from other sources in New Zealand viz., uncovered landfills, coal mine vents, wastewater treatment plants and natural gas leaks. This soil biofilter technology will help reduce the escape of CH_4 into the atmosphere and thus contribute towards reducing overall CH_4 production from New Zealand and worldwide. Though this thesis indicated that N gets assimilated into the soil over time, however more regimented studies

needs be conducted understand and enumerate the removal of other compounds like NH_3, H_2S and volatile compounds from effluent ponds. Potentially, the thick crust formed on the surface of dairy and piggery effluent ponds could also be explored as an biofilter material in future.

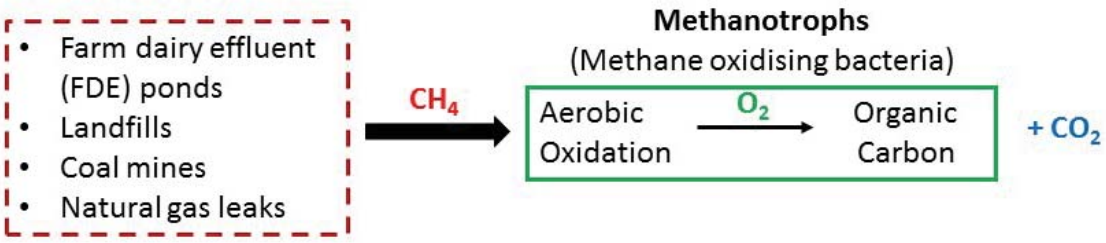


Figure 7.3 Using methanotroph biofiltration technology to mitigate high concentrated CH_4 sources in New Zealand.

7.8.2 Mitigating low concentrated CH_4 emissions

The experiments in this PhD were conducted to understand the capability of the soils and different materials to remove high concentrations of CH_4 (> 3 300 ppm). However, the ability of the materials to remove much lower concentrations of CH_4 – 150 ppm (levels of CH_4 measured in barns/animal sheds) has not yet been explored. Recent studies by Landcare Research New Zealand Ltd (not part of this Thesis) indicated that the volcanic pumice soil used as a standard in the work described in this thesis, and other soils effective at removing high CH_4 concentrations were not effective at removing these relatively much lower concentrations of CH_4. It is speculated that this may be attributed to: (i) competition between methanotrophs and other microorganisms present in the soil for resources (energy and nutrients) – and the inability of the low amount of CH_4 (concentration) to boost the population of methanotrophs to compete with other microorganisms present in the soil and/or (ii) suppressed growth and activity of type II methanotrophs due to high N contents and the presence of inhibitory substances/conditions, or predation. Therefore, future studies should focus on – screening

readily available New Zealand soils (e.g., forest soils) capable of removing low CH_4 concentrations; and on understanding the ecology of these methanotrophs by extracting RNA from soils and using molecular techniques (qPCR, T-RFLP and DGGE) to identify the active group involved in oxidising low CH_4 concentrations. These methanotrophs could offer a potential solution for reducing CH_4 emissions from housed grazing animals (in barns/animal sheds), where CH_4 is sourced from waste as well as enteric emissions.

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Appendices

Appendix I

Inoculation transfer experiments:

The objective of these short experiments was to determine the best method to inoculate the micro-organisms (including methanotrophs) to the potential biofilter materials tested. The experiments using different biofilter materials is described in Chapter 6 of this thesis.

Two approaches were designed to transfer the inoculum to the potential biofilter materials – (1) Direct mixing of the inoculum (i.e., volcanic pumice soil and perlite mixture (50/50), subsequently called "volcanic pumice") with the biofilter material (Biochar) (2) Buffer dispersion technique, where the inoculum was washed in three different buffers and the solution containing bacteria was added to Biochar

Below experiments were carried out in duplicate in gas tight 1.8 L AGEE™ jars. The total volume of the materials tested was kept constant at 100 ml. Moisture content of the materials were kept at 50% (dry wt) and incubation temperature was kept constant at 25 °C. Every batch period lasted for 24 hr, where CH_4 was injected into the jar to maintain ambient concentration of 3 300 ppm. Oxygen was supplied by opening the lid of the jar and passively letting fresh air diffuse into the jar for about 20 minutes at the beginning of each batch period. The physico-chemical properties of the volcanic pumice soil and biochar are listed in Table 6.1 (Chapter 6).

Mixing technique:

Two different volumes of inoculum were mixed with the biochar – 10% and 20%. Methane (3 300 ppm) was fed every 24 hours. After 4 days of incubation, CH_4 concentration was measured on at 0, 2, 4 and 6 hrs to determine the CH_4 oxidation rate. Figure I.1 shows the amount of CH_4 oxidised over time.

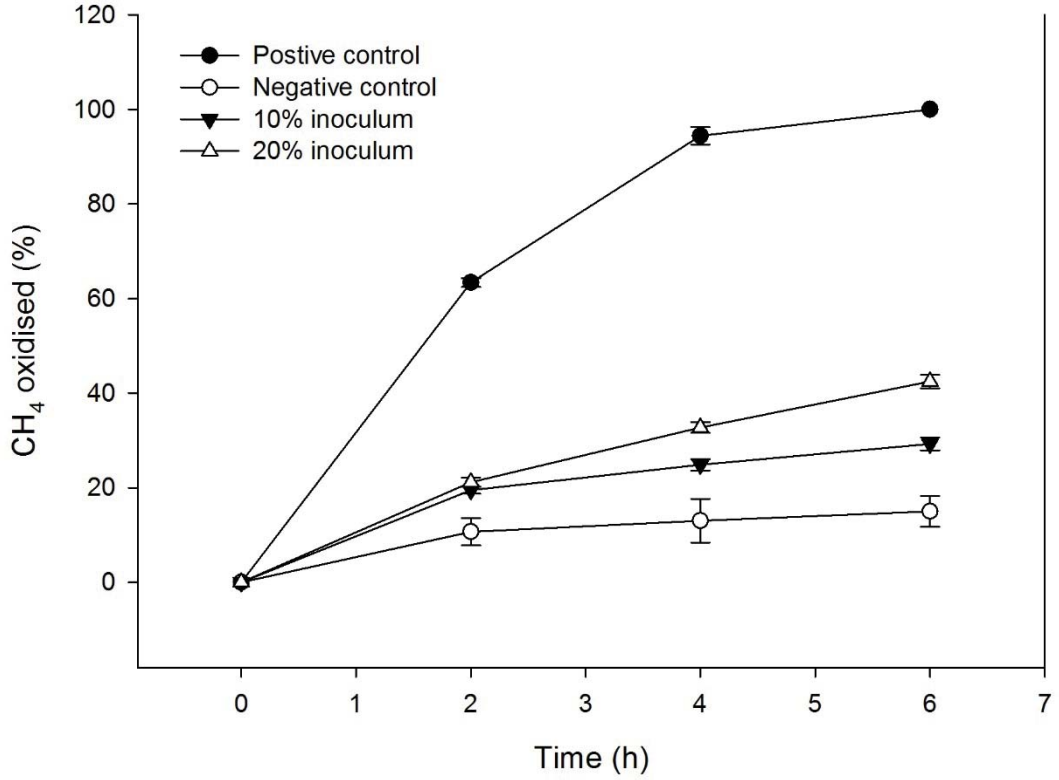

Figure I.1 Methane oxidized by the biochar materials with different volumes of added inoculum. Positive control indicates – volcanic pumice soil (100% inoculum); Negative control indicates biochar without added inoculum. Error bars represents SD from the mean of triplicate measurements (n=3).

Results indicated that there was considerable difference in the CH_4 removing capability of 10% and 20% inoculated biochar. The highest CH_4 oxidation achieved by the 20% inoculated biochar was 40%. Therefore, 20% inoculum was chosen as a standard mixing ratio for carrying out further batch experiments with different biofilter materials (See Chapter 6).

Dispersion technique:

Three different buffers – Reverse osmosis water, 0.1 M phosphate buffer saline (PBS) and 0.01 M calcium chloride ($CaCl_2$) were tested to identify the best solution to suspend

the micro-organisms (including methanotrophs). The technique involved washing 10ml of inoculum with 20 ml of buffer.

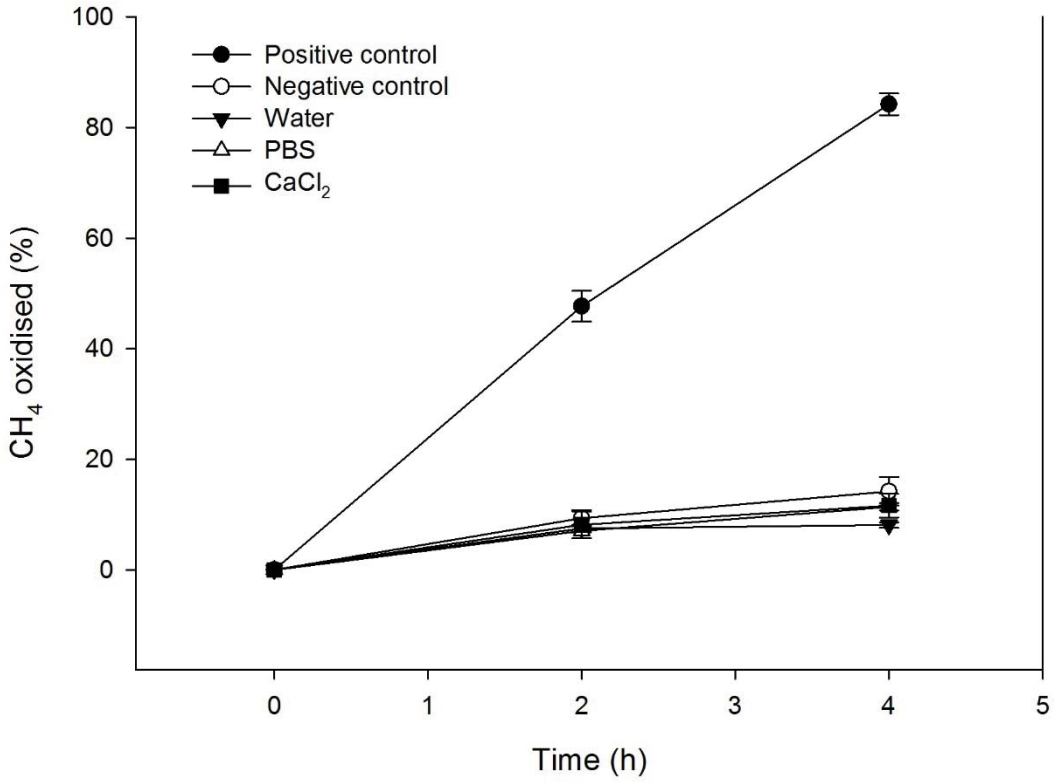

Figure I.2 Methane oxidized by the materials after the inoculum was dispersed in different buffers. Error bars represents SD from the mean of triplicate measurements (n=3).

The suspension was then mixed gently and centrifuged at 1000 rpm for 45s. The supernatant was pipetted out and saved in a separate falcon tube. Pellet was again re-dispersed in the buffer and centrifuged at 1000 rpm for 1 min. The supernatant from both the steps was mixed and centrifuged at 2000 rpm for 10 min. Supernatant (clear solution) now containing just buffer was discarded. The pellet containing bacteria was dispersed in 10ml of buffer and the dispersion liquid was added to 90 ml of biofilter material (biochar). Methane and oxygen was fed as previously described in section 3.7.1. After four days of incubation, gas concentration was measured up to 4 hours and there was no significant

difference between the different washes (Figure I.2). However, PBS (0.1M) buffer was chosen as the ideal buffer to disperse the inoculum, as it is widely used in the biotechnology industry for suspending live bacterial cells.

Dispersion using PBS buffer:

Above described procedure was used to test the CH_4 removal capability of 10% and 20% inoculated biochar. The 10% and 20% inoculated biochar mixture was prepared by dispersing 10 ml and 20 ml of inoculum, respectively in 10 ml of PBS.

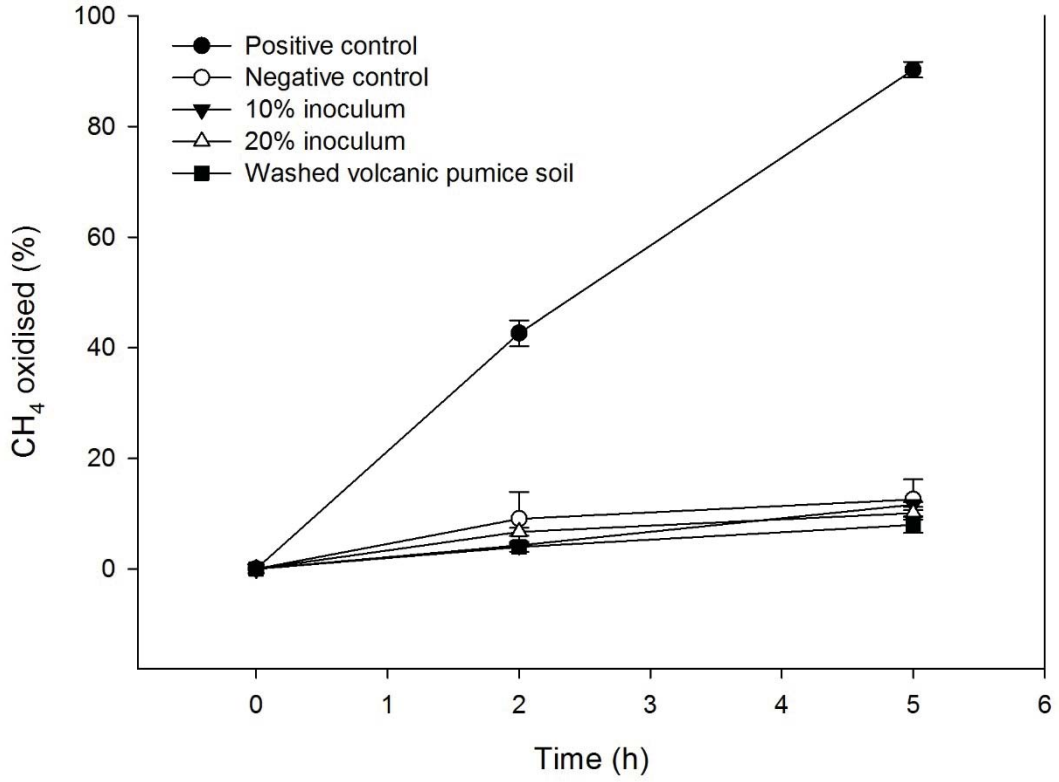

Figure I.3 Methane oxidized by the biochar inoculated with PBS buffer containing varying amounts of inoculum. Volcanic pumice soil is 100% of inoculum. Error bars represents SD from the mean of triplicate measurements (n=3).

Figure I.3 shows the amount of CH_4 oxidised after four days of periodic incubation of the materials. During the washing procedure (described above), the volcanic pumice soil

(after washing) was air dried and wetted to 50 % (dry wt) moisture content and assessed for CH_4 removal as well. There was no significant oxidation of CH_4 in the dispersed materials and no notable difference between different inoculum mixtures as well, indicating that the dispersion technique might not be a best method to carry further experiments. The washed soil was also not removing CH_4 indicating that the washing procedure was done efficiently and were dispersed successfully in the washing buffer. The reason behind the micro-organisms not successfully acclimatising to the new environment is yet unknown.

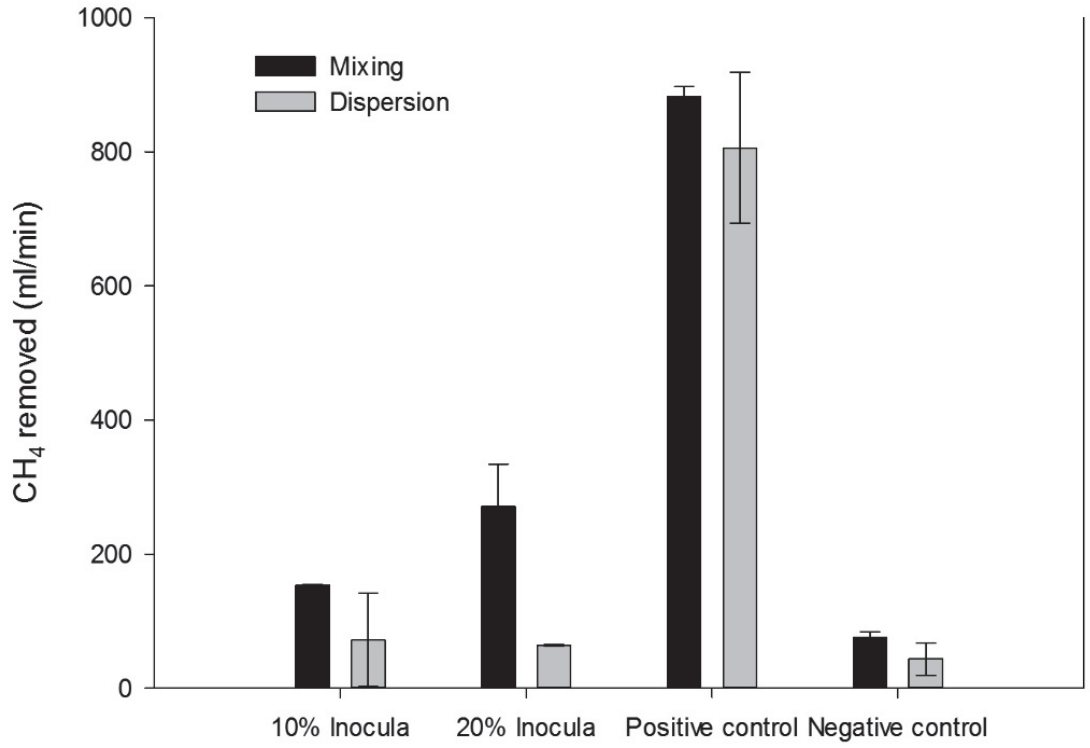

Figure I.4 Methane oxidized by the biochar inoculated via mixing and dispersion techniques. Error bars represents SD from the mean of triplicate measurements (n=3).

The CH_4 removal rates (ml min^{-1}) of the above described techniques (mixing and dispersion) were compared in the Figure I.4. The results clearly indicate that direct mixing of the inoculum with the biofilter material is the quick and most efficient way of

inoculation. In addition, the mixing technique could be easily scale-able without requiring additional equipment.

Appendix II

PowerSoil® DNA Isolation Kit

To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.

1. Gently vortex to mix.
2. Check Solution C1. If Solution C1
3. Solution C1 and invert several times or vortex briefly.
4. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
5. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
6. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
7. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4oC for 5 minutes.
8. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*
9. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
10. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4oC for 5 minutes.
11. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*
12. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
13. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

14. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.

    Note: A total of three loads for each sample processed are required.
15. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
16. Discard the flow through.
17. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
18. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
19. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
20. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
21. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

Appendix III

PCR Clean up Kit (NucleoSpin® Gel and PCR Clean-up)

The following protocol is suitable for PCR clean-up as well as DNA concentration and removal of salts, enzymes, etc. from enzymatic reactions (SDS < 0.1 %).

1. Adjust DNA binding condition: For very small sample volumes < 30 µL adjust the volume of the reaction mixture to 50–100 µL with water. It is not necessary to remove mineral oil. Mix 1 volume of sample with 2 volumes of Buffer NTI (e.g., mix 100 µL PCR reaction and 200 µL Buffer NTI). Note: For removal of small fragments like primer dimers dilutions of Buffer NTI can be used instead of 100 % Buffer NTI. Please refer to section 2.3.
2. Bind DNA: Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µL sample. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube. Load remaining sample if necessary and repeat the centrifugation step.
3. Wash silica membrane: Add 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
   Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and improve A260/A230 values (see section 2.7 for detailed information).
4. Dry silica membrane: Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube. Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.
5. Elute DNA: Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube (not provided). Add 15–30 µL Buffer NE and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000

x g. Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min.

Appendix IV

Cloned sequences from reconstituted biofilter study (Chapter 4). Genbank Accession numbers (KT424049 – KT424060)

> *pmoA* clone RSKB-AVP1

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>*pmoA* clone RSKB-AVP2

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Cloned sequences from the floating biofilters study (Chapter 5). Genbank accession numbers – KU840813 – KU84082

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TTGGCATACGGTTTGTTATTCTATCCAGGTAACTGCCAATCATTGCTCCATTA
CATGTTCCTGTAGAATATAACGGCATGATGATGACACTGGCTGACTTGCAA
GGTTACCACTATGTTAGGACCGGCACACCTGAATACATCAGAATGGTTGAA
AAAGGTACCTTGAGAACTTTCGGTAACGACGTTGCCC

> *pmoA* clone RSKB-FBF10
GGTGACTGGGACTTCTGGACCGACTGGAAAGATAGACGTCTATGGGTAACC
GTAGCTCCAATCGTTTCAATCACTTTCCCTGCTGCTGTTCAAGCAATCTTGTG
GTATCGCTACCGACTGCCTTTCGGTGCAGTTCTTTGTATTTTAGGTCTGCTCT
TGGGTGGGTGGGTCAACAGATACATGAACTTCTGGGGCTGGACATATTTCC
CTGTTAACTTCTGCTTCCCATCAAACTTGATGCCAGGTGCTATCGTACTTGA
CGTTATCCTGATGCTGTCTGGCAGTATGACATTGACTGCCGTTATCGGTGGC
TTGGCATGGGGTCTGTTGTTCTATCCAGGTAACTGGCCAATCATTGCTCCAT
TACATGTTCCTGTTGAATACAACGGCATGATGATGACTCTGGCTGACTTACA
AGGTTACCACTAAGTAAGAACTGGTACACCTGAGTACATCAGAATGGTTGA
AAAAGGTACACTGAGAACATTCGGTAACGACGTTGCC

> *pmoA* clone RSKB-FBF11
CCGGGGCAACGTCGTTACCGAAAGTTCTCAAGGTACCTTTTTCAACCATTCT
GATGTACTCTGGAGTACAAGTTCTAACATAGTGGTAACCTTGCAAGTCAGCC
AGGGTCATTACCATGCCGTTGTATTCAACAGGAACGTGCAGAGGAGCAATT

ACAGGCCAGTTGCCGGGATAGAACAACAAACCGTATGCCAAGCCGCCCAGA
ACCGCAGTCAACTGCATGCTGTTAGACAGCATCAGAATGACGTCAAGAACG
ATTGCGCCTGGAACGAATTGTGATGGGAACACAAAGTTAACTGGGAAGTAA
GTCCATCCCCAGAAGTTGAAATATCTGTTAACCCATTCACCGAACAACAGG
CCCAGAACAGCCAGCATTGCGCCGAATGGCAGCTTCCAACGATACCACACA
ACTGCTTGAACAGCGGCCGGGAAAGTAATACCAACGATGGGCAATACAGTT
ACCCACAGACGACGGTCTTTCCAGTCAGTCCAGAAGTCCCAGT

Cloned sequences from Chapter 6 (Assessment of potential biofilter materials). Genbank accession numbers: KU215855 – KU215865.

>*pmoA* clone RSKB–1
GGTGACTGGGACTTCTGGACTGACTGGAAAGACCGCCGTCTGTGGGTAACC
GTATTGCCAATCGTCGGCATTACCTTCCCAGCCGCTGTTCAAGCAGTTCTGT
GGTATCGTTGGAAACTGCCATTTGGCGCAATGTTGGCCGTTTTGGGCTTGCT
GTTCGGCGAATGGGTTAACAGATACTTCAACTTCTGGGGATGGACTTACTTC
CCAGTTAACTTTGTATTCCCATCACAATTCGTCCCAGGCGCTCTGGTACTGG
ACGTAATCCTGATGTTGTCTAACAGCATGCAGTTGACTGCTGTTCTGGGTGG
CTTGGCTTATGGTTTGTTGTTCTATCCTGGCAACTGGCCTGTCATCGCTCCAT
TGCACGTGCCAGTTGAATACAACGGCATGGTAATGACTCTGGCTGACTTGC
AAGGTTACCACTATGTAAGAACCGGTACTCCAGAATATACCCGGATGGTTG
AAAAAGGTACTCTGAGAACTTTCGGTAACGACGTTGCC

> *pmoA* clone RSKB–2
CCGGGGCAACGTCGTTACCGAAAGTTCTTAGTGCACCTTTTTCAACCATTCT
GATGTACTCAGGTGTACCAGTTCTTACATAGTGGTAACCTTGTAAATCAGCC
AGTGTGAACATCATGCCATTGTATTCAACAGGAATATGTAATGGCGCGATG
ATAGGCCAGCTGCCAGGGTAGAACAACAGACCCCAAGCCATGCCACCAACA
ACCGCAGTCAATGTCATGCTGCCAGACAGCATCAAAACAACATCAAGTACG
ATAGCGCCTGGCATCAGGTTTGAAGGGAAACAGAAGTTAACTGGGAAATAT
GTCCATCCCCAGAAGTTCAGGTATCTGTTAACCCACTCACCCAGGAGCAGA
CCTAAGACAGCAACTACAGCGCCGAAAGGCAAACGGAAACGATACCACAG

CGTGGCTTGTACAGCAGCAGGGAAAGTGACTGAAACGATAGGTGCTACAGT
TACCCATAGGCGTCTATCTTTCCAGTCTGTCCAGAAGTCCCAGT

> *pmoA* clone RSKB–3

GGTGACTGGGACTTCTGGACAGACTGGAAAGATAGACGTCTATGGGTAACT
GTAGCACCTATCGTTTCAATCACTTTCCCTGCGGCTGTTCAAGCAAGCTTGT
GGTACCGTTACCGTCTGCCTTTCGGCGCGGTTGTTTGTGTCTTAGGTCTGTTC
CTGGGTGAGTGGGTTAACAGATACCTGAACTTCTGGGGATGGACATATTTCC
CAGTTAACTTCTGCTTCCCTTCAAACCTGATGCCAGGCGCTATCGTTCTTGAT
GTTGCTTTGATGCTGTCTAACAGCATGACATTGACAGCGGTTGTTGGTGGTA
TGGCTTGGGGTCTGTTGTTCTATCCTGGCAACTGGCCAATCATCGCGCCATT
ACACATTCCTGTTGAATACAATGGCATGATGTTTACTCTGGCTGACTTACAA
GGTTACCACTACGTAAGAACTGGTACTCCTGAGTACATCAGAATGGTTGAA
AAAGGTACACTAAGAACTTTCGGTAACGACGTTGCC

> *pmoA* clone RSKB–4

CCGGGGCAACGTCGTTACCGAAAGTTCTCAGAGTACCTTTTTCAACCATCCG
GATATATTCTGGAGTACCGGTTCTTACATAGTGGTAACCTTGCAAGTCAGCC
AGAGTCGTTACCATGCCGTTGTATTCAACTGGCACGTGCAATGGAGCGATG
ACAGGCCAGTTGCCAGGATAGAACAACAAACCATAAGCCAAGCCACCCAG
AACAGCAGTCAACTGCATGCTGTTAGACAACATCAGGATTACGTCCAGTAC
CAGAGCGCCTGGGACGAATTGTGATGGGAATACAAAGTTAACTGGGAAGTA
AGTCCATCCCCAGAAGTTGAAGTATCTGTTAACCCATTCGCCGAACAGCAA
GCCCAAAACGGCCAACATTGCGCCAAATGGCAGTTTCCAACGATACCACAG
AACTGCTTGAACAGCGGCTGGGAAGGTAATGCCGACGATTGGCAATACGGT
TACCCACAGACGGCGGTCTTTCCAGTCAGTCCAGAAGTCCCAGT

> *pmoA* clone RSKB–5

GGGGACTGGGACTTCTGGACTGACTGGAAAGATAGACGTCTATGGGTAACC
GTGGCACCAATCGTTTCAATCACTTTCCCTGCAGCGGTTCAAGCGGTACTTT
GGTGGCGCTACCGTATCGCATGGGGTGCAACTCTGTGTGTTTTAGGTCTGTT
ACTGGGTGAGTGGGTCAACAGATACTTCAACTTCTGGGGTTGGACATATTTC

CCAGTTAACTTCGTATTCCCATCTAACCTGATGCCAGGCGCCATCGTATTAG
ACGTCATTCTGATGCTTTCTAACAGCATGACTCTGACTGCGGTTGTCGGTGG
TTTGGCTTACGGCTTATTGTTCTATCCAGGTAACTGGCCAATCATTGCTCCAT
TACATGTTCCTGTTGAATACAACGGCATGATGATGACTTTGGCTGACTTACA
AGGTTACCACTATGTTAGAACTGGTACTCCTGAGTACATCCGTATGGTTGAA
AAAGGAACACTGAGAACATTCGGTAACGACGTTGCCCC

> *pmoA* clone RSKB–6
GGTGACTGGGACTTCTGGACAGACTGGAAAGATAGACGCCTATGGGTAACT
GTAGCACCTATCGTTTCAATCACTTTCCCTGCTGCTGTACAAGCCACACTGT
GGTATCGTTTCCGTTTGCCTTTCGGCGCTGTAGTTGCTGTCTTAGGTCTGCTC
CTGGGTGAGTGGGTTAACAGATACCTGAACTTCTGGGGATGGACATATTTCC
CAGTTAACTTCTGTTTCCCTTCAAACCTGATGCCAGGCGCTATCGTACTTGA
TGTTGTTTTGATGCTGTCTGGCAGCACGACATTGACTGCGGTTGTTGGTGGC
ATGGCTTGGGGTCTGTTGTTCTACCCTGGCAACTGGCCTATCATCGCGCCAT
TACATATTCCTGTTGAATACAATGGCATGATGTTCACACTGGCTGATTTACA
AGGTTACCACTATGTAAGAACTGGTACACCTGAGTACATCAGAATGGTTGA
AAAAGGTACACTAAGAACTTTCGGTAACGACGTTGCCCC

> *pmoA* clone RSKB–7
GGGGACTGGGACTTCTGGACTGACTGGAAAGACCGCTGTCTGTGGGTAACC
GTATTGCCAATCGTCGGCATTACCTTCCCAGCCGCTGTTCAAGCAGTTCTGT
GGTATCGTTGGAAACTGCCATTTGGCGCAATGTTGGCCGTTTTGGGCTTGCT
GTTCGGCGAATGGGTTAACAGATACTTCAACTTCTGGGGATGGACTTACTTC
CCAGTTAACTTTGTATTCCCATCACAATTCGTCCCAGGCGCTCTGGTACTGG
ACGTAATCCTGATGTTGTCTAACAGCATGCAGTTGACTGCTGTTCTGGGTGG
CTTGGCTTATGGTTTGTTGTTCTATCCTGGCAACTGGCCTGTCATCGCTCCAT
TGCACGTGCCAGTTGAATACAACGGCATGGTAATGACTCTGGCTGACTTGC
AAGGTTACCACTATGTAAGAACCGGTACTCCGGAATATATCCGGATGGTTG
AAAAAGGTACTCTGAGAACTTTCGGTAACGACGTTGCCCC

> *pmoA* clone RSKB–8

CCGGGGCAACGTCGTTACCGAATGTTCTCAGTGTACCTTTTTCAACCATTCT
GATGTACTCAGGTGTACCAGTTCTTACATAGTGGTAACCTTGTAAGTCAGCC
AGAGTCATCATCATGCCGTTGTACTCAACAGGAACATGTAATGGAGCAATG
ATTGGCCAGTTACCTGGATAGAACAACAGACCCCATGCCAAGCCACCGATA
ACGGCAGTCAATGTCATACTGCCAGACAGCATCAGGATAACGTCAAGTACG
ATAGCACCTGGCGTCAAGTTTGATGGAAAGCAGAAGTTAACAGGGAAATAT
GTCCAGCCCCAAAAGTTCATGTATCTGTTGACCCGCTCACCCAAGAGCAGA
CCTAAAATACAAATAACTGCACCGAAAGGCAGACGGTAGCGATACCACAA
GACTGCTTGAACAGCAGCAGGAAAAGTGATTGAAACGATTGGAGCTACGGT
TACCCATAGACGTCTATCTTTCCAGTCGGTCCAGAAGTCCCAGTC

> *pmoA* clone RSKB–9

CCGGGGCAACGTCGTTACCGAAAGTTCTTAGTGTACCTTTTTCAACCATTCT
GATGTACTCAGGAGTACCAGTTCTTACGTAGCGGTAACCTTGTAAGTCAGCC
AGAGTAAACATCATGCCATTGTATTCAACAGGAATGTGTAATGGCGCGATG
ATTGGCCAGTTGCCAGGATAGAACAACAGACCCCAAGCCATACCACCAACA
ACCGCTGTCAATGTCATGCTGTTAGACAGCATCAAAACAACATCAAGAACG
ATAGCGCCTGGCATCAGGTTTGAAGGGAAGCAGAAGTTAACTGGGAAATAT
GTCCATCCCCAGAAGTTCAGGTATCTGTTAACCCACTCACCCAGGAGCAGA
CCTAAGACACAAACAACCGCGCCGAAAGGCAGACGGTAACGGTACCACAA
GCATGCTTGAACAGCCGCAGGGAAAGTGATTGAAACGATAGGTGCTACAGT
TACCCATAGACGTCTATCTTTCCAGTCTGTCCAGAAGTCCCAGTC

> *pmoA* clone RSKB–11

GGTGACTGGGACTTCTGGGTTGACTGGAAGGATCGCCGTATGTGGCCGACG
GTCGTGCCGATTCTCGGCGTGACCTTCTGCGCGGCGGCGCAGGCGTTCTGGT
GGGCGAACTTCCGTCTGCCGTTTGGCGCGGTGTTCGCGGCTCTGGGCCTTCT
GATTGGCGAGTGGATCAACCGCTACGTCAACTTCTGGGGCTGGACCTACTTC
CCGATCAGCCTTGTGTTCCCGTCTGCGCTGATCGTTCCGGCGATCTGGCTTG
ACGTGATCCTGCTTCTGTCGGGCTCCTATGTGATCACGGCGATTGTTGGTTC
GCTGGGTTGGGGTCTGTTGTTCTACCCGAACAACTGGCCGGCGATCGCGGC
GTTCCATCAGGCGACGGAGCAGCATGGACAGCTGATGACGCTTGCGGATCT

GATCGGCTTCCACTTCGTCCGCACGTCGATGCCTGAATATATCCGCATGGTC
GAGCGCGGCACGCTGCGCACCTTCGGTAACGACGTTGCCCC

> *pmoA* clone RSKB–12

CCGGGGCAACGTCGTTACCGAAAGTTCTTAGTGTACCTTTTTCAACCATTCT
GATGTACTCAGGAGTACCAGTTCTTACGTAGTGGTAACCTTGTAAGTCAGCC
AGAGTAAACATCATGCCATTGTATTCAACAGGAATGTGTAATGGCGCGATG
ATTGGCCAGTTGCCAGGATAGAACAACAGACCCCAAGCCATACCACCAACA
ACCGCTGTCAATGTCATGCTGTTAGACAGCATCAAAACAACATCAAGAACG
ATAGCGCCTGGCATCAGGTTTGAAGGGAAGCAGAAGTTAACTGGGAAATAT
GTCCATCCCCAGAAGTTCAGGTATCTGTTAACCCACTCACCCAGGAGCAGA
CCTAAGACACAAACAACCGCGCCGAAAGGCAGACGGTAACGGTACCACAA
GCTTGCTTGAACAGCCGCAGGGAAAGTGATTGAAACGATAGGTGCTACAGT
TACCCATAGACGTCTATCTTTCCAGTCTGTCCAGAAGTCCCAGTC