Response of methanotroph-heterotroph consortia to different environmental factors

Thesis submitted by

Karthigeyan Chidambaram Padmavathy (M.Sc.,)

In

January 2017

For the degree of Doctor of Philosophy

In the College of Science and Engineering

James Cook University

Townsville, Queensland, Australia

Statement of the Contribution of others

- Fees
    - Tuition fee waiver, James Cook University
- Stipend support
    - Advanced Manufacturing Cooperative Research Centre (AMCRC) grants
    - Postgraduate Research Scholar Award, James Cook University
    - Write-up scholarships, Graduate Research School, James Cook University
- Supervision
    - A/Prof. Kirsten Heimann, College of Science and Engineering, James Cook University
    - Dr. Obulisamy Parthiba Karthikeyan, College of Science and Engineering, James Cook University
- Editorial assistance
    - A/Prof. Kirsten Heimann, College of Science and Engineering, James Cook University
    - Dr. Obulisamy Parthiba Karthikeyan, College of Science and Engineering, James Cook University
- Research assistance
    - Dr. Obulisamy Parthiba Karthikeyan for training me in methanotroph culture establishment, characterization and molecular analysis
    - A/Prof. Patrick Lee, City University of Hong Kong and Dr. Gregory Maes, James Cook University for their intellectual assistance in NGS.
    - Dr. Roger Huerlimann for assistance with NGS data analyses
    - Mr. Stan Hudson for training me in general lab instrument usage
    - Mr. Saravanan Natarajan for assistance with the setup of reactors for chapter 4.
- Project costs
    - AMCRC grant
    - North Queensland Algal Identification/Culturing Facility
    - A/Prof Kirsten Heimann
- Use of infrastructure external to organisational unit within JCU
    - MEEL and AAC lab

Acknowledgements

I take this opportunity to express my profound sense of reverence to my supervisor, Associate Professor Kirsten Heimann, for her constant support, guidance, encouragement and for her belief in me. I equally thank my co-supervisor Dr. Obulisamy Parthiba Karthikeyan for his guidance, positiveness and for his moral support during my PhD. This thesis would not have been possible without their help and support.

I am very grateful for the scholarships I received from Advanced Manufacturing Cooperative Research Centre (AMCRC), tuition fee waiver and write-up grants provided to me by the Graduate Research School and James Cook University.

I would like to thank Mr. Tom Beard, Waste Management Officer, Townsville Waste Services, Townsville City Council for his help and support in collection of cover soil samples from Stuart landfill, Townsville.

I sincerely thank Dr. Roger Huerlimann, for his help and guidance in analysing the 16s rRNA data sets. I would like to convey my hearty thanks to A/Prof. Patrick Lee and Dr. Gregory Maes for their advice in next-generation sequencing and Dr. Shane Askew for his technical assistance in GC analysis.

I am thankful to all my lab members and research assistants of the NQAIF lab. I want to especially thank my lab managers Mr. Stan Hudson for training me on the correct use of general lab equipment's and Mr. Saravanan Natarajan for assisting in gas monitoring systems and operations.

It is my pleasure to acknowledge all my roommates for their moral and financial support at tough times, fun chats, weekend trips and thanks for making my PhD life in Australia memorable.

I am extremely thankful and indebted forever to my parents and siblings for their continuous support at all times. Without them, I would not be where I am now. Finally, I thank the Almighty God for all the blessings and for the opportunities.

DEDICATED TO MY

PARENTS

AND

SUPERVISORS

Abstract

Methane (CH_4) is a potent greenhouse gas with a global warming potential of 28-34 times that of carbon dioxide (CO_2) over a 100-year period according to IPCC 2014. Globally, landfills are the third largest anthropogenic source of CH_4 emissions (~760 $MMTCO_2eq$), accounting for 11 % of total emissions (6,875 $MMTCO_2eq$). Landfill soils are shown to be the active sink for CH_4 due to the presence of CH_4 oxidising bacteria (methanotrophs), which act as natural bio-filters. Theoretically, landfill CH_4 can be re-routed through commercial bio-filters for CH_4 remediation. The microbial biomass generated could then be utilised for valuable co-product production, such as fatty acids, polyhydroxybutyrate (PHB, a key ingredient in the production of biodegradable plastics). Re-routing CH_4 for PHB production has significant advantages compared to fossil fuel-derived plastics or sugar-derived bioplastics due to the simple concept of killing two birds with one stone; i.e. reducing the carbon footprint and producing renewable biodegradable PHB-based plastics.

Research based on the bio-chemistry and the mechanism involved in the CH_4 oxidation process, bioremediation potential and biofuel/bioplastic production utilising axenic mono-species methanotroph cultures has been widely studied in the last decades. Little is however, known about the feasibility of utilising mixed cultures (methanotrophs and non-methanotrophs) for such purposes, as responses to environmental conditions are poorly studied. This knowledge is vital, as mono-species cultures are unlikely to be maintained in industrial outdoor/landfill scenarios. Therefore, characterising the community composition of methanotrophs and the co-habiting non-methanotrophs in landfill cover soils and their resilience and responses to different nutrient-governed environmental conditions was the primary focus of this thesis. Specifically, responses of mixed consortia to factors such as variable CH_4 and oxygen (O_2) concentrations, as these vary with time and age in landfills, potentially affecting methanotroph diversity, and variable concentrations of essential trace elements,

primarily copper (Cu^{2+}) and iron (Fe^{2+}), as levels of these govern expression and activity of the key enzymes of the CH_4 oxidation pathway, affecting CH_4 oxidation capacity (MOC) of methanotrophs.

In this Ph.D, I aimed to

1) establish methanotroph-heterotroph consortia (mixed consortia) from different landfill cover soils,
2) examine the robustness of community composition of these consortia to variable CH_4:O_2 (air) and Cu^{2+}/Fe^{2+} ratios and investigate the flow on impacts on CH_4 removal efficiency, oxidation capacity, fatty acid and PHB content.

To achieve these aims, four different landfill cover soils (vegetated cover, mulch cover, fresh and compost soils) were collected from Stuart landfill, Townsville, Australia (chapter 2). Initially landfill soil slurries were subjected to high CH_4 environments (50:50 and 20:80 (v/v) of CH_4: air) to measure MOC and total microbial/ methanotrophic diversity using next-generation sequencing. Mulch soil showed higher MOC and was rich in methanotroph diversity compared to other soils and compost soil, the most commonly used landfill cover, had acceptable levels of both type -I and -II methanotrophs, as well as MOC. Therefore, based on MOC and methanotrophic diversity of the soil slurries, mulch - (LB) and compost soil (CB) were selected to establish a mixed consortium. While the enriched consortia (LB and CB) showed similar methanotrophic community profiles (dominated by *Methylosarcina*, a type -I methanotroph), the co-habiting non-methanotrophs differed. Irrespective of varying microbial structure in both LB and CB, the established consortia showed more or less similar MOC and CH_4 removal efficiencies. These findings suggest that such microbial consortia could be used for biological landfill CH_4 emission abatement.

The robustness and responses of these established mixed consortia to variable CH_4:O_2 ratios (10-50 % CH_4 in air) was then further investigated to assess potential for biological CH_4 mitigation, as these gases change in proportion in landfill environments

(chapter 3). PHB-content was also examined to evaluate potential for co-production of biodegradable plastics. Interestingly, CH_4/O_2 ratios did not affect the dominance profile of the mixed consortia, which were dominated by *Methylosarcina* (50-70 %) and *Chryseobacterium* (10-30 %) and an average removal efficiency of ~50 % was achieved. Due to the low abundance of type –II methanotrophs PHB content of both LB (~2.5 %) and CB (~1.4 %) was lower than expected. The sustained co-dominance of *Methylosarcina* and *Chryseobacterium* suggests that there may be advantageous biological interactions between these microbes, such as assistance in CH_4 oxidation by *Chryseobacterium* through potential horizontal gene transfer.

In an attempt to increase PHB accumulation, cultivation conditions known to favour growth of type -II methanotrophs (nutrient-limitation, high CH_4 concentration, low pH), the established mixed consortia (LB and CB) were sequentially enriched in nutrient-deficient conditions under high CH_4 concentrations. These further enriched consortia were then then cultivated as sequentially varying Cu^{2+}/Fe^{2+} ratios (chapter 4). Irrespective of the similar methanotroph structure (LB and CB) and the same cultivation conditions, the responses of the mixed community to changing Cu^{2+}/Fe^{2+} ratios differed. *Methylosarcina* and *Chryseobacterium* was initially dominant in both LB and CB, but increased in Cu^{2+}/Fe^{2+} ratios led to dominance of *Azospirillum* and *Sphingopyxis* over methanotrophs in LB and dominance of *Sphingopyxis* and *Methylosinus* (a type -II methanotroph) in CB. Irrespective of the reduced presence of methanotrophs, MOCs and CH_4 removal efficiencies were maintained in LB. Overall, the dominance of type -II methanotrophs and, consequently PHB content, was negatively affected by increasing Cu^{2+}/Fe^{2+} ratios in both LB and CB. The outcome of this research highlights that single-species responses differ significantly to those of mixed consortia and that more in depths research is warranted to understand allelopathic, metabolic and molecular interactions in mixed microbial assemblages, particularly when subjected to variable environmental conditions.

In conclusion, my research has increased knowledge on microbial community structure of mixed consortia and their responses to variable CH_4/O_2 (air) and Cu^{2+}/Fe^{2+} ratios. Results suggest that the non-methanotrophs co-dominated with methanotrophs may offer beneficial services to methanotrophs in reducing toxic metabolite concentrations and potentially thrive through utilisation of secreted complex carbohydrates (extracellular polymeric substances (EPS)), as no complex carbon sources were provided in the growth medium. Future enzymatic/physiological studies are required to further unravel metabolic interactions and functional relationships in mixed microbial communities. Such studies will help to develop community models to evaluate potential for CH_4 emission abatement a priori. The case-scenario on PHB production potential from landfill CH_4, presented in the general discussion, shows that the cost of PHB production can be reduced to 1.5–2.0 AUD meeting the market value of synthetic plastics by increasing production volumes through building a centralised extraction and refinement facility suitable for large metropolitan cities. However, the scenario is based on optimal bio-filter performance in terms of CH_4 abatement and PHB contents, which had not been reached in the above mixed consortia environmental variable response studies. This demonstrates that future studies need to couple biochemical profiling with optimised bio-filter designs and microbial networks to ascertain the true potential for co-development of renewable products like bioplastics (PHB). Such work will lead to establishing techno-economic models for optimised bio-filter performance with regards to CH_4 abatement in general and co-product potential specifically.

Table of Contents

List of Graphical abstracts

List of Tables

List of Figures

Abbreviations

µg	Microgram
µM	Micro molar
ANME	Anaerobic methanotrophs
AnOM	Anaerobic oxidation of methane
ANOVA	Analysis of variance
AOM	Aerobic oxidation of methane
ATP	Adenosine triphosphate
CB	Compost biomass
CBB	Calvin-Benson-Basham
CH_3OH	Methanol
CH_4	Methane
CO	Carbon monoxide
CO_2	Carbon dioxide
CO_2eq	CO_2 equivalents
CSTR	continuous stirred tank reactor
DGGE	Denaturing gradient gel electrophoresis
DNMS	Diluted Nitrate mineral salts
DW	Dry weight
FADH	Flavin adenine dinucleotide
FAME	Fatty acid methyl ester
FDH	Formate dehydrogenase
FID	Flame Ionisation Detector
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
GHG	Greenhouse gas
GWP	Global warming potential

H_2S	Hydrogen sulfide
HCHO	Formaldehyde
HCOOH	Formate
Kg	Kilogram
Km	Kilometre
LB	Landfill biomass
LFG	Landfill gas
mg	Milligram
MMO	Methane monooxygenase
$MMTCO_2eq$	Million Metric tons of CO_2 equivalents
MOC	Methane oxidation capacity
Mt	Million tonnes
N_2O	Nitrous oxide
NADH	Nicotinamide adenine dinucleotide
NGS	Next-generation sequencing
NMS	Nitrate mineral salts
O_2	Oxygen
OD	Optical density
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PLA	Polylacticacid
PLFA	Phospho lipid fatty acid
pMMO	Particulate methane monooxygenase
PP	Polypropylene
PPB	Parts per billion
PS	Polystyrene

QS	Quorum sensing
RuMP	Ribulose monophosphate
sMMO	Soluble methane monooxygenase
TCA	Tricarboxylic acid
TCD	Thermal conductivity detector
TCE	Trichloroethylene
Tg	Tetragram
TN	Total nitrogen
TOC	Total organic carbon
TP	Total Phosphorus
TS	Total solids
V/V	Volume/Volume
VS	Volatile solids
W/V	Weight/Volume

Refereed Thesis Publications

Review articles

1. **Chidambarampadmavathy K**, Karthikeyan OP & Heimann K (2016) Sustainable bioplastic production through landfill methane recycling. Renewable & Sustainable Energy Reviews 71: 555-562. (https://doi.org/10.1016/j.rser.2016.12.083).
2. Karthikeyan OP, **Chidambarampadmavathy K**, Cirés S & Heimann K (2015) Review of sustainable methane mitigation and biopolymer production. Critical Reviews in Environmental Science and Technology 45: 1579-1610 (http://dx.doi.org/10.1080/10643389.2014.966422).
3. **Chidambarampadmavathy K**, Karthikeyan OP & Heimann K (2015) Role of copper and iron in methane oxidation and bacterial biopolymer accumulation. Engineering in Life Sciences 15: 387-399. (doi:10.1002/elsc.201400127).

Research articles

4. **Chidambarampadmavathy K**, Karthikeyan OP, Huerlimann R, Maes EG & Heimann K (2016) Responses of mixed methanotrophic communities to variable Cu^{2+}/Fe^{2+} ratios. Journal of Environmental Management 197: 159-166.(https://doi.org/10.1016/j.jenvman.2017.03.063).
5. **Chidambarampadmavathy K**, Karthikeyan OP, Huerlimann R, Maes EG & Heimann K (2016) Response of mixed methanotrophic consortia to different methane to oxygen ratios. Waste Management 61: 220-228 (http://dx.doi.org/10.1016/j.wasman.2016.11.007).
6. **Chidambarampadmavathy K**, Karthikeyan OP & Heimann K (2015) Biopolymers made from methane in bioreactors. Engineering in Life Sciences 15: 689-699. (doi:10.1002/elsc.201400203).

Conference presentations at national and international meetings

1. **Chidambarampadmavathy K**, Karthikeyan, OP & Heimann K (2016). Bacterial synergy in methane oxidizing environments. Asia-Pacific Conference on Biotechnology for Waste Conversion, Hong Kong December 06-08. (Research paper and poster presentation).
2. Heimann K., **Chidambarampadmavathy K** & Karthikeyan, OP (2016). Sustinable bioplastic production through landfill methane recycling. Bioenergy Conference, Brisbane Australia November 14-16. (Oral and poster presentation).
3. **Chidambarampadmavathy K**, Karthikeyan, OP & Heimann K (2015). Effect of methane concentration on biopolymer content and methanotrophic community structure. International Conference on Solid Wastes: Knowledge Transfer for Sustainable Resource Management, Hong Kong May19-23. (Research paper and oral presentation)
4. **Chidambarampadmavathy K**, Karthikeyan, OP & Heimann K (2014). Methane mitigation and biopolymer production by methanotrophs enriched from landfill top cover soils: effect of Cu^{2+}/Fe^{2+} Biology in the Tropics. School of Marine & Tropical Biology Postgraduate Student Conference, Townsville, Australia June 12-13. (Oral presentation).
5. **Chidambarampadmavathy K**, Karthikeyan, OP & Heimann K (2014). Bacteria-mediated bioremediation of methane to biopolymer production in

bioreactors. North Queensland Festival of Life Sciences, Townsville, Australia November 4. (Poster presentation)

6. Heimann K., **Chidambarampadmavathy K** & Karthikeyan, OP (2014). Bioreactor-produced biopolymers using methane: effect of Cu^{2+}/Fe^{2+}. Biomaterials from Bioreactors, Radebeul, Germany May 26-28. (Research paper and oral presentation).
7. Heimann K., Karthikeyan, OP, **Chidambarampadmavathy K** & Cirés, S. A biotechnological approach to convert methane into biopolymer. Greenhouse Conference. Adelaide October 8-11. (Poster presentation).

[1]Chapter 1: General Introduction

[1] This chapter has been adapted from:

Chidambarampadmavathy K, Karthikeyan OP & Heimann K (2016) Sustainable bio-plastic production through landfill methane recycling. Renewable & Sustainable Energy Reviews 71:555-562 (https://doi.org/10.1016/j.rser.2016.12.083).

Chidambarampadmavathy K, Karthikeyan OP & Heimann K (2015) Role of copper and iron in methane oxidation and bacterial biopolymer accumulation. Engineering in Life Sciences 15: 387-399. (doi:10.1002/elsc.201400127).

Karthikeyan OP, **Chidambarampadmavathy K**, Cirés S & Heimann K (2015) Review of sustainable methane mitigation and bio-polymer production. Critical Reviews in Environmental Science and Technology 45: 1579-1610 (http://dx.doi.org/10.1080/10643389.2014.966422).

1.0 Preface

The general introduction outline the knowledge on landfill methane (CH_4) emissions, importance of biological remediation of methane using methanotrophs and the enzymes involved in this oxidation process with special emphasis on the need for bio-product development particularly biodegradable plastics, i.e. polyhydroxybutyrate (PHB). In regard to the latter, the introduction outlines the problems of plastics with regards to landfill methane generation and waste management practices, emphasizing the urgent need for alternatives, i.e. PHB, (microbially-derived bioplastics), which could be addressed by employing the services of methanotrophs. As such, the information provided below is essential for understanding the experimental approach decided on for my PhD research. While the general introduction does not provide any detail about mixed methanotrophic consortia, so as to not repeat information provided in the introductions and discussions of later chapters of the thesis, it must be highlighted here that at industrial scale, CH_4 emission abatement and co-product development (PHB) using axenic cultures will not be economically feasible. Therefore, my research focussed on establishing methanotroph-heterotroph consortia (mixed consortia) from landfill soils and to characterise their responses to and impacts of critical environmental variables (CH_4 and O_2 concentrations, Cu^{2+}/Fe^{2+} ratios) on consortium structures, which influence CH_4 oxidation and ultimately PHB accumulation.

1.1 Objectives of this study.

As reviewed in detail below, there are number of environmental factors that govern CH_4 oxidation capacities, biomass growth and microbial/ methanotrophic consortia interactions that influence biomass end product yields/ quality (PHB). In this study, I aimed to test the responses of two environmental variables (gases and heavy metals), i.e. different CH_4 and oxygen (O_2) concentrations and copper and iron (Cu^{2+}/Fe^{2+}) ratios in mixed consortia. CH_4 is an essential substrate for methanotrophs, while O_2 is important for both methanotrophs and co-existing non-methanotrophs (i.e. biological O_2 demand), the latter providing benefits to methanotrophs through metabolic conversion of toxic intermediates from CH_4 oxidation pathways (Karthikeyan et al., 2015a). Generally, type -I methanotrophs dominate in niches where CH_4 levels are low and O_2 levels are high, with the opposite being the case for type -II methanotrophs (López et al., 2014; Wei et al., 2015). Low O_2 concentrations favour type -II, as the nitrogenase complex responsible for N_2-fixation is sensitive to O_2 (Whittenbury and Dalton, 1981). It is critically important to consider CH_4 and O_2 ratios, as the responses of methanotrophs may vary, potentially affecting oxidation capacity in-turn, depending on the type enriched. Understanding CH_4 and O_2 ratio-induced microbial community shifts is also important for developing/customising bio-filters that work economically well for CH_4 mitigation in the varying CH_4 and O_2 ratio fluxes, as experienced in landfills. In addition, optimal copper or iron concentrations reported for their growth are species-specific, different species will require different levels of copper and iron for maximal PHB accumulation. Therefore, interactive effects of copper and iron should also be investigated to develop robust and highly productive mixed consortia cultures.

In this context, the overarching aim of this thesis was to enrich robust methanotrophic consortia from indigenous landfill communities and to test different environmental factors on consortia establishment and longevity, methane oxidation

capacity (MOC) and CH_4 removal efficiency, growth, fatty acid contents and PHB accumulation potential.

The data chapters in this thesis are divided into three chapters. The aims of each chapter are presented below:

Chapter 2: The aims were to enrich and establish a robust consortium from different indigenous landfill soils and to initially characterise these for selection of the best performing and/or species-rich consortia for chapters 3 and 4.

Chapter 3: The aims were to evaluate the effect of variable CH_4/O_2 (air) ratios (10-50 % CH_4 in air) on established consortia and to quantify flow on impacts on MOC, fatty acid contents and profiles, and PHB accumulation capacity.

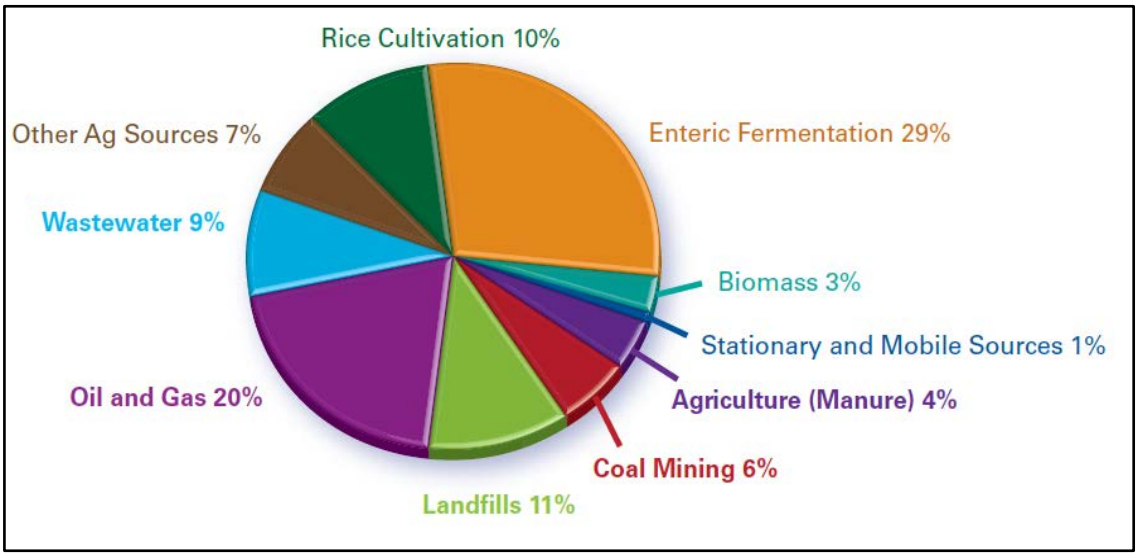
Chapter 4: The aims were to investigate the interactive effect of Cu^{2+}/Fe^{2+} ratios on the growth and community structure of mixed consortia and effects on methane oxidation capacity (MOC), fatty acid contents and profiles, and PHB accumulation capacity.

Chapter 5: Summarises major findings and synthesises these with an industrial perspective, i.e. a case-scenario based on optimal bio-filter performance in terms of CH_4 abatement and PHB contents for PHB production potential from landfill CH_4.

1.2 Methane and emission sources.

For the past 60 years (1951–2016), many studies focused on increase in greenhouse gas (GHG) emissions-induced global warming and the impacts associated with it. Of the three GHGs, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), CH_4 contributes with ~20 % to the global GHG emission and is the most potent GHG, remaining for 12 ± 3 years in the atmosphere (IPCC, 2007, 2014). It has a global warming potential (GWP) of 84-86 over 20 years, 28-36 over 100 years and 7.6 over 500 years (EPA, 2016; IPCC, 2007, 2014; Neubauer, 2014). This difference in GWP with increasing time in the atmosphere is due to oxidation of CH_4 by hydroxyl radicals (OH) resulting in water and CO_2 (Levy, 1971).

The atmospheric concentration of CH_4 has increased considerably from a pre-industrial level of ~715 to ~1840 ppb in 2015 (Butler and Montzka, 2016; EEA, 2016; IPCC, 2014). More than 63 % (avg. 346 Tetragram (Tg).Y^{-1}) of the global atmospheric CH_4 levels are emitted from anthropogenic sources such as agriculture, coal mines, landfills, oil and natural gas, enteric fermentation and wastewater treatments and the remaining 37 % (avg. 202 $Tg.Y^{-1}$) by natural sources such as wetlands, anoxic soil and sediments, peat lands, fresh water lakes, oceans, hyper saline lakes and ponds (GMI, 2010; IPCC, 2007, 2014; Karthikeyan et al., 2015a; Solomon et al., 2007). In 2010, global anthropogenic CH_4 emissions were estimated as 6,875 million metric tons of CO_2 equivalents ($MMTCO_2eq$) and projected to increase by 15 % reaching 7,904 $MMTCO_2eq$ in 2020 (GMI, 2010). Of this, oil and natural gas, enteric fermentation, landfills, agriculture, coal mines and wastewater alone contribute approximately 80-90 % (Figure 1.1).



Source - Global Methane Index, GMI (2010)

Figure 1.1 Global anthropogenic methane emissions

1.3 Methane emission from landfills

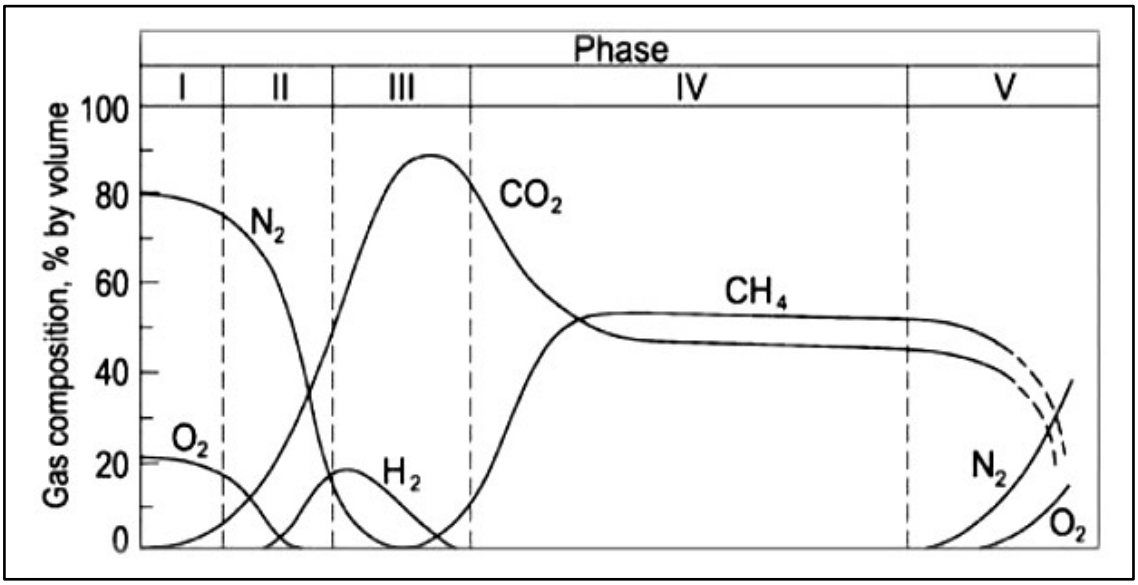
Globally, landfills are considered to be the third largest anthropogenic source of CH_4, contributing ~760 $MMTCO_2eq$ as of 2010 (IPCC, 2014), which is projected to increase by 10 % by 2020 (GMI, 2010). Biogenic CH_4 emissions from landfills occur due to microbial anaerobic degradation of organic matter and varies at different time intervals with respect to landfill age (Scheutz et al., 2009).

Under anaerobic conditions, organic components are biochemically converted into CH_4 via series of steps like hydrolysis, acidogenesis, acetogenesis and methanogenesis (Appels et al., 2008; Nikiema et al., 2007; Peck, 2007). Typical landfill gas (LFG) contains 30 to 70 % of CH_4 and 20 to 50 % of CO_2, with small amounts of non-CH_4 organic compounds as shown in Table 1.1 (Nikiema et al., 2007). The emission of LFG is a continuous process and occurs anytime in different phases of landfill operations and, therefore the gas composition changes with maturation time (Figure 1.2) (Peck, 2007).

Table 1.1 Composition of bio-gas produced in landfills

Components	Percentage (%)
Methane	30 - 70
Carbon dioxide	20 - 50
Nitrogen	1 - 5
Oxygen	0.1 - 1.0
Ammonia	0.1 - 1.0
Sulfur compounds	0.0 – 0.2
Hydrogen	0.0 – 0.2
Carbon monoxide	0.0 – 0.2
Other trace compounds	0.01 – 0.6

Source - Nikiema et al. (2007)



Source - Peck (2007).

Figure 1.2 Landfill gas compositional changes over different phases of wastes degradation

1.4 Landfills in Australia

Australia mainly depends on landfills for their solid waste management practices (Australian Bureau of Statistics, 2010). A total of 1168 landfills exist in Australia, with highest landfill densities in New South Wales (Table 1 2) (BERE consultancy, 2013). Landfills in Australia vary greatly in size; small landfills receive on average 5,000 tonnes of wastes, whereas large modern waste-gas managed landfills receive more than 100,000 tonnes of waste annually (BDA Group, 2009; BERE consultancy, 2013).

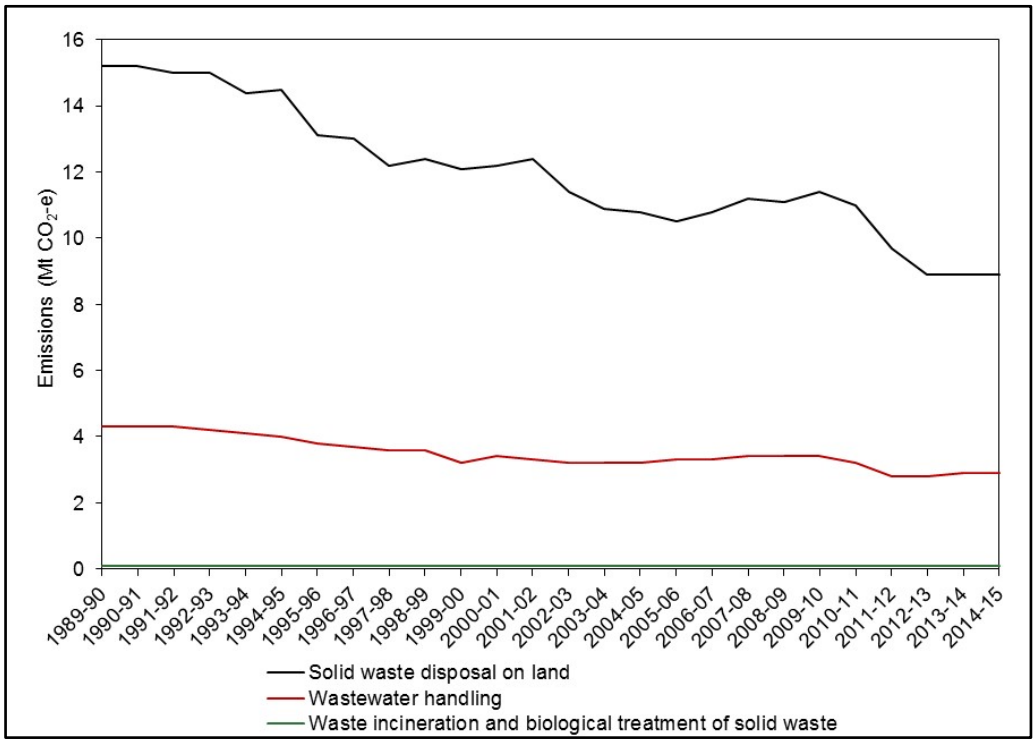
Table 1 2 Australia's waste management infrastructure

Jurisdiction	NSW	VIC	Qld	WA	SA	TAS	NT	ACT
Number of Landfills	369	92	265	187	117	19	118	1
Resource Recovery Facility	121	233	88	86	2471	14	10	6
Transfer Station	166	239	236	26	133	67	4	1

Source - BERE consultancy (2013)

NSW - New South Wales; Vic – Victoria; QLD – Queensland; WA - Western Australia; SA - South Australia; TAS – Tasmania; NT - Northern Territory; ACT - Australian Capital Territory

Annually, 48 million tonnes (Mt) of solid waste are generated in Australia, of which 40 % (~20 Mt) are disposed in landfills. Of the 20 Mt of waste, 14 Mt are organic waste (BERE consultancy, 2013), releasing 0.65–1.53 tonnes of CO_2eq per tonne of waste (BDA Group, 2009). Methane emissions from solid waste are estimated based on the first order decay method (Eggleston et al., 2006) and, in general, a 28-36 times multiplier is applied (EPA, 2016; IPCC, 2007, 2014; Scheutz et al., 2009) for converting CH_4 emissions to CO_2eq to create a comparative base for all GHG emissions. As of 2015, the net emission was ~12 Mt CO_2eq (Figure 1.3), which includes the emission from solid waste disposal, waste incineration and waste water handling (Australia's National Greenhouse Accounts, 2016).



Source - Australia's National Greenhouse Accounts (2016), reproduced with permission.

Figure 1.3 Waste by sector, annual emissions, 1989-90 to 2014-15

1.5 Methods employed to mitigate CH_4 emissions in landfills

To reduce the impact of CH_4 emissions from landfills, several different treatment approaches have been published (EPA, 2011; Huber-Humer, 2004; Huber-Humer et al., 2008; Humer and Lechner, 1999). In Australia, a major route for CH_4 abatement from landfills is extraction/purification for power generation; the appropriateness, however, depends on landfill age (CH_4 concentrations of the gas mixture). Other options for abatement have also been developed, like flaring of LGF at landfill sites, diversion of degradable material and sorting. Most of these options are expensive, the merits and de-merits of each technology are summarized in Table 1.3.

Comparatively, biological approaches are relatively simple, reliable, cost-effective and robust, which suit CH_4-abatement from LFG well, as they are more independent of changing conditions with landfill age (EPA, 2011; Huber-Humer, 2004; Huber-Humer et al., 2008; Humer and Lechner, 1999), compared to combustion and

biofuel techniques, providing a long term solution to mitigate CH_4 in LFG. Biological CH_4 mitigation is a microorganism-driven technology, whereby CH_4 is oxidised to CO_2 by methane oxidising bacteria (methanotrophs). Information on the type of methane oxidation and the pathways involved in methane oxidation is given in section 1.9.

Table 1.3 Methods employed to mitigate CH_4 emissions

Methods	Advantages	Disadvantages
Combustion	Fast destruction of CH_4; Recovery of energy possible from the hot gases	Emits secondary pollutants (e.g. NO_x and CO.
Extract and produce biofuel	Can be used for generating alternative fuels for electricity production to meet rising demand	Cost and performance varies.For use as an energy source, pre-treatment (to remove sulfur compounds, such as hydrogen sulfide (H_2S), and siloxanes) of LFG is required.
Biological treatment	Suitable as a long term solution as no minimum amount of CH_4 is required	Depends on landfill characteristics and climate.

1.6 Plastics disposal in landfills

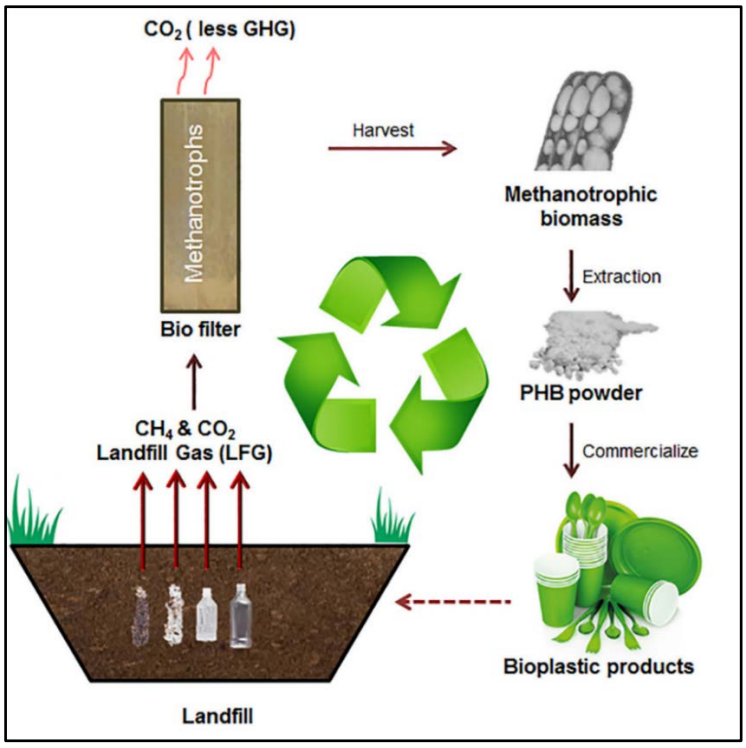
Another, major issue associated with the landfill operation and management is linked with plastic depositions. Plastics are indispensable commodities in our day-to-day life and the unprecedented human population growth will ultimately be mirrored in an increased need for plastics. The annual production of plastics has increased dramatically from 1.5 Mt (1950) to 300 Mt (as of 2013) (Plastic the Facts, 2015) and a four- to five-fold market growth has been predicted by the end of this century (Lemstra, 2012; Philp, 2014a). Eight percent of the global oil and gas is used for plastic production, 4 % as feed-stock and ~3-4 % as energy for manufacturing and transportation (Hopewell et al., 2009). Fossil fuel resources are non-renewable as they take millions of year to form naturally and, as our economy entirely relies on their use, depletion is becoming a global problem. It is estimated that 20-25 % of current crude

oil production (93 million barrels per day) (EIA, 2014) will be required for plastics manufacturing by the end of this century (Philp, 2014b).

Annually, millions of tonnes of plastics (60 % of the produced plastic) are being disposed of in landfill (Hopewell et al., 2009). Globally, plastic accounts for 18-20 % of the waste volume in landfill (Criddle et al., 2014; Ishigaki et al., 2004). From a sustainability perspective, there are some major drawbacks to waste management of plastics in landfills, i.e. leachates percolate through plastics are toxic, adversely affecting landfill and soil microbial flora, leading to soil infertility and/or ground water and water supply contamination (Oehlmann et al., 2009; Teuten et al., 2007). Toxins and/or the packaging of organic waste in bio-inert and impervious plastic bags result in persistence of easily biodegradable waste (Thompson et al., 2009; Wiles and Scott, 2006). Even targeted degradation in composting facilities still requires the separation of degradable plastics from non-degradable ones. Considering the current methods employed to mitigate CH_4 emissions, and the need for environmentally sustainable alternatives for plastics, microbial waste management practices should be seriously considered. Since fossil fuels are not renewable and fossil fuels-derived plastic are not biodegradable, research has focussed on alternative methods for producing plastics using renewable materials like CH_4. Economically sustainable production of microbial derived plastics (bioplastics) has gained renewed interest, as this approach promises to solve landfilling problems of non-degradable plastics and positively manage fossil fuel use and GHGs emissions.

Bioplastics are defined as polymeric materials derived from renewable carbon sources that decompose naturally to CO_2 and water under aerobic condition or CH_4, CO_2 and water under anaerobic condition (Lackner, 2015; Pei et al., 2011), making them sustainable and eco-friendly. Biodegradability of bioplastics depends entirely on the type of bio-plastic and the environmental conditions they are exposed to. For example, bioplastics generally degrade within 50-100 days (90% aerobic

biodegradation) in compost, whilst it takes 100 to 300 days (65 – 90% anaerobic biodegradation) in soils (Emadian et al., 2017). The theoretical concept behind this approach is the cradle-to-grave-to-cradle approach stemming from landfilling of bioplastics and recreating bioplastic through sustainable approaches using methanotrophs (Figure 1.4).



Source - Chidambarampadmavathy et al. (2016b)

Figure 1.4 Cradle-to-grave-to-cradle approach for PHB production

However from an industrial point of view, maintaining an axenic culture in large scale bio-filters (for CH_4 abatement and bioplastic production) will not be economically feasible, so there is a need to develop robust consortia that can withstand various environmental conditions in order to improve CH_4 oxidation-coupled bioplastic production. Furthermore, the main challenges for bioplastics is retention of the physico-chemical properties of petroleum-derived plastics, whilst keeping the properties of biocompatibility and biodegradability (Keshavarz and Roy, 2010; Lackner, 2015; Reddy et al., 2003). The most promising bioplastics that are considered as viable alternatives to synthetic plastics are polyhydroxyalkanoate (PHA).

1.7 Polyhydroxyalkanoates

Polyhydroxyalkanoates are aliphatic polyesters produced by microbes via conversion of various carbon substrates for intracellular carbon and energy storage (Keshavarz and Roy, 2010; Lee, 1996a; Reddy et al., 2003). PHAs are mainly classified based on the number of carbon atoms and the type of monomeric units, e.g. polymers with 3–5 carbon atoms are considered short chain length PHA, whilst medium chain length PHA contain 6-14 carbon atoms (Reddy et al., 2003). Among the biodegradable plastics, PHAs have similar material properties to conventional plastics (Steinbüchel and Füchtenbusch, 1998), with physical (Harding et al., 2007; Ojumu et al., 2004) and mechanical properties being tuneable by changing co-polymer contents (Harding et al., 2007; Lee, 1996a). PHA can be used in a wide range of short-term packaging applications, such as plastic films for bags and other diverse packaging applications, containers and paper coatings, in disposable articles (personal care products, surgery clothes), upholstery (Lee, 1996a; Ojumu et al., 2004). As PHAs are biodegradable and immunologically inert, they have a promising future in medical applications, despite expensive production (Keshavarz and Roy, 2010; Lee, 1996a; Ojumu et al., 2004). The most commonly studied PHA is polyhydroxybutyrate (PHB), produced by several Gram-positive and Gram-negative bacteria, including methanotrophs (Karthikeyan et al., 2015a; Ojumu et al., 2004; Pieja et al., 2011a; Wendlandt et al., 2010). PHB polymers have desirable characteristics similar to polypropylene (PP) such as moisture resistance, water insolubility, O_2 impermeability, resistance to UV weathering, indefinite stability in air and high optical purity (Chanprateep, 2010; Reddy et al., 2003). Taken production into consideration, being a microbial carbon and energy store, PHBs are suitable for a full cradle-to-grave-to-cradle waste management approach (Figure 1.4). Compared to commercially available biodegradable and synthetic plastics, production of PHB requires only 44.7 MJ/kg being 50 to 70 % lower than for synthetic plastic production, emitting 2.6 kg of

CO_2eq.kg^{-1} of polymer. Whilst synthetic plastics like high density polyethylene, low density polyethylene, polypropylene, nylon, polyethylene terephthalate, polystyrene production emits 4.8, 5.1, 3.4, 7.6, 4.9, and 6.0 kg of CO_2eq.kg^{-1} polymer, respectively (Chanprateep, 2010; Gironi and Piemonte, 2011; Harding et al., 2007; Lee, 1996b; Ojumu et al., 2004; Wendlandt et al., 2010).

1.8 Synthesis of Polyhydroxyalkanoates

Polyhydroxyalkanoates are synthesised *via* bacterial fermentation, in genetically modified plants, and using enzymatic catalysts in cell-free systems. At present, microbially synthesised PHA production (mainly PHB) is the only method available at industrial-scale. Microbial PHBs are commercially produced using a number of carbon-sources as feed-stocks such as wheat bran, whey, molasses, cane starch, palm oil, cassava waste, sucrose, glucose (Gerngross and Slater, 2000; Harding et al., 2007), which constitute more than 30 to 50 % of the production cost (Li et al., 2009). Feed-stock provision for PHB producing microbes is therefore the limiting and cost-prohibitive factor for mass production of microbial PHB. As the price of PHB is strongly influenced by the substrates and bacterial strain used, costs of PHB can differ between USD 4-16 per kg of polymer (Keshavarz and Roy, 2010; Reddy et al., 2003). For example PHB produced form *Cupriavidus necator* (formerly *Alcaligenes eutrophus*) costs USD 16 per kg of polymer, which is more expensive than polypropylene, whilst production using recombinant *Escherichia coli* reduces costs to USD 4 per kg of polymer, which is comparable to the production costs of other biodegradable plastic materials such as polylactic acid and aliphatic polyesters. Despite production cost challenges, the global bioplastic production is expected to increase from 890,000 tonnes to 2.5 Mt from 2012 to 2017 (Roland-Holst et al., 2013), expected to reach 1.5-4.4 Mt by 2020, globally (Li et al., 2009).

Production area requirements for feed-stocks also hamper viability (Li et al., 2009; Roland-Holst et al., 2013), as increase in production could lead to competition

for arable land between food and feed-stocks crops. According to the Food and Agriculture Organization (FAO) of the United Nations, world food prices reached record highs in 2010 with a Food Price Index of 214.7 points comparable to the food crisis in 2008 (213.5 points) (BBC News, 2008).

Given the above, re-routing of unutilized/wasted CH_4 from landfills and other sources, as a carbon source for PHB production may have significant advantages for reducing production costs associated with feed-stock, land use, and energy requirements (Karthikeyan et al., 2015a; Listewnik et al., 2007; Rostkowski et al., 2012; Wendlandt et al., 2010). Using CH_4-fed methanotrophic bacteria for PHB production has significant advantages, such as sequestering the GHGs CH_4 and CO_2 (~60 % of oxidised CO_2 from CH_4 is incorporated for biomass growth (Yang et al., 2013)), higher accumulation of PHB (maximal ~70 % of dry biomass) and more than 50-56 % on an industrial-scale (Wendlandt et al., 2010)).

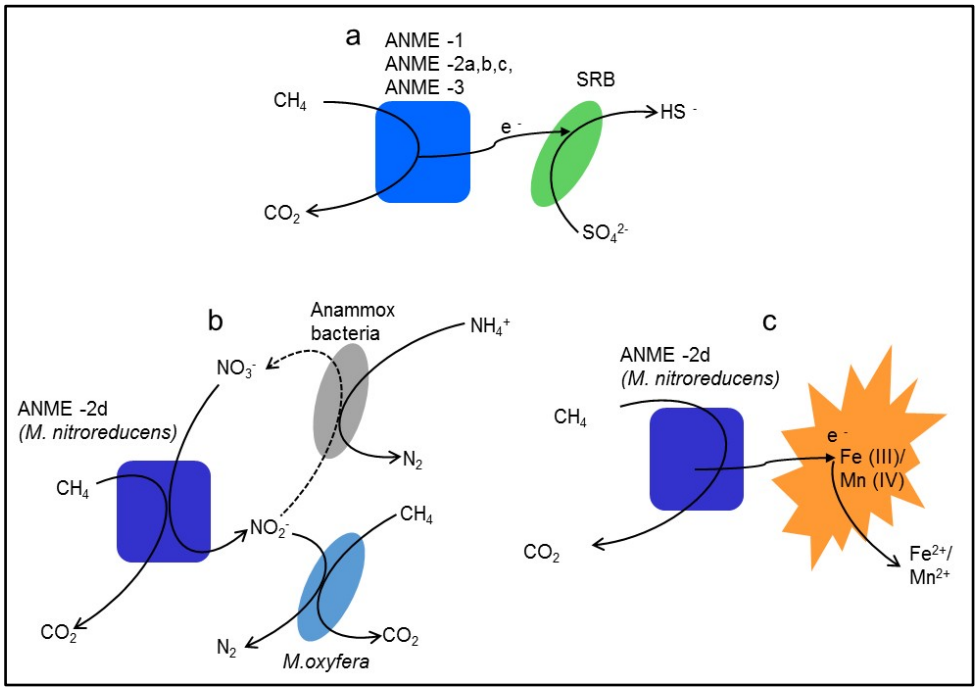
1.9 Methanotrophs

Methanotrophs are key players in global CH_4 mitigation and are grouped into aerobic and anaerobic methanotrophs based on their physiological traits. The aerobic oxidation of CH_4 to CO_2 by methanotrophs is well studied, while the anaerobic pathway is still unresolved.

1.9.1 Anaerobic methanotrophs

Anaerobic oxidation of CH_4 (AnOM) is carried out in anoxic marine sediments by a group of archaea belonging to the Euryarchaeota commonly known as anaerobic methanotrophs (ANME). To date, three groups of ANME (ANME-1, ANME-2 a,b,c,d and ANME-3) have been identified which are closely related to the orders *Methanosarcinales* and *Methanomicrobiales* (Boetius et al., 2000; Hinrichs and Boetius, 2003; Knittel and Boetius, 2009; Oni and Friedrich, 2016). The ecology and physiological traits of these ANME is not fully understood, since difficulties in establishing pure isolates are encountered (Girguis et al., 2005). With regards to

managing non-anthropogenic sources of CH_4, however, understanding the mechanism of AnOM is considered to be one of the most important research focus, because 90 % (20 to 300 Tg $CH_4.Y^{-1}$) of ocean-derived CH_4 is oxidized by ANME *(Hinrichs and Boetius, 2003)*. Based on the results of field observations and enrichment experiments, metagenomic and proteomic studies, Knittel and Boetius (2009) proposed that the key enzymes of methanogenesis are deployed in reverse for CH_4 oxidation by ANME. In general, ANME bacteria oxidize CH_4 to CO_2 with sulfate (SO_4^{2-}) as the final electron acceptor, which in turn is utilized by the syntrophic partner, sulfate-reducing bacteria (SRB), who gain energy by reducing SO_4^{2-} to hydrogen sulfide. However, recently AnOM coupled to iron, manganese and nitrate reduction also been demonstrated in ANME (Beal et al., 2009; Haroon et al., 2013; Oni and Friedrich, 2016) (Figure 1.5).



Source - Oni and Friedrich (2016), reproduced with permission from the journal.

Anaerobic oxidation of methane (AnOM) coupled to (a) sulfate reduction. (b) nitrate reduction (c) reduction of Fe(III) or Mn(IV)

Figure 1.5 Electron-accepting processes coupled to anaerobic CH_4 oxidation

1.9.2 Aerobic methanotrophs

Aerobic oxidation of CH_4 to CO_2 is carried out by aerobic methanotrophic bacteria. They are a diverse group of Gram-negative bacteria belonging to the proteobacteria group and utilize CH_4 as their sole carbon and energy source (Hanson and Hanson, 1996; Whittenbury et al., 1970). Methanotrophs are classified into two different types, based on (i) the concentration of CH_4 and O_2 they require for growth: high affinity and low affinity methanotrophs (Graham et al., 1993; Hanson and Hanson, 1996); (ii) the structures of intra-cytoplasmic membranes (ICM); (iii) pathways of carbon assimilation and ability to fix nitrogen; (iv) mole percent G+C content of their DNA and (v) other morphological and physiological traits (Hanson and Hanson, 1996; Karthikeyan et al., 2015a; Smith and Murrell, 2008).

A gammaproteobacterial type -I (subtypes are type -Ia (previously type -X), type -Ib and type -Ic) belonging to the families of Methylococcaceae and Methylothermaceae. Methylococcaceae include the following genera: *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylogaea*, *Methyloglobulus*, *Methylomagnum*, *Methylomarinum*, *Methylomicrobium*, *Methylomonas*, *Methyloparacoccus*, *Methyloprofundus*, *Methylosarcina*, *Methylosoma*, *Methylosphaera*, *Methylovulum*, *Methylothermus*, and *Methylohalobius*. Whilst Methylothermaceae include the genera.*Methylohalobius*, *Methylomarinovum*, and *Methylothermus*. Type –II methanotrophs belong to the alphaproteobacteria, which contain the families Methylocystaceae and Beijerinckiaceae. The Methylocystaceae include the genera *Methyloferulla*, *Methylocystis* and *Methylosinus*) whilst the Beijerinckiaceae contain the genera *Methylocapsa* and *Methylocella* (Bowman, 2014; Chidambarampadmavathy et al., 2015a; DiSpirito et al., 2016; Hanson and Hanson, 1996; Webb et al., 2014). Other than these, some other species of methanotrophs have also been grouped in the Verrucomicrobiae group (Bowman, 2006; Dunfield et al., 2007; Hanson and Hanson, 1996; Hirayama et al., 2014; Hirayama et al., 2013; Iguchi et al., 2011a; Jiang et al.,

2010; Semrau, 2011; Stoecker et al., 2006). Other than these, there are filamentous methanotrophs belonging to gammaproteobacteria, genera include *Crenothrix* and *Clonothrix* (Stoecker et al., 2006), and other non-proteobacterial (verrucomicrobial) methanotrophs genera include *Methylacidimicrobium* and *Methylacidiphilum* (Pol et al., 2007; van Teeseling et al., 2014). Recently, a new type of methanotroph has also been discovered, closely related to *Methylomirabilis oxyfera* belonging to the NC 10 phylum, performing anaerobic methane oxidation, but unlike AnOM, here the process is coupled with a denitrification process. Also, unlike other ANME, the newly discovered anaerobic methanotroph does not adopt the reverse methanogenic process and does not utilize environmental O_2 to oxidize CH_4 as described for aerobic methanotrophs. Instead, O_2 for aerobic CH_4 oxidation using methane monooxygenase enzyme (MMO) is produced through intracellular conversion of nitrite under anaerobic environmental conditions (Wu et al., 2011). The use of intracellularly produced O_2 justifies its inclusion in the aerobic methanotrophs.

1.10 Enzymes and pathways of aerobic oxidation of methane (AOM)

Methane is one of the most inert hydrocarbons, requiring 104 kcal.mol^{-1} of energy for breaking the C-H bonds. The MMO in aerobic methanotrophic bacteria are capable of oxidizing C-H bonds to C-OH groups *in-vivo* by incorporating one O_2 atom (eq.1.1 and 1.2) of the O_2 molecule into CH_4 to form methanol (CH_3OH) at the first step, while the other atom forms water (Bowman, 2006; Ito et al., 2014). The CH_3OH is further oxidized to formaldehyde, a cytotoxic intermediate (HCHO) by methanol dehydrogenase (MDH) at the second step. This HCHO is either assimilated into biomass or converted to formate (HCOOH) by formaldehyde-dehydrogenase (FADH) and finally to CO_2 by formate-dehydrogenase (FDH) providing reducing equivalents for the cell (Semrau et al., 2010) (eq.1.3) (the pathways involved in formaldehyde assimilation is explained further below). These reactions involve bundles of enzymes which are categorized into two groups, i.e. $NADH^+$-specific and cytochrome-linked

enzymes (Hanson and Hanson, 1996). In type -I methanotrophs, 3-hexulosephosphate synthase (HPS) catalyses formaldehyde fixation into (D-arabino)-3-hexulose-6-phosphate. The reaction requires magnesium (Mg^{2+}) and/or manganese (Mn^{2+}) as a cofactor(s) for enzyme activation (Trotsenko and Murrell, 2008). Subsequently, D-arabino-3-hexulose-6-phosphate is converted into different intermediate products and then to CO_2 via an incomplete tricarboxylic acid (TCA) cycle.

In contrast, formaldehyde oxidation is activated by the pterin cofactor (H4MPT) catalysed by tetrahydrofolate (H4F) (THF)-enzymes in type -II methanotrophs (Vorholt, 2002). The THF-enzymes provide the formaldehyde acceptor, called N5, N10 methylene-H4F to support the reaction between formaldehyde with glycine to form serine. These THF-enzymes mainly regulate the oxidation or assimilation of formaldehyde especially in type -II methanotrophs. Subsequently, type -II methanotrophs proceed with either the TCA or the PHB cycle under nutrient-sufficient or -deficient conditions respectively, and produce CO_2 (Figure 1.6). The enzymes involved in the PHB pathway are explained in section 1.11.

$$CH_4 + O_2 + 2e^- + 2H^+ \rightarrow CH_3OH + H_2O \qquad (eq.\,1.1)$$

$$CH_4 + NAD(P)H_2 + O_2 \rightarrow CH_3OH + NAD(P^+) + H_2O \qquad (eq.\,1.2)$$

$$CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2 \qquad (eq.\,1.3)$$

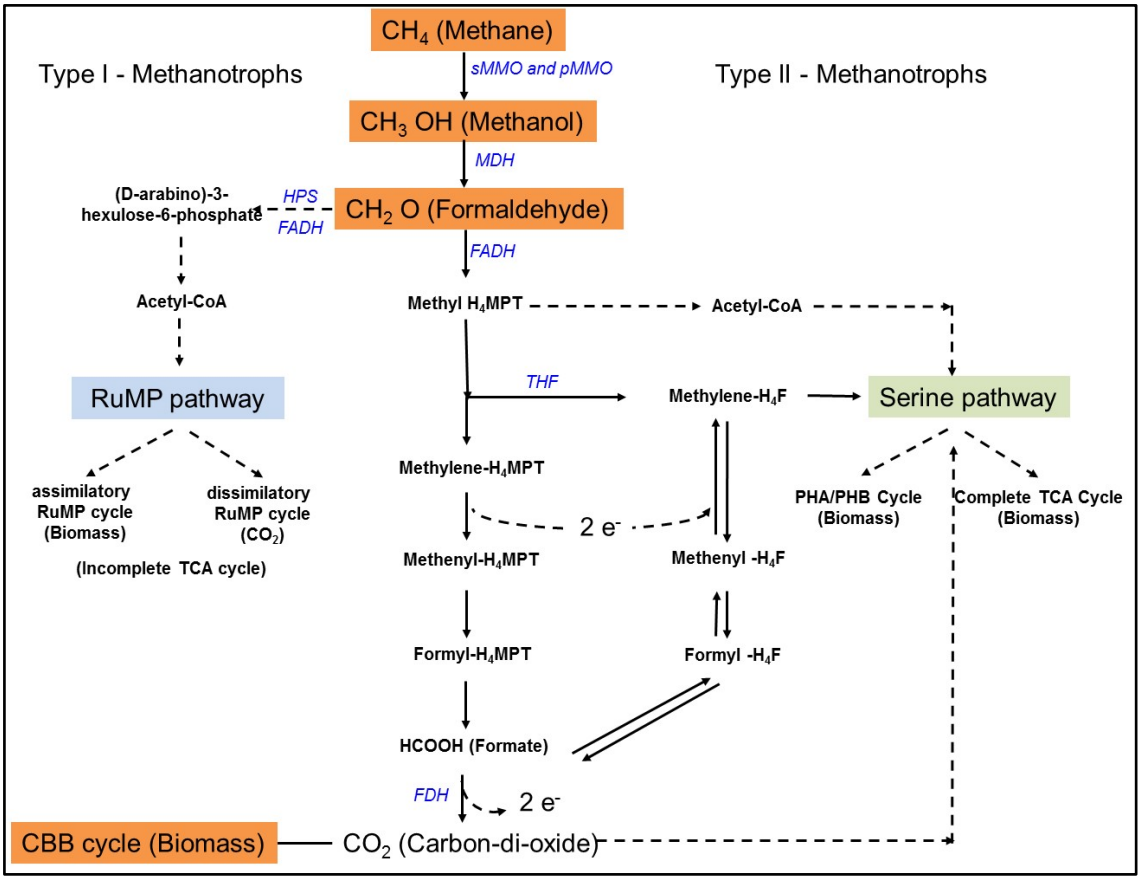
Formaldehyde assimilation into biomass occurs by two different pathways, depending on the type of methanotroph, namely the ribulose monophosphate (RuMP) pathway for type -I or the serine pathway for type -II methanotrophs.

RuMP pathway:

$$CH_4 + 1.50\ O_2 + 0.118NH_4^+ \rightarrow 0.118\ (C_4H_8O_2N) + 0.529\ CO_2 + 1.71\ H_2O + 0.118\ H^+ (eq.\,1.4)$$

Serine pathway:

$$CH_4 + 1.57\ O_2 + 0.102\ NH_4^+ \rightarrow 0.102\ (C_4H_8O_2N) + 0.593\ CO_2 + 1.75\ H_2O + 0.102\ H^+ (eq.\ 1.5)$$



Source - Karthikeyan et al. (2015a).

Figure 1.6 Methane oxidation pathways by methanotrophic bacteria

Type -I methanotrophs are found to be more efficient and require only 1 ATP for 3 molecules of formaldehyde formed through CH_4 oxidation. While, type -II methanotrophs are advantageous in terms of storing CH_4 as phospholipid fatty acids (PLFA; e.g.18:1ω8c) and biopolymers (mainly PHB) (Hanson and Hanson, 1996; Higgins et al., 1981; Semrau et al., 2010). The process is, however, more energy- and reducing equivalent expensive, as it requires 3 ATP and 2 NADH to fix two molecules of formaldehyde and one CO_2 (Scheutz et al., 2009). Under nutrient-limiting conditions (nitrogen, potassium, phosphate, copper, etc.), type -II methanotrophs convert CH_4 into biopolymers and CO_2 emissions are also reduced further (Higgins et al., 1981;

Whittenbury and Dalton, 1981). It was reported that type -II methanotrophs can assimilate ~60 % of CO_2-carbon into biomass, compared to 5–15 % for type -I methanotrophs. The enzyme phosphoenol-pyruvate carboxylase (PEP) is responsible for CO_2 fixation in both type -I and -II methanotrophs, while in type -1a methanotrophs, the Calvin–Benson–Basham (CBB) cycle and related enzymes are mainly involved in CO_2 fixation (Trotsenko and Murrell, 2008). Metabolic differences are mainly associated with the arrangement of intracytoplasmic membranes, especially type -I methanotrophs possess bundles of vesicular disks distributed throughout the cell, while in type -II these are paired peripheral layers (Semrau et al., 2010). Further, type -I methanotrophs lack functionally active enzymes, such as *α*-ketoglutarate dehydrogenase, isocitrate lyase, and malate synthase, leading to a horseshoe like TCA cycle (Trotsenko and Murrell, 2008).

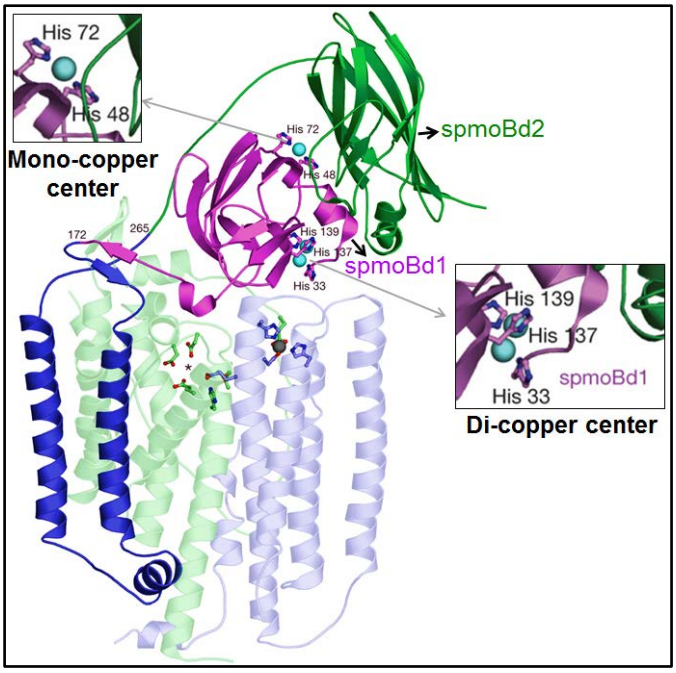
1.11 Methane monooxygenases

MMOs (i.e. pMMO-particulate methane monooxygenase and sMMO-soluble methane monooxygenase) are two structurally and biochemically distinct enzymes, with a critical role in CH_4 oxidation (Murrell et al., 2000; Murrell and Smith, 2010). Differential expression of these enzymes depends on the type of methanotroph, CH_4 and nitrate concentrations, biomass density and copper availability.

1.11.1 Particulate methane monooxygenase

The pMMOs are membrane-integral/transmembrane metallo-enzymes present in most methanotrophs. X-ray crystallographic models of pMMO from *Methylosinus trichosporium* OB3b (Hakemian et al., 2008), *Methylococcus capsulatus* Bath (Lieberman and Rosenzweig, 2005a, 2005b)*, Methylocystis* sp. strain M (Smith et al., 2011) revealed it to be a protein complex with a trimeric structure, $\alpha_3\beta_3\gamma_3$ consisting each of three polypeptide subunits, pmoB, pmoA, and pmoC, respectively (Figure 1.7). The trimeric subunits of the complex together construct a pore with a width of 11 Å in the soluble periplasmic part and 22 Å in the transmembrane part of the protein

complex (Culpepper et al., 2012a; Culpepper and Rosenzweig, 2012b). The pMMO from *M. capsulatus* (Bath) differs from the other two methanotrophs, i.e. *M. trichosporium* OB3b and *Methylocystis* sp. strain M, by lacking a transmembrane helix adjacent to the pmoC subunit indicating this helix is specific to pMMO from type -II methanotrophs. In addition, three metal binding sites have been identified in pMMOs; two copper - (mono- and di-nuclear, respectively) and one zinc-binding centre. (Culpepper and Rosenzweig, 2012b; Smith et al., 2011).



Source - Balasubramanian et al. (2010), reproduced with permission from the journal.

pmoA - faint light green; pmoB - blue - transmembrane helices of pmoB; purple - amino-terminal domain of pmoB (spmoBd1); green - carboxy-terminal domain of pmoB (spmoBd2); pmoC - faint light blue.

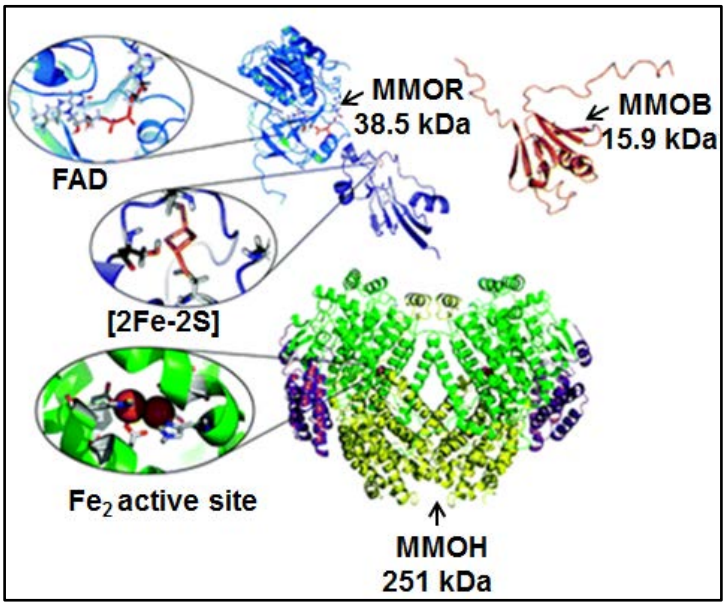
Figure 1.7 Structure of pMMO

The mono-nuclear copper centre is co-ordinated by two histidine residues (His-48 and His-72) and present only in *M. capsulatus* (Bath) but absent in *M. trichosporium* OB3b and *Methylocystis* sp. strain M (Hakemian et al., 2008; Smith et al., 2011), while the dinuclear copper centre co-ordinated by three highly conserved histidine residues (His-33, His-137 and His-139) is present in the soluble region of pmoB, designated as spmoB (Culpepper et al., 2012a).

The active site and metal content of pMMO, and whether the active site utilises copper or iron for its function has remained a puzzle for many years. Research using the soluble portion of the pmoB by Balasubramanian et al. (2010) identified the di-nuclear copper containing portion in the soluble N-terminal part of the spmoB as sufficient for CH_4 oxidation, ending the copper-versus-iron debate and additional research by Culpepper (Culpepper et al., 2012a) showed O_2 and CH_4 binding, confirming the spmoB di-nuclear copper site as the catalytically active site of the pMMO complex. The third metal centre is located between hydrophilic residues within the membrane pmoC subunit and is occupied by zinc/copper depending on the species (Balasubramanian et al., 2010; Culpepper and Rosenzweig, 2012b; Lieberman and Rosenzweig, 2005b; Smith et al., 2011)

1.11.2 Soluble methane monooxygenase

The sMMO is a multi-component cytoplasmic enzyme well characterized from *M. capsulatus* (Bath) (Green and Dalton, 1985), *Methylocystis sp*. strain M (Nakajima et al., 1992) and *M. trichosporium* OB3b (Fox et al., 1989). It consists of three components *viz*., hydroxylase (MMOH), reductase (MMOR), and the regulatory protein (MMOB), which is responsible for coupling NADH oxidation with CH_4 hydroxylation (Figure 1.8). The MMOH is a dimer with three subunits namely $(\alpha\beta\gamma)_2$ (Grosse et al., 1999; Rosenzweig et al., 1993), which contains two di-iron centres and serves as the active site of the enzyme where CH_4 is oxidized to CH_3OH. The genes *mmoX mmoY* and *mmoZ* encode α, β and γ subunits, respectively with all three genes being involved in the CH_4 oxidation process. The MMOR is a NADH-dependent [2Fe-2S], flavin adenine dinucleotide (FAD)-containing component and is encoded by *mmoC*. It is mainly involved in transporting electrons from NADH to the active site of MMOH. The regulatory protein, MMOB, encoded by *mmoB* assists in regulating the electron transfer from MMOR to MMOH (Jahng and Wood, 1996; Merkx et al., 2001).



Source - Friedle et al. (2010), reproduced with permission from the journal.

α subunits are shown in green, the β subunits are shown in purple, and γ subunits are shown in light green. The active site iron ions are shown as orange spheres.

Figure 1.8 Structure of sMMO

In contrast to these genes, the sMMO operon containes an additional open reading frame (*orfY*) between *mmoZ* and *mmoC* which was named *mmoD* (Merkx and Lippard, 2002). The primary function of this polypeptide was unknown, but Merkx and Lippard (2002) speculated that it might be involved in sensing copper and iron levels. Semrau et al. (2013) suggested instead that the protein MmoD could play a key role in regulating gene expression.

Although the two MMO enzymes (sMMO and pMMO) perform the same function, oxidize CH_4 to CH_3OH, they differ completely in their molecular structure, active site composition, requirements for metal ions (please refer to section 1.10.3), substrate-specificity and CH_4 oxidation kinetics (Murrell et al., 2000; Murrell and Smith, 2010; Semrau et al., 2010; Zhang et al., 2009). These MMOs can degrade a variety of saturated, unsaturated and aromatic hydrocarbons and also co-oxidize their halogenated derivatives (Wendlandt et al., 2010), but the rate of degradation depends on the type expressed (Semrau, 2011). sMMOs have broad substrate specificity and

can degrade a wide range of alkanes, alkenes, aliphatic and aromatic hydrocarbons, while pMMOs have a narrower substrate specificity and can degrade only smaller hydrocarbons. Chlorinated hydrocarbons like trichloroethylene (TCE) are the most common secondary pollutants in landfills, which can be co-metabolised and converted into CO_2 by the MMOs. But the co-oxidation efficiency of CH_4 and these pollutants depends on the concentration of the pollutants present, as both, the growth substrate (CH_4) and co-metabolic substrate, compete for MMO enzymes (Wendlandt et al., 2010). Some notable differences between these two enzymes are given in Table 1.4 (Balasubramanian et al., 2010; Glass and Orphan, 2012; Murrell and Smith, 2010; Semrau, 2011).

Table 1.4 Significant differences between pMMO and sMMO

Parameteres	pMMO	sMMO
Structure	pMMO has three subunits, pmoB (α), pmoA (β), and pmoC (γ)	Non-haem, iron-containing enzyme complex consisting of a hydroxylase, a reductase and a regulatory protein .
Enzyme Location	Intracytoplasmic membrane	Cytoplasm
Active site	Di-copper site in the spmoB portion of pmoB subunit	Di-iron site in MMOH
Metal ion requirement.	Copper	Iron
Electron donor	NADH and duroquinol	NADH
CH_4 oxidation efficiency	High	Low
Substrate specificity & degradation of hydrocarbons	Narrow; degrade only C4 hydrocarbons or smaller	Broad; degrade a wide range of alkanes, alkenes, aliphatic & aromatic hydrocarbons

Source - Chidambarampadmavathy et al. (2015a)

1.11.3 Metal ions involved in regulation of MMOs

Metal ions, mainly copper and iron, are known to regulate MMO activity. These transition metals also play a vital role in CH_4 oxidation, as the MMO enzymes (pMMO and sMMO), responsible for this oxidation process, use copper or iron depending on

copper availability in the first step of CH_4 oxidation and subsequent steps rely on iron (Glass and Orphan, 2012). Low copper concentrations (~0.3 µM) support the activity of both pMMO and sMMO (Choi et al., 2003). While pMMO is expressed at higher copper concentrations; exceedingly high copper concentrations (>50 µM), however, inhibit pMMO activity (Balasubramanian et al., 2010; Yu et al., 2003) by reacting with the reductant generating hydrogen peroxide, which reversibly inhibits the pMMO. In general, high copper (> 0.85 µM) to biomass concentrations inhibit sMMO activity, whereas excess addition of iron has been reported to stimulate the expression of sMMO (Begonja and Hršak, 2001). In particular, at high copper to biomass ratios, i.e. >0.86 µmol $Cu.g^{-1}$ dry-weight, CH_4 oxidation takes place in the periplasmic membrane space catalyzed by pMMO, while at <0.86 µmol $Cu.g^{-1}$ dry-weight, oxidation of CH_4 is catalyzed by sMMO in the cytosol (Chan et al., 2004; Murrell et al., 2000).

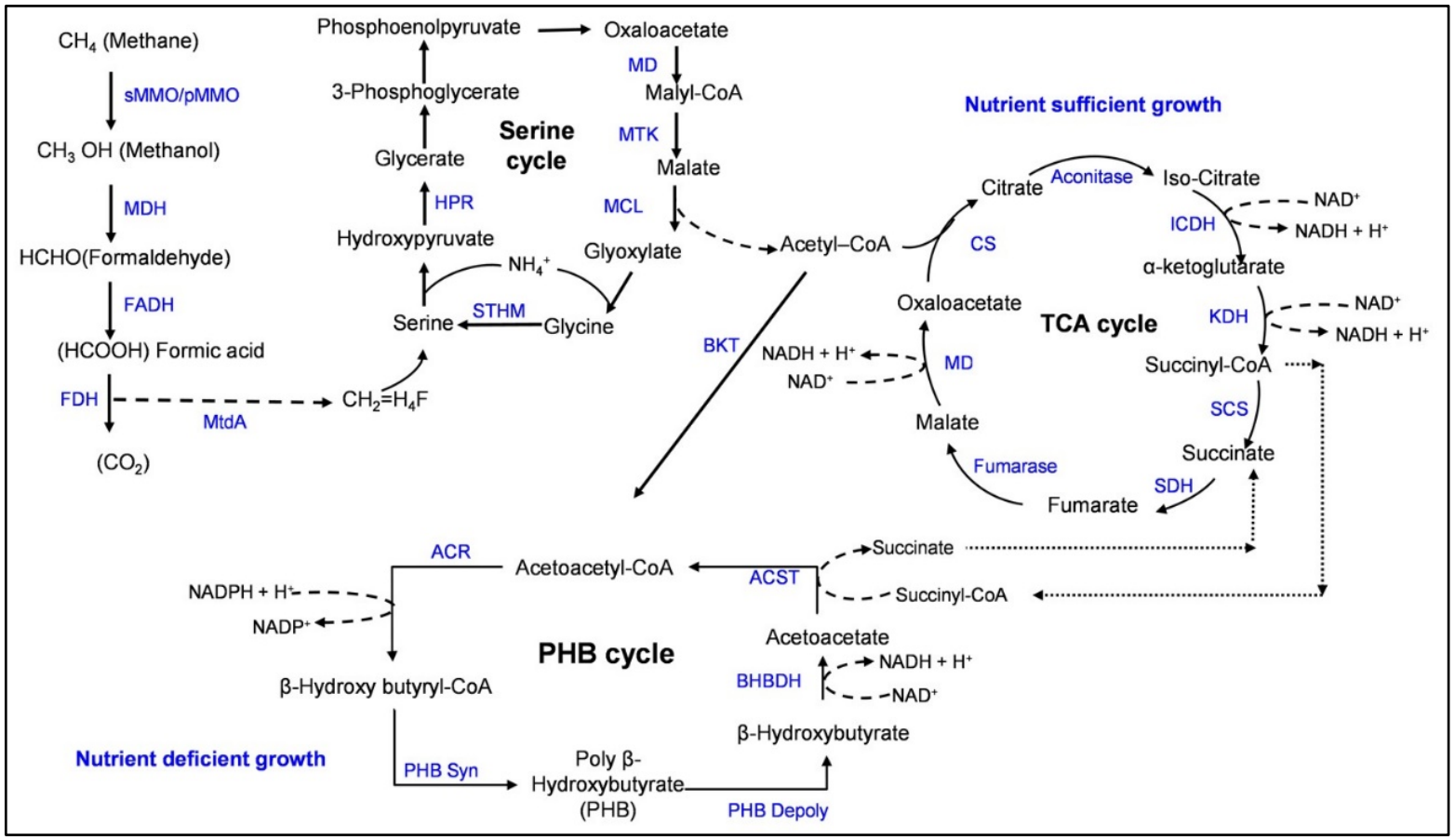
Loss of sMMO activity at high copper concentrations is due to binding of copper to the [2Fe-2S] centres of the MMOR component of sMMO, inhibiting electron transfer from FAD to MMOH (Jahng and Wood, 1996). Lack of sMMO activity ultimately leads to the expression of pMMO in the plasma membrane thereby activating the CH_4 oxidation process. Here, copper functions as a metabolic activator for the expression of pMMO, termed the “copper switch” (Chan et al., 2004; Murrell et al., 2000; Stanley et al., 1983). Stanley et al. (1983) first reported this copper switch for *M. capsulatus* (Bath).

However, the exact mechanisms by which copper interacts and regulates pMMO expression were unclear. Methanobactin (Mb), a chalkophore (a siderophore-like molecule), appears to play a key role in regulation of this copper switch, where Mb co-ordinate with the MmoD protein, to regulate this copper switch (Dassama et al., 2017; Semrau et al., 2013). Methanobactin has been proposed to be essential for uptake of copper into the cell through chelation of copper forming the copper-Mb complex (Kenney and Rosenzweig, 2011). The precise uptake mechanism for the

Copper-Mb complex was explained by Balasubramanian et al. (2011). They reported that copper uptake occurs via passive diffusion, whilst Copper-Mb uptake occurs via active transport in *M. trichosporium* OB3b. Porins allow for the diffusion of smaller solutes, which is a possible route for uptake of unchelated copper. Whilst, the uptake of Mb has been reported to involve TonB-dependent transporters (Gu et al., 2016). Though Mb has a high specificity for copper, Mb also binds other transition metal ions like Au, Hg, Ag, Cd, Pb, Mn, Fe, Co, and Ni (Dassama et al., 2017; DiSpirito et al., 2016).

1.12 The PHB cycle in type -II methanotrophs

The general ability of type -II methanotrophic bacteria to convert CH_4 to PHB was first described by (Whittenbury et al., 1970). Later, it was well documented that PHB accumulation occurred primarily under excess carbon but nutrient/trace element-deplete conditions (Helm et al., 2008; Helm et al., 2006; Karthikeyan et al., 2015b; Listewnik et al., 2007; Pieja et al., 2011a; Pieja et al., 2011b; Zhang et al., 2008). PHB also acts as a storage pool for reducing equivalents (Pieja et al., 2011b). In type -II methanotrophs, where the serine cycle is used to convert oxidised CH_4 into organic carbon, have a functioning tricarboxylic acid -TCA/PHB cycle, where methylene-tetrahydrofolate (CH_2=H_4F) is formed via formic acid from CH_4. This CH_2=H_4F is converted to serine from which acetyl-CoA is derived. Acetyl-CoA enters the PHB synthesis cycle under nutrient-deficient conditions or the TCA cycle under nutrient-sufficient conditions (Figure 1.9). PHB is easily polymerised and depolymerised via acetyl-CoA. Reactions involved in the conversion of acetyl-CoA into PHB and the enzymes responsible for catalyzing these conversions are *β*-ketothiolase, acetoacetyl-CoA-reductase, and PHB synthetase (Asenjo and Suk, 1986). Enzymes like PHB depolymerase, *β*-hydroxybutyrate dehydrogenase, and acetoacetate succinyl-CoA transferase are involved in the depolymerisation and catabolism of PHB into acetyl-CoA required for restoring biomass activity.



Source - Chidambarampadmavathy et al. (2015a).

Figure 1.9 PHB accumulation pathways in type -II methanotrophic bacteria

The net equation for PHB accumulation in type -II methanotrophs is given below (eq. 1.6).

$$2CH_4 + 3O_2 + 6NADH_2 + 2CO_2 \rightarrow C_4H_6O_2\,(\text{PHB momomer}) + FPH_2 \qquad (\text{eq. } 1.6)$$

It has been reported that cultivation of methanotrophs in diluted nitrate mineral salts medium with an acidic pH range (5.0-6.0) favours the growth of type -II methanotrophs and increased PHB accumulation (Bussmann et al., 2004; Pieja et al., 2011a).

There are a number of other factors affect PHB accumulation capacity in methanotrophs (Table 1.5) such as temperature, pH, gases (CH_4, O_2, CO_2, and N_2) and their mixtures, as well as availability of macro- and micro-nutrients (Karthikeyan et al., 2015a; Pieja et al., 2011a; Pieja et al., 2012; Rostkowski et al., 2012). Nitrogen - and phosphorus-deficient conditions re-route carbon utilisation (CH_4 & CO_2) to storage (accumulation as PHB) (Helm et al., 2006; Pieja et al., 2012), whereas magnesium and potassium deficiency increases the molecular weight of accumulated polymers (Helm et al., 2008). Trace metals like copper and iron have been reported to affect the PHB accumulation capacity indirectly (Helm et al., 2008; Jahng and Wood, 1996), as these metals are responsible for regulating the activity of MMO (Chidambarampadmavathy et al., 2015a).

High pMMO activity is beneficial for the production of PHB in methanotrophs. Pieja et al. (2011a) reported an increase in PHB accumulation of up to 25 % in the presence of 5 µM copper. Zhang et al. (2009) indicated that higher pMMO activity was observed in the presence of 10 µM copper and it increased the PHB content to 8.07 % from 4.22 % in mixed consortia. Increase in PHB content to 8.3 % was seen in *M. trichosporium* IMV 3011, when grown in 30 µM copper (Dong, 2013). In addition to copper, ferric and ferrous ions have also been reported to improve the activity of pMMO (Takeguchi et al., 1999). However, as methanotrophic biomass increases throughout the growth period, the copper to biomass ratio changes in favour of

expression and activity of sMMO (Zhang et al., 2009). At this stage, iron is essential for sMMO expression. Helm et al. (2008) reported iron-deficiency to limit PHB synthesis. Park et al. (1991) reported 40 µM iron to be optimal for significant cell growth. Thus, in order to obtain high yields of PHB and growth, managing copper and iron concentrations are important.

Table 1.5 Factors affecting PHB accumulation in methanotrophic bacteria

Conditions	Effects	Optimum values	Reference
pH	Low pH is expected to select preferentially for type -II methanotrophs	5 - 6	(Pieja et al., 2011a)
CO_2	As a gas or as bicarbonate addition, increases biomass productivity	1 mM	(Park et al., 1991)
Nitrogen	Under nitrogen limitation, the carbon was redirected to PHB production	-	(Pieja et al., 2011a)
Nitrogen and methane limitations	Repeated nitrogen and methane limitations favoured PHB accumulation	-	(Pieja et al., 2012)
Phosphate	Optimum for cell growth, sMMO and MDH activity	25 mM (25 - 40 mM)	(Bowman and Sayler, 1994)
	PHB produced is of very high quality having high molecular mass of up to 2.5×10^6 Da.	Nil	(Wendlandt et al., 2005)
Iron	Iron deficiency decreased the formation PHB	40 - 80 µM	(Helm et al., 2008)
Copper, Nickel and Zinc	Inhibits sMMO which may reduce growth and PHB accumulation	1 – 10 µM	(Jahng and Wood, 1996)
Sodium formate	Addition of formate is favourable for PHB accumulation.	10 mM	(Pieja et al., 2012)
Potassium	Potassium deficiency resulted in the accumulation of high quality PHB with a Mw of 3.1 ± 0.02 MDa.	-	(Helm et al., 2008)

Source - Karthikeyan et al. (2015a)

1.13 Mixed methanotrophic cultures

Most previous research used mono-species cultures; only limited research has been carried out with mixed consortia to date (Helm et al., 2006; Ho et al., 2016; Ho et al., 2014; Karthikeyan et al., 2015b; Listewnik et al., 2007; López et al., 2014; Pfluger et al., 2011; van der Ha et al., 2013; Wei et al., 2015). From an industrial point of view, maintaining an axenic culture will not be commercially viable, so research should focus on understanding the responses of mixed microbial cultures to various environmental factors to subsequently improve the CH_4 oxidation capacities for biotechnological applications, for e.g. PHB production coupled with continuous CH_4 mitigation in bio-filters.

Many studies have reported the association between methanotrophic and non-methanotrophic bacteria (methylotrophs - microbes that use methanol as a carbon source and heterotrophs - microbes that use complex organic substrates as carbon source) in both natural environments and mesocosm studies (Beck et al., 2013; Ho et al., 2016; Ho et al., 2014; Iguchi et al., 2011b; Oshkin et al., 2015). Complex interactions occur within these microbial communities. This interaction is generally linked to the cross-feeding of metabolites, where methanotrophs and non-methanotrophs coexist through carbon sharing, exerting a synergistic relationship. The cross-feeding metabolites are mainly methanol, formaldehyde and organic acids. This synergistic relationship can be explained by a scenario (Figure 1.10), where the co-habiting non-methanotrophic bacteria consume toxic and over-produced metabolites (for e.g. methanol) thereby potentially improving methanotrophic activity and methane oxidation potential (Oshkin et al., 2015). Co-existing non-methanotrophs also provide some essential vitamins (cobalamin), required for methanotrophic growth (Iguchi et al., 2011b).

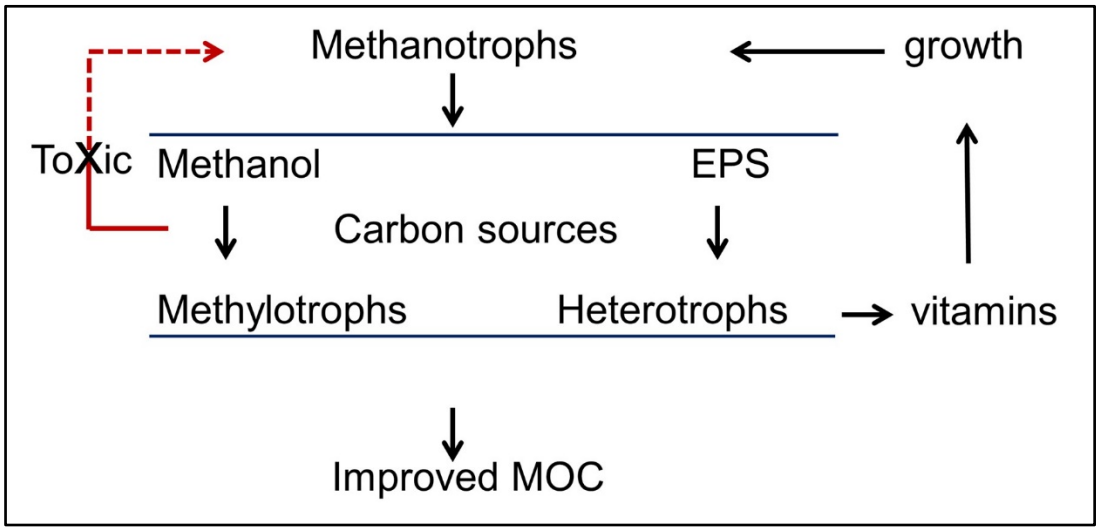
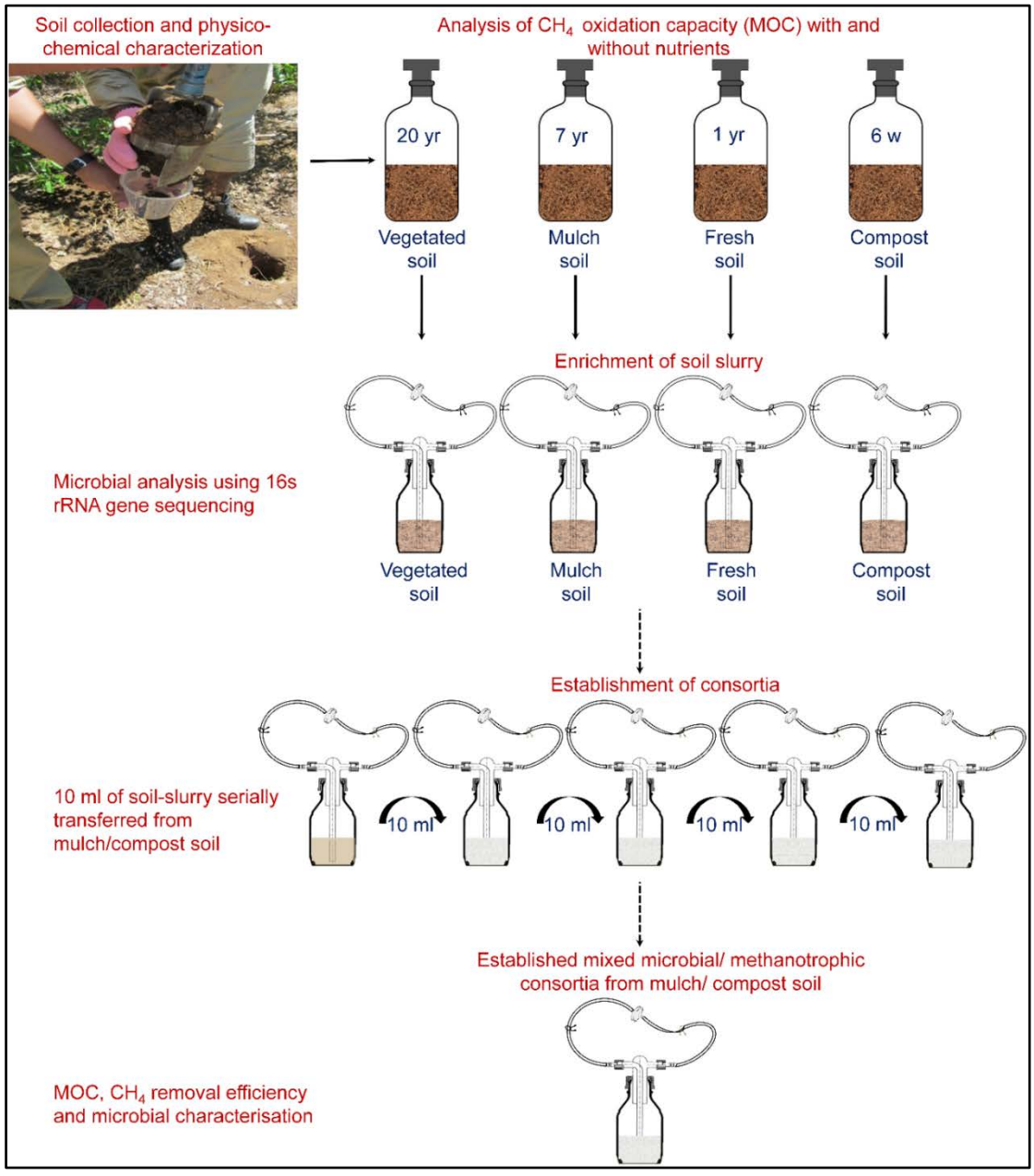


Figure 1.10 Concept of synergistic services between methanotroph and non-methanotrophs

Chapter 2: Enrichment and establishment of mixed consortia



Graphical Abstract 1 Establishment of mixed consortia - chapter 2 overview

2.0 Abstract

Methanotrophs play a unique role in the biological fixation of one-carbon compounds, e.g. CH_4 a potent greenhouse gas. High rates of CH_4 oxidation by diverse consortia of methanotrophs have been reported in aerobic parts of landfill cover soils. In natural, as well as in engineered ecosystem (i.e. landfill cover soils), methanotrophs exist in a synergistic relationship with non-methanotrophs (methylotrophs and heterotrophs), the mechanisms that govern the community structure are not well understood. Therefore, this study aimed to characterise the methanotroph and non-methanotroph community structure from landfill cover soils of different ages using next-generation sequencing after initial enrichment of soil slurries (CH_4:air (20:80%) for 20 days)and for consortia establishment (CH_4:air (20:80%) for 20 days). As 16S rRNA-based approaches may provide a better understanding of methanotrophic and non-methanotrophic community structure in these soils, test samples (vegetated cover, mulch cover, fresh and compost soils) were collected from Stuart landfill, Australia and characterized for their physico-chemical characteristics, MOC, and total microbial diversity. Of the different soils tested, mulch cover soil from a 7 year-old landfill showed a high MOC (16.33 $\mu g.g^{-1}DW_{soil}.h^{-1}$), followed by fresh cover - (11.46 $\mu g.g^{-1}DW_{soil}.h^{-1}$) and compost soil (5.57 $\mu g.g^{-1}DW_{soil}.h^{-1}$), while the vegetated soil from a 20 year-old landfill had low MOC (3.57 $\mu g.g^{-1}DW_{soil}.h^{-1}$). This observation correlated well with the studied community structures. Type -I methanotrophs were more abundant in all soils compared to type -II methanotrophs, but heterotroph diversity varied between the soil samples. The results suggest that, at least during enrichment, age and nutrient status of the soils may have influenced community structure of methane oxidizing consortia still evident after the enrichment period in high CH_4 environments. Best performing and typically used cover soils were selected for establishing two obligate mixed consortia, which appeared to have similar methanotroph community profiles (type -I *Methylosarcina* was dominant), but the cohabiting non-methanotrophs widely

differed. These consortia yielded the starting material for investigating the effects of variable CH_4/O_2 (air) and Cu^{2+}/Fe^{2+} ratios in chapters 3 and 4, respectively.

2.1 Background

Biogenic CH_4 emissions from landfills occur due to microbial anaerobic degradation of organic matter *via* a series of steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) (Bareither et al., 2013; Shalini et al., 2010; Wise et al., 1999). In addition, high rates of CH_4 oxidation have also been reported in oxic portion of landfill cover soils (Cébron et al., 2007; Henneberger et al., 2012; Jugnia et al., 2009; Su et al., 2014; Wise et al., 1999). Around 50 % of the CH_4 produced from landfills are oxidized to CO_2 by methanotrophs before entering the atmosphere (Whalen et al., 1990), however the percentage of CH_4 oxidized in these top cover soils is not constant and vary depending on soil type, climatic conditions, CH_4 concentration and other physio-chemical parameters (Chanton et al., 2011). Chanton et al. (2010) reported an average of ~37 % of CH_4 to be captured within landfill soil covers. Profiling and quantifying methanotroph and co-habiting non-methanotroph abundances and heterogeneity in the soil covers are a prerequisite for establishing efficient methane-oxidising bio-filters to abate emissions and develop bio-products.

Soil microbial communities are incredibly diverse; within soil microbial populations only a minor fraction (~1 % of the microbial communities) of the microbes has been studied and cultured (Torsvik et al., 1998), with a large amount of uncultured microbes awaiting discovery. To unveil these uncultured microbes various molecular techniques like cloning followed by Sanger-sequencing (Chen et al., 2007; Dowd et al., 2008), denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) (Dowd et al., 2008; Jugnia et al., 2009; Wise et al., 1999), terminal restriction fragment length polymorphism (T-RFLP) (Gebert et al., 2009; Henneberger et al., 2012; Stralis-Pavese et al., 2006), fluorescence in situ hybridization (FISH) (Kalyuzhnaya et al., 2006), DNA-based stable isotope probing (Cébron et al., 2007; Héry et al., 2008) and diagnostic microarray technologies (Bodrossy et al., 2003; Gebert et al., 2009; Kumaresan et al., 2009; Stralis-Pavese et al., 2004) have been used to characterize

these soil communities in the past. However, next-generation sequencing (NGS), using 16S rRNA gene has become an attractive tool for analysis of microbial diversity from complex environmental samples (Samarajeewa et al., 2015). The main disadvantage with traditional Sanger-sequencing or T-RFLP is that only a small portion of the genome (one read maximum ~1Kb) can be sequenced at a time. DGGE/TGGE can separate mixed DNAs based on molecular weight, but requires excessive caution in excision of the bands from the polyacrylamide gels, but a single band may still represent multiple species, and quantitative representation of specific microbial genera is difficult, since the abundance of specific taxa, is estimated based on the intensity of each band. In contrast, NGS can read a massive amount of genomes (Gb of reads) in a single run. Moreover, NGS can quantify the presence of original, unculturable organisms *in situ*, and can be reproduced in a more accurate way, which is a limiting factor with micro arrays due to the complex preparative methodologies, data unavailability and insufficient information reported in analytical approaches (Ioannidis et al., 2009). An additional advantage of NGS is the sensitivity of the technique (nanograms of DNA is sufficient for sequencing), whereas microarrays requires micrograms of DNA (Hurd and Nelson, 2009; Samarajeewa et al., 2015; Shendure and Ji, 2008).

A number of studies recently characterized methanotrophic communities from landfill top cover soils (Cébron et al., 2007; Chanton et al., 2011; Chen et al., 2007; Gebert et al., 2009; Henneberger et al., 2012; Jugnia et al., 2009; Spokas and Bogner, 2011). However, none has attempted to explore the complete methanotroph- non-methanotroph community structure necessary for unravelling complex interactions in such environments. The microbiomes of landfills capable of CH_4 oxidation are still in need of investigation to further our understanding of how different community structures impact on CH_4 bioremediation in landfill soils.

Therefore, as a baseline study, I aimed to

i. assess MOC of landfill top cover soils differing in age and nature,
ii. characterise community structures of enriched soil-slurry using 16s rRNA gene sequencing, and
iii. establish robust methanotrophic consortia from landfill cover soils that can be later tested to improve process designs for CH_4 abatement.

2.2 Materials and methods

2.2.1 Field site

Stuart landfill (latitude 19°34'3"S / longitude 146°86'8"E) is the main solid waste site for Townsville, Australia, commencing operation in 1998 with an estimated life span until 2033. An average of ~130,000 tonnes of mixed wastes are disposed every year consisting primarily of organic wastes along with clean fill, ferrous and non-ferrous metals, white goods, tyres, domestic recycling products and a range of construction and building materials. The landfill base is primarily clay lined; and the cover is capped with clay, loam top soil and compost materials (LAWMAC, 2014; Meinhardt, 2000).

2.2.2 Soil collection and characterization

Landfill soil covers naturally act as sinks for CH_4 and in addition, compost soil covers are used as landfill cover soils for enhancing CH_4 oxidation. In order to characterise the microbes present in the soils and to establish a robust consortia, four different types of landfill top cover soil samples were collected from Stuart landfill. Samples of vegetative (latitude 19°20'5"S / longitude 146°52'2"E) and mulch cover soil (latitude 19°20'8"S / longitude 146°52'2"E) were obtained from 0.2, 0.6 and 1.0 m depths, to account for microbes present in micro-aerobic and aerobic parts of the soils. In contrast, no depth profiles were obtained from the fresh landfill cover (less than one year old cover soil, latitude 19°20'7"S / longitude 146°52'1"E) and biosolid compost (6weeks old; supplied to the site by McCahills landscaping supplies, latitude 19°20'2"S

/ longitude 146°51'9"E) (Figure 2.1).

Figure 2.1 Sample collection from landfills – auger sampling

The vegetated soil cover was 20 years old covered with trees and grass and was previously used for landfilling wastes, while the mulch soil was 7 years old containing a mixture of biosolids and wood chips. Similar to mulch soil, compost soil consisted of 60 % biosolid wastes and 40 % woodchips and had been previously used for enhancing CH_4 oxidation, while the fresh soil was primarily a compacted clay lined soil (Figure 2.2). Collected samples were immediately transported to the laboratory and stored at 4^{o}C. For further analysis, soils taken from different depths of the same location were combined and treated as a single sample to obtain a single population profile following enrichment. Sub-samples of soils were taken in triplicate for analysing pH, total and volatile solids (TS and VS; gravimetric method), total organic carbon (TOC; analyser), total nitrogen (TN), total phosphorus (TN) and essential metal contents.

The pH of the soils were measured in slurries prepared by mixing 1 g soil with 2 mL of distilled water and using a pH electrode (WP-81, Themofisher Scientific, Scoresby, Vic, Australia). For TS and VS, 25 g of soil samples were weighed in ceramic crucibles

(in triplicates) and dried at 105^{o}C for 24 h, before ashing at 500^{o}C for 8 h (APHA, 2005). The differences between dry weight (DW) of soil and ash were calculated for individual samples to obtain total solids (TS), volatile solids (VS) and moisture contents, all expressed as percent. TOC, TN and TP were outsourced to TropWater, James Cook and analysed using American Public Health Association-APHA 4500 N_{org}/NO3 - and 4500 P-B&F methods, respectively (APHA, 2005).

Figure 2.2 Samples collected from different landfill cover soils

2.2.3 Methane oxidation capacity of soils

The collected soil samples were further characterized for MOC. Briefly, 5 g of each soil were added to 100 mL serum vials and the headspace of the bottles were purged with CH_4: air mixture at a ratio of 1:1(v/v) to measure baseline MOC capacity

without any external addition of nutrients. A 50:50 (CH_4:air) ratio was chosen to avoid potential for the development of anaerobic condition. In parallel tests, 5 g of samples were sprinkled and moistened with 5 mL of Nitrate Mineral Salts (NMS, Table 2.1) medium (Whittenbury et al., 1970) to improve MOC through increased moisture and nutrient availability for methanotrophs (Figure 2.3).

Table 2.1 Composition of Nitrate Mineral Salts media (L^{-1})

$MgSO_4.7H_2O$	1 g
KNO_3	1 g
$CaCl_2.6H_2O$	0.2 g
KH_2PO_4	0.272 g
$Na_2HPO_4 \cdot 12H2O$	0.717 g
Ferric sodium EDTA	5 mg
Trace element solution	1 mL
Composition of trace element solution (L^{-1})	
Na_2EDTA	0.5 g
$FeSO_4.7H_2O$	0.2 g
$ZnSO_4.7H_2O$;	0.01 g
H_3BO_3	0.03 g
$CoCl_2.6H_2O$;	0.02 g
$MnCl_2.4H_2O$	0.003 g
$CuSO_4.5H_2O$;	0.003 g
$NiCl_2.6H_2O$;	0.002 g
$Na_2MoO_4.2H_2O$.	0.003 g

The serum vials were incubated in room temperature and the MOC was measured every 24 h for four days.

Figure 2.3 MOC analysis of different soil samples in serum vials

100 µL of head space CH_4 was sampled every 24 h (sampled using an air-tight Hamilton syringe; 100 µL, Model 1710 RN; Grace Davison Discovery Sciences, Rowville, VIC, Australia) from the two different batches and concentrations were quantified using gas chromatography equipped with thermal conductivity and flame ionization detectors (GC-TCD-FID, Varian-CP 3800, Mulgrave, Vic, Australia) fitted with a fused silica column (BR-Q PLOT; 30 m x 0.53 mm x 20 µm (Bruker Pty., Ltd., Vic., Australia). Helium was used as carrier and make up gas and delivered at a flow rate of 1 $mL.min^{-1}$. Column temperature was programmed 50 °C for 1 min, followed by ramping to 250 °C at a rate of 20 $°C.min^{-1}$ every five minutes. Injector temperature was set to 200 °C with a split ratio of 15. The GC was calibrated using standard CH_4 gas (10-50 %) and a regression factor was calculated. CH_4 oxidation capacities of soils were calculated as per Eq. 2.1 and are expressed as $\mu g.g^{-1}.DW_{soil}.h^{-1}$.

$$MOC = \frac{dCH_4}{dt} \times \frac{V_{bottle} \times MM_{CH_4} \times 10}{V_{molar\ gas} \times dw_{soil} \times 24} \qquad (eq.\ 2.1)$$

Where, MOC = CH_4 oxidation capacity ($\mu g.g_{soil}^{-1}.h^{-1}$)

$\frac{dCH_4}{dt}$ = slope of change in CH_4 concentration (vol %) over time (day)

V_{bottle} = Gas volume of bottle (mL)

MM_{CH_4} = Molar Mass of CH_4 = 16 g.mol^{-1}

$V_{molar\ gas}$ = molar gas volume at given temperature (L)

dw_{soil} = ash free dry-weight of soil (g)

2.2.4 Microbial diversity and consortia establishment

To screen the microbial diversity, all soil samples were individually enriched in 500 mL mini-bench top gas tight reactors (filter disc porosity 1-gas-wash bottle from Schott-Duran, VWR International, Murarrie, QLD, Australia) with 200 mL working volume of NMS and purged with 20:80 (v/v) of CH_4:air, the selected CH_4:air ratio was chosen to avod the overgrowth of non-methanotrophs at very low CH_4 concentration The inlet and outlet of the gas vents were connected with flexible Tygon tubing and sealed after every purging. Sterile air filters (0.2 µM, PTFE, Acro®50; VWR International International, Murarrie, QLD, Australia) were fitted in the gas vents to avoid contaminations while purging/collecting the samples. Calibrated mass flow meters from the BioFlo®310 fermenters (New Brunswick, John Morris Scientific, Australia) were used for mixing compressed air and 99.9 % pure CH_4 to the desired concentrations during enrichments. To improve the gas transfer rate, the reactor contents were continuously stirred at 200 rpm and incubated at room temperature (25 ºC). Soil cultures were kept at a neutral pH to favour methanotroph establishment. Soil slurries collected after 20 days were used for extracting genomic DNA (gDNA) and microbial sequencing was carried out by amplifying the V3-V4 region of 16s rRNA gene as detailed in section 2.2.5. For consortia establishment, soil slurries of 10 g after enrichment from mulch soil and compost soil was prepared with 200 mL sterile NMS; in a 500 mL sterile mini-bench-top gas tight reactors (Figure 2.4) as described above. The reactors were purged with 20:80 (v/v) of CH_4:air in the headspace, every 24 h for 20 days. Every 20 days, 10 mL of inoculum was transferred into fresh NMS medium in

new mini-bench-top reactors and the final mixed consortia were obtained after 4 sequential transfers of cultures (after 100 days). Biomass growth (OD_{600}; optical density at 600 nm; Enspire-2300, PerkinElmer, Australia) were used to confirm growth of the mixed cultures during the last enrichment cycle.

Figure 2.4 Enrichment/ establishment of mixed consortia in 500 mL gas tight reactors

Biomass protein was determined spectrophotometrically (Enspire, details as above) at 600 nm every two days using the Lowry method (TP0300, Sigma Aldrich, NSW, Australia). MOC was also calculated as per equation 2.1. Here the sample gas volume was 1000 µL, which was injected by an auto-sampler (Bruker Pty., Ltd., Vic., Australia), while CH_4 removal efficiency were calculated based on eq.2.2.

$$\text{Removal efficiency}\ (\%) = \frac{CH_4\ \text{IN} - CH_4\ \text{OUT}}{CH_4\ \text{OUT}}\ \text{x}\ 100 \qquad (\text{eq. } 2.2)$$

Mother cultures obtained after the final enrichment cycle were labelled as LB and CB to designate the soil type from which they were established; LB: landfill top cover soil-derived biomass and CB: compost soil-derived biomass, respectively. Established mixed consortia (LB and CB) were also characterized by amplifying the V3-V4 region of 16s rRNA gene as detailed in section 2.2.5.

2.2.5 Extraction of DNA from soils and PCR amplification

Soil slurries (after 20 day enrichment) and biomass collected from the established consortia of LB and CB (after day 5 and day 20 of the final enrichment cycle) were used for extracting genomic DNA (gDNA). In short 50 mL of the soil-slurry/ culture was collected under aseptic conditions and centrifuged at 3220 rcf (Eppendorf 5810 R, VWR International, QLD, Australia) for 20 min at room temperature. The supernatant was discarded and the biomass pellets were re-suspended in 200 µL of nuclease-free water. Genomic DNA of the biomass was extracted using the PowerSoil DNA extraction kit (MO-BIO Laboratories Pty., Ltd., GeneWorks, SA, Australia) following the manufacturer's instructions. Subsequently, DNA quality was assessed using agarose (1.5 %) gel electrophoresis and quantified using Nanodrop® 146 (Nanodrop 1000, ThermoFisher Scientific, USA).

Microbial sequencing was carried out by amplifying the V3-V4 region of 16s rRNA gene using 16S Amplicon PCR forward and reverse primers (16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC), and overhang adapter sequences are added to the locus-specific primer (Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus- specific sequence] Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus- specific sequence]) and sequenced using the Miseq® (Reagent kit V3 600 cycles PE, Illumina, USA) Illumina platform. PCR conditions, product clean-up and sequence library preparations were carried out, as outlined in the 16S metagenomics sequencing library preparation guide supplied by Illumina (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_prepar

ation.html). Forward sequences were used for the analysis. The raw reads were treated for quality analysis, read depth of the soil slurries ranged from 28306 to 64609 reads, while for the established biomass culture, the read depth were from 28181 to 47877. Primer and adapter sequences were removed, and the sequences were quality checked with average quality cut off of Q15 using (version 1.7.0, http://qiime.org/) Sequences shorter than 100 base pairs were filtered using filter_short_reads.py (Tony Walters, https://gist.github.com/walterst/7602058) and rarefied to ~19,000 reads for soil samples and ~26000 for mother cultures (rarefied to the level of the sample with least number of reads in each biomass) using subsample_fasta.py in QIIME (version 1.7.0, http://qiime.org/) (Caporaso et al., 2010). Taxonomic assignment and clustering into OTUs was carried out in RDP classifier (https://pyro.cme.msu.edu/classifier/form.spr) (Cole et al., 2013). Mean abundance of the mixed consortia were prepared using Calypso, version 5.6 (http://cgenome.net/calypso/) (Al-Shehri et al., 2016; Hagen et al., 2015). Raw sequences were deposited in the European Nucleotide Archive (EMBL-EBI, UK), project accession of PRJEB14093 and PRJEB17609 (http://www.ebi.ac.uk/ena/data/view/PRJEB14093 and http://www.ebi.ac.uk/ena/data/view/PRJEB17609) respectively.

2.2.6 Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich, NSW, Australia and CH_4 (99.9 % pure CH_4), CH_4 calibration gases (i.e. 10-70 % for GC-TCD-FID), helium and compressed air of instrument grade (N_2-78.08 % and O_2-20.94 %) were supplied by BOC, a member of the Linde group, Townsville, QLD, Australia. All gases were ISO certified for purity.

2.3 Results

2.3.1 Physico-chemical characteristics and methane oxidation capacity of soils after 24 and 96 h in high methane atmospheres

The physical and chemical characteristics of the soil samples are listed in Table 2.2.

Table 2.2 Physico-chemical characteristics of the soil

Physicochemical characteristics	Vegetated. Soil	Mulch Soil	Fresh Soil	Compost Soil
pH	7.82±0.3	7.47±0.6	7.85±0.5	7.86±0.3
Moisture (%)	14.19±0.3	18.0±0.2	11.6±0.2	16.8±0.1
Total Solids (%)	85.81±1.8	60.78±1.3	88.40±2.0	69±1.5
Volatile Solids (%)	4.11±0.3	5.76±0.5	4.54±0.3	48±2.6
Total Nitrogen as N ($mg.kg^{-1}$)	1000±40	5970±120	1030±53	10600±0280
Total Organic Carbon (%)	0.60±0.1	20.0±0.6	0.45±0.1	25.8±0.7
Total Phosphorus as P ($mg.kg^{-1}$)	272±6	1300±12	197±4	4530±18
Ammonia as N ($mg.kg^{-1}$)	<20±1	<20±1	<20±1	<20±1
Nitrite as N (Sol.) ($mg.kg^{-1}$)	<0.1	3.0±0.5	<0.1	<0.2
Nitrate as N (Sol.) ($mg.kg^{-1}$)	4.2±0.3	1.3±0.1	9.4±0.5	<0.1
Nitrite + Nitrate as N (Sol.) ($mg.kg^{-1}$)	4.2±0.2	4.3±0.3	9.4±0.6	<0.2
Sulfate as SO_4^{2-} ($mg.kg^{-1}$)	<50±2	<50±2	<50±2	1760±36
Cu ($mg.kg^{-1}$)	38.3±0.8	123±0.9	35.0±0.3	166±0.8
Fe ($mg.kg^{-1}$)	1800±3	1930±11	1400±5	2160±9
Ni ($mg.kg^{-1}$)	14.2±0.3	9.7±0.2	11.9±0.3	14.6±0.3
Zn ($mg.kg^{-1}$)	83.5±2	261±8	60.3±5	572±6
Cr ($mg.kg^{-1}$)	8.2±0.1	18.6±0.5	22.7±0.4	37.8±0.6
Mn ($mg.kg^{-1}$)	579±1.9	454±1.9	567±2.4	316±1.6

All soil samples were circum-neutral in pH ranging from 7.4 to 7.8, while the moisture content varied from 11.6 % to 18 % with mulch and compost soil relatively rich in moisture (18 % and 16.8 %). Most of the soil properties of mulch soil and compost soil were similar with high TOC (20 and 25.8 %), TN (5970 and 10600 $mg.kg^{-1}$), TP (1300 and 4530 $mg.kg^{-1}$). In contrast, TOC (0.60 and 0.45 %), TN (1000 and 1030 $mg.kg^{-1}$) and TP (272 and 197 $mg.kg^{-1}$) contents of vegetated and fresh cover soil were very low. Except for compost soil which had high sulphate content (1760 $mg.kg^{-1}$), soils typically contained traces of sulphate (<50 $mg.kg^{-1}$). In addition, the soil samples contained metal ions such as copper, iron, nickel, zinc, chromium and manganese. Copper, iron and zinc were found be high in mulch (123, 1930 and 261 $mg.kg^{-1}$) and compost soil (166, 2160 and 572 $mg.kg^{-1}$), while nickel contents were high in vegetated (14.2 $mg.kg^{-1}$) and compost soil (14.6 $mg.kg^{-1}$) and manganese concentrations were high in vegetated (578 $mg.kg^{-1}$) and fresh soil (567 $mg.kg^{-1}$) (Table 2.2).

Figure 2.5a shows the MOCs of soils (unmodified controls) without addition of any nutrients or change of moisture contents, while Figure 2.5b represents the MOCs of soils after addition of moisture/ nutrients (5 mL NMS). Soils without nutrient addition showed low and similar MOCs ranging from ~3 to 5 $\mu g.g^{-1}DW_{soil}.h^{-1}$, with mulch soil having the highest MOC (5.36 $\mu g.g^{-1}DW_{soil}.h^{-1}$) after 96 h incubation in a 50:50 (CH_4: air) atmosphere. Nutrient enrichment of soils improved MOCs by 5-fold for mulch soil and fresh soil, while the MOCs did not increase considerably in vegetated and compost soil (Figure 2.5a, b). Mulch cover soil showed the highest MOC (16.33 $\mu g.g^{-1}DW_{soil}.h^{-1}$), followed by fresh cover (11.46 $\mu g.g^{-1}DW_{soil}.h^{-1}$) and compost soil (5.57 $\mu g.g^{-1}DW_{soil}.h^{-1}$), while the vegetated soil from a 20 years old landfill continued to show low MOC (3.57 $\mu g.g^{-1}DW_{soil}.h^{-1}$) after 24 h incubation in CH_4:air (50:50 %) (Figure 2.5). MOCs of all the soils decreased after 96 h in the methane-rich atmosphere and did not correlate with the age of soils.

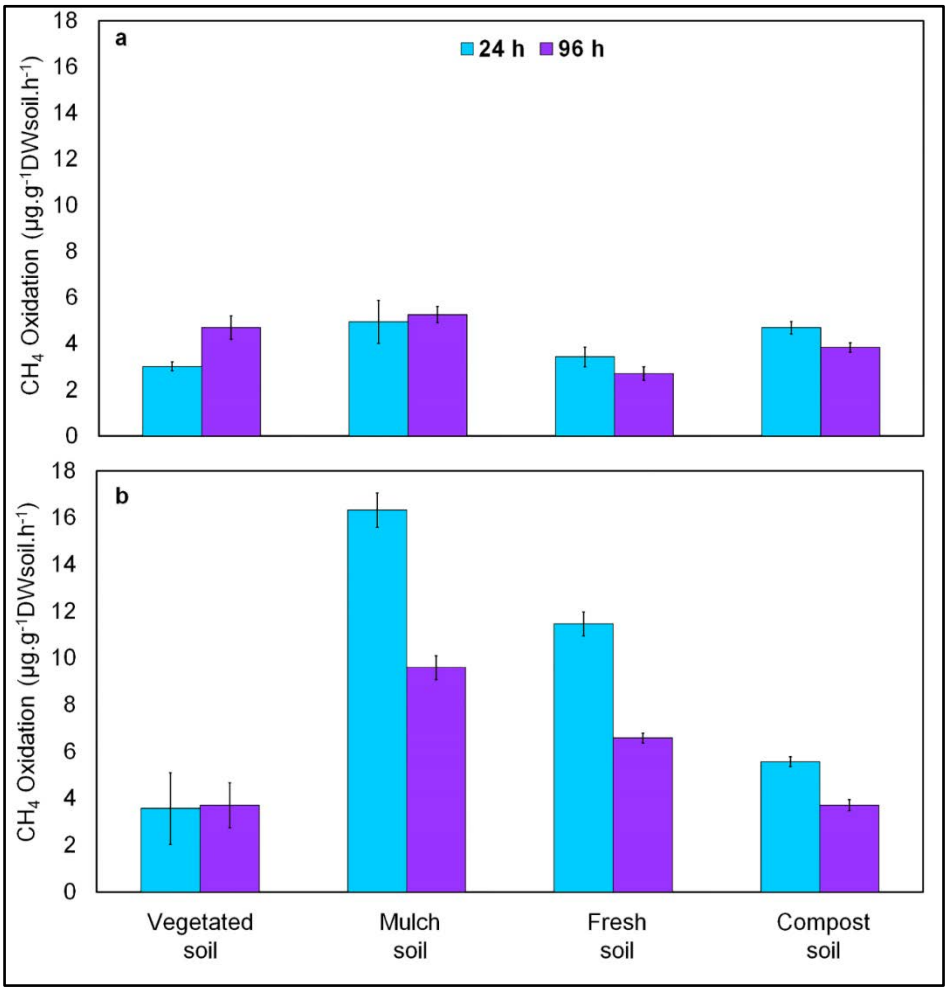


Figure 2.5 MOCs of different landfill cover soils a) without nutrients; b) with nutrients

2.3.2 Microbial community structure of different landfill cover soils slurries after 20-day enrichment

16s rRNA gene sequencing showed Proteobacteria as the dominant phylum (80-85 %) in all soil types investigated, followed by Bacteroidetes (6-10 %) (Figure 2.6a). Among the methanotrophs, type -I were more abundant in mulch and compost soils (Figure 2.6b), but mulch soil was dominated by *Methylosarcina* (~65 %), while the abundance of this genus was very low in compost soil (~2 %), which was dominated by *Methylobacter* (~75 %) instead.

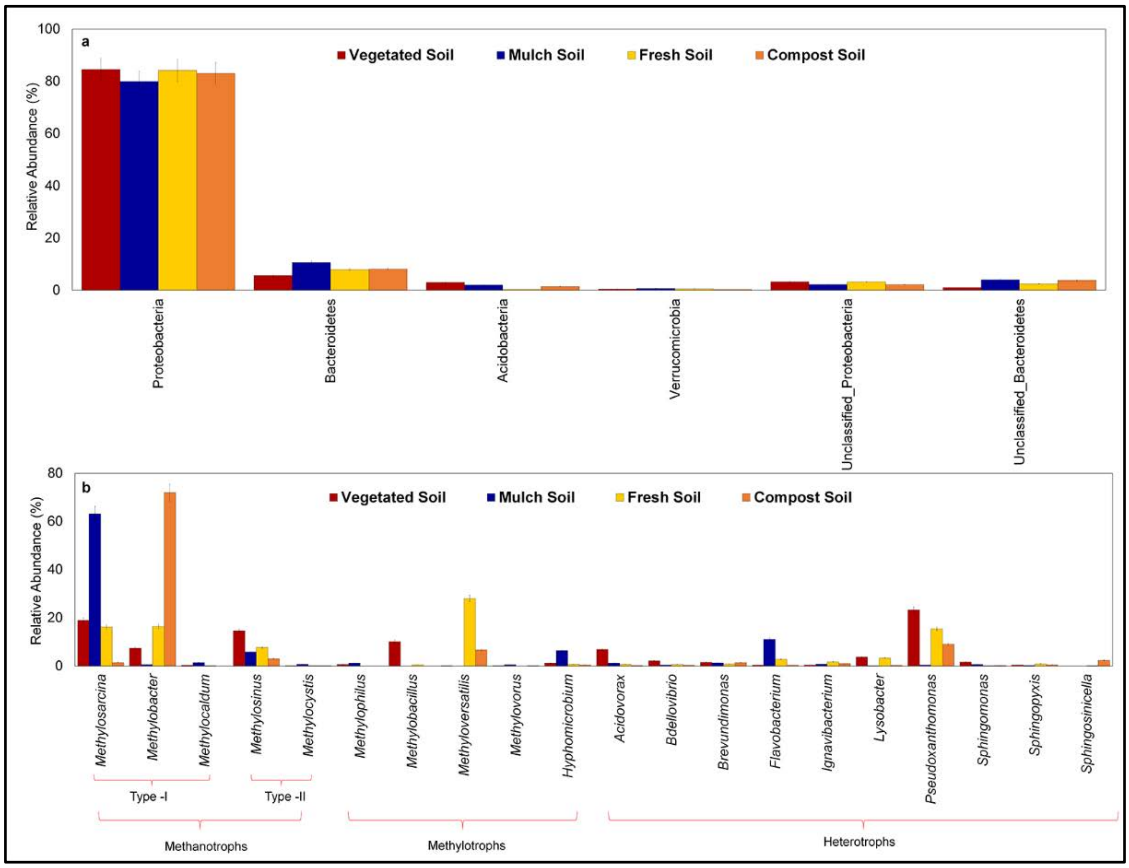


Figure 2.6 Microbial diversity of different landfill cover soils a) phylum level, b) genus level

Methylosarcina were also present with similar abundance in vegetated - and fresh soil (~19 %), while *Methylobacter* was co-dominant with *Methylosarcina* in fresh (~18 %), but not in vegetated soil (~8 %). Of the type -II methanotrophs, *Methylosinus* were also highly abundant in vegetated (~17 %) (~ equal abundance with type -I) and fresh soil (~11 %). Other methanotrophs, such as *Methylocaldum* (type -I) and *Methylocystis* (type -II) were also detected in low abundances in mulch soil samples. While, methanotrophs dominated the mulch and compost soils, non-methanotrophs were co-dominant in vegetated and fresh soils. The heterotroph *Pseudoxanthomonas* (~24 %) and the methylotroph *Methylobacillus* (~15 %) were present in high abundances in vegetated soil, while fresh soil showed high abundances of the methylotroph *Methyloversalitis* (~30 %) and *Pseudoxanthomonas* (~15 %). In contrast, in compost soil, *Methyloversalitis* and *Pseudoxanthomonas* showed low abundances

(5 %). The heterotrophic genera *Flavobacterium* (~10 %) *and Hyphomicrobium* (~5 %) were present in noticeable abundances in mulch cover soil. Other non-methanotrophs such as the methylotrophic genera *Methylophilus*, *Methylovorous* and the heterotrophic genera *Acidovorax*, *Bdellovibrio*, *Brevundimonas*, *Ignavibacterium*, *Lysobacter*, *Sphingomonas* and *Sphingopyxis* were also detected in the soil samples but in very low abundances (Figure 2.6b)

2.3.3 Methane oxidation capacity, removal efficiency and changes in community composition of the mixed consortia

Average CH_4 removal efficiency of established mother cultures (LB and CB) were 56 % and 62 %, respectively (Figure 2.7a).

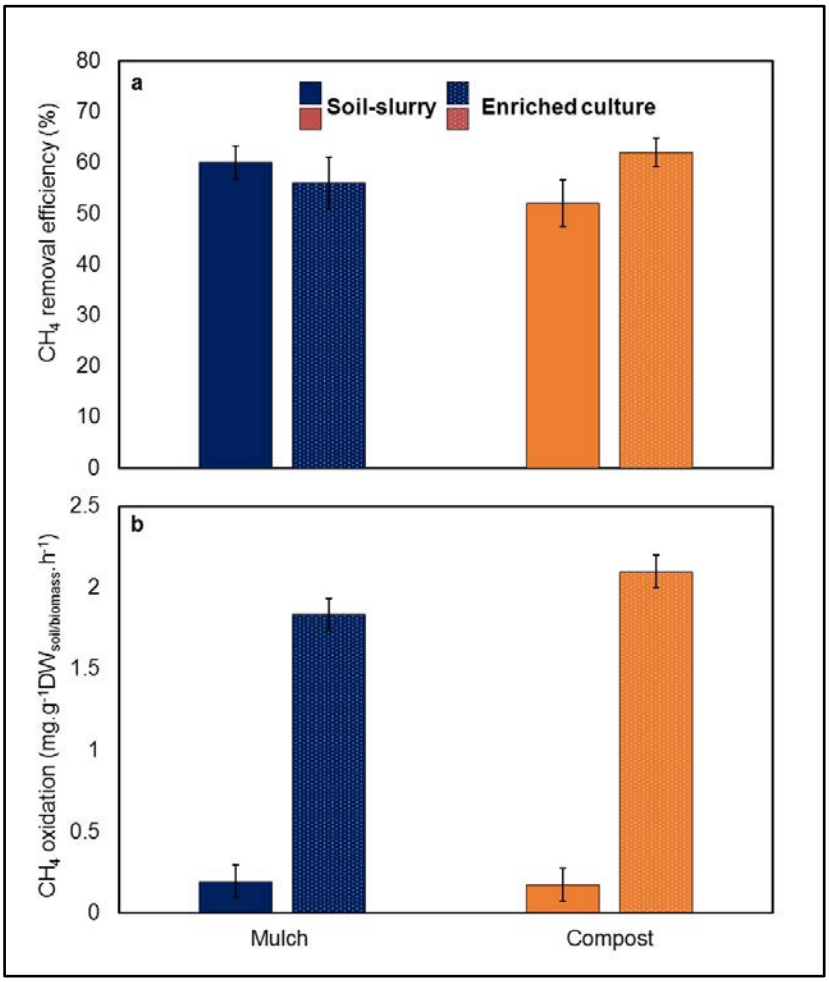


Figure 2.7 CH_4 removal efficiency and MOCs of soil slurries and established consortia a) CH_4 removal efficiency; b) CH_4 oxidation capacity.

While the average MOCs of LB and CB were 1.83 and 2.10 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ respectively, showing an improvement of 10-fold compared to the enriched soil slurries (Figure 2.7b). Similar to the soil-slurry, 16s rRNA gene sequencing showed Proteobacteria as the dominant phylum in LB (~75 %) and CB (~80 %), followed by Bacteroidetes (8-10 %) (Figure 2.8).

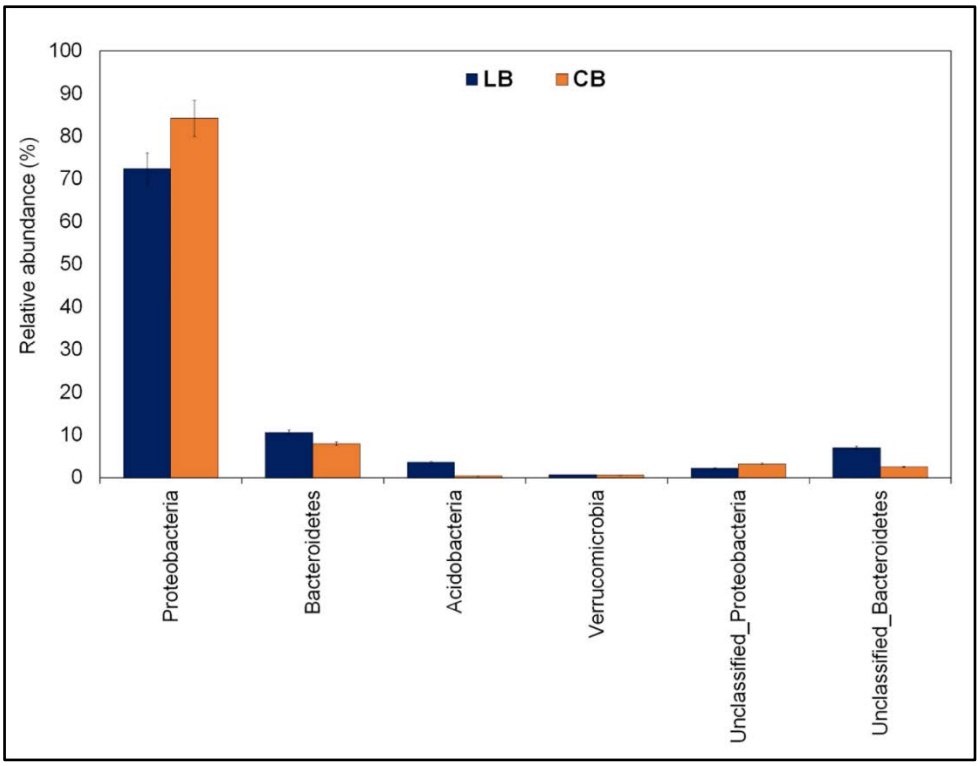


Figure 2.8 Microbial diversity of established consortia of Landfill biomass (LB) and Compost biomass (CB) at phylum level

Established consortia (LB and CB) showed similar methanotrophic community profiles, but the co-habiting heterotrophs and methylotrophs differed (Figure 2.9a, b) in day 5 and day 20. At day 5, *Methylosarcina* was highly abundant in both LB (~85 %) and CB (~60 %), but co-habiting non-methanotrophs showed a very low abundances (<5 %) in LB and CB except *Methylophaga* which was found to be more abundant (~30 %) in CB (Figure 2.9b). While in LB at day 20, *Methylosarcina* (~25 %) and *Pseudoxanthomonas* (~30 %) were dominant, while *Methylosarcina* (~30 %) and *Methylophaga* (~27 %) were dominant in CB. In LB, the heterotrophic bacterial genera,

Azospirillum, Azotobacter and *Pelomonas* were present in equal abundance (~7-10 %), while *Flavobacterium*, *Chryseobacterium*, *Brevundimonas*, *Sphingopyxis* were found at much lower abundances (<5 %). In contrast, in CB, *Flavobacterium* and *Chryseobacterium* were highly abundant (~10-12 %). *Pesudomonas*, *Pseudoxanthomonas*, *Sphingopyxis* and *Sphingomonas* were also detected but with lower abundances (<5 %).

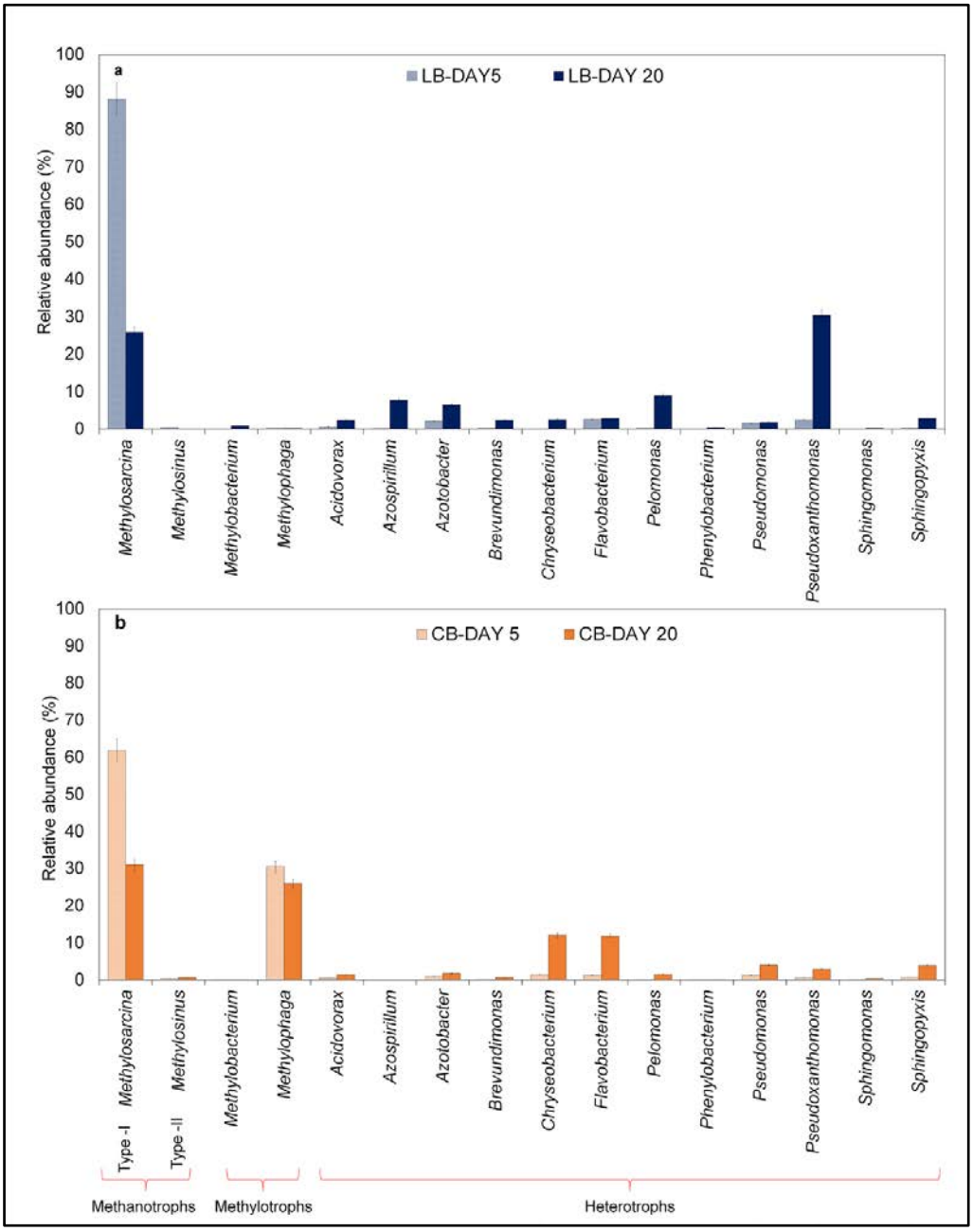


Figure 2.9 Microbial diversity of established consortia differences at day 5 and day 20 a) Landfill biomass (LB); b) Compost biomass (CB) at genus level

2.4 Discussion

Landfills are one of the major anthropogenic sources of methane (Huber-Humer et al., 2008; IPCC, 2014). Methanotrophs present in landfill top cover soils act as natural bio-filters. Microbial ecologists have long been interested in the methanotrophic community structure of landfills due to their key role in oxidizing CH_4 to CO_2 (Hanson and Hanson, 1996; Henneberger et al., 2012) and their intricate relationships with the co-existing organisms (Ho et al., 2016; Ho et al., 2014; Hršak and Begonja, 2000; Iguchi et al., 2011b; van der Ha et al., 2013). As an applied microcosm study, the aims were to characterise microbial consortia structures after initial enrichment of landfill soil slurries and after establishing mixed cultures from the soil slurry supernatants under high CH_4 concentrations, which represents the culturable bacterial community. Due to low water solubility of CH_4 (Duan et al., 1992), high CH_4 concentrations were used for our enrichment studies to ensure that sufficient CH_4 was available for methanotroph growth. Moreover, high CH_4 concentrations would selectively enrich microbes capable of survival in high CH_4 environments like landfills which would be a prerequisite for developing an efficient microbial based bio-filter.

Four landfill cover soils were chosen and their physico-chemical characteristics were analysed. All soils were circum-neutral in pH, which is generally reported to be optimal for CH_4 oxidation and growth of most methanotrophs when provided with sufficient CH_4 (Semrau, 2011; van der Ha et al., 2010). Of these four soils, mulch cover soil and compost had high TOC contents, indicating they contained more biodegradable carbon, which is commonly reported to harbour active methanotrophic communities (Lu et al., 2011; Pariatamby et al., 2015; Spokas and Bogner, 2011). Generally, high organic content enhances the soil's capacity to hold water which in turn enhances CH_4 oxidation (Chanton et al., 2011; Huber-Humer et al., 2008). This is supported by high MOCs of mulch soil (Figure 2.5), but compost soil showed comparatively low MOC after addition of nutrients (Figure 2.5) despite high TOC and

similar moisture content. High organic content can also result in CH_4 formation under micro-aerobic conditions (Chanton et al., 2011), which cannot be ruled out, as initial enrichments were done in batch cultures. High TN content in these soils also indicates they are more suitable for the growth of type -I methanotrophs, which is evident in the 20-day CH_4 atmosphere enrichment study (Figure 2.6b). In contrast, vegetated and fresh soil had lower TOC and TN contents and showed co-dominance of type -I and type -II methanotrophs, as expected (Figure 2.6b) (Pieja et al., 2011a). Although, the overall research presented in this thesis also investigated PHB production capacity, a trait of type -II methanotrophs, vegetated and fresh cover soils were not chosen for further enrichment and culture establishment of mixed consortia. Instead, as discussed below, mulch and biosolid compost soils were selected, despite lower abundances of type -IIs, based on MOCs/ methanotrophic richness and general use as landfill cover, respectively, because the aim was to establish robust consortia that can survive and perform well under variable CH_4/O_2 and Cu^{2+}/Fe^{2+} ratios, respectively. In addition, some of the heavy metals (copper, iron, manganese and zinc) present in the soils have been identified as essential trace elements for the growth of methanotrophic bacteria (Glass and Orphan, 2012). The two heavy metals, copper and iron, present in high concentrations in mulch and compost cover soils (Table 2.2), play a vital role in the expression of pMMO and sMMO enzymes (Chidambarampadmavathy et al., 2015a; Glass and Orphan, 2012). Therefore, these two soils could be more favourable for establishment of robust mixed microbial/ methanotrophic consortia compared to the vegetated and fresh soils.

Methane oxidation patterns of the different soils (Figure 2.5b) correlated well with enriched community structures of soil slurries (Figure 2.6b). Despite similar physico-chemical characteristics of mulch and compost soils, microbial diversity and MOCs were quite different. The observed high MOC in mulch soil could be due to co-existence with *Flavobacterium* (synthesize essential vitamins) and *Hyphomicrobium*

(utilise methanol, excreted by methanotrophs during methane oxidation thereby avoiding methanol-toxicity), reported to increase methanotroph activity (Beck et al., 2013; Iguchi et al., 2011b; Oshkin et al., 2015; van der Ha et al., 2013). *Methylobacter*, on the other hand, dominated in compost soil at the expense of non-methanotrophs, which could explain the low MOCs achieved.

Although enriched fresh cover soil did not show distinct dominance of a specific methantroph genus, high MOCs were achieved, which could be the result of co-dominance of type -I methanotrophs and a 10 % abundance of the type -II methanotroph genus *Methylosinus*. In addition, co-existence with the methylotroph *Methyloversalitis*, may reduce methanol toxicity (Beck et al., 2013; Oshkin et al., 2015), indirectly supporting high MOCs. In contrast, a non-methanotroph (*Pseudoxanthomonas*) dominated vegetated soil characterised by low abundance of methanotrophs, which explains the low MOCs achieved. In summary, the presence of methylotrophs in mulch and fresh soil correlated well with high MOCs, possibly due to reduced methanol toxicity. *Flavobacterium*, *Pseudoxanthomonas*, *Pseudomonas* were commonly reported to co-exist with methanotrophs in natural ecosystem as well as in microcosm studies (Hernandez et al., 2015; Ho et al., 2014; Iguchi et al., 2011b; Oshkin et al., 2015; van der Ha et al., 2013). Overall, differences in MOCs of the soil are reflected in microbial community structures, possibly influenced by the differing nutrient/ metal contents. The reported MOCs for mulch soil, as well as for the soil-slurry were higher than for other reported soil types (Börjesson et al., 2004; Einola et al., 2007; Tate et al., 2012).

Generally, depending on the inoculum size (i.e. g soil or sediment as seed), growth rate and other physico-chemical conditions, cultures can be successfully enriched within days or weeks, but in case of slow growing/ metabolising organisms, the enrichment process can take several weeks or even months (Kelly and Wood, 2010). In order to establish robust consortia, the soil-slurries obtained from mulch

cover soil and compost soil were further enriched at 20 % CH_4 through five 20-day-inoculi serial transfers of microbial-containing supernatants after gravity settling of the soil particles. The established consortia (LB and CB) were characterised for their MOC, CH_4 removal efficiency and microbial diversity. Established mixed consortia showed slightly higher MOCs in CB than LB and MOCs were much higher in established cultures compared to the respective soil slurry (Figure 2.7). The mixed consortia were dominated by type -I methanotrophs, while the type -II methanotroph *Methylosinus* was present in very low abundances. Community profiles of LB and CB changed significantly following serial dilution for establishing the mixed consortia (compare Figure 2.6 and Figure 2.9). *Methylosarcina,* which were more abundant in mulch soil reduced in abundance in established consortia (LB), while *Methylobacter* which were dominant in compost soil, were not detected in the established consortia cultures (CB), but *Methylosarcina* became dominant (Figure 2.9). Oshkin et al. (2015) also reported the persistence of specific type -I methanotrophic genera in higher abundance in various microcosm experiments.

Observed community shifts are likely due to the continuous enrichment with nutrients and culturing conditions (liquid void of solids vs. soil particle slurries) leading to the preferential growth of certain methanotrophic genera. One of the limitations of comparing microbial composition of soil particle slurry and liquid cultures is that liquid enrichment cultures favour the growth of fast growing organisms and due to high substrate concentrations used (20 % CH_4 and NMS medium), slow growing organisms are outcompeted by fast growing organisms resulting in the dominance of certain genotype (Dunbar et al., 1997). Moreover, in this study, establishment of culture was not carried under continuous cultivation conditions, rather as mentioned earlier, enrichment cycles were carried out through five 20-day-inoculi serial transfers under semi-continuous conditions which would favour fast growing organisms. This bias is

well reflected in changes in diversity between enriched soil-slurry and liquid culture (compare Figure 2.6 and Figure 2.9).

Community profile of non-methanotrophs also changed significantly when compared to the soil-slurry. *Chryseobacterium*, *Flavobacterium* and *Methylophaga*, not detected in the compost soil-slurry, are now present in high abundances in CB. Similarly, *Azospirllum*, *Azotobacter*, *Pseudoxanthomonas* and *Pelomonas* which were not detected or were present in very low abundance in the mulch soil-slurry, dominated after enrichment (LB). Irrespective of continuous serial transfers in high CH_4 environment with growth conditions favourable for methanotrophs, observed co-dominance of non-methanotrophs in established consortia are likely due to metabolic exudates and secretion of extracellular polymeric substances by methanotrophs favouring their growth.

Metabolites are the currency of microbial interaction (Ho et al., 2016) and cross feeding of certain metabolites (methanol, formaldehyde, sugars or organic acids) from methanotrophs to non-methanotrophs have been widely reported (Beck et al., 2013; Ho et al., 2016; Kalyuzhnaya et al., 2013; Khmelenina et al., 2015; Oshkin et al., 2015; van der Ha et al., 2013). The differences in abundances and diversity patterns of non-methanotrophs between the soil-slurry and established consortia, might be due to the availability of different metabolic products produced by methanotrophs, which would have created synergistic relationships (Beck et al., 2013; van der Ha et al., 2010) as a result of continuous enrichment. The impact of effect of accumulation of exudates/ extracellular polymeric substances on diversity profiles was confirmed through community profile comparison of samples at day 5 and day 20 of LB and CB (Figure 2.9). At day 5 (5 days after inoculum transfer for the final enrichment cycle into fresh NMS), *Methylosarcina* was relatively more abundant in both LB and CB, but co-habiting non-methanotrophs showed low abundances. At day 20, abundances of methanotrophs had decreased, while co-habiting non-methanotrophs increased

(Figure 2.9). As no complex carbon sources were provided for their growth, it is likely that increased availability of metabolites secreted by methanotrophs favoured their dominance. Similarly, van der Ha et al. (2013) reported a sustained presence non-methanotrophs richness in mixed cultures with methanotrophs, in the absence of complex carbon sources. Very recently, Ho et al. (2016) performed a network analyses on microbial/ methanotrophic communities by mining various datasets covering diverse environments and reported that co-existence of non-methanotrophs are site - and methanotroph-species-specific. A few other recent studies also suggested that co-existence community profiles are not random and are influenced by the specific environmental conditions and/ or bacterial species dominance; methanotrophs synergistically co-habited with either methylotrophs or heterotrophs (Beck et al., 2013; Oshkin et al., 2015), as observed here.

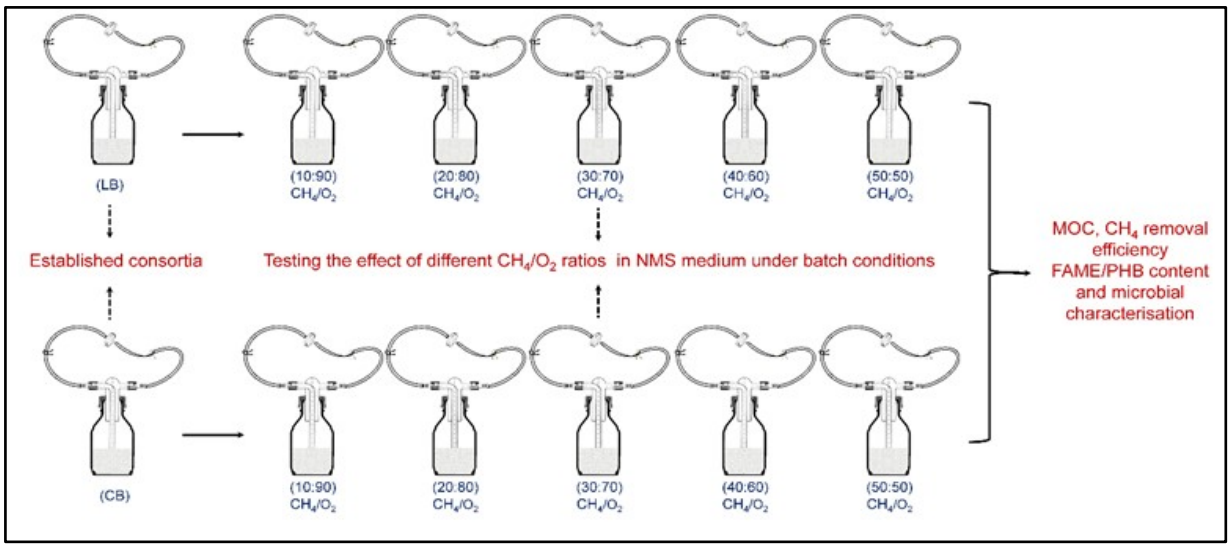
2.5 Major findings from chapter 2:

In summary mulch soil showed higher MOC than compost soils and was rich in methanotroph diversity in enriched soil-slurry. The established consortia (LB and CB) showed high abundance of *Methylosarcina* at day 5 and co-habiting non-methanotrophs, reported to increase methanotrophic activity initially present in low abundance following inoculum transfer – increased in abundance over the last 15 days of the 20 days enrichment cycle. Irrespective of varying methanotroph abundance, the established consortia showed more or less similar MOC and removal efficiency, which suggests potential of such microbial consortia for biological landfill CH_4 emission abatement.

In landfill environments, however, CH_4, CO_2 and O_2 ratios vary with time, climatic condition and location within the landfill depth profile, as do essential trace metal concentrations. These variable conditions will undoubtedly influence microbial profiles of landfill top cover soils. Understanding the responses of mixed communities is therefore a prerequisite for developing efficient bio-filters for landfill CH_4 emission

abatement. Therefore, in order to ascertain the robustness of the established type -I dominated LB and CB mixed consortia, my PhD research investigated responses (MOCs, CH_4 removal efficiencies and microbial community structures) to variable CH_4/O_2 concentrations (mimicking landfill gas mixture concentrations) in chapter 3 as follow on to chapter 2 In addition, fatty acid methyl esters (FAME) contents, FAME profiles and PHB contents of these established consortia were analysed, to evaluate potential cost-benefits derived from biological landfill CH_4 emission abatement approaches.

[2]Chapter 3: Response of mixed consortia to different CH_4/O_2 ratios



Graphical Abstract 2 Responses of mixed consortia to different CH_4/O_2 ratios - chapter 3 overview

[2] This chapter is based verbatim on the following publication and has been reformatted to fit the overall thesis format and to create the link to the next chapter:

Chidambarampadmavathy K, Karthikeyan OP, Huerlimann R, Maes EG & Heimann K (2016) Response of mixed methanotrophic consortia to different methane to oxygen ratios. Waste Management 61: 220-228 (https://doi.org/10.1016/j.wasman.2016.11.007).

To avod repeating materals and methods, methods previously described in the thesis have been removed and have been referred to appropriate sections and has been reformatted to fit the format of this thesis.

3.0 Abstract

Methane (CH_4) and O_2 (in air) concentrations affect the CH_4 oxidation capacity (MOC) and mixed community structures in compost (fresh) and landfill (age old) top cover soils. A change in the mixed community structure in response has implications for landfill CH_4 bio-filter remediation and possible bio-product outcomes (i.e. FAME content and profiles and PHB contents). Therefore, the study aimed to evaluate the effect of variable CH_4/O_2 ratios (10-50 % CH_4 in air) on mixed community structures of LB and CB and to quantify flow on impacts on MOC, total FAME contents and profiles, and PHB accumulation. A stable consortium developed achieving average MOCs of 3.0±0.12, 4.1±0.26, 6.9±0.7, 7.6±1.3 and 9.2±1.2 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ in LB and 2.9±0.04, 5.05±0.32, 6.7±0.31, 7.9±0.61 and 8.6±0.48 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ in CB for a 20 day cultivation period at 10, 20, 30, 40 and 50 % CH_4, respectively. CB at 10 % CH_4 had a maximal FAME content of 40.5±0.8 $mg.g^{-1}$ $DW_{biomass}$, while maximal PHB contents (25 $mg.g^{-1}$ $DW_{biomass}$) was observed at 40 % CH_4 in LB. Despite variable CH_4/O_2 ratios, the mixed methanotrophic community structures in both LB and CB were relatively stable, dominated by *Methylosarcina* sp., and *Chryseobacterium*, suggesting that a resilient consortium had formed which can now be tested in bio-filter operations for CH_4 mitigations in landfills.

3.1 Background

Contributing to ~ 20 % of the worldwide GHG emissions, CH_4 is considered a potent GHG together with CO_2 (IPCC, 2007, 2014). The atmospheric concentration of CH_4 has increased considerably from a pre-industrial level of ~ 715 to ~1840 ppb in 2015. (Butler and Montzka, 2016; EEA, 2016; IPCC, 2014). Both natural and anthropogenic sources contribute to CH_4 emissions, while anthropogenic activities alone contribute to 50 to 65 % of total CH_4 emissions, causing a sharp rise in GHG emissions (GMI, 2010; Karthikeyan et al., 2015a). Landfill is the third largest anthropogenic source of CH_4, contributing ~600 $MMTCO_2eq$ as of 2010, which is projected to increase by 10 % by 2020 (GMI, 2010). Biogenic CH_4 emissions from landfills occur due to microbial anaerobic degradation of organic matter and varies at different time intervals with respect to landfill age (Scheutz et al., 2009). Landfill soil covers naturally act as sinks for CH_4 (Henneberger et al., 2012; Scheutz et al., 2009; Staley and Barlaz, 2009). In addition, various types of compost/mulch materials are being used as landfill cover soil for enhancing CH_4 oxidation (Humer and Lechner, 1999). In addition to methane emission mitigations, the deposition of fossil fuel-derived non-biodegradable plastics into landfills also causes landfill management issues. Despite all recycling efforts for non-biodegradable plastics, approximately 20 % (w/v) are deposited in landfills (Staley and Barlaz, 2009), posing a threat to adjacent environments, impeding environmentally sustainable landfill management practices. Therefore, reducing deposition of non-biodegradable plastics is of equal importance to developing GHG mitigation strategies. As a cradle-to-grave/ cradle approach, the emanated CH_4 from landfill could potentially be re-routed for bioplastic production using methanotrophs (Karthikeyan et al., 2015a; Rostkowski et al., 2012). Methanotrophs, a group of Gram-negative bacteria utilize landfill CH_4 as a carbon source and can store the CH_4-carbon in form of PHB, a key ingredient for the production of biodegradable plastics (Hanson and Hanson, 1996; López et al., 2014;

Pieja et al., 2011a). To date, two major groups of methanotrophs have been identified: type -I methanotrophs belonging to the gamma proteobacteria (includes sub types -Ia and –Ib,family Methylococcaceae; 14 genera), and type -II methanotrophs belonging to the alpha proteobacteria group (family Methylocystaceae; 4 genera) (Bowman, 2014; Chidambarampadmavathy et al., 2015a; Hanson and Hanson, 1996; Semrau, 2011). Compared to type -I methanotrophs, type -II are capable of fixing atmospheric nitrogen, utilize CO_2, produce C18 as dominant fatty acids and are capable of storing carbon as PHB. Type -II methanotrophs are therefore beneficial for industrial bio-product development (Pieja et al., 2011a; Rostkowski et al., 2012; Strong et al., 2015; Wendlandt et al., 2010). Other than these, some methanotrophs are also grouped under Verrucomicrobia and NC10 phylum, with the latter reported to perform CH_4 oxidation coupled with denitrification process (Op den Camp et al., 2009; van Teeseling et al., 2014; Wu et al., 2011).

Studies on MOC and production of PHB have been previously carried out with pure cultures of methanotrophs, but research utilizing mixed microbial communities are rare (Helm et al., 2006; Karthikeyan et al., 2015b; López et al., 2014). Furthermore, complex interactions occur within the mixed microbial communities, whereby the co-habiting bacteria consume toxic and over-produced metabolites of methanotrophs such as methanol and formaldehyde, thereby potentially improving methanotrophic activity and PHB accumulation (Ho et al., 2016; Ho et al., 2014; Hršak and Begonja, 2000; Wendlandt et al., 2010). Provision of methanol and formaldehyde to heterotrophs could prove beneficial for methanotrophs in terms of supply with vitamins in exchange. Such a scenario is possible given the evidence that heterotrophs provide cobalamin to methanotrophs (Ho et al., 2014; Iguchi et al., 2011b). To achieve these beneficial outcomes, sufficient supply of O_2 is vital for supporting CH_4 oxidation and heterotrophic growth.

Theoretically, methanotrophs require 2 moles of O_2 to oxidize 1 mole of CH_4. Generally type -I methanotrophs dominate in niches where CH_4 levels are low and O_2 levels are high, with the opposite being the case for type -II methanotrophs (López et al., 2014; Wei et al., 2015). Low O_2 concentrations favour type -II, as the nitrogenase complex responsible for N_2-fixation is sensitive to O_2 (Whittenbury and Dalton, 1981). Another reason may be the formation of exopolysaccharides/ extracellular polymeric substance by type -I methanotrophs, which, in mixed natural samples, may limit supply of O_2, thereby favouring the growth of type -II (Whittenbury and Dalton, 1981). As stated in the general introduction (section 1.1), CH_4: O_2 ratios vary with landfill age and conditions, potentially negatively affecting MOCs of bio-filters, and production of co-products such as PHB, the latter potentially important for production of biodegradable plastic to offset costs and provide a financial incentive to remediate fugitive CH_4 emissions from landfills. Therefore, it is critically important to consider CH_4/O_2 ratios, as the responses of methanotrophs may vary, potentially affecting MOC in-turn, depending on the type enriched. Understanding CH_4/O_2 ratio-induced microbial community shifts is also important for developing/ customizing bio-filters (a man-made device to capture pollutants, in this case CH_4 from landfills) that work economically well for CH_4 mitigation in the varying CH_4/O_2 ratio fluxes, as experienced in landfills.

In this context, the present study aimed to evaluate the impact of variable CH_4/O_2 ratios on MOC, FAME and PHB content of established consortia (LB and CB). Specifically, we examined the community composition of methanotrophs and non-methanotrophs of LB and CB in varying CH_4/O_2 ratios.

3.2 Materials and methods

Mother cultures obtained after enrichment were labelled as LB and CB to designate the soil type from which they were established (refer section 2.2.4 for details) were used to study the effect of different CH_4/O_2 ratios.

3.2.1 Influence of CH_4/O_2 ratio on mixed consortia

To study the effect of CH_4/O_2 ratios, 20 x 500 mL mini-bench top reactors (Schott-Duran® gas-wash bottle, VWR International, QLD, Australia) were used for establishing microbial cultures (Figure 3.1).

Figure 3.1 Testing the effect of different CH_4/O_2 ratio on mixed consortia in 500 mL gas tight reactor

Of the 20 reactors, 15 reactors were used to set up triplicate cultures to study the effects of five CH_4/O_2 ratios (10:90, 20:80, 30:70, 40:60 and 50:50 %) in a working volume of 200 mL NMS, inoculated with 5 mL of each mother culture, yielding a biomass concentration of 0.090±0.015 and 0.110±0.009 (OD_{600}) with a protein content of 37.93±0.06 $\mu g.mL^{-1}$ and 40.69±0.08 (Lowry method, TP0300, Sigma-Aldrich, NSW, Australia) for LB and CB, respectively. As landfill soils, especially mulch (LB) and compost soils (CB) are particularly rich in nitrogen (Table 2.2), NMS medium was used for the cultivation of the mixed consortia. The other five reactors served as abiotic control without biomass to understand the natural CH_4 dissolution rates. The bench top reactors head space (~300 mL) was purged with 10:90, 20:80, 30:70, 40:60 and 50:50 % of CH_4 to O_2 (i.e. 0.20–1.6 CH_4/O_2 ratios) every 24 h for 20 days. CO_2 was not specifically added to the gas mix, because a bio-filter for the mitigation of CH_4 would be deployed, where exploitation for biogas production would become uneconomical due to low CH_4 concentration and increasingly aerobic conditions. Furthermore, CH_4

oxidation activity by the consortia will generate CO_2 the extent of which is unknown. Therefore adding CO_2 as a priori could jeopardise consortia establishment and productivity through CO_2 poisoning.

Biomass growth (OD_{600}) was monitored spectrophotometrically (Enspire-2300, PerkinElmer, USA) every two days and biomass protein was measured every five days using the Lowry method (TP0300, Sigma-Aldrich, Castle Hill, NSW, Australia). Dry-weight was measured every 5^{th} (5, 10, 15 and 20) day by harvesting 10 mL of culture sample and sample loss was compensated for by adding fresh NMS medium. Head space CH_4 samples were collected in vacuumed screw cap GC vials using an air tight syringe (Hamilton; 100 µL, Model 1710 RN, Grace Davison Discovery Science, Vic., Australia) and analysed by GC-TCD-FID. Methane removal efficiencies and MOC were calculated based on eq 2.1 and 2.2 as explained in section 2.2.3. FAME and PHB contents of the biomass was measured at day 5, 10, 15 and 20 as detailed in section 3.2.3. All results are expressed in $mg.g^{-1}DW_{biomass}$.

3.2.2 Molecular characterization.

Biomass of both LB and CB from the different CH_4/O_2 treatment samples collected on the 20^{th} day was used for genomic DNA extraction (gDNA). Microbial sequencing was carried out as explained in section 2.2.5. Forward sequences were used for the analysis. The read depth ranged from 14,248 to 197,900 reads for LB and 17,874 to 218,911 reads for CB. Sequences were rarefied to ~14,000 reads for LB and ~17,000 reads for CB (rarefied to the level of the sample with least number of reads in each biomass). Taxonomic assignment and clustering into OTUs was carried out in RDP classifier and mean abundance of the mixed consortia were prepared using Calypso, version 5.6 (http://cgenome.net/calypso/) as detailed in section 2.2.5. The raw sequences have been deposited in the European Nucleotide Archive (EMBL-EBI, UK) with the project accession of PRJEB10389 for LB

(http://www.ebi.ac.uk/ena/data/view/PRJEB10389) and PRJEB14087 for CB (http://www.ebi.ac.uk/ena/data/view/PRJEB14087) respectively.

3.2.3 Extraction and quantification of PHB and FAME

Sub-samples of 50 mL biomasses (LB and CB) from different CH_4/O_2 treatment samples were aseptically collected on 20th day and centrifuged (Eppendorf 5810 R, VWR International, QLD, Australia) at 3220 × g for 20 mins at 24 ˚C. The supernatants were discarded and the biomass pellets were frozen at -80 ˚C and freeze-dried (VirTis, Scitek, Vic., Australia) for PHB extraction (as detailed in (Pieja et al., 2011a)). In brief, 10 mg of freeze-dried biomass was weighed into clean screw cap extraction vials and extracted with 2 mL of acidified methanol (3 % v/v of sulphuric acid) containing 1 g.L^{-1} of benzoic acid and 2 mL.L^{-1} of chloroform. The vials were agitated gently and extracted at 100 ˚C for 3.5 h. After cooling at room temperature, 1 mL of triple-distilled deionized water was added to each vial. The vials were vortexed for 30-60 s and allowed to stand for phase separation. The organic phase was collected (100 µL) and filtered (through 0.2 µm, PTFE membrane, Agilent) for gas chromatography-mass spectrometry (GC-MS, Agilent 7890). For trans-esterification of fatty acids, biomass was solvent-extracted as above and trans-esterified following von Alvensleben et al. (2013).

The GC-MS (Agilent 7890GC – 5975MS, Vic., Australia) was fitted with a DB-23 capillary column (0.15 µm cyanopropyl stationary phase, inner diameter - 60 m × 0.25 mm ID) and equipped with FID. The split ratio was 1/50 and helium was used as the carrier gas. Injector, FID inlet and GC column temperatures were programmed following (David et al., 2002). For PHB analysis, a standard curve was prepared (range 0.1 - 6 mg) using pure PHB standards (Sigma-Aldrich, NSW, Australia). Fatty acids were quantified by comparison of peak areas of authentic standards (Sigma Aldrich, NSW, Australia). Benzoic acid and C19:0 was used as the internal standards for PHB and FAME analyses to correct for recovery and results were expressed in

$mg.g^{-1}$ $DW_{biomass}$.

3.2.4 Statistical analysis.

Statistical analyses used in Calypso are standard t-test and standard ANOVA, where the taxonomic units are normalized by total-sum normalization (TSS). Normalized data was compared by standard ANOVA. Figures show all taxa with significantly differential abundance between different CH_4 groups. A significance level of alpha=0.05 was applied. Pair-wise comparisons were done by standard t-tests of transformed and normalized taxonomic counts. P-values are annotated as *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

3.3 Results

3.3.1 Influence of CH_4/O_2 ratio on mixed consortia.

Proteobacteria dominated at all CH_4/O_2 ratios in both LB and CB (60-80 % abundance in LB and 60-70 % in CB) and the rest of the community belonged to the Bacteroidetes (20-35 %, for LB and 25-30 % in CB) (Figure 3.2). Irrespective of CH_4/O_2 ratios, the community structure showed a strong dominance of the genus *Methylosarcina* (type -I) in both LB and CB, however, the abundance pattern differed. In LB, 60-70 % of the abundance was retained in 20 % to 50 % CH_4 concentration, while abundances were only ~20 % at 10 % CH_4. In contrast, abundance of *Methylosarcina* was more or less constant (~45-55 %) for CB for all CH_4/O_2 ratios. In addition to *Methylosarcina*, the consortium also included other methanotrophs such as *Methylobacter*, *Methylomicrobium*, (type -I), *Methylocystis* and *Methylosinus* (type -II) at very low abundance levels (Table 3.1).

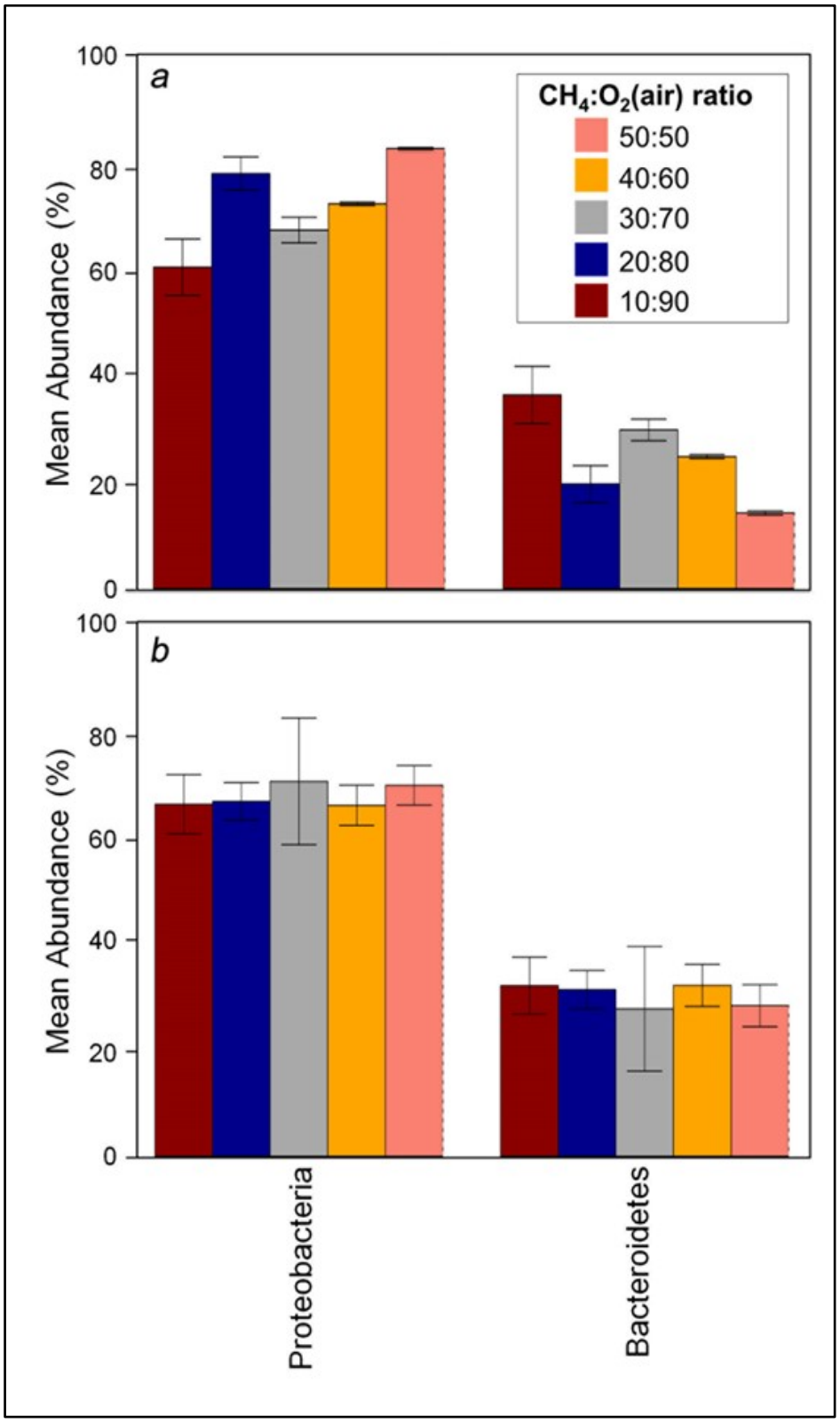


Figure 3.2 Influence of CH_4/O_2 ratio on the microbial diversity at phylum level a) Landfill biomass (LB); b) Compost biomass (CB)

Table 3.1 Methanotrophic strains detected in different CH_4/O_2 ratios

	10:90		20:80		30:90		40:60		50:50	
Methanotrophs	LB	CB	LB	CB	LB	CB	LB	CB	LB	CB
Methylosarcina	+	+	+	+	+	+	+	+	+	ND
Methylomicrobium	ND	ND	ND	+	+	+	ND	+	ND	ND
Methylobacter	+	+	+	+	+	+	ND	+	ND	ND
Methylocystis	+	+	ND	+	ND	+	+	+	+	+
Methylosinus	+	+	+	+	+	+	+	+	+	+

+ - Presence, ND - Not detected. Except *Methylosarcina*, rest other methanotrophic strains were detected in very low abundance (~1 %) in all CH_4/O_2 ratios; Landfill biomass (LB); Compost biomass (CB)

CH_4/O_2 ratios affected the abundance profiles of co-habiting non-methanotrophs. *Methylosarcina* abundance was followed by high abundances of *Chryseobacterium* in both LB (10-35 %) and CB (20-25 %) at all CH_4/O_2 ratios except at 10 % CH_4 in CB, where *Flavobacterium* (30 %) dominated and *Chryseobacterium* abundance was low (~1 %). In LB, other non-methanotrophs such as *Phenylobacterium and Pseudoxanthomonas* showed abundance of ~10-15 % in 10 % CH_4, but were not detected in considerable abundances at other CH_4/O_2 ratios, while *Hyphomirobium* abundance increased with CH_4/O_2 ratios (~2 to 5 %) (Figure 3.3a).

In contrast, *Phenylobacterium*, *Pseudoxanthomonas*, *Hyphomirobium* were not present or not detected in considerable abundances in CB. *Flavobacterium* dominated in 10 % CH_4, but abundances were lower (~5 %) and remained constant at other CH_4/O_2 ratios. Interestingly, the abundances of *Sphingopyxis* (~10 %) was not affected by varying CH_4/O_2 ratios, while *Pseudomonas* abundances oscillated ~1 to 10 % in CB, which did not correlate with CH_4/O_2 ratios (Figure 3.3b).

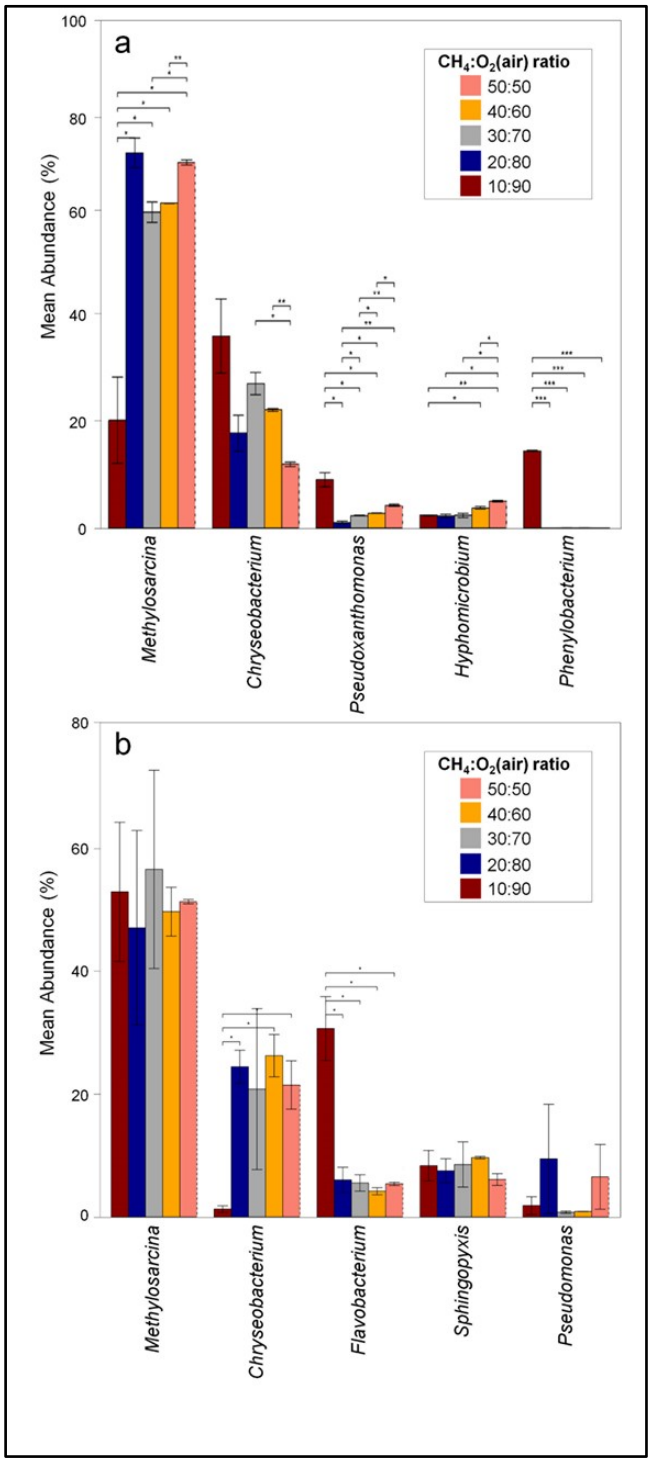


Figure 3.3 Influence of CH_4/O_2 ratio on the microbial diversity at genus level. a) Landfill biomass (LB); b) Compost biomass (CB). P-values are annotated as *: p<0.05; **: p<0.01; ***: p<0.001.

3.3.2 Influence of CH_4/O_2 ratio on methane removal efficiency and oxidation capacity.

CH_4 removal efficiency stabilised with time especially in CB (after 5-days), with little fluctuations in LB, but decreased with increasing CH_4 concentration (Figure 3.4a, b). The mixed consortia showed an average removal efficiency of ≤ 50 % for the 20-day cultivation period irrespective of CH_4/O_2 ratios in both LB and CB, except at 10 % CH_4 where a maximal removal efficiency of ~80 % was observed (Figure 3.4a, b). In contrast, MOCs increased with increasing CH_4 concentration (Figure 3.4c, d) with average MOCs of 3.0±0.12, 4.2±0.26, 6.9±0.7, 7.6±1.3 and 9.2±1.2 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ for LB and 2.9±0.04, 5.05±0.32, 6.7±0.31, 7.9±0.61 and 8.6±0.48 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ for CB at 10 %, 20 %, 30 %, 40 % and 50 % CH_4, respectively for the 20 day cultivation period. Comparatively, LB showed better MOC than CB with a maximum MOC of 12.04±1.2 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ at 50 % CH_4 on day 5. The biomass dry-weight of LB and CB at all CH_4/O_2 ratios were more or less similar, with average dry-weight of 1.51±0.12, 1.55±0.11, 1.54±0.08, 1.58±0.09, 1.52±0.09 $g.L^{-1}$ for LB and 1.31±0.10, 1.33±0.12, 1.33±0.06, 1.34±0.09, 1.34±0.08 $g.L^{-1}$ for CB at 10 %, 20 %, 30 %, 40 % and 50 % CH_4, respectively for a 20 day cultivation period. Comparatively, LB showed higher biomass dry-weight than CB (Figure 3.4e, f). In contrast culture optical density (OD_{600}) increased with time in both LB and CB of CH_4/O_2 ratios (Figure 3.5a, b).

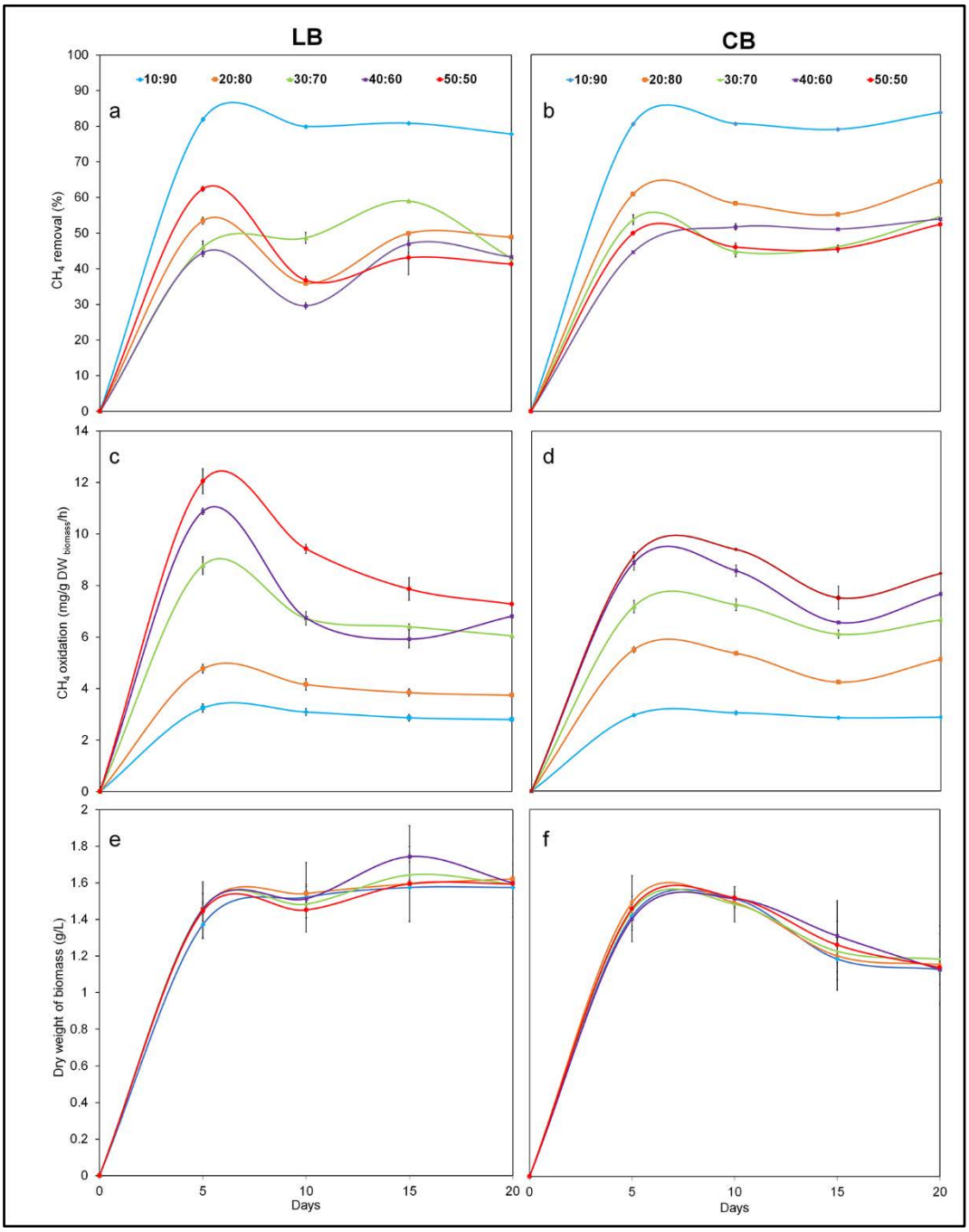


Figure 3.4 Influence of CH_4/O_2 ratio on (a and b) CH_4 removal efficiency; (c and d) CH_4 oxidation capacity; (e and f) dry-weight of landfill biomass (LB) and compost biomass (CB) respectively.

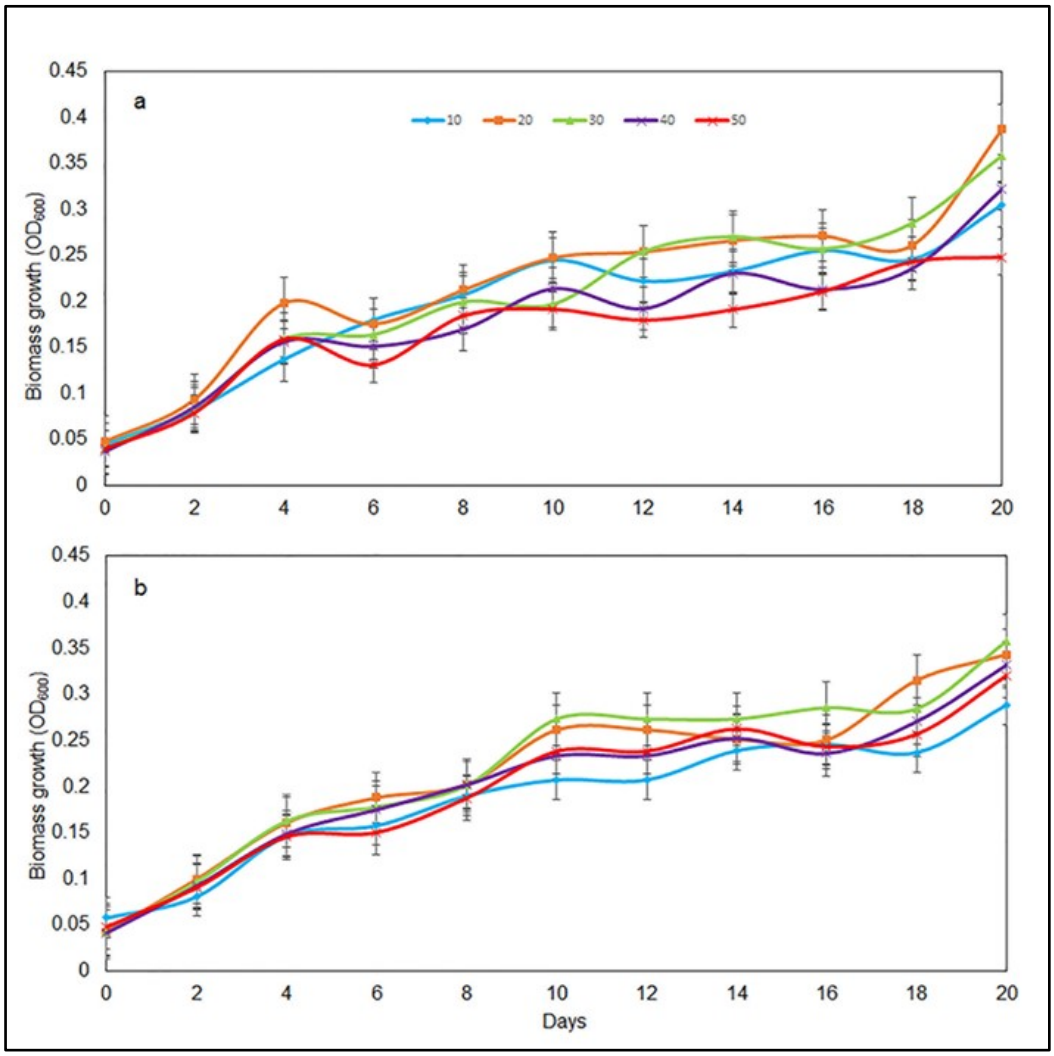


Figure 3.5 Biomass growth – OD_{600} for a) Landfill biomass (LB); b) Compost biomass (CB)

3.3.3 Influence of CH_4/O_2 ratios on FAME profiles and biopolymer content

FAME profiles and total fatty acid contents varied slightly between LB and CB, where LB showed a higher FAME content than CB except at 10 % CH_4. A maximum and minimum FAME content was observed at 20 % and 10 % CH_4 for LB and 10 % and 50 % CH_4 for CB (Figure 3.6).Total FAME content ($mg.g^{-1}.DW_{biomass}$) at day 20 for LB and CB was 32.5±3.1, 39.9±0.8, 35.1±0.9, 38.1±0.2, 34.9±0.2 and 40.5±0.8, 33.0±2.1, 32.6±0.8, 32.6±1.1, 31.1±1.2 at 10 %, 20 %, 30 %, 40 % and 50 % CH_4. In this study, relatively high amounts of $C_{16:1}\omega7c$ and $C_{16:1}$ ω9c (~70 %) were observed in all CH_4/O_2 ratios, while amounts of $C_{16:0}$ (~15 %) and $C_{18:1}$ ω9c (~10 %) remained low

in both LB and CB. Other than the above mentioned fatty acids, $C_{14:0}$ and $C_{15:0}$ fatty acids were also detected in low amounts (~3 %) at all CH_4/O_2 ratios in both LB and CB (Figure 3.6).

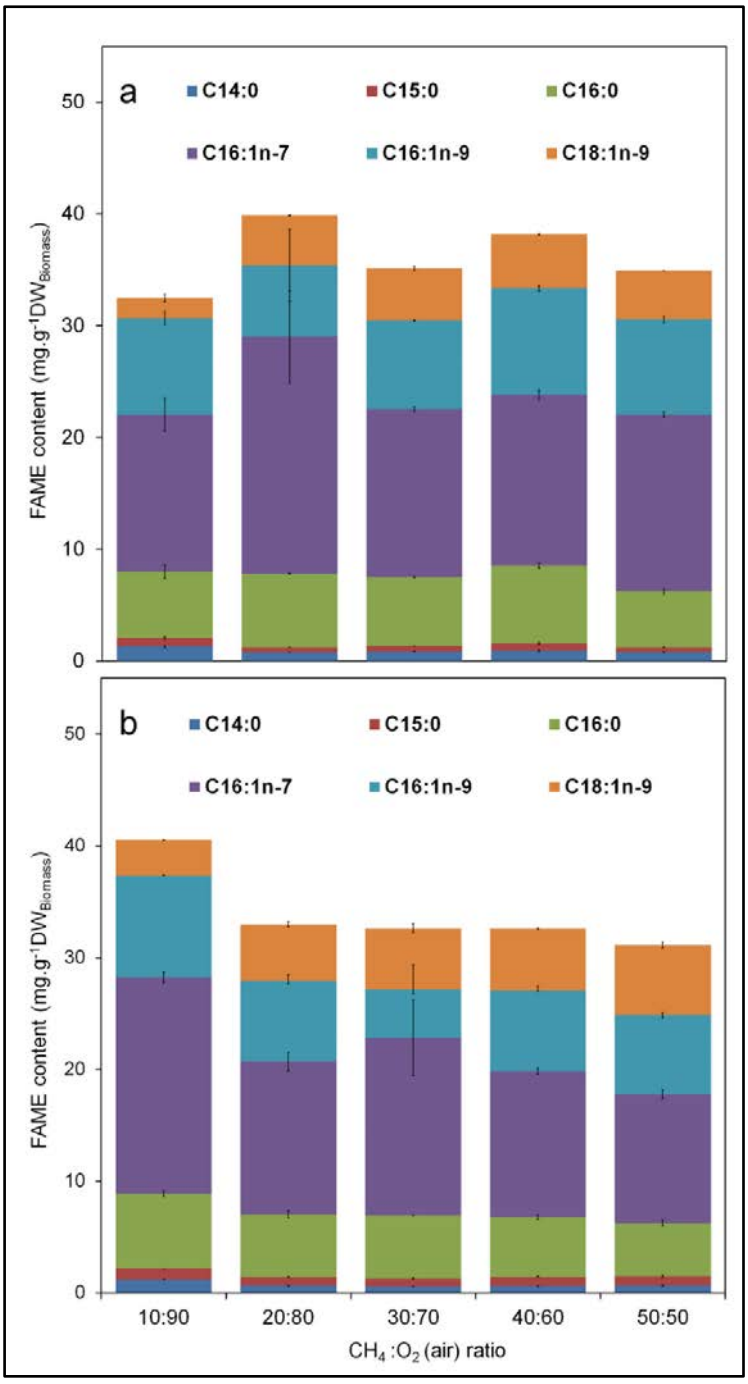


Figure 3.6 Influence of CH_4/O_2 ratio on FAME content a) Landfill biomass (LB); b) Compost biomass (CB) .

Maximal PHB contents achieved in my study were 24.6±0.7 (2.5 %) mg PHB.g^{-1} $DW_{biomass}$ at 40 % CH_4 for LB and 14.2±0.9 (1.4 %) mg PHB.$g^{-1}DW_{biomass}$ at 50 % CH_4 for CB (Figure 3.7). Comparatively, LB showed much higher PHB content than CB with increasing accumulation being observed up to 40 % CH_4 (up to 2.5 % in LB) which declined sharply (1.2 %) at 50 % CH_4. Minimal PHB contents of ~3.4±0.17 (0.3 %) mg PHB.g^{-1} $DW_{biomass}$ were observed at 10 % CH_4 in both LB and CB.

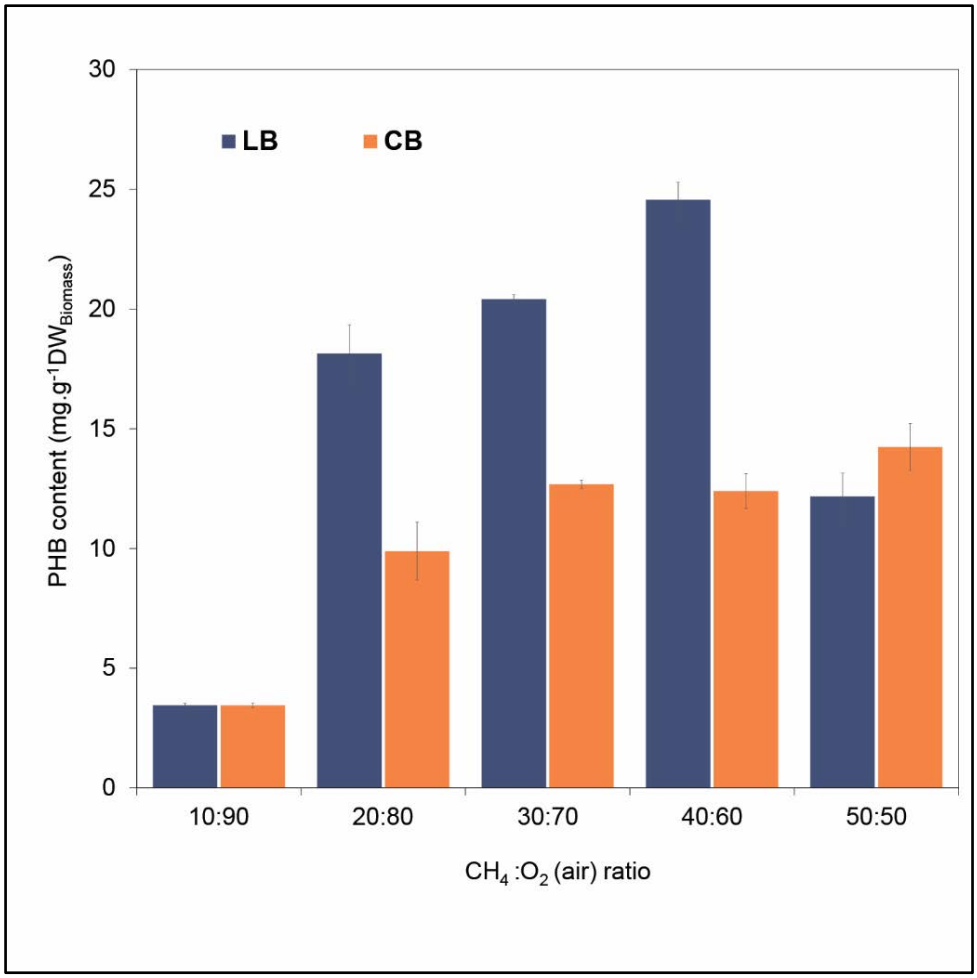


Figure 3.7 Influence of CH_4/O_2 ratio on PHB accumulation potential of Landfill biomass (LB) and Compost biomass (CB).

3.4. Discussion

Dominance of *Methylosarcina* has commonly been reported in landfill top cover and compost soils (Henneberger et al., 2012; Kim et al., 2013; Seghers et al., 2003; Su et al., 2014), suggesting that this might be a common genus. The ability to form cysts and produce capsules (diffuse slime layers) (Henneberger et al., 2012) enhances the survival of *Methylosarcina*, making them key players for CH_4 oxidation even in environments like landfills. Other factors such as neutral pH of the culture medium (Bowman, 2014; Henneberger et al., 2012; Su et al., 2014) and higher nutrient availability may have also contributed to sustain dominance of *Methylosarcina* in both LB and CB (Hernandez et al., 2015). This is the first study that observed a resilience of *Methylosarcina* under varying CH_4/O_2 ratios in both LB and CB (Figure 3.3) which could be explained by survival strategy. Generally, type -II methanotrophs outcompete type -I methanotrophs at high CH_4 concentrations. pH of the culture media was likely slightly acidic through the production of CO_2 during CH_4 oxidation which at high CH_4 concentration should have additionally favoured establishment of type -II methanotrophs. Here, however, the strong dominance of *Methylosarcina* was observed for all CH_4/O_2 ratios in both LB and CB except at 10 % CH_4 in LB, where abundances were slightly lower. As the biomass for both soil types (LB; aged cover soil and CB; fresh cover) were subjected to the same enrichment conditions for 100 days, it is unlikely that soil-inherent compositional structures and length of exposure to landfill conditions would have carried over into the different responses to 10 % CH_4 observed here for the two soil types (Figure 3.3). Likewise, oxygen levels experienced by the bacteria would be comparable. The most likely explanation is that the mother culture from CB had a higher abundance of methanotrophs (Figure 2.9) and there is the distinct possibility that species/strain differences with regards to oxygen tolerance and abundance patterns of other heterotrophs could have also influenced observed differences in abundance patterns. There are few other studies reporting the

dominance of type -I methanotrophs under variable CH_4/O_2 ratios. Similarly to our results, Henckel et al. (2000), studied the effect of CH_4 and O_2 concentrations on methanotrophic community structure in soil, also reported persistence and dominance of type -I populations under high CH_4 and low O_2 concentration. Using a denaturing gradient gel electrophoresis (DGGE) approach, López et al. (2014) studied the effects of different CH_4/O_2 ratios on a mixed consortium enriched from landfill cover soil and activated sludge and found that type -I methanotrophs (*Methylosarcina, Methylomicrobium, Methylosoma* and *Methylobacter*) completely dominated the community structure. Enrichment of type -I methanotrophs was interpreted to be due to high copper concentrations present in the medium. This is in line with our results, as the soils used for enrichment in this study also showed high total nitrogen and copper contents. In contrast, a similar study carried out with a different consortium enriched from marine soil showed dominance of the type -II methanotroph *Methylocytis* irrespective of CH_4/O_2 ratios (Karthikeyan et al., 2015b), which was potentially due to rather different physio-chemical characteristics of the soil (low total nitrogen and high iron content). This study demonstrates that type -I methanotrophs can outcompete type -II methanotrophs in nitrogen-rich conditions irrespective of CH_4/O_2 ratios.

Continuous enrichment changed abundance patterns for *Pseudoxanthomonas* in LB and *Methylophaga* in CB, which were dominant in mother cultures (Figure 2.9), but declined strongly under the treatment conditions here (Figure 3.3). After enrichment, at 10 % CH_4, LB showed a higher abundance of the co-habiting *Chryseobacterium* (35 %), whereas CB showed a higher abundance of *Flavobacterium* (30 %), but only 1 % of *Chryseobacterium*. This swapping pattern between dominance of *Chryseobacterium* and *Flavobacterium* could indicate that these two genera occupy the same ecological niche, with *Chryseobacterium* typically outcompeting *Flavobacterium*. *Chryseobacterium* and *Flavobacterium*, belong to the same phylum Bacteroidetes, and *Chryseobacterium* was previously classified as

Flavobacterium, until Vandamme et al. (1994) delineated the two genera based on characteristic differences. Abundances of non-methanotrophic bacteria (*Chryseobacterium*, *Phenylobacterium*, *Pseudoxanthomonas* in LB and *Flavobacterium* and *Pseudomonas* in CB (Figure 3.3b) were variable at higher CH_4 concentrations but generally lower than at 10 %, except for *Chryseobacterium* and *Sphingopyxis* in CB, which remained constant (Figure 3.3b). Higher CH_4 levels decrease availability of O_2, potentially restricting growth of heterotrophs (Hršak and Begonja, 2000). Even though abundance changes of *Hyphomicrobium* were not significant in LB, increase in abundance with increasing CH_4 concentration was observed (Figure 3.3a). This may be explained by their ability to utilize methanol, a metabolite that could accumulate during CH_4 oxidation, allowing it to persist and grow at low O_2 concentrations (Poindexter, 2006). A patent study by Quan et al. (2012) suggested that *Chryseobacterium* sp. JT03 isolated from a landfill was able to survive and degrade high concentrations of CH_4 (40 %). To date, no other studies reported the prevalence of *Chryseobacterium* in correlation with methanotrophic activity/ CH_4 metabolism, but Lee et al. (2012) reported that *Sphingopyxis* exhibited methanotrophic activity when grown in CH_4 as a sole carbon source. In my study, *Sphingopyxis* was not abundant except in CB (Figure 3.3b). The presence of these co-habiting bacteria *Chryseobacterium*, *Sphingopyxis*, *Flavobacterium*, *Phenylobacterium*, *Pseudoxanthomonas* and *Hyphomicrobium* could be of benefit in landfill bio-filter operations, as they have been reported to degrade many polycyclic aromatic hydrocarbons present in landfills (Bernardet et al., 2006; Eberspächer and Lingens, 2006; Poindexter, 2006). In particular, *Chryseobacterium* strains have potential applications in the clean-up of various environmental contaminants like pentachlorophenol, aniline and carbofuran (Bernardet et al., 2006), and *Hyphomicrobium* has been shown to promote the growth of methanotrophs by removing excess methanol (Poindexter, 2006).

The established consortia (LB and CB) showed an average removal efficiency of ≤ 50 % at all CH_4/O_2 ratios, except at 10 % CH_4. It appears that the dissolution of CH_4 into the medium under batch cultivation conditions could be the limiting factor negatively affecting CH_4 removal efficiencies at higher CH_4 concentrations. In addition, at 10 % CH_4, higher amounts of O_2 could have enabled more efficient methane utilization (Van Bodegom et al., 2001). This is consistent with other reports (Karthikeyan et al., 2015b; Wei et al., 2015). While MOC initially positively correlated with methane concentrations on day 5, it did not change significantly with culture time for 10 and 20 % CH_4, but dropped significantly for the higher CH_4 concentrations. Again, limited CH_4 dissolution in batch cultivation could have negatively affected MOCs. This is supported by MOC being not significantly different for 30-50 % methane after 20 days of cultivation. Irrespective of the biomass concentration, CH_4 consumption was stable and high. At low O_2 concentrations, methanotrophs have been reported to switch to fermentation mode leading to formation of more organic compounds like formate, pyruvate, acetate, succinate, lactate as end products and convert only a small amount of CH_4 to biomass (Kalyuzhnaya et al., 2015) which would explain our data. For the first five days, the sharp increase in CH_4 removal efficiency and MOC correlated with strong biomass increase (Figure 3.4e, f). Biomass increase was not correlated to CH_4/O_2 ratios and MOC, suggesting a maximal utilization for growth at 10 % CH_4 concentrations. Thereafter, biomass and MOC increases were not observed, supporting the conclusion that surplus CH_4 carbon was no longer utilized for growth with observed MOC responses likely reflecting an increased contribution *via* fermentation. In contrast to LB, CB decreased after 10 days for all CH_4 concentrations, while culture optical density (OD_{600}) increased with time (Figure 3.5). The increase in OD_{600} can possibly be explained by increases in extracellular polymeric substances, which has been reported in other studies (Karthikeyan et al., 2015b; López et al., 2014). As biofilm formation was observed in our reactors, the accuracy of the

quantified growth by biomass dry-weight and OD_{600} could be limited. In any case, CH_4 and O_2 diffusion has been reported to be obstructed in dense biofilms, making significant contributions of the biofilm biomass to MOC unlikely.

The reported MOC of type -I-dominated consortia in LB and CB was higher at all tested CH_4/O_2 ratios (1.64 ± 0.12, 2.56 ± 0.43, 2.34 ± 0.41, 6.54 ± 1.23. 6.64 ± 0.45 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ at 10 %, 20 %, 30 %, 40 % and 50 % CH_4) than reported MOC of type -II methanotroph-dominated mixed consortium enriched from marine soil (Karthikeyan et al., 2015b). Some type -II methanotrophs like *Methylocytis* sp. and *Methylosinus* sp. possess both pMMO and sMMO with expression of these enzymes correlating with low and high CH_4 concentrations, as pMMO has a higher affinity for CH_4 than sMMO (Semrau et al., 2013). Irrespective of soil type used in this study, *Chryseobacterium* was always abundant along with methanotrophs, a pattern that was maintained in subsequent sub-culturing tests. Under our experimental conditions, we are the first to report higher MOC in *Chryseobacterium*-assisted type -I methanotroph consortia. As reported by Lee et al. (2012) horizontal gene transfer of CH_4 oxidation genes to co-habiting heterotrophs can occur. For example, *Sphingopyxis* sp.MD2 exhibited methanotrophic activity and expressed pMMO after co-cultivation with methanotrophs, when growing re-isolates with CH_4 as a sole carbon source. Such gene transfer mechanism may also explain the higher MOC in our *Chryseobacterium*-assisted type -I dominated consortium.

FAME profiles and total fatty acid contents were not affected by CH_4/O_2 ratios used (Figure 3.6). Compared to heterotrophs, methanotrophs produce higher amounts of C_{16} and C_{18} unsaturated PLFA (Bodelier et al., 2009). Specifically, the dominance profile of fatty acid chain length differs between type -I and -II methanotrophs, with the former mainly synthesizing the saturated and monounsaturated fatty acids (MUFA) $C_{16:0}$ and $C_{16:1}$, respectively, while the latter produce predominantly C_{18}:$_1$ MUFA (Bowman, 2014). Hence, PLFA analysis can be used as a marker for abundance shifts

in type -I and -II methanotrophs within a mixed consortium. High amounts of $C_{16:1}$ observed in all CH_4/O_2 ratios in both LB and CB, supports the 16s rRNA gene analysis-based dominance of type -I methanotrophs. *Chryseobacterium* has been reported to accumulate mostly with higher proportion of $C_{15:0,}$ and low proportion of $C_{16:0,}$ $C_{17:0}$ and $C_{17:1}$ fatty acids and not $C_{16:1}\omega7c$ or $C_{18:1}$ ω9c (Bernardet et al., 2006; Boone et al., 2011; Vandamme et al., 1994), while *Methylosarcina* can form $C_{16:0}$ (10–20 %) and $C_{16:1}$ fatty acids with isomers of $C_{16:1}\omega8c$ (14–34 %), $C_{16:1}\omega7c$ (18–32 %), $C_{16:1}\omega6c$ (7–9 %) and $C_{16:1}\omega5t$ (19–30 %) (Bowman, 2014; Wise et al., 2001) supporting the high amounts of $C_{16:1}\omega7c$ (>50 %) observed. Presence of $C_{16:1}\omega9c$, however, may be due to the other methanotrophs or heterotrophs present in the consortium. Increasing CH_4 concentrations were expected to lead to higher amounts of $C_{18:1}$ fatty acids, as observed by (Henckel et al., 2000) and (Karthikeyan et al., 2016b), reflecting the dominance of the type -II methanotroph *Methylocystis* in the latter study. C_{14}, C_{15} PLFA contributed less than ~5 % to the total fatty acid content irrespective of CH_4/O_2 ratios, which might represent a contribution by non-methanotrophs co-habiting the methanotrophs.

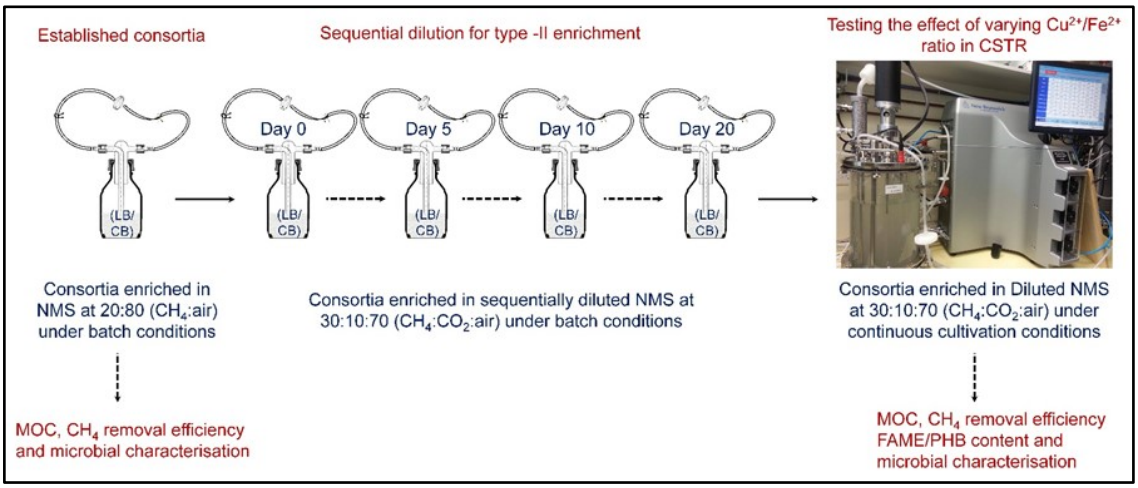
Generally, type -II methanotrophs accumulate PHB while this is not a defining characteristic of type -I (Pieja et al., 2011a). It is therefore not surprising that PHB accumulation was low. The achieved PHB in both LB and CB was low compared to contents achieved in a type -II *Methylocystis*-dominated mixed consortium (Karthikeyan et al., 2015b). This can be explained by the dominance of the type -I methanotroph *Methylosarcina*. Wise et al. (2001) reported internal PHB inclusions in cells of *Methylosarcina* sp., making it even more difficult to assign PHB production to specific bacterial groups seen in our consortia. It is possible that type -II methanotrophs (*Methylosinus*/ *Methylocystis*) present in LB and CB were primarily responsible for the observed low PHB accumulation. However, contributions by certain

non-methanotrophs (e.g. *Pseudomonas, Sphingopyxis and Hyphomicrobium*) to the PHB content cannot be ruled out (Poindexter, 2006).

3.5 Major findings from Chapter 3

Overall, while the mixed consortia structure was surprisingly stable in both LB and CB under different CH_4/O_2 ratios, the heterotrophic population changed significantly in response to changing CH_4/O_2 ratios for both LB and CB. The general stability and unprecedented interactions between *Methylosarcina* and *Chryseobacterium* offer a strong potential for development as a continuous bio-filter for sustainable landfill CH_4 mitigation, particularly for CB, where dominance profiles were least affected by CH_4/O_2 ratios above 10 % CH_4. We expected, type -II methanotrophs will dominate at higher CH_4 concentrations with possible increase in PHB content. But the observed ~3 % (w/w) PHB content of the mixed consortia was lower than expected but in line with the type -I methanotroph dominance. However, PHB accumulation of the mixed consortia could be improved by selecting conditions favouring dominance of type -II methanotrophs, such as low pH and/or inducing nutrient-limitation and by adding copper and iron at ratios supportive of sMMO expression (Chidambarampadmavathy et al., 2015a; Karthikeyan et al., 2015a). Optimising pH, nutrients, copper to iron ratios could change the mixed community structure in favour of type -II methanotrophs dominance, potentially providing an economic incentive for landfill CH_4 bio-filter remediation and biopolymer development. Therefore, in chapter 4, to enrich type -II methanotrophs and thereby improve PHB accumulation potential, the established mixed consortia (LB and CB) were sequentially enriched in nutrient-deficient conditions under high CH_4 concentrations (30 %) along with CO_2 (10 %) (CO_2 as a gas or as bicarbonate reported to favour type -II growth) and were then then subjected to varying Cu^{2+} and Fe^{2} ratios to activate pMMO and/or sMMO activity for improving PHB accumulation.

[3]Chapter 4: Responses of mixed consortia to variable Cu^{2+}/Fe^{2+} ratios



Graphical Abstract 3 Responses of mixed consortia to different Cu^{2+}/Fe^{2+} ratios - chapter 4 overview

[3] This chapter represents the combined data from the below publications with text verbatim as per Chidambarampadmavathy et al. (2017) Journal of Environmental Management. Lead over statements to the general discussion have been included:

Chidambarampadmavathy K, Karthikeyan OP, Huerlimann R, Maes EG & Heimann K (2016) Responses of mixed methanotrophic communities to variable Cu^{2+}/Fe^{2+} ratios. Journal of Environmental Management 197: 159-166 (https://doi.org/10.1016/j.jenvman.2017.03.063).

A fraction of this data has been published as

Chidambarampadmavathy K, Karthikeyan OP & Heimann K (2015) Biopolymers made from methane in bioreactors. Engineering in Life Sciences 15: 689-699 (10.1002/elsc.201400203).

To avod repeating materals and methods, methods previously described in thesis have been removed and have been referred to appropriate sections and has been reformatted to fit the format of this thesis.

4.0 Abstract

Methane mitigation in landfill top cover soils is mediated by methanotrophs whose optimal methane (CH_4) oxidation capacity is governed by environmental and complex microbial community interactions. Optimization of CH_4 remediating bio-filters need to take microbial responses into account. Divalent copper (Cu^{2+}) and iron (Fe^{2+}) are present in landfills at variable ratios and play a vital role in MOC and growth of methanotrophs. This study, as a first of its kind, therefore quantified effects of variable Cu^{2+} and Fe^{2+} (5:5, 5:25 and 5:50 µM) ratios on the established mixed consortia (LB and CB)). MOC, CH_4 removal efficiencies, FAME content/profiles and PHB contents were also analysed to quantify performance and potential co-product development. Mixed cultures were raised in 10 L continuous stirred tank reactors (CSTRs, Bioflo® & Celligen® 310 Fermentor/Bioreactor; John Morris Scientific, Chatswood, NSW, Australia). Community structure was determined by amplifying the V3-V4 region of 16s rRNA gene. Community structure and, consequently, fatty acid-profiles changed significantly with increasing Cu^{2+}/Fe^{2+} ratios, and responses were different for LB and CB. Effects on MOC and PHB contents were similar in the LB- and CB-CSTR, decreasing with increasing Cu^{2+}/Fe^{2+} ratios, while biomass growth was unaffected. In general, high Fe^{2+} concentration favoured growth of the type -II methanotroph *Methylosinus* in the CB-CSTR, but methanotroph abundances decreased in the LB-CSTR. Increase in Cu^{2+}/Fe^{2+} ratio increased the growth of *Sphingopyxis* in both systems, while *Azospirllum* was co-dominant in the LB- but absent in the CB-CSTR. After 13 days, MOC and PHB content decreased by ~50 % and more in response to increasing Fe^{2+} concentrations. Although methanotroph abundance was ~2 % in the LB- (compared to >50 % in CB-CSTR), MOC were comparable in the two systems, suggesting that MOC was maintained by the dominant *Azospirllum* and *Sphingopyxis* in the LB-CSTR. Despite similar methanotroph inoculum community composition and controlled environmental variables, increasing Cu^{2+}/Fe^{2+} ratios resulted in significantly

different microbial community structures in the LB- and CB-CSTR, indicative of complex microbial interactions. In summary, our results suggest that a detailed understanding of allelopathic interactions in mixed consortia is vital for constructing robust bio-filters for CH_4 emission abatement.

4.1. Background

Methanotrophs are physiologically distinct proteobacteria capable of oxidizing CH_4, the second largest greenhouse gas. The CH_4 to CO_2 conversion usually takes place *via* two different pathways i.e. ribulose monophosphate (type -I) and/or serine (type -II) pathways depending on the type of methanotrophs (Hanson and Hanson, 1996). To date, two different types of methanotrophs have been classified, a gammaproteobacterial Type -I (subtypes are Type -Ia, Type -Ib and Type -Ic) belonging to the family of Methylococcaceae (genera include *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylogaea*, *Methyloglobulus*, *Methylomagnum*, *Methylomarinum*, *Methylomicrobium*, *Methylomonas*, *Methyloparacoccus*, *Methyloprofundus*, *Methylosarcina*, *Methylosoma*, *Methylosphaera*, *Methylovulum*, *Methylothermus*, and *Methylohalobius*) and Methylothermaceae family (genera include *Methylohalobius*, *Methylomarinovum*, and *Methylothermus*) and alphaproteobacterial Type -II, belonging to the family Methylocystaceae (genera include *Methyloferulla*, *Methylocystis* and *Methylosinus*) and Beijerinckiaceae (genera include *Methylocapsa* and *Methylocella*) (Bowman, 2014; Chidambarampadmavathy et al., 2015a; DiSpirito et al., 2016; Hanson and Hanson, 1996; Webb et al., 2014). Type -II methanotrophs assimilate CH_4 *via* the serine pathway, are known for their capacity to assimilate atmospheric nitrogen, to accumulate biopolymers (PHB), and to utilize CO_2 (~60 % of oxidized CO_2 is reutilized in the serine cycle) for cellular growth (Karthikeyan et al., 2015a; Pieja et al., 2011a; Strong et al., 2016; Wendlandt et al., 2010; Yang et al., 2013). Re-routing CH_4 as a carbon source for methanotrophs to produce PHB can be an economic and environmentally friendly greenhouse gas remediation approach.

Theoretically, methanotrophs yield ~60-70 % PHB, which is higher than for other commercially available heterotrophic bacteria (Karthikeyan et al., 2015a; Rostkowski et al., 2012; Wendlandt et al., 2010). PHB accumulation is primarily driven by macro nutrient-deficiency (nitrogen, potassium, phosphate), but trace metals like

copper and iron can also indirectly increase PHB accumulation capacity (Helm et al., 2008; Pieja et al., 2011a; Pieja et al., 2011b, 2012; Wendlandt et al., 2010). Copper and iron regulate the activity of MMOs, enzymes which mediate the first step in CH_4 oxidation. Higher MMO activities are positively correlated with PHB accumulation, in nitrogen-limited cultures, whilst actively growing cultures utilize CH_4 for growth (Zhang et al., 2008). Methane monooxygenases exist in two forms, a Cu^{2+}-dependent particulate form (pMMO) and an iron-dependent soluble form (sMMO) (DiSpirito et al., 2016). In general, all methanotrophs express pMMO, but the ability to express sMMO is observed only in very few type -II and type -Ia methanotrophs (Chidambarampadmavathy et al., 2015a; DiSpirito et al., 2016). In methanotrophs that possess both forms of MMO, the expression of sMMO is regulated by extracellular Cu^{2+} concentration. At low Cu^{2+} concentrations (<1 µM), sMMO activity was detected at a biomass of 0.8 $gDW_{biomass}.L^{-1}$, while at high Cu^{2+} concentrations, pMMO activity was initially high, while sMMO activity was induced when medium Cu^{2+} levels were <1 µM (Hanson and Hanson, 1996; Sullivan et al., 1998). In addition to low Cu^{2+} concentrations, sMMO activity can be further increased by increasing Fe^{2+} concentrations (>30 µM) and using continuous cultivation (Begonja and Hršak, 2001; Morton et al., 2000). It needs to be considered though that the reported individual metal ion concentrations for pMMO and sMMO expressions are for pure methanotroph cultures, and concentrations, as well as ratios, may vary for the expression and activity of MMOs in mixed culture. The individual effect of Cu^{2+} and Fe^{2+} on MMO expression has been studied extensively, while only a few studies investigated effects of Cu^{2+} and Fe^{2+} concentrations on growth and PHB accumulation in methanotrophs. Compared to pure methanotroph cultures, physiological growth conditions are improved in mixed cultures, as some co-habiting bacteria can utilize toxic by-products of CH_4 oxidation, e.g. methanol, formaldehyde etc.(Beck et al., 2013; Ho et al., 2016; Ho et al., 2014). In mixed cultures, however, competition for trace elements exists, which can lead to poor

CH_4 oxidation rates and methanotroph growth. The combined effect of Cu^{2+}/Fe^{2+} ratios on CH_4 oxidation, PHB accumulation, and specifically on community structure of mixed consortia has not been investigated to date. Since, the use of mixed cultures has been proposed as a cost-effective approach for large-scale biopolymer production in industrial settings (Wendlandt et al., 2010), the present study investigated the

i. interactive effect of Cu^{2+}/Fe^{2+} on growth and community structure of mixed consortia enriched from landfill (LB) - and compost (CB) cover soils along with
ii. MOCs, FAME contents and profiles, and PHB accumulation capacities under varying Cu^{2+}/Fe^{2+} ratios.

4.2. Materials and Methods

4.2.1. Inoculum-enrichment and growth conditions

The inoculum used in this experiment was a mixed community, enriched from a top cover soil taken from a 7 year-old landfill and a 6 week-old compost soil (refer section 2.2.1 and 2.2.2).The microbial community obtained after enrichment from landfill (LB) and compost soils (CB) were then subjected to periodic nutrient stress for 20 days to enrich type -II methanotrophs in a 500 mL mini-bench top reactors (Schott-Duran gas-wash bottle, VWR International, QLD, Australia) with a working volume of 200 mL NMS medium.50 mL of the grown culture medium was replenished with sterile 50 mL of diluted nitrate mineral salts (DNMS, 1:5 dilution of NMS) medium every 5^{th} day of the cultivation cycle to induce nutrient-depletion. During the nutrient-stress cultivation period, the head space of the mini-bench-top reactors were purged with 30:10:60 of CH_4:CO_2:air (vol. %) every 24 h, continuously stirred at 200 rpm by magnetic stirrer and the reactor bottles were incubated under 25°C. Biomass obtained on day 20 (2.5 % cells v/v) was then used as an inoculum for testing the interactive effect of Cu^{2+}/Fe^{2+} on PHB, MOC and microbial diversity in a continuous stirred tank reactor (CSTR).

4.2.2. Fate of Cu^{2+}/Fe^{2+} ratio in continuous stirred tank reactor

Two 15L CSTRs (BioFlo®310, Eppendorf, New Brunswick, USA) were operated aseptically as continuous batch reactors (Figure 4.1). Containers, tubing and ports, and media were autoclaved prior to use. Gases were filter-sterilized before supply to the reactors. Reactor temperature was maintained at 25ºC, while pH was not controlled, but monitored with an inline pH meter. Gas flows of CH_4, CO_2, and air into the reactors (at a ratio of 30:10:60) were controlled by mass flow controllers (Red-y, Vogtlin, WI, USA) calibrated in the range of 0–500 $mL.min^{-1}$ for the respective gas delivered at a final flow rate of 250 $mL.min^{-1}$ (Dywer instruments, Pty. Ltd., NSW, Australia).

Figure 4.1 Continuous stirred tank reactor

The gas was injected from the bottom into the medium via a u-shaped 1cm diameter ring sparger of 6 cm outer and 5 cm inner diameter with 9 central holes with a diameter of 1.5 mm, each. This ring sparger provided the gas mixture to a 15 L vessel of a height of 43 cm with culture fill height of 32 cm. Reactor content was stirred at 200 rpm throughout the experiment. The inoculum (2.5 % cells v/v) of LB and CB obtained after the nutrient-stress was transferred separately to the two 15 L CSTRs (LB-CSTR and CB-CSTR) with a working volume of 10 L of DNMS medium (Dedysh and Dunfield, 2011; Dunfield et al., 2003). The reactors were continuously supplied with CH_4:CO_2:air at the ratio of 30:10:60 and the reactors were operated for a period of 20

days. While gas supply was continuous, operation of the CSTRs was in batch mode. Every fifth day (5^{th} day, 10^{th} day, and 15th day), 2 L of the culture broth were replenished with 2 L of sterile DNMS medium and Cu^{2+} (as $CuSO_4$) and Fe^{2+} (as $FeSO_4$) was added at a concentration of 5:5 µM on day 5, 5:25 µM on day 10 and 5:50 µM on day 15 for both reactors (LB- and CB-CSTR). Biomass samples were taken from both CSTRs on days 5, 10, 15 and 20 from 2 L harvested medium to determine growth rate, Cu^{2+} and Fe^{2+} content, PHB accumulation and FAME profiles as detailed in section 3.2.3. The amount of CH_4 oxidized was measured using a quadrupole mass spectrometer (MKS Instruments, Inc. MA, USA), CH_4 removal efficiency and the MOCs of the biomass were calculated based on eq 2.1 and removal efficiency was calculated based on eq 2.2 as detailed in section 2.2.3. Total DNA was extracted from samples taken and 16S rRNA gene sequencing was performed to examine community composition and changes in response to Cu^{2+} and Fe^{2+} fertilisation.

4.2.3 DNA extraction and 16S rRNA gene sequencing.

Biomass collected after day 5, 10, 15 and 20 from the LB-CSTR- and CB-CSTR was used for extracting genomic DNA (gDNA). Microbial sequencing was carried out as explained in section 2.2.5. Raw reads were treated for quality analysis, read depth ranged from 58,688 to 202,835 reads for LB-CSTR and 34,315 to 168,883 for CB-CSTR. After trimming, sequences were rarefied to 29,000 reads for LB-CSTR and 25,000 reads for LB-CSTR (rarefied to the level of the sample with least number of reads in each biomass). Taxonomic assignment and clustering into OTUs was carried out using RDP and mean abundance of the mixed consortia were prepared using Calypso, version 5.6 (http://cgenome.net/calypso/) as detailed in section 2.2.5. The raw sequences have been deposited in the European Nucleotide Archive (EMBL-EBI, UK) with the project accession of PRJEB13407 (http://www.ebi.ac.uk/ena/data/view/PRJEB13407).

4.2.4. Growth estimation of the mixed consortium

Biomass concentration was determined using optical density and gravimetrically by dry-weight. Biomass concentration was quantified on a UV/VIS spectrophotometer (OD_{600}) (Enspire-2300, PerkinElmer, USA) with NMS or DNMS (as per sample origin) as blanks; and biomass standard curves. In addition, total protein was determined spectrophotometrically (OD_{600}) using the Micro Lowry method following the manufacturer's instructions (Peterson's method, TP0300, Sigma-Aldrich, Castle Hill, NSW, Australia). For dry-weight, 10 mL of cell suspension was taken in a pre-dried beaker, and the biomass was dried at 105℃ for 48 h. Ash free fry weight was calculated by ashing at 500ºC in muffle furnace overnight. Weight differences between dried biomass and ash content were calculated for individual samples to obtain the dry-weight.

4.2.5 PHB and FAME extraction and quantification

Collected biomasses at day 5, 10, 15 and 20 from LB-CSTR and CB-CSTR were centrifuged (Eppendorf 5810 R, VWR International, QLD, Australia) at 3,220 rcf for 20 min at 4°C. The supernatants were discarded and the biomass pellets were frozen at –80°C and freeze-dried (VirTis, Scitek, Vic., Australia) for FAME and PHB extraction as detailed in 3.2.3.

4.2.6 Metal analysis

Analysis of copper and iron were outsourced to the Advanced Analytical Centre, James Cook University, Townsville, Australia. Briefly, samples were pre-filtered using 0.2 µm PTFE membrane filter (Agilent Technologies, Mulgrave, VIV 3170, Australia) and diluted ten-fold with deionized water. Filtered samples (devoid of cells) were acidified (2 % nitric acid final concentration) and analysed using ICP-OES 5100 (Agilent Technologies, Vic., Australia).). A series of multi-element (copper as Cu, and iron as Fe, included) standard solutions were used to calibrate the instrument, containing 50 ppb of high purity mixed standards (Cat # ICP-200.7-6, EPA method

200.7 standard 6, Solution A, Choice Analytical, Sydney, NSW, Australia with a concentration of Cu and Fe of 20 µg mL^{-1}, each) were used for point calibration of the instrument. Standard and sample solutions were introduced into the ICP-OES by a glass concentric nebulizer for analysis. The metal ions were determined based on the emission wavelength (Cu 324.345 nm, Fe 259.940 nm) and quantified based on their intensity of the emission line.

4.2.7 Statistical analysis

Statistical analyses used in Calypso are standard t-test and standard ANOVA, where the taxonomic units are normalised by total-sum normalization (TSS). Since the experiments are done in high volume CSTRs without replicates, a standard error of ±5 % was applied to our MOCs, CH_4 removal efficiencies, FAME and PHB data values as suggested by Box et al. (2005).

4.3. Result

4.3.1 Effect of Cu^{2+}/Fe^{2+} ratio on mixed consortia

The effect of Cu^{2+}/Fe^{2+} ratios on community structure was assessed for methanotrophs and other co-inhabiting bacteria LB and CB, respectively (Figure 4.2). While the community composition was more or less similar for both soil type-derived consortia, abundance profiles differed. Type -I methanotrophs dominated both consortia until day 5. Overall, Cu^{2+}/Fe^{2+} addition did not enhance or even decreased methanotrophs abundances in the LB-CSTR (Figure 4.2a), whereas the type -I *Methylosarcina* showed a variable response and the type -II *Methylosinus* increased at higher and highest Fe^{2+} concentrations in the CB-CSTR, respectively (Figure 4.2b). In detail, *Methylosarcina* was dominant initially (on day 5), but declined to 16 % in LB-, whereas it increased to 65 % in CB-CSTR (65 %) (Figure 4.2a, b). *Methylobacter*, another type -I methanotroph - was also more abundant in the CB-CSTR, while abundances of the type -II methanotrophs, *Methylosinus* and *Methylocystis* was low initially in both systems (Figure 4.2a, b).

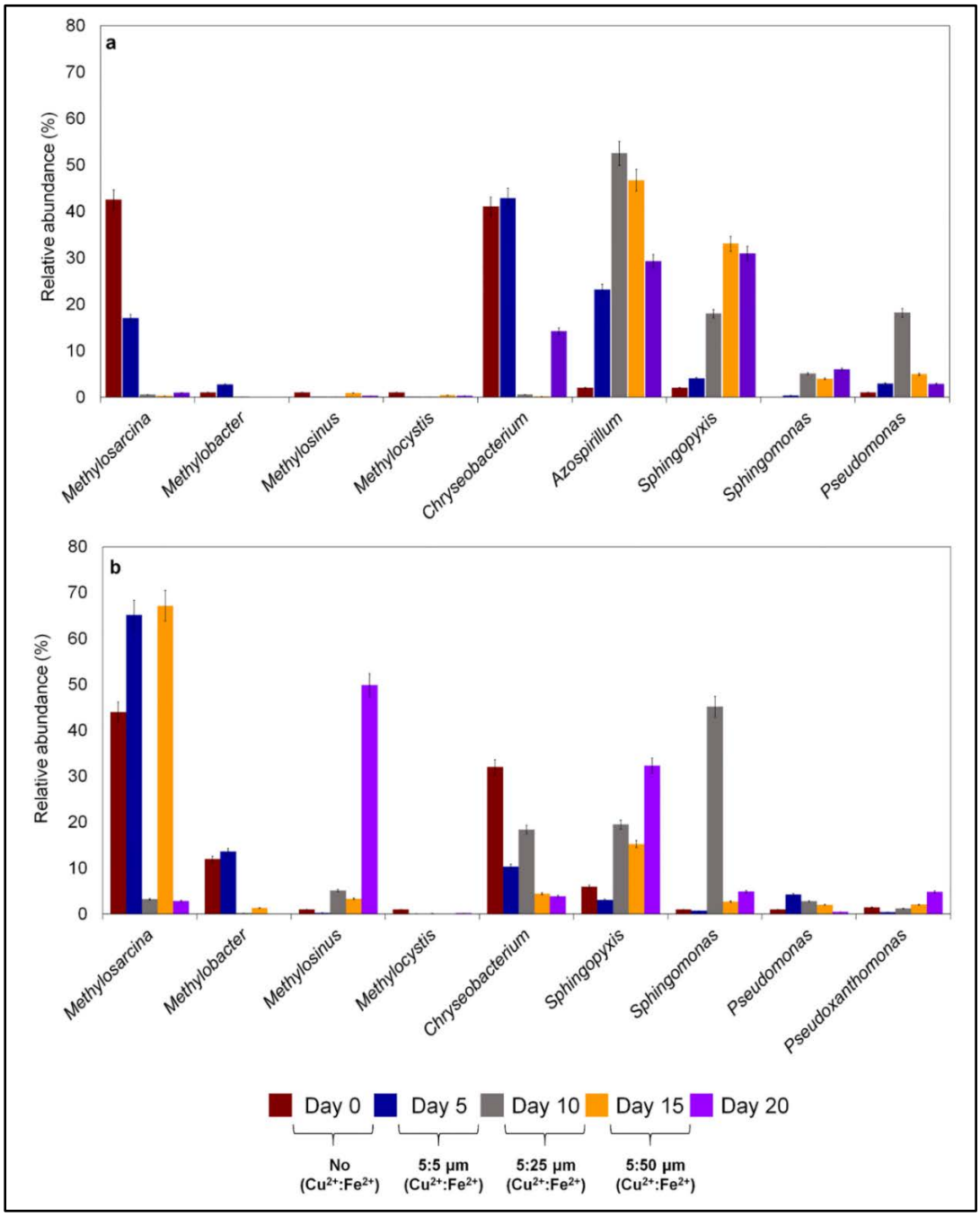


Figure 4.2 Effect of Cu^{2+}/Fe^{2+} ratio on genus-level microbial diversity. (a) microbial abundance profiles in Landfill biomass (LB–CSTR); (b) microbial abundance profiles in Compost biomass (CB–CSTR).

In the CB-CSTR, *Methylosarcina* remained dominant until day 15, while *Methylosinus* dominated on day 20 (50 %) after addition of 5:50 µM Cu^{2+}/Fe^{2+} on day 15 of the cultivation period (Figure 4.2b). Cu^{2+}/Fe^{2+} ratios also affected abundance profiles of methylotrophs and heterotrophs (Figure 4.2a, b). *Chryseobacterium* (~43 %) was a dominant genus in the LB-CSTR until Cu^{2+}/Fe^{2+} addition on day 5, which resulted in a strong decline in abundance to 0.1 % on days 10 and 15 (Figure 4.2a). Interestingly, further supplementation with 5:50 µM Cu^{2+}/Fe^{2+} on day 15, resulted in recovery to 14 % at day 20. Cu^{2+}/Fe^{2+} addition had similar effects, but not as pronounced population trajectories, on *Chryseobacterium* in the CB-CSTR (Figure 4.2b). In contrast, in the LB-CSTR, *Azospirillum* which was present at low abundance (~3 %) on day 0, responded to Cu^{2+}/Fe^{2+} addition (5:5 µM Cu^{2+}/Fe^{2+} on day 5) with an increase in abundance to 50 %, but the population declined to 30 % following further increases in the Fe^{2+} concentrations of the medium (Figure 4.2a). On the other hand, in the CB consortium, *Azospirillum* was neither detected in the CSTR inoculum (Figure 2.9b) nor during the CSTR study. *Pseudomonsas*, was present in both consortia, and, although not as abundant as *Azospirillum*, behaved similarly in the LB-CSTR (Figure 4.2a), while Cu^{2+}/Fe^{2+} concentrations had comparatively little impact in the CB-CSTR (Figure 4.2b). Interestingly, *Sphingomonas* responded differently to Cu^{2+}/Fe^{2+} addition in the two consortia. In the LB-CSTR, abundancies remained below 5 %, but responded positively (Figure 4.2a), while in the CB-CSTR, *Sphingomonas* abundance increased sharply to ~40 % after 5:5 µM Cu^{2+}/Fe^{2+}, declining strongly at higher Fe^{2+} concentrations (Figure 4.2b). *Sphingopyxis* was the only genus present at noticeable abundances, responding positively to high Fe^{2+} concentrations in both the LB- and CB-CSTRs, increasing to ~35 % (Figure 4.2a, b).

4.3.2 Effect of Cu^{2+}/Fe^{2} ratio on growth and MOCs

Average CH_4 removal efficiency was 16 % for the 18-day-cultivation period (the last two days were omitted due to instrument error) for LB- and CB-CSTRs, with

highest removal efficiency of 28 % recorded on day 13 (Figure 4.3a). Irrespective of differences in community structure and abundance profiles, both LB- and CB-CSTR showed a similar trend in removal efficiency. Addition of 5:5 μM Cu^{2+}/Fe^{2+}(day 5) increased removal efficiency to 21 % on days 7 to 8, which further increased to 28 % on days 12 to 13 after addition of 5:25 μM Cu^{2+}/Fe^{2+} on day 10 (Figure 4.3a). LB-CSTR showed a higher MOC than CB-CSTR for the first 10 days of the cultivation period (Figure 4.3b). The highest MOCs of 613.9 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ for LB-CSTR and 599.7 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ for CB-CSTR were observed after day 10 following addition of 5:25 μM Cu^{2+}/Fe^{2+}. CH_4 oxidation and removal efficiency decreased sharply after day 13 (Figure 4.3a, b). Decline of CH_4 removal efficiency was slowed, while the decline of MOC was rescued following addition of at 5:50 μM Cu^{2+}/Fe^{2+} on day 15 (Figure 4.3a, b). On day 15, MOC and CH_4 removal efficiency were ~244 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ and 10 %, in LB- and CB-CSTR respectively. In contrast to maximal MOCs and CH_4 removal efficiency being reached on day 13, growth of the mixed consortia increased sharply for the first day of cultivation, and was more or less steady thereafter, showing no significant responses to Cu^{2+}/Fe^{2+} ratios (Figure 4.3c). An average biomass growth of 0.37 and 0.38 $g.L^{-1}$ was observed in LB- and CB-CSTRs (Figure 4.3c) over the 20 day cultivation period. In contrast to biomass dry-weight, culture optical density (OD_{600}) increased with time, as did total protein content (Figure 4.4 and Figure 4.5). Increase in culture age of non-continuous cultivation of aquatic microscopic organisms (bacteria, microalgae, yeast etc.) leads to accumulation of exudates and/or pH shift-induced precipitation of media ingredients, resulting in higher medium turbidity and increase in absorbance (Xuerning et al., 1999). This limits the usefulness of this indirect proxy of culture growth to young, actively growing cultures, unless calibration curves between OD and cell numbers are established for the experimental conditions used.

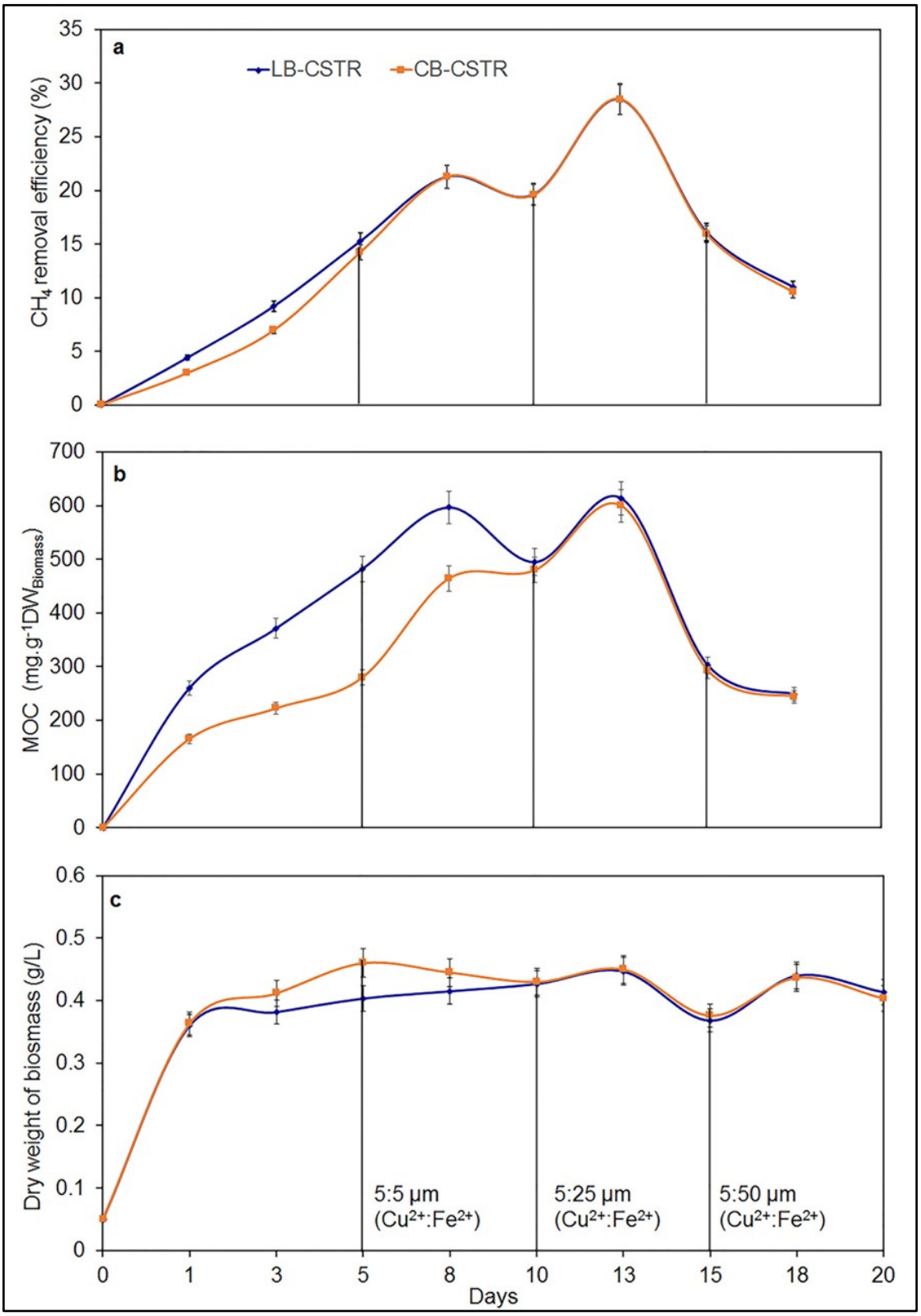


Figure 4.3 Effect of Cu^{2+}/Fe^{2+} ratio on (a) CH_4 removal efficiency; (b) CH_4 oxidation capacity; (c) dry-weight of biomass of Landfill biomass (LB–CSTR) and Compost biomass (CB–CSTR).

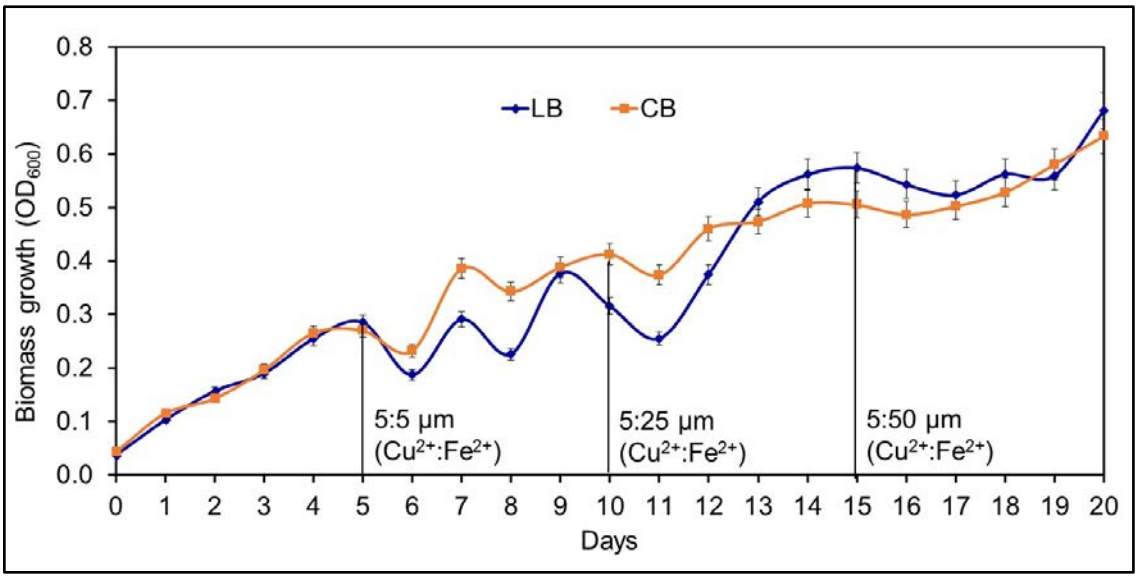


Figure 4.4 Biomass growth of Landfill biomass (LB–CSTR) and Compost biomass (CB–CSTR)

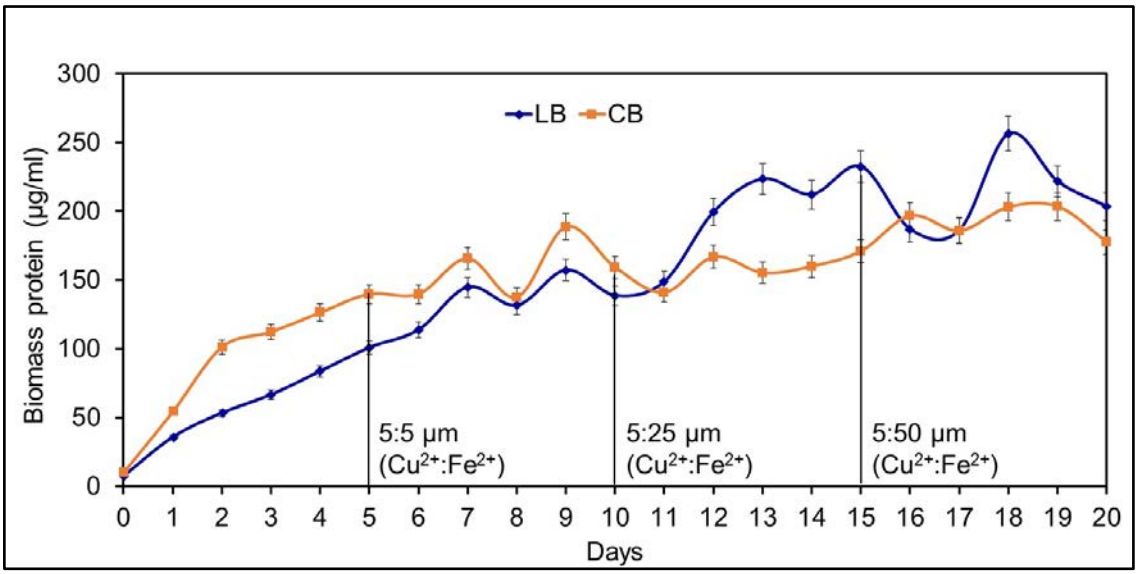


Figure 4.5 Total protein content of Landfill biomass (LB–CSTR) and Compost biomass (CB–CSTR)

4.3.3 Effect of Cu^{2+}/Fe^{2+} ratio on FAME and PHB accumulation

Methanotrophs are capable of using CH_4 as a carbon source for fatty acid synthesis and can store excess carbon; i.e. when population growth ceases, as PHB. Specific fatty acid enrichment in biomass can be used as biomarkers for presence/dominance of type -I and type -II methanotrophs (see discussion). Therefore,

the fatty acid profiles of LB- and CB- CSTR were analysed in response to Cu^{2+}/Fe^{2+} ratios (Figure 4.6).

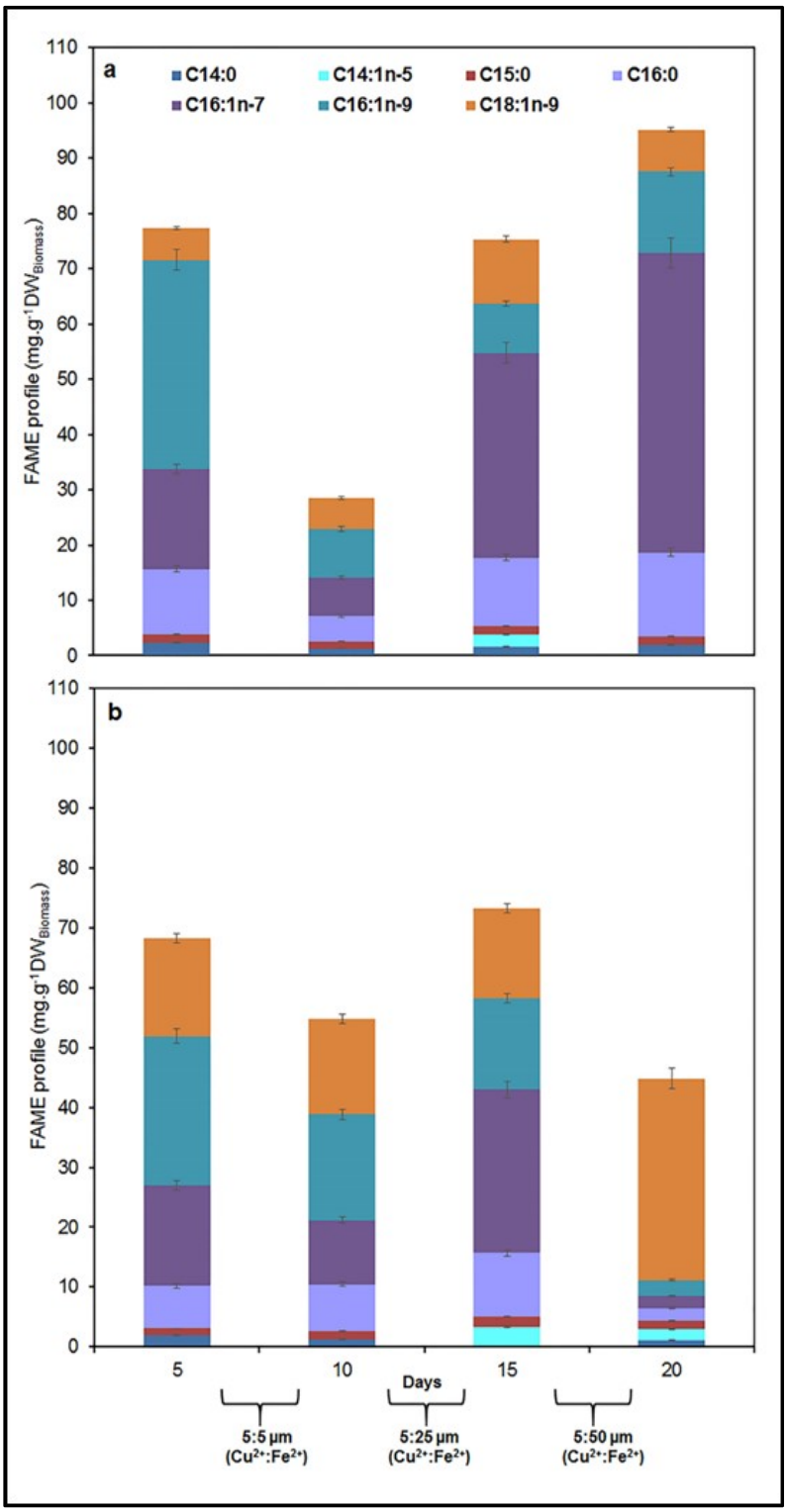


Figure 4.6 Effect of Cu^{2+}/Fe^{2+} ratio on FAME content a) Landfill biomass (LB–CSTR), b) Compost biomass (CB–CSTR)

Total FAME content at day 5 was 77.3 mg.g^{-1}.DW$_{biomass}$ for LB-CSTR and 68.3 mg.g^{-1}.DW$_{biomass}$ for CB-CSTR (Figure 4.6a, b). Addition of 5:5μM Cu^{2+}/Fe^{2+} decreased total fatty acid contents by 63 % (from 77.3 to 28.5 mg.g^{-1}.DW$_{biomass}$) for LB- and 20 % (68.3 to 54.8 mg.g^{-1}.DW$_{biomass}$) for CB-CSTR at day 10. Total FAME contents recovered to more or less day 5 levels (75.4 and 73.3 mg.g^{-1}.DW$_{biomass}$, respectively) for both systems following addition of 5:25 μM Cu^{2+}/Fe^{2+} (day 15) (Figure 4.6a, b). In contrast, an increase of the Cu^{2+}/Fe^{2+} to 5:50 μM elicited a different response in the two systems, with a small increase seen in LB- (to 95.1 mg.g^{-1}.DW$_{biomass}$) and a strong decrease in CB-CSTR (44.8 mg.g^{-1}.DW$_{biomass}$) (Figure 4.6a, b). $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ were the major fatty acids found in LB- and CB-CSTR. C14 and C15 fatty acid was also detected, but in very low amounts (~1 to ~3 mg.g^{-1}.DW$_{biomass}$) and did not show much variations with different Cu^{2+}/Fe^{2+} ratio for both LB- and CB-CSTR. High Fe^{2+} concentration increased the $C_{16:0}$ (11.8 to 15 mg.g^{-1}.DW$_{biomass}$) and $C_{16:1}$ω7c contents (18 to 54.2 mg.g^{-1}.DW$_{biomass}$) in LB-CSTR, but amounts decreased in the CB-CSTR (7 to 2.1 and 16.7 to 2 mg.g^{-1}.DW$_{biomass}$). $C_{16:1}$ω9c fatty acid contents also decreased at high Cu^{2+}/Fe^{2+} ratios (from ~37.8 mg.g^{-1}.DW$_{biomass}$ for LB-CSTR and 24.9 mg.g^{-1}.DW$_{biomass}$ for CB-CSTR at day 5 to 14.6 mg.g^{-1}.DW$_{biomass}$ for LB-CSTR and 2.6 mg.g^{-1}.DW$_{biomass}$ for CB-CSTR). Increase in Cu^{2+}/Fe^{2+} ratios did not affect the $C_{18:1}$ω9c content in LB-CSTR, while contents increased by 75 % in the CB-CSTR (from 16.3 to 33.6 mg.g^{-1}.DW$_{biomass}$) at day 20.

Total PHB content for LB- and CB- CSTR on day 5 was 37.03 and 25.28 mg PHB.g^{-1} DW$_{biomass}$, respectively (Figure 4.7). Addition of 5:5 μM Cu^{2+}/Fe^{2+} increased PHB content in LB-CSTR, while CB-CSTR recorded a slight decrease. Increasing Cu^{2+}/Fe^{2+} ratios 5:25 μM led to strong declines in PHB contents, particularly for LB-CSTR, and to a lesser extent in CB-CSTR. Furthermore, increasing the Cu^{2+}/Fe^{2+} ratio to 5:50 μM did have no further effect on CB-CSTR, while further loss of PHB content was observed for LB-CSTR.

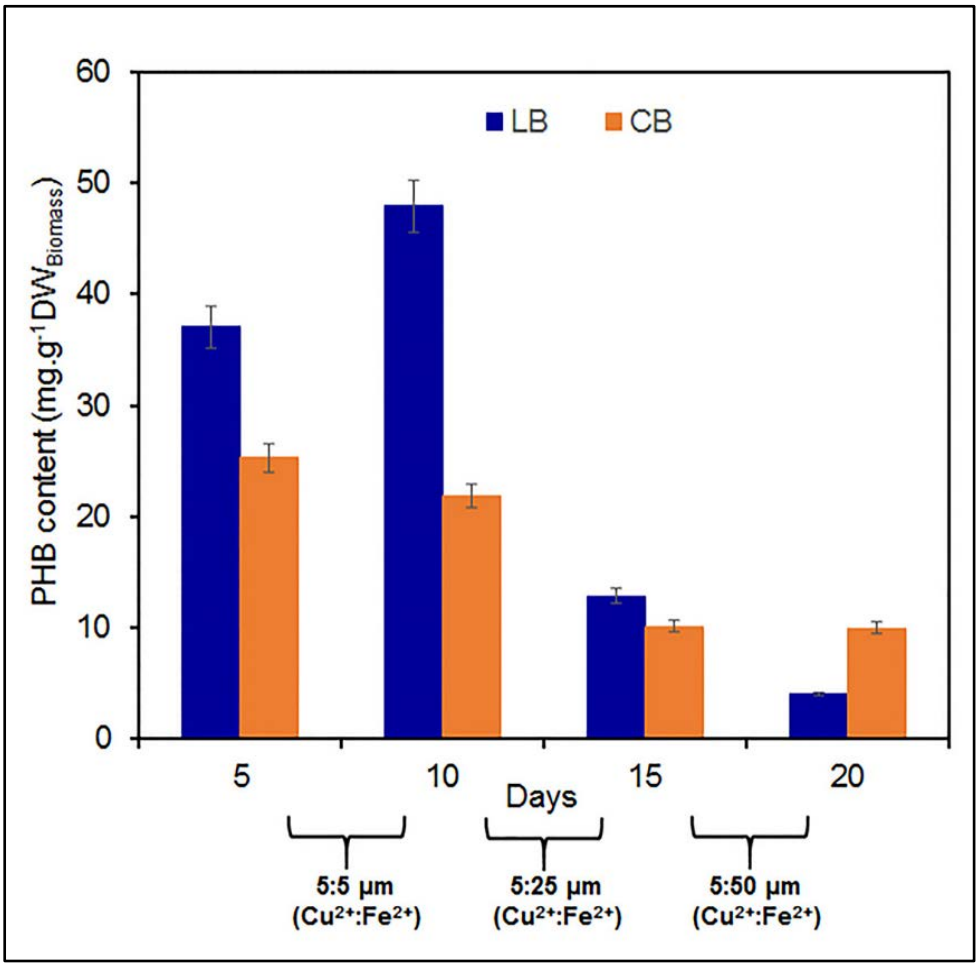


Figure 4.7 Effect of Cu^{2+}/Fe^{2+} ratio on PHB accumulation potential of Landfill biomass (LB–CSTR) and Compost biomass (CB–CSTR).

4.4. Discussions

This study is an extension of our previously published paper (Chidambaramapadmavathy et al., 2015b), where we reported the effects of 5:5µM Cu^{2+}/Fe^{2+} on MOCs, CH_4 removal efficiencies, FAME and PHB contents of LB- and CB-CSTR only (data for the first 10 days). This study extended the previous study and investigated the responses to higher Cu^{2+}/Fe^{2+} ratios (5:25 and 5:50 µM) and included the analysis of effects on the microbial community structure of LB- and CB-CSTR. The initial microbial consortium structure after enrichment from soils was also presented in our previous manuscript (Chidambarampadmavathy et al., 2016a). Both LB and CB had similar methanotrophic community structure, while the non-methanotrophic communities differed in structure and in abundances (Figure 2.9). As concluded

previously, overall low MOCs and CH_4 removal efficiencies, as well as biomass yields are likely due to the high gas flow rate (0.25 $L.min^{-1}$, minimal gas flow rates of the CSTRs) (Karthikeyan et al., 2016b) applied to the reactors, resulting in insufficient gas residence times.

The two transition metals Cu^{2+} and Fe^{2+} play a vital role in the regulation of the MMO enzymes, which regulate the first step in CH_4 assimilation and other redox reactions (Begonja and Hršak, 2001; DiSpirito et al., 2016; Semrau et al., 2010). Not surprisingly, 87 % of the supplied Fe^{2+} was quickly absorbed by the biomass in the LB-CSTR and 72 to 96 % in the CB-CSTR (Figure 4.8), but Cu^{2+} uptake by the consortium appeared to be slow, and consequently uptake was incomplete. Cu^{2+} or Fe^{2+} are co-factors of MMO enzymes (pMMO and sMMO). Expression and activity of either enzyme is regulated by Cu^{2+} availability, i.e. pMMO expression increases at higher Cu^{2+}, while sMMO decreases. Furthermore, following CH_4 to methanol oxidation, requires iron as a co-factor, as do many biological redox reactions (Glass and Orphan, 2012). This may explain why Fe^{2+} uptake was more efficient in both LB- and CB-CSTR. Generally high copper concentration is toxic to cells (Trevors and Cotter, 1990). A study investigating the effect of Cu^{2+} on optimal pMMO expression in mono-species methanotroph cultures showed uptake of 40-60 µM of Cu^{2+}, while highest expression occurred at 30 µM of Cu^{2+} (Yu et al., 2003) but biomass concentration was not provided. Cantera et al. (2016) investigated the effect of Cu^{2+} (0.05 and 25 µM) in a mixed culture and recorded higher biomass content (1.2 $g.L^{-1}$) at 25 µM Cu^{2+}. Unfortunately, comparisons to our results are impeded by lack of information on biomass concentration (g dry-weight) or Cu^{2+} uptake, respectively and significantly differing enrichment conditions and cultivation period (8 % CH_4 and an enrichment period 45 days) for the latter. Under the cultivation conditions used here, only 1/3 of the biomass content was achieved, with only ~50 % of Cu^{2+} being uptaken. The data suggest that Cu^{2+} was oversupplied, whilst Fe^{2+} supplies met the actual demand. This

shows that microbial community structure and biomass per unit volume have significant impacts not only on Cu^{2+} and Fe^{2+} requirements, but also on MOCs and CH_4 removal efficiencies.

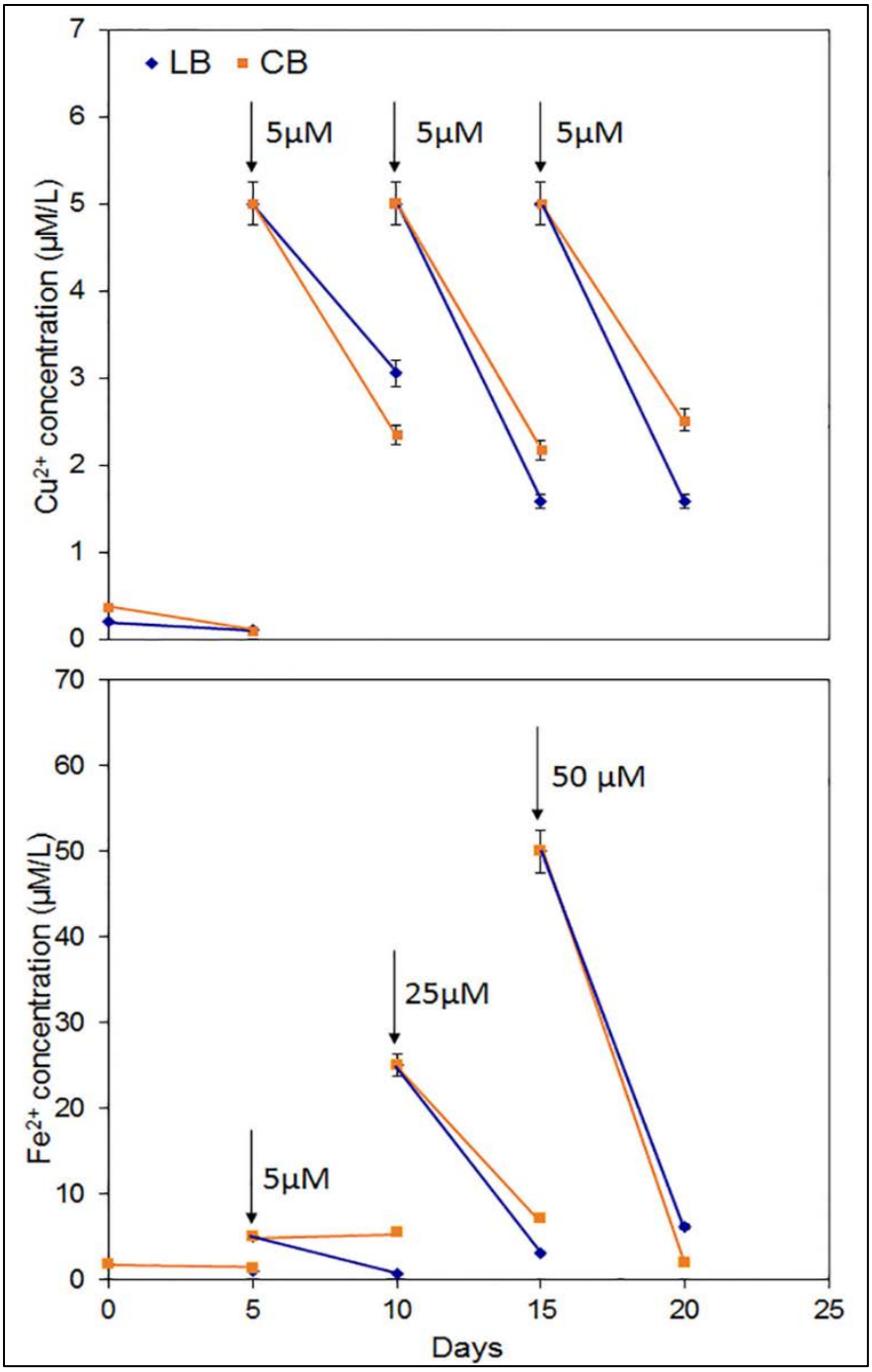


Figure 4.8 Copper and Iron concentration in the medium

Therefore, we will discuss performances in response to Cu^{2+}/Fe^{2+} ratios in conjunction with changes in the microbial profile.

Surprisingly, Cu^{2+}/Fe^{2+} ratios had no large effects on MOCs and CH_4 removal efficiencies in both systems, although there were significant differences in the consortium community structure, i.e. CB-CSTR had a prolific abundance of methanotrophs, while LB-CSTR was methanotroph-poor. However observed low MOCs and CH_4 removal efficiencies, as well as biomass yields are likely due to the high gas flow rate (0.25 $L.min^{-1}$, minimal gas flow rates of the CSTRs) (Karthikeyan et al., 2016b) applied to the reactors, resulting in insufficient gas residence times. Other studies also observed that irrespective of non-methanotroph richness and combination, their presence increased CH_4 oxidation rates (Ho et al., 2016; Ho et al., 2014; Iguchi et al., 2011b; Jeong et al., 2014). *Azospirillum* dominated the mixed consortium in the LB-CSTR, which has been also observed in previous studies, suggesting that the microbial community benefits through removal of metabolic products secreted by methanotrophic bacteria and potential assimilation of CO_2 (Doroshenko et al., 2007; Slobodova et al., 2006). Further to these suggestions, it is important to note that methanobactin (a critical component of the pMMO enzyme) genes with high sequence homologies to methanotroph genes have been discovered in *Azospirillum* (Haft et al., 2012; Krentz et al., 2010; Semrau et al., 2013). Methanobactin has a functional role in the cellular uptake of copper (Cu^{1+}/Cu^{2+}) (Semrau et al., 2013). This would suggest that dominance of *Azospirillum* in the LB-CSTR was perhaps facilitated by the availability of Cu^{2+} and metabolic products of methanotrophs. As the CB-CSTR was void of *Azospirillum*, it is not surprising that system was dominated by methanotrophs.

Chryseobacterium co-dominance with type -I methanotrophs has been reported for both LB and CB batch enrichment cultures without Cu^{2+} and Fe^{2+} fertilization (refer third chapter results). As previously reported, initial MOCs and CH_4 removal efficiencies could be ascribed to *Chryseobacterium* co-dominance with *Methylosarcina*. However, as abundances decreased and remained low after day 5 of

cultivation in both LB- and CB-CSTRs, it appears that Cu^{2+} and Fe^{2+} fertilization of this mixed consortium under continuously stirred cultivation conditions does not support *Chryseobacterium* dominance and unchanged MOCs and CH_4 removal efficiencies must be supported by other components of the mixed consortium. *Sphingopyxis*, which only had a 10 % abundance in CB and very low abundances in LB batch cultures not supplemented with Cu^{2+} or Fe^{2+} (refer third chapter results), became dominant in both systems following Cu^{2+} or Fe^{2+} fertilization under continuously stirred cultivation. Jeong et al. (2014) reported positive effects of *Sphingopyxis* on CH_4 utilisation of methanotrophs (e.g. enhanced expression of key genes involved in the CH_4 oxidation pathway (MMO, MDH and FADH). Furthermore, Lee et al. (2012) reported methanotrophic activity and expression of pMMO in *Sphingopyxis* sp. MD2 when grown on CH_4 as a sole carbon source, which suggests that *Sphingopyxis* acquired CH_4 oxidation genes via horizontal gene transfer. It thus appears that *Sphingopyxis* could also be responsible for maintaining MOCs and CH_4 removal efficiencies. In the methanotroph-poor LB-CSTR, this could be assisted by co-dominance of *Azospirillum*, whilst in the CB-CSTR, *Sphingopyxis* could assist the dominant other *Methylosarcina* and *Methylosinus* present at different Cu^{2+}/Fe^{2+} ratios.

Correspondingly, Cu^{2+}/Fe^{2+}-induced cell death results in PLFA hydrolysis Yao et al. (2015), changes in FAME profiles can potentially be co-related with changes in methanotrophic community structure. In particular, key biomarkers for type -I and type -II methanotrophs are the monounsaturated $C_{16:1}\omega8c$ and $C_{16:1}\omega5t$ and $C_{18:1}\omega8c$, respectively (Bodelier et al., 2009; Bowman, 2014). FAME profiles obtained for both systems, however, did not show the presence of these biomarkers at detectable limits. Instead, $C_{16:1}\omega7c$ and $C_{16:1}\omega9c$ were dominant in both LB-CSTR and CB-CSTR. Although $C_{16:1}\omega7c$ is also one of the key fatty acids for type -I methanotrophs, it has also been reported in other bacteria like *Azospirillum*, *Sphingomonas* and *Pseudomonas* (Dworkin et al., 2006a, 2006b). $C_{16:1}\omega9c$ has not reported for the

dominant bacteria of the described consortia, but has been reported for other bacteria like *Marinobacter* and *Alkalimarinus* not found here. This suggests that it might be a representative fatty acid also for type -I methanotrophs, e.g. *Methylosarcina* or *Methylobacter*. This conclusion is supported by a strong decrease of this fatty acid in the LB-CSTR, which correlates with the breakdown of the dominant *Methylosarcina* following continued Cu^{2+}/Fe^{2+} fertilization. As expected, the type -II specific $C_{18:1}\omega9c$ content was low in the LB-CSTR, but increased significantly with Cu^{2+}/Fe^{2+} fertilization in the CB-CSTR, reflecting the shift in dominance to *Methylosinus*.

In general, type -II methanotrophs accumulate PHB while this is not a defining characteristic of type -I (Karthikeyan et al., 2015a; Pieja et al., 2011a). However, information on PHB accumulation for type -I and -II are contradictory. Bowman (2014) reported type -I methanotrophs (*Methylosarcina* and *Methylobacter*) to accumulate PHB, whereas Pieja et al. (2011a) clearly state that PHB synthesis is linked to the serine pathway of type -II methanotrophs and no type -I methanotroph produced measurable PHB accumulation. So in the absence of significant abundances of type -II methanotrophs in the LB-CSTR, the low PHB content could be due to dominance of *Azospirillum* and *Pseudomonas*, who have both reported to accumulate PHB (Dworkin et al., 2006b; Hartmann and Baldani, 2006). In contrast, a sudden increase in the type -II methanotroph *Methylosinus* upon 5:50 µM Cu^{2+}/Fe^{2+} fertilisation should have been accompanied by increased PHB accumulation in the CB-CSTR. The observed low PHB content at day 20 could reflect use of PHB for the production of reducing equivalents to aid the growth of *Methylosinus*, specifically for maintaining activity of the serine pathway (Pieja et al., 2012).

4.5 Major findings from chapter 4.

In conclusion, the present study focused on responses of mixed consortia to Cu^{2+}/Fe^{2+} ratios. It was expected that the cultivation conditions would favour dominance of type -II methanotrophs, which was only observed for the CB-CSTR at a

ratio of 5:50 µM Cu^{2+}/Fe^{2+}. Irrespective of the similar methanotrophic structure and the same cultivation conditions, this study highlights that responses of mixed microbial community differ. These differences are potentially driven by hitherto largely unexplored allelopathic interactions within the mixed consortia and/or horizontal gene transfers. For example, dominance of *Azospirillum* and *Sphingopyxis* over methanotrophs and the maintained MOCs and CH_4 removal efficiencies in the LB-CSTR could indicate acquired capacity for CH_4 utilisation by these heterotrophs. In contrast, 5:50 µM Cu^{2+}/Fe^{2+} ratio favoured growth of the type -II methanotroph *Methylosinus* in CB-CSTR, as expected, but overall dominance of type -I methanotrophs was negatively affected by high Cu^{2+}/Fe^{2+} ratios in both systems. Differences in responses of the two mixed consortia will require further enzymatic/physiological studies to unravel metabolic interactions and functional relationships, i.e. potential competition and/or tolerance thresholds for Cu^{2+}/Fe^{2+} uptake. Such studies will help to develop simulative community models to investigate potential for CH_4 emission abatement ***a priori***. Coupling of these studies with biochemical profiling would then also enable to assess potential for co-development of renewable products like bioplastics from PHB.

[4]Chapter 5: General Discussion

[4] Case study of this chapter has been adapted from:

Chidambarampadmavathy K, Karthikeyan OP & Heimann K (2016) Sustainable bio-plastic production through landfill methane recycling. Renewable & Sustainable Energy Reviews 71: 555-562 (https://doi.org/10.1016/j.rser.2016.12.083).

5.1 Thesis findings and broader implications

This thesis was part of the AMCRC-funded research project (grant number 2.3.4.) entitled “Bioremediation of methane from mine ventilation air”. The project aimed to develop a dual culture system to convert CH_4 into green fuels using methanotrophic bacteria and nitrogen-fixing cyanobacteria in novel bioreactors. My PhD project aimed to establish robust mixed consortia from indigenous landfill soils to abate CH_4 and to study their responses under different gas regimes and nutrient-governed environmental conditions, whilst giving consideration for possible bio-product outcomes, especially PHB. Publications on mixed methanotrophic culture were rare, at the conception of my research, and only a few studies have recently been published, highlighting the need for community interaction models and response analyses of mixed microbial communities. These studies indicate that methanotrophs synergistically co-habit with non-methanotrophs (either methylotrophs or heterotrophs) depending on environmental conditions (Beck et al., 2013; Ho et al., 2016; Ho et al., 2014; Oshkin et al., 2015; van der Ha et al., 2013).

It has become apparent that industrial bio-filters for the remediation of waste gases and water cannot be operated in axenic modes for long periods and community interactions of mixed microbial consortia can benefit the remediation process (Iguchi et al., 2011a; Stock et al., 2013). Therefore, understanding microbial community responses to different environmental factors is very important, especially in a mixed culture system for developing efficient microbial-based bio-filters. Within this context, I aimed to establish robust mixed consortia from four indigenous landfill cover soils (vegetated soil, mulch soil, fresh land cover and a compost soil) (chapter 1). Of these, mulch and compost cover soils, were selected for further enrichment and response studies. Mulch-based landfill cover soil (LB) were selected, because of higher MOC and methanotroph diversity compared to other soils and compost soil (CB) being the most commonly used landfill cover had acceptable levels of both type -I and -II

methanotrophs, as well as MOC. The associated non-methanotrophs differed in diversity and abundances between the enriched soil-slurries and established consortia. The entire microbial community relied on CH_4 as their only carbon source, as no other complex source of organic carbon was provided for growth, suggesting CH_4 is converted into complex organic substrates by the methanotrophs that are further utilised by the non-methanotrophs.

The established consortia were further used to test their responses to different CH_4/O_2 (in air) ratios (chapter 3) and in varying Cu^{2+}/Fe^{2+} ratios (chapter 4). To the best of my knowledge, no previous studies investigated responses of mixed methanotrophic populations in a wide range of CH_4 and O_2 concentrations, mimicking landfill gas concentration variability as well as in varying Cu^{2+}/Fe^{2+} ratios. In order to develop an efficient bio-filter, it is important to study these responses, as these gases change in proportion in landfill environments as do the metal ion concentrations. My research was the first to demonstrate the resilience of type -I methanotrophs dominated by *Methylosarcina*, which was sustained even at high CH_4 concentrations. Moreover, the established consortia withstood variable CH_4/O_2 ratios. Furthermore, this research documented co-dominance and continues persistence of certain non-methanotrophic bacteria, such as *Chryseobacterium* and *Sphingopyxis* at variable CH_4/O_2 (air) ratios.

At the applied high CH_4/ low O_2 concentrations, a dominance of type -II methanotrophs was expected based on published references (Amaral and Knowles, 1995; Graham et al., 1993; Hanson and Hanson, 1996; López et al., 2014; Wei et al., 2015). Surprisingly, type -II were not abundant, in an attempt to enrich type –II methanotrophs and thereby to improve the PHB accumulation potential of the established mixed consortia (LB and CB), nitrate was sequentially limited once every 5 days over 20 days, as type -II methanotrophs have been reported to fix atmospheric nitrogen, and CH_4 enriched atmosphere (30 %) supplemented with CO_2 (10 %) was

provided. Addition of CO_2 as a gas or as bicarbonate has been reported to increase biomass productivity, encourages specifically type -II methanotroph growth, and is one of the prerequisite component for the serine pathway, a physiological characteristic of type -II methanotrophs (Karthikeyan et al., 2015a; Park et al., 1991; Pieja et al., 2011a). Cultures were then subjected to varying Cu^{2+}/Fe^{2+} ratios every 5 days to sequentially activate pMMO and then sMMO to improve PHB accumulation. Although the type -II methanotroph *Methylosinus* was abundant at later stages (at high Cu^{2+}/Fe^{2+} ratio) in the CB-CSTR, abundances in the LB-CSTR were very low. Instead, *Azospirillum* dominated this consortium. Community composition responses to different CH_4/O_2 ratios (chapter 3) as well as to varying Cu^{2+}/Fe^{2+} ratios (chapter 4) indicated that methanotrophs and non-methanotrophs interact in such a way that MOCs are stabilised. A schematic overview synthesising the most important results from all three chapters is presented in Figure 5.1.

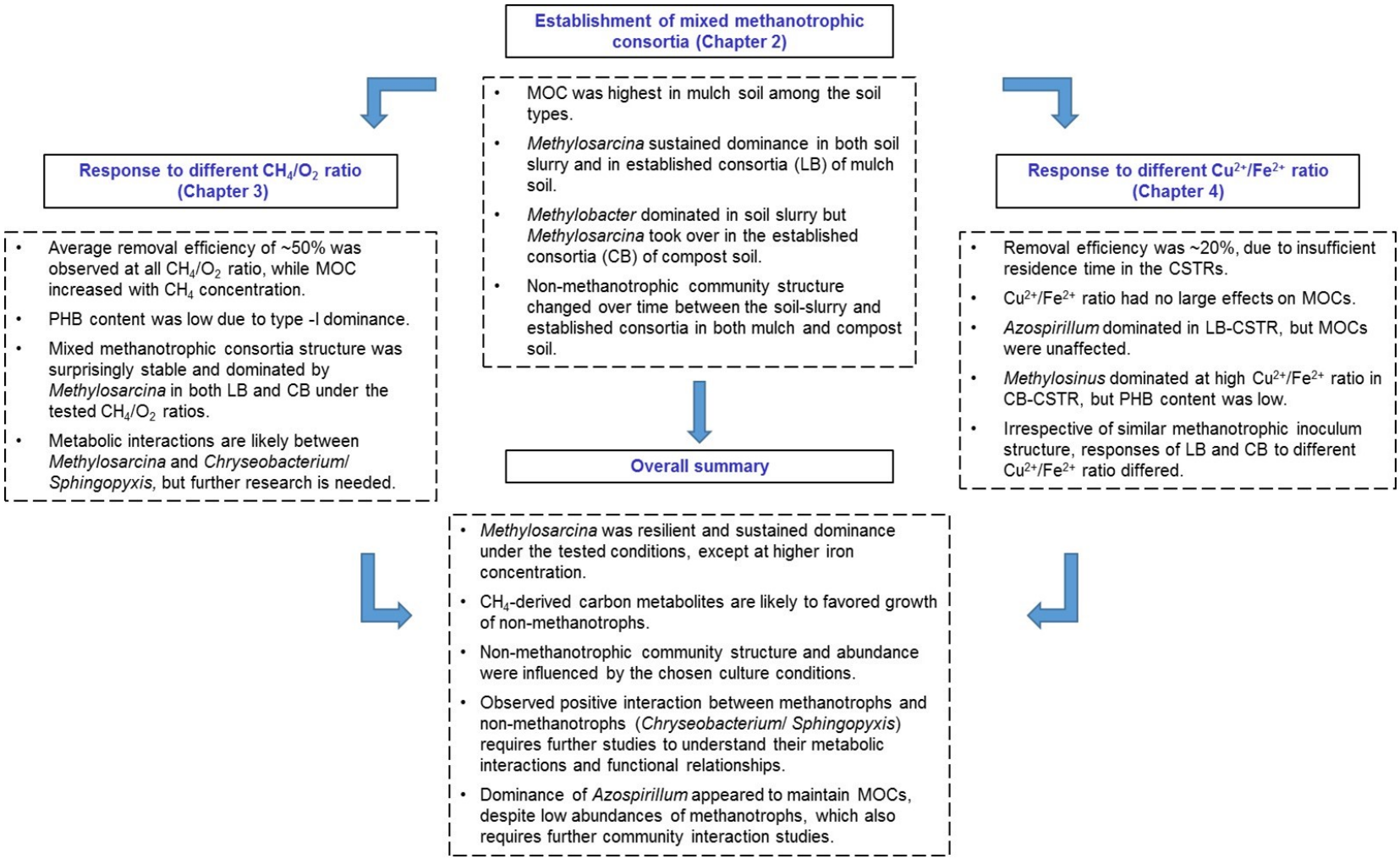


Figure 5.1 Schematic overview of thesis findings

In general, microbes establish community consortia for a number of reasons (Faust and Raes, 2012), for example

- Mutualism or syntrophic associations, where both the interacting species benefit each other through exchange of metabolites.
- Amensalism - one species benefit at the expense of others.
- Commensalism - one species benefit, while the other species neither benefits nor are they harmed.

Syntrophic relationships with non-methanotrophs have been predominantly reported for methanotrophs. A pure culture study by Iguchi et al. (2011b) reported cobalamin produced by a non-methanotroph (*Rhizobium*) enhanced growth and was utilised by the methanotroph. Likewise Jeong et al. (2014) reported positive effects (enhanced methanotrophic activity and expression of key genes involved in the CH_4 oxidation pathway) of *Sphingopyxis* when co-cultivated with methanotrophs. In contrast, negative effects on the MOC of methanotrophs by non-methanotrophs (i.e. *Pseudomonas*) have also been reported (Syamsul Arif et al., 1996; Van Bodegom et al., 2001). However, in this study, interactions between methanotrophs and non-methanotrophs were positive, as MOCs were maintained. *Chryseobacterium* and *Sphingopyxis* enhanced MOC of *Methylosarcina* (chapter 3), while *Azospirillum* and/ or *Sphingopyxis* maintained the MOC of LB-CSTR (chapter 4), irrespective of low methanotroph abundances. At present, knowledge of interactions within these mixed communities is rather limited, as such studies were beyond the scope of this thesis, nonetheless the co-existence of methanotrophs and non-methanotrophs were not random and influenced by the environmental conditions tested. *Methylobacter*, which were abundant in compost soil-slurry (Figure 2.6b), were not found in noticeable abundances in the established consortia of CB enriched in nutrient-sufficient medium (Figure 2.9b), but dominated consortia enriched under low nutrients conditions (Figure 4.2b). This response can be explained by the described presence of certain genes with

ascribed function in di-nitrogen assimilation (Hernandez et al., 2015). Similarly *Azospirillum*, which were found in low abundance in the established consortia of LB (Figure 2.9a), dominated under nutrient-limited conditions and high Cu^{2+}/Fe^{2+} ratios in the LB-CSTR (Figure 4.2a). Dominance might have been triggered by high availability of Cu^{2+}, CO_2, and metabolic products of methanotrophs (Haft et al., 2012; Krentz et al., 2010; Slobodova et al., 2006). It must be noted that my experimental designs did not provide organic carbon sources for the growth of non-methanotrophs, instead conditions were selected to specifically enhance growth of methanotrophs. This made CH_4 the only carbon source provided, thus the observed co-dominance of non-methanotrophs in different experiments is likely due to the exchange of metabolites, indicating a close association between these communities. The mode of association or the mechanism involved in these interaction was beyond the scope of this PhD, but very recently, Puri et al. (2016) identified a quorum sensing (QS) molecule, N-3-hydroxydecanoyl-L- homoserine lactone, which is believed to aid communication between Burkholderiales and Pseudomonads. This molecule also regulates the secretion of organic compounds by *Methylobacter tundripaludum*. The research team stated that this QS molecule is not common to methanotrophs and hypothesised that *M*. *tundripuludum* acquired this trait from the environment.

As stated earlier, microbial community interaction mechanisms research was beyond the scope of this thesis, but my research established the robust methanotrophic consortia, viable under different gas mixtures and Cu^{2+}/Fe^{2+} ratios with sustained MOCs. Despite all attempts to enrich specifically for type -II methanotrophs, consortia were primarily dominated by the type -I methanotroph *Methylosarcina*, which negatively affected PHB accumulation potential. Nonetheless, published data on CH_4 utilisation, average PHB accumulation capacity of type -II methanotrophs were used to ascertain criteria for economically viable commercial PHB production (explained in the later section). Hence, I have applied these data to examine the feasibility for PHB

production from landfill gas. At the very least, this study provides a baseline for commercial criteria that need to be considered for the production of biodegradable plastics (PHB) in a landfill gas emission abatement scenario.

5.2 Case-study on bioplastic (PHB) production from methane

Based on predicted population growth and finite fossil fuel resources, economically and environmentally sustainable production of biodegradable bioplastics using completely renewable resources will be mandatory for future generations. Microbial production of PHB can meet the future requirements only, if produced by mixed methanotrophs using waste GHGs, as this avoids competition for agricultural food products and land use. Disposing the bioplastics in landfills is considered the best option, because gases released from landfills usually contain 30 to 70 % of CH_4 and 20 to 50 % of CO_2, but the gas composition changes with landfill age (Rostkowski et al., 2012). Release of CH_4 due to the degradation of biopolymers induces a cradle-to-grave-to-cradle production approach, as the emitted CH_4 can be effectively used for renewed PHB production. This study did not achieve high PHB contents, due to limitations outlined before, so in order to evaluate the theoretical merits of rerouting CH_4 for PHB production using methanotrophs, a case study was carried out on Australian landfill waste management practices based on optimised conditions of axenic cultures (Table 5.1), which is likely to be achievable with type -II dominated mixed consortia.

Landfills in Australia vary greatly in size; small landfills receive on average 5,000 tonnes of wastes, whereas large modern waste-gas managed landfills receive more than 100,000 tonnes of waste annually (BDA Group, 2009; BERE consultancy, 2013). Annually, 48 Mt of solid waste are generated in Australia, of which 40 % (~20 Mt) are disposed in landfills. Of the 20 Mt of waste, 14 Mt are organic waste (BERE consultancy, 2013), releasing 0.65-1.53 tonnes of CO_2eq per tonne of waste (BDA Group, 2009). Methane emissions from solid waste are estimated based on the first

order decay method (Eggleston et al., 2006) and in general, a 25 times multiplier is applied based on earlier IPCC reports (IPCC, 2007). However, it should be noted that, the current GWP potential of CH_4 has been increased to 28-36 for a100 year period (EPA, 2016; IPCC, 2014). In this study, we used a factor of 25 for converting CH_4 emissions to calculate CO_2eq and maintained this estimate in this thesis, as the below is part of my recently published research (Chidambarampadmavathy et al., 2016b). Thus, 14 Mt of waste emit 9.1 to 21.42 Mt of CO_2eq, resulting in an average of 15.26 Mt of CO_2eq, (i.e. 0.6 Mt of CH_4 are emitted annually in Australia). In order to effectively use the emitted CH_4 for PHB production, the following key factors have to be considered:

Life of each landfill is estimated to be 30 years and CH_4 can be collected from the time of opening and up to 30 to 50 years after closure of each landfill (BDA Group, 2009). CH_4 emission will vary depending on the climate and the age of the landfill. Using gas collection systems from well-engineered landfills, a maximum of 95 % with an average of 75 % of emitted CH_4 can be recovered (BDA Group, 2009). The energy required to run a PHB production plant can be derived using 18-26 % of the captured CH_4, leaving 74-82 % for use as feedstock for PHB production. Using 1.13 g of CH_4, 0.5-0.6 g of PHB can be produced (Roland-Holst et al., 2013; Rostkowski et al., 2012). The following factors were used to calculate one tonne PHB production from CH_4

- One tonne organic waste releases 0.65 to 1.53 tonnes of CO_2eq (average 1.09 tonnes of CO_2eq). Thus multiplying 14 Mt of waste and with average 1.09 tonnes of CO_2eq emission results in 15.26 Mt of CO_2eq i.e. 0.6 Mt of CH_4 (total CO_2eq emission divided by 25, GWP of CH_4 for 100 years).
- Of the 0.6 Mt of CH_4, 0.45 Mt of CH_4 can be recovered based on 75 % CH_4 recovery.
- To produce one tonne of PHB at a yield rate of 0.56 g PHB.$g^{-1}CH_4$, 1.79 tonnes of CH_4 are required as a carbon source for production and another 0.52 tonnes

of CH_4 for energy (Table 5.1) for the production process (Roland-Holst et al., 2013). Thus the production of one tonne PHB requires a total of 2.3 tonnes of CH_4. Based on 0.45 Mt of total CH_4 input and the ratio of CH_4 needed for production and energy, a maximum of ~196 Kilo tonnes of PHB can be produced annually.

Table 5.1 Base factors for net PHB calculation

CH_4 recovery* (%)		75
Yield of PHB ** **(g_{PHB} g^{-1} CH_4)**		0.56
Amount of CH_4 required for PHB production* **(per tonne)**	As carbon source	1.78
	As energy	0.52

Source - Chidambarampadmavathy et al. (2016b)

- On an average 75 % of CH_4 can be recovered (BDA Group, 2009).
- **1.13 g of CH_4, can produce 0.56 g of PHB
- *** Refer Roland-Holst et al. (2013)for the model calculation.

A small landfill, with average annual disposal of 5,000 tonnes of wastes, 163 tonnes of CH_4 can be recovered annually using the same model calculations applied before, which can yield 71 tonnes of PHB, whilst medium and large landfills with average annual disposal of 35, 000 and 230,000 tonnes of wastes and 1138 and 7480 tonnes of CH_4 recovery can produce 495 and 3252 tonnes of PHB (refer to Table 5.1 and Table 5.2 for base factor inputs). Listewnik et al. (2007) and co-workers published a process design for synthesis of PHB using methanotrophs fed with natural gas (96 % CH_4) as a carbon source. Estimated costs to produce 1 kg of PHB in a plant producing 500 tonnes PHB per year was AUD ~10.50.

Table 5.2 Annual waste disposal in Australian landfills

Landfill size	**Amount of waste disposed** (tonnes / year)	**Assumed annual disposal** (tonnes / year)	**CH_4 emissions** (tonnes / year)	**Average CH_4 recovery** (tonnes / year)	**Net PHB production** (tonnes / year)	**Energy used** (tonnes / year)
Small	< 10,000	5,000	216	163	71	65
Medium	10,000 – 100,000	35,000	1518	1138	495	455
Large	> 100,000	230,000	9973	7480	3252	2993

Source: Chidambarampadmavathy et al. (2016b).

However, by increasing production of PHB, the cost can be considerably reduced. Criddle et al. (2014) and co-workers estimated that the costs per kg of PHB can be reduced to 1.5 to 2.0 AUD based on using natural gas as a feedstock and increasing the production volumes of PHB from 500 tonnes to 5,000 or 10,000 tonnes per year. While in a recent study, Levett et al. (2016) published a detailed techno-economic analysis of PHB from CH_4 in a 100,000 tonnes plant and estimated the production cost as 4.6 to 7.6 AUD per kg of PHB, which can be further reduced by using thermophilic methanotrophs to 3.6–6.1AUD per kg of PHB. However, the main drawback in this study is, the cost analysis is calculated by offsetting the refrigerant cost by using thermophilic methanotrophs for producing PHB. It should be noted that the PHB content of a thermophilic methanotroph has not been reported to date. Furthermore, most thermophilic methanotrophs use the RuMP pathway, while PHB synthesis in methanotrophs is linked to the serine pathway of type -II methanotrophs (Pieja et al., 2011a). Based on these limitations of their study and considering the realistic data of PHB content of methanotroph (0.56 $gPHB.g^{-1}CH_4$) available (Criddle et al., 2014; Listewnik et al., 2007; Wendlandt et al., 2010), the techno economic model analysis of Criddle et al. (2014) and co-workers were adopted and the production cost of PHB using CH_4 emitted from Australian landfills is described and discussed.

A comparison with the market price of starch-based bioplastics and other petroleum-based polymers confirms that PHB production using CH_4 could be an attractive option even to date (Table 5.3), when prices for the raw material are most likely considerably lower than they would be once fossil fuel reserves become severely limiting, resulting in higher prices for material inputs into non-degradable plastics. Based on current prices, PHB production in small and medium landfills would be possible, but the low volumes of PHB produced would result in non-competitive pricing of the product.

Table 5.3 Market prices for various polymers

Polymer Type	PS	PP	PET	PLA	PHB (sugar)	PHB (CH_4)
Market Price ($/kg)	1.87-2.14	1.38-1.43	1.7-1.9	2.0-2.5	3.5-5.0	1.5 - 2.0

Source - Criddle et al. (2014).

All prices are in AUD, market value based on 2012. PS - polystyrene; PP - polypropylene; PET - polyethylene terephthalate; PLA – polylacticacid; PHB - polyhydroxybutyrate

However, larger landfills can produce a minimum of 3252 tonnes of PHB making the process more economical. In large metropolitan areas, it may be possible to create a central PHB extraction and refining facility, which would allow to pool PHB containing biomass from a variety of landfills, which should make the product cost-effective even based on today's low prices. Major metropolitan cities with high population densities like Sydney, Melbourne, Perth, and Brisbane have many large landfills. This concept was applied to a case scenario for Sydney.

Sydney is one of the largest and most populated Australian cities (4.67 million people as of 2012) (BDA Group, 2009). The daily amount of waste produced per person in Australia is 2.2 Kg per person (Hyder Consulting, 2012). Of this, nearly ~50 % is of organic waste (Australian Bureau of Statistics, 2013), resulting in 1.1 Kg per person per day of waste suitable for CH_4 rerouting into PHB production. This yields ~5000 tonnes of waste per day or 1.8 Mt per year. Almost 75 % of generated wastes in Sydney are disposed of primarily in five larger landfill (Figure 5.2) or smaller or medium landfills in and around it (not considered in this case study). Life cycle analyses for biofuel and bio-refinery feedstock indicate a maximum 50- 80 kilometre (km) radius for a central biomass receiving facility as economical and energy-sustainable considering transport logistics (Daystar et al., 2013; Elliott et al., 2014; Energy). Therefore, as the price for PHB is somewhat comparable to that for biofuels,

this case analysis assumed a planned central PHB extraction and refining facility within a radius of ~50 km for the five larger landfills (Figure 5.1). The biomass grown using emitted CH_4 at these five larger landfills can be transported to this central location for extraction of PHB yielding ~25,500 tonnes per year (assumed PHB production based on above calculations) which would ensure PHB can be produced at a market value of 1.5 to 2.0 AUD (Criddle et al., 2014).

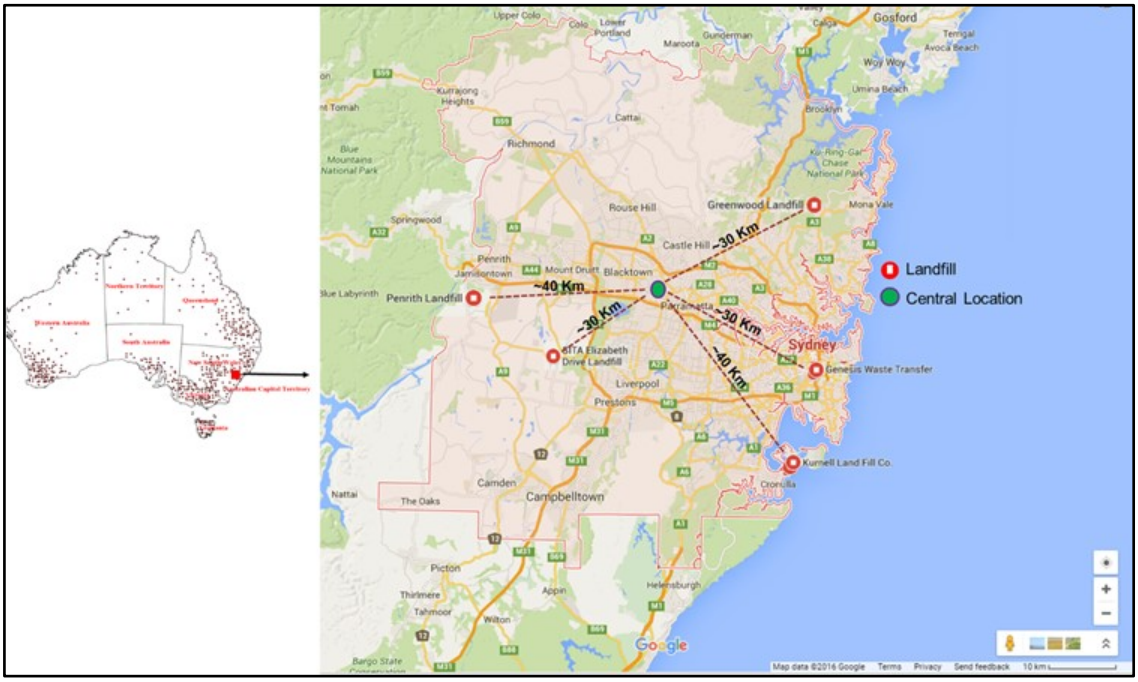


Figure 5.2 Model scenario for a centrally located biomass receiving PHB extraction and refining facility in Sydney, Australia

5.3 Limitations and future directions of this research

Future research should be conducted to identify the exchange of metabolites and characterise the microbes involved in these complex associations. This can be achieved by using stable isotopes of CH_4 to trace its assimilation products and must be coupled with community structure and abundance analyses via next-generation sequencing. For example, the mechanism underpinning the described positive interaction between the non-methanotrophs (*Chryseobacterium* and *Sphingopyxis*) and methanotrophs (*Methyloarcina* or *Methylosinus*) need to be investigated, as do the negative impacts on methanotrophs, when consortia are dominated by *Azospirillum*

surprisingly whilst maintaining MOCs at much reduced methanotroph abundances. Furthermore, enzyme activity and expression analyses of the MMOs (pMMO and sMMO) should be conducted to confirm changes in methanotrophic diversity especially under increased Cu^{2+}/Fe^{2+} ratios. Also this research could not investigate certain other factors such as gas transfer efficiency (i.e. gas residence time could not be increased due to research infrastructure limitations available to my project), year-long outdoor cultivation to understand impacts of season on the community structure and associated bio-product potential in large scale bio-reactors due to time - and resource limitations. While my research laid the required foundation for utilising mixed consortia for industrial use, future research should utilise these and other published data to establish techno-economic models for optimised bio-filter performance with regards to CH_4 abatement in general and co-product potential (PHB) specifically.

6. References

Al-Shehri, S.S., Sweeney, E.L., Cowley, D.M., Liley, H.G., Ranasinghe, P.D., Charles, B.G., Shaw, P.N., Vagenas, D., Duley, J.A., Knox, C.L., 2016. Deep sequencing of the 16S ribosomal RNA of the neonatal oral microbiome: a comparison of breast-fed and formula-fed infants. Scientific Reports 6, 1-13.

Amaral, J.A., Knowles, R., 1995. Growth of methanotrophs in methane and oxygen counter gradients. FEMS Microbiology Letters 126, 215-220.

APHA, 2005. Standard methods for the examination of water and wastewater. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Appels, L., Baeyens, J., Degrève, J., Dewil, R., 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. Progress in Energy and Combustion Science 34, 755-781.

Asenjo, J.A., Suk, J.S., 1986. Microbial conversion of methane into poly-β-hydroxybutyrate (PHB): growth and intracellular product accumulation in a type II methanotroph. Journal of Fermentation Technology 64, 271-278.

Australia's National Greenhouse Accounts, 2016. Quarterly update of Australia's national greenhouse gas inventory: December 2015, Commonwealth of Australia., Australia. , pp. 1-38.

Australian Bureau of Statistics, A., 2010. Measures of Australia's Progress, in: Cat.non.1370.0. (Ed.). Accessed: April 20, 2013, Australia.

Australian Bureau of Statistics, A., 2013. Waste Account, Australia, Experimental Estimates. 2013, http://www.abs.gov.au/ausstats/abs@.nsf/Latestproducts/4602.0.55.005Main%20Features42013?opendocument&tabname=Summary&prodno=4602.0.55.005&issue=2013&num=&view=, Accessed: May 05, 2015.

Balasubramanian, R., Kenney, G.E., Rosenzweig, A.C., 2011. Dual pathways for copper uptake by methanotrophic bacteria. Journal of Biological Chemistry 286, 37313-37319.

Balasubramanian, R., Smith, S.M., Rawat, S., Yatsunyk, L.A., Stemmler, T.L., Rosenzweig, A.C., 2010. Oxidation of methane by a biological dicopper centre. Nature 465, 115-119.

Bareither, C.A., Wolfe, G.L., McMahon, K.D., Benson, C., H, 2013. Microbial diversity

and dynamics during methane production from municipal solid waste. Waste Management 33, 1982-1992.

BBC News, 2008. World Food Prices at Fresh High, Says UN.

BDA Group, 2009. The full cost of landfill disposal in Australia, in: Department of the Environment, W., Heritage and the Arts (Ed.), BDA Group, Canberra, Australia, http://www.environment.gov.au/protection/national-waste-policy/publications/full-cost-landfill-disposal-australia. Accessed: May 03, 2015.

Beal, E.J., House, C.H., Orphan, V.J., 2009. Manganese-and iron-dependent marine methane oxidation. Science 325, 184-187.

Beck, D.A.C., Kalyuzhnaya, M.G., Malfatti, S., Tringe, S.G., del Rio, T.G., Ivanova, N., Lidstrom, M.E., Chistoserdova, L., 2013. A metagenomic insight into freshwater methane-utilizing communities and evidence for cooperation between the Methylococcaceae and the Methylophilaceae. PeerJ 1, e23.

Begonja, A., Hršak, D., 2001. Effect of growth conditions on the expression of soluble methane monooxygenase. Food Technology and Biotechnology 39, 29-35.

BERE consultancy, B.E.a.R.E., 2013. National Waste Reporting: Waste generation and resource recovery in Australia, http://www.environment.gov.au/topics/environment-protection/nwp/reporting, Accessed: May 05, 2015.

Bernardet, J.F., Hugo, C., Bruun, B., 2006. The genera *Chryseobacterium* and *Elizabethkingia*, in: Martin Dworkin, S.F., Eugene Rosenberg, Karl-Heinz Schleifer, Erko Stackebrandt (Ed.), The Prokaryotes. Springer, New York, USA, pp. 638-676.

Bodelier, P.L.E., Gillisen, M.-J.B., Hordijk, K., Damsté, J.S.S., Rijpstra, W.I.C., Geenevasen, J.A.J., Dunfield, P.F., 2009. A reanalysis of phospholipid fatty acids as ecological biomarkers for methanotrophic bacteria. The ISME Journal 3, 606-617.

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

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jørgensen, B.-B., Witte, U., Pfannkuche, O., 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407, 623-626.

Boone, D.R., Castenholz, R.W., Garrity, G.M., Krieg, N.R., 2011. Bergey's Manual of Systematic Bacteriology: Volume 4: The Bacteroidetes, Spirochaetes, Tenericutes

(Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes. Springer, New York, NY.

Börjesson, G., Sundh, I., Svensson, B., 2004. Microbial oxidation of CH_4 at different temperatures in landfill cover soils. FEMS Microbiology Ecology 48, 305-312.

Bowman, J., 2006. The methanotrophs—the families Methylococcaceae and Methylocystaceae, in: Rosenberg E., S.E., Thompson F., Lory S., DeLong E. (Ed.), The Prokaryotes. Springer, New york, pp. 266-289.

Bowman, J.P., 2014. The Family Methylococcaceae, The Prokaryotes: Gammaproteobacteria, in: Rosenberg, E.F.D., Lory, S., Stackenbrandt, E., Thompson, F. (Eds.), The Prokaryotes : Prokaryotic Biology and Symbiotic Associations. Springer New York, New York, USA, pp. 411-441.

Bowman, J.P., Sayler, G.S., 1994. Optimization and maintenance of soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b. Biodegradation 5, 1-11.

Box, G.E.P., Hunter, J.S., Hunter, W.G., 2005. Statistics for experimenters: design, innovation, and discovery. Wiley, New York, USA.

Butler, J., Montzka, S.A., 2016. The NOAA annual greenhouse gas index (AGGI), Earth System Research Laboratory, Global Monitoring Dvision Website. . University of Colorado., Colorado., pp. Accessed: November 25, 2016.

Cantera, S., Lebrero, R., García-Encina, P.A., Muñoz, R., 2016. Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures. Journal of Environmental Management 171, 11-20.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Cébron, A., Bodrossy, L., Chen, Y., Singer, A.C., Thompson, I.P., Prosser, J.I., Murrell, J.C., 2007. Identity of active methanotrophs in landfill cover soil as revealed by DNA-stable isotope probing. FEMS Microbiology Ecology 62, 12-23.

Chan, S.I., Chen, K.H.C., Yu, S.S.F., Chen, C.L., Kuo, S.S.J., 2004. Toward delineating the structure and function of the particulate methane monooxygenase from methanotrophic bacteria. Biochemistry 43, 4421-4430.

Chanprateep, S., 2010. Current trends in biodegradable polyhydroxyalkanoates.

Journal of Bioscience and Bioengineering 110, 621-632.

Chanton, J., Abichou, T., Langford, C., Hater, G., Green, R., Goldsmith, D., Swan, N., 2010. Landfill methane oxidation across climate types in the US. Environmental Science and Technology 45, 313-319.

Chanton, J., Abichou, T., Langford, C., Spokas, K., Hater, G., Green, R., Goldsmith, D., Barlaz, M.A., 2011. Observations on the methane oxidation capacity of landfill soils. Waste Management 31, 914-925.

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

Chidambaramapadmavathy, K., Karthikeyan, O.P., Heimann, K., 2015b. Biopolymers made from methane in bioreactor. Engineering in Life Sciences 15, 689-699.

Chidambarampadmavathy, K., Karthikeyan, O.P., Heimann, K., 2015a. Role of copper and iron in methane oxidation and bacterial biopolymer accumulation. Engineering in Life Sciences 15, 387-399.

Chidambarampadmavathy, K., Karthikeyan, O.P., Heimann, K., 2016b. Sustainable bio-plastic production through landfill methane recycling. Renewable and Sustainable Energy Reviews 71, 555-562.

Chidambarampadmavathy, K., Karthikeyan, O.P., Huerlimann, R., Maes, G.E., Heimann, K., 2016a. Response of mixed methanotrophic consortia to different methane to oxygen ratios. Waste Management 61, 220-228.

Choi, D.-W., Kunz, R.C., Boyd, E.S., Semrau, J.D., Antholine, W.E., Han, J.-I., Zahn, J.A., Boyd, J.M., Arlene, M., DiSpirito, A.A., 2003. The membrane-associated methane monooxygenase (pMMO) and pMMO-NADH: quinone oxidoreductase complex from *Methylococcus capsulatus* Bath. Journal of Bacteriology 185, 5755-5764.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2013. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research, gkt1244.

Criddle, C.S., Billington, S.L., Frank, C.W., 2014. Renewable bioplastics and biocomposites from biogas methane and waste-derived feedstock: Development of enabling technology, life cycle assessment, and analysis of costs, http://www.calrecycle.ca.gov/Publications/Documents/1502%5C20141502.pdf. Stanford University, California, p. 169.

Culpepper, M.A., Cutsail III, G.E., Hoffman, B.M., Rosenzweig, A.C., 2012a. Evidence for oxygen binding at the active site of particulate methane monooxygenase. Journal of the American Chemical Society 134, 7640-7643.

Culpepper, M.A., Rosenzweig, A.C., 2012b. Architecture and active site of particulate methane monooxygenase. Critical Reviews in Biochemistry and Molecular Biology 47, 483-492.

Dassama, L.M.K., Kenney, G.E., Rosenzweig, A.C., 2017. Methanobactins: from genome to function. Metallomics 9, 7-20.

David, F., Sandra, P., Wylie, P.L., 2002. Improving the analysis of fatty acid methyl esters using retention time locked methods and retention time databases. Agilent Technologies-Application. Agilent Technologies, Palo Alto, CA.

Daystar, J., Gonzalez, R., Reeb, C., Venditti, R.A., Treasure, T., Abt, R., Kelley, S., 2013. Economics, environmental impacts, and supply chain analysis of cellulosic biomass for biofuels in the southern US: Pine, eucalyptus, unmanaged hardwoods, forest residues, switchgrass, and sweet sorghum. BioResources 9, 393-444.

Dedysh, S.N., Dunfield, P.F., 2011. Facultative and obligate methanotrophs: how to identify and differentiate them. Methods in Enzymology 495, 31-44.

DiSpirito, A.A., Semrau, J.D., Murrell, J.C., Gallagher, W.H., Dennison, C., Vuilleumier, S., 2016. Methanobactin and the Link between Copper and Bacterial Methane Oxidation. Microbiology and Molecular Biology Reviews 80, 387-409.

Dong, J., 2013. The role of copper in the growth of *Methylosinus trichosporium* IMV 3011 and poly-β-hydroxybutyrate biosynthesis. Applied Mechanics and Materials 268, 221-224.

Doroshenko, E.V., Boulygina, E.S., Spiridonova, E.M., Tourova, T.P., Kravchenko, I.K., 2007. Isolation and characterization of nitrogen-fixing bacteria of the genus Azospirillum from the soil of a Sphagnum peat bog. Microbiology 76, 93-101.

Dowd, S.E., Sun, Y., Secor, P.R., Rhoads, D.D., Wolcott, B.M., James, G.A., Wolcott, R.D., 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiology 8, 43.

Duan, Z., Møller, N., Greenberg, J., Weare, J.H., 1992. The prediction of methane solubility in natural waters to high ionic strength from 0 to 250 C and from 0 to 1600 bar. Geochimica et Cosmochimica Acta 56, 1451-1460.

Dunbar, J., White, S., Forney, L., 1997. Genetic diversity through the looking glass:

effect of enrichment bias. Applied and Environmental Microbiology 63, 1326-1331.

Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Dedysh, S.N., 2003. *Methylocella silvestris* sp. nov., a novel methanotroph isolated from an acidic forest cambisol. Int. J. Syst. Evol. Microbiol. 53, 1231-1239.

Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S., Ly, B., Saw, J.H., Zhou, Z., Ren, Y., 2007. Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. Nature 450, 879-882.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., 2006a. The Prokaryotes:Vol. 5: Proteobacteria: Alpha and Beta Subclasses. . Springer, Springer, New York, NY, USA.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., 2006b. The Prokaryotes:Vol. 6: Proteobacteria: Gamma Subclass. Springer Science & Business Media, Springer, New York, NY, USA.

Eberspächer, J., Lingens, F., 2006. The genus *Phenylobacterium*, in: Martin Dworkin, S.F., Eugene Rosenberg, Karl-Heinz Schleifer, Erko Stackebrandt (Ed.), The Prokaryotes. Springer, New York, USA, pp. 250-256.

EEA, E.E.A., 2016. Atmospheric concentration of carbon dioxide, methane and nitrous oxide http://www.eea.europa.eu, Accessed: August 05 , 2016.

Eggleston, H.S., Buendia, L., Miwa, K., Ngara, T., Tanabe, K., 2006. IPCC guidelines for national greenhouse gas inventories. Institute for Global Environmental Strategies, Hayama, Japan.

EIA, U., 2014. International energy statistics, US Energy and Information Administration, Washington, DC Accessed: March 15, 2016, https://www.eia.gov/cfapps/ipdbproject/iedindex3.cfm?tid=5&pid=53&aid=1&cid=ww,&syid=2014&eyid=2015&unit=TBPD ; .

Einola, J.-K.M., Kettunen, R.H., Rintala, J.A., 2007. Responses of methane oxidation to temperature and water content in cover soil of a boreal landfill. Soil Biology and Biochemistry 39, 1156-1164.

Elliott, J., Sharma, B., Best, N., Glotter, M., Dunn, J.B., Foster, I., Miguez, F., Mueller, S., Wang, M., 2014. A spatial modeling framework to evaluate domestic biofuel-induced potential land use changes and emissions. Environmental Science & Technology 48, 2488-2496.

Emadian, S.M., Onay, T.T., Demirel, B., 2017. Biodegradation of bioplastics in natural

environments. Waste Management 59, 526-536.

Energy, U.S.D.o., Final Environmental Impact Statement for the Proposed Abengoa Biorefinery Project near Hugoton, Stevens County, Kansas (Doe/Eis-0407), in: https://www.energy.gov/sites/prod/files/EIS-0407-FEIS-01-2010.pdf (Ed.), 20.10.2015.

EPA, U., 2011. Available and emerging technologies for reducing greenhouse gas emissions from municipal solid waste landfills in: Agency, U.S.E.P. (Ed.). EPA 430-R-11-005, North Carolina

EPA, U., 2016. Understanding Global Warming Potentials, US Energy and Information Administration, Washington, DC https://www.epa.gov/ghgemissions/understanding-global-warming-potentials#Learn why. Acessed: October 15, 2016.

Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nature Reviews Microbiology 10, 538-550.

Fox, B.G., Froland, W.A., Dege, J.E., Lipscomb, J.D., 1989. Methane monooxygenase from *Methylosinus trichosporium* OB3b. Purification and properties of a three-component system with high specific activity from a type II methanotroph. The Journal of Biological Chemistry 264, 10023-10033.

Gebert, J., Singh, B.K., Pan, Y., Bodrossy, L., 2009. Activity and structure of methanotrophic communities in landfill cover soils. Environmental Microbiology Reports 1, 414-423.

Gerngross, T.U., Slater, S.C., 2000. How green are green plastics? Scientific American 283, 36-41.

Girguis, P.R., Cozen, A.E., DeLong, E.F., 2005. Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. Applied and Environmental Microbiology 71, 3725-3733.

Gironi, F., Piemonte, V., 2011. Bioplastics and petroleum-based plastics: strengths and weaknesses. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects 33, 1949-1959.

Glass, J., Orphan, V.J., 2012. Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. Frontiers in Microbiology 3, 61.

GMI, G.M.I., 2010. Global methane emissions and mitigation opportunities, www. globalmethane. org Acessed: August, 17, 2015.

Graham, D.W., Chaudhary, J.A., Hanson, R.S., Arnold, R.G., 1993. Factors affecting

competition between type I and type II methanotrophs in two-organism, continuous-flow reactors. Microbial Ecology 25, 1-17.

Green, J., Dalton, H., 1985. Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). A novel regulatory protein of enzyme activity. The Journal of Biological Chemistry 260, 15795-15801.

Grosse, S., Laramee, L., Wendlandt, K.-D., McDonald, I.R., Miguez, C.B., Kleber, H.-P., 1999. Purification and characterization of the soluble methane monooxygenase of the type II methanotrophic bacterium *Methylocystis* sp. Strain WI 14. Applied and Environmental Microbiology 65, 3929-3935.

Gu, W., Haque, M.F.U., Baral, B.S., Turpin, E.A., Bandow, N.L., Kremmer, E., Flatley, A., Zischka, H., DiSpirito, A.A., Semrau, J.D., 2016. A TonB-Dependent Transporter Is Responsible for Methanobactin Uptake by *Methylosinus trichosporium* OB3b. Applied and Environmental Microbiology 82, 1917-1923.

Haft, D.H., Selengut, J.D., Richter, R.A., Harkins, D., Basu, M.K., Beck, E., 2012. TIGRFAMs and genome properties in 2013. Nucleic Acids Research 41, D387-D395.

Hagen, L.H., Vivekanand, V., Pope, P.B., Eijsink, V.G.H., Horn, S.J., 2015. The effect of storage conditions on microbial community composition and biomethane potential in a biogas starter culture. Applied Microbiology and Biotechnology 99, 5749-5761.

Hakemian, A.S., Kondapalli, K.C., Telser, J., Hoffman, B.M., Stemmler, T.L., Rosenzweig, A.C., 2008. The metal centers of particulate methane monooxygenase from *Methylosinus trichosporium* OB3b. Biochemistry 47, 6793-6801.

Hanson, R.S., Hanson, T.E., 1996. Methanotrophic bacteria. Microbiological Reviews 60, 439-471.

Harding, K.G., Dennis, J.S., Von Blottnitz, H., Harrison, S., 2007. Environmental analysis of plastic production processes: Comparing petroleum-based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid using life cycle analysis. Journal of Biotechnology 130, 57-66.

Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z., Tyson, G.W., 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500, 567-570.

Hartmann, A., Baldani, J.I., 2006. The Genus *Azospirillum*, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes:Vol. 5: Proteobacteria: Alpha and Beta Subclasses. . Springer, Springer, New York, USA, pp.

115-140.

Helm, J., Wendlandt, K.D., Jechorek, M., Stottmeister, U., 2008. Potassium deficiency results in accumulation of ultra-high molecular weight poly-β-hydroxybutyrate in a methane-utilizing mixed culture. Journal of Applied Microbiology 105, 1054-1061.

Helm, J., Wendlandt, K.D., Rogge, G., Kappelmeyer, U., 2006. Characterizing a stable methane-utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system. Journal of Applied Microbiology 101, 387-395

Henckel, T., Roslev, P., Conrad, R., 2000. Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. Environmental Microbiology 2, 666-679

Henneberger, R., Lüke, C., Mosberger, L., Schroth, M.H., 2012. Structure and function of methanotrophic communities in a landfill-cover soil. FEMS Microbiology Ecology 81, 52-65.

Hernandez, M.E., Beck, D.A.C., Lidstrom, M.E., Chistoserdova, L., 2015. Oxygen availability is a major factor in determining the composition of microbial communities involved in methane oxidation. Peer J. 3, e801.

Héry, M., Singer, A.C., Kumaresan, D., Bodrossy, L., Stralis-Pavese, N., Prosser, J.I., Thompson, I.P., Murrell, J.C., 2008. Effect of earthworms on the community structure of active methanotrophic bacteria in a landfill cover soil. The ISME Journal 2, 92-104.

Higgins, I.J., Best, D.J., Hammond, R.C., Scott, D., 1981. Methane-oxidizing microorganisms. Microbiological Reviews 45, 556.

Hinrichs, K.-U., Boetius, A., 2003. The anaerobic oxidation of methane: new insights in microbial ecology and biogeochemistry, in: Wefer, G., Billett, D., Hebbeln, D., Jørgensen, B.-B., Schlüter, M., van Weering, T.C.E. (Eds.), Ocean Margin Systems. Springer Berlin Heidelberg, pp. 457-477.

Hirayama, H., Abe, M., Miyazaki, M., Nunoura, T., Furushima, Y., Yamamoto, H., Takai, K., 2014. *Methylomarinovum caldicuralii* gen. nov., sp. nov., a moderately thermophilic methanotroph isolated from a shallow submarine hydrothermal system, and proposal of the family Methylothermaceae fam. nov. International Journal of Systematic and Evolutionary Microbiology 64, 989-999.

Hirayama, H., Fuse, H., Abe, M., Miyazaki, M., Nakamura, T., Nunoura, T., Furushima, Y., Yamamoto, H., Takai, K., 2013. *Methylomarinum vadi* gen. nov., sp. nov., a methanotroph isolated from two distinct marine environments. International Journal of

Systematic and Evolutionary Microbiology 63, 1073-1082.

Ho, A., Angel, R., Veraart, A.J., Daebeler, A., Jia, Z., Kim, S.Y., Kerckhof, F.-M., Boon, N., Bodelier, P.L.E., 2016. Biotic Interactions in Microbial Communities as Modulators of Biogeochemical Processes: Methanotrophy as a Model System. Frontiers in Microbiology 7, 1285.

Ho, A., De Roy, K., Thas, O., De Neve, J., Hoefman, S., Vandamme, P., Heylen, K., Boon, N., 2014. The more, the merrier: heterotroph richness stimulates methanotrophic activity. The ISME Journal 8, 1945-1948.

Hopewell, J., Dvorak, R., Kosior, E., 2009. Plastics recycling: challenges and opportunities. Philosophical Transactions of the Royal Society B 364, 2115-2126.

Hršak, D., Begonja, A., 2000. Possible interactions within a methanotrophic-heterotrophic groundwater community able to transform linear alkylbenzenesulfonates. Applied and Environmental Microbiology 66, 4433-4439.

Huber-Humer, M., 2004. International research into landfill gas emissions and mitigation strategies – IWWG working group “CLEAR”. Waste Management 24, 425-427.

Huber-Humer, M., Gebert, J., Hilger, H., 2008. Biotic systems to mitigate landfill methane emissions. Waste Management and Research 26, 33-46.

Humer, M., Lechner, P., 1999. Alternative approach to the elimination of greenhouse gases from old landfills. Waste Management and Research 17, 443-452.

Hurd, P.J., Nelson, C.J., 2009. Advantages of next-generation sequencing versus the microarray in epigenetic research. Briefings in Functional Genomics, elp013.

Hyder Consulting, P.L., 2012. Waste and recycling in Australia, in: Department of sustainability, e., water, pollution and communities. (Ed.), Regional Population Growth, Australia, 2011-12, Australia.

Iguchi, H., Yurimoto, H., Sakai, Y., 2011a. *Methylovulum miyakonense* gen. nov., sp. nov., a type I methanotroph isolated from forest soil. International Journal of Systematic and Evolutionary Microbiology 61, 810-815.

Iguchi, H., Yurimoto, H., Sakai, Y., 2011b. Stimulation of methanotrophic growth in cocultures by cobalamin excreted by rhizobia. Applied and Environmental Microbiology 77, 8509-8515.

Ioannidis, J.P.A., Allison, D.B., Ball, C.A., Coulibaly, I., Cui, X., Culhane, A.C., Falchi, M., Furlanello, C., Game, L., Jurman, G., 2009. Repeatability of published microarray

gene expression analyses. Nature Genetics 41, 149-155.

IPCC, 2007. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, in: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (Eds.), Climate Change 2007: The Physical Science Basis, Cambridge and New York., pp. 93-129.

IPCC, 2014. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, in: Stocker, T., Qin, D., Plattner, G., Tignor, M., Allen, S., Boschung, J., Nauels, A., Xia, Y., Bex, B., Midgley, B. (Eds.), Cambridge University Press,Cambridge, United Kingdom and New York, NY, USA, p. 1535

Ishigaki, T., Sugano, W., Nakanishi, A., Tateda, M., Ike, M., Fujita, M., 2004. The degradability of biodegradable plastics in aerobic and anaerobic waste landfill model reactors. Chemosphere 54, 225-233.

Ito, H., Mori, F., Tabata, K., Okura, I., Kamachi, T., 2014. Methane hydroxylation using light energy by the combination of thylakoid and methane monooxygenase. Royal Society of Chemistry Advances 4, 8645-8648.

Jahng, D., Wood, T., 1996. Metal ions and chloramphenicol inhibition of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. Applied Microbiology and Biotechnology 45, 744-749.

Jeong, S.-Y., Cho, K.-S., Kim, T.G., 2014. Density-dependent enhancement of methane oxidation activity and growth of Methylocystis sp. by a non-methanotrophic bacterium *Sphingopyxis* sp. Biotechnology Reports 4, 128-133.

Jiang, H., Chen, Y., Jiang, P., Zhang, C., Smith, T.J., Murrell, J.C., Xing, X.-H., 2010. Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. Biochemical Engineering Journal 49, 277-288.

Jugnia, L.-B., Aït-Benichou, S., Fortin, N., Cabral, A.R., Greer, C.W., 2009. Diversity and dynamics of methanotrophs within an experimental landfill cover soil. Soil Science Society of America Journal 73, 1479-1487.

Kalyuzhnaya, M.G., Puri, A.W., Lidstrom, M.E., 2015. Metabolic engineering in methanotrophic bacteria. Metabolic Engineering 29, 142-152.

Kalyuzhnaya, M.G., Yang, S., Rozova, O.N., Smalley, N.E., Clubb, J., Lamb, A., Gowda, G.-A.N., Raftery, D., Fu, Y., Bringel, F., 2013. Highly efficient methane biocatalysis revealed in a methanotrophic bacterium. Nature communications 4.

Kalyuzhnaya, M.G., Zabinsky, R., Bowerman, S., Baker, D.R., Lidstrom, M., Chistoserdova, L., 2006. Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. Applied and Environmental Microbiology 72, 4293-4301.

Karthikeyan, O.P., Chidambarampadmavathy, K., Cirés, S., Heimann, K., 2015a. Review of sustainable methane mitigation and bio-polymer production. Critical Reviews in Environmental Science and Technology 45, 1579-1610.

Karthikeyan, O.P., Chidambarampadmavathy, K., Nadarajan, S., Lee, P.K., Heimann, K., 2015b. Effect of CH_4/O_2 ratio on fatty acid profile and polyhydroxybutyrate content in a heterotrophic–methanotrophic consortium. Chemosphere 141, 235-242.

Karthikeyan, O.P., May, J.S.Y., Rajasekar, B., 2016b. Gradient packing bed bio-filter for landfill methane mitigation. Bioresource Technology 217, 205-209.

Kelly, D., Wood, A., 2010. Isolation and characterization of methanotrophs and methylotrophs: diversity of methylotrophic organisms and of one-carbon substrates, Handbook of Hydrocarbon and Lipid Microbiology. Springer, pp. 3827-3845.

Kenney, G.E., Rosenzweig, A.C., 2011. Chemistry and biology of the copper chelator methanobactin. ACS chemical biology 7, 260-268.

Keshavarz, T., Roy, I., 2010. Polyhydroxyalkanoates: bioplastics with a green agenda. Current Opinion in Microbiology 13, 321-326.

Khmelenina, V.N., Rozova, O.N., But, S.-Y., Mustakhimov, I., Reshetnikov, A.S., Beschastnyi, A.P., Trotsenko, Y.A., 2015. Biosynthesis of secondary metabolites in methanotrophs: Biochemical and genetic aspects (Review). Applied Biochemistry and Microbiology 51, 150-158.

Kim, T.G., Moon, K.E., Yun, J., Cho, K.S., 2013. Comparison of RNA-and DNA-based bacterial communities in a lab-scale methane-degrading biocover. Applied Microbiology and Biotechnology 97, 3171-3181.

Knittel, K., Boetius, A., 2009. Anaerobic oxidation of methane: progress with an unknown process. Annual Review of Microbiology 63, 311-334.

Krentz, B.D., Mulheron, H.J., Semrau, J.D., DiSpirito, A.A., Bandow, N.L., Haft, D.H., Vuilleumier, S., Murrell, J.C., McEllistrem, M.T., Hartsel, S.C., 2010. A comparison of methanobactins from *Methylosinus trichosporium* OB3b and *Methylocystis* strain SB2 predicts methanobactins are synthesized from diverse peptide precursors modified to create a common core for binding and reducing copper ions. Biochemistry 49, 10117-

10130.

Kumaresan, D., Abell, G.-C.J., Bodrossy, L., Stralis-Pavese, N., Murrell, J.C., 2009. Spatial and temporal diversity of methanotrophs in a landfill cover soil are differentially related to soil abiotic factors. Environmental Microbiology Reports 1, 398-407.

Lackner, M., 2015. Bioplastics. Kirk-Othmer Encyclopedia of Chemical Technology, 1-41.

LAWMAC, 2014. Townsville update, Local authority waste managment advisory comitee.

Lee, J.-H., Kim, T., Cho, K.-S., 2012. Isolation and characterization of a facultative methanotroph degrading malodor-causing volatile sulfur compounds. Journal of Hazardous Materials 235, 224-229.

Lee, S.Y., 1996a. Bacterial polyhydroxyalkanoates. Biotechnology and Bioengineering 49, 1-14.

Lee, S.Y., 1996b. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends in Biotechnololgy 14, 431-438.

Lemstra, P.J., 2012. Petro-vs. bio-based polymers, Second International Plastics Conference: Trends in Bioplastics, September 17–18, Ljubljana, Slovenia.

Levett, I., Birkett, G., Davies, N., Bell, A., Langford, A., Laycock, B., Lant, P., Pratt, S., 2016. Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane—The case for thermophilic bioprocessing. Journal of Environmental Chemical Engineering 4, 3724-3733.

Levy, H., 1971. Normal atmosphere: Large radical and formaldehyde concentrations predicted. Science 173, 141-143.

Li, S., Juliane, H., Martin, K.P., 2009. Product overview and market projection of emerging bio-based plastics, in: Bioplastics., R.f.E.P.N.o.E.E.a.E. (Ed.), Utrecht University, The Netherlands, pp. 1–227.

Lieberman, R.L., Rosenzweig, A.C., 2005a. Crystal structure of a membrane-bound metalloenzyme that catalyses the biological oxidation of methane. Nature 434, 177-182.

Lieberman, R.L., Rosenzweig, A.C., 2005b. The quest for the particulate methane monooxygenase active site. Dalton Transactions, 3390-3396.

Listewnik, H.F., Wendlandt, K.D., Jechorek, M., Mirschel, G., 2007. Process Design for

the Microbial Synthesis of Poly-β-hydroxybutyrate (PHB) from Natural Gas. Engineering in Life Sciences 7, 278-282.

López, J.C., Quijano, G., Pérez, R., Muñoz, R., 2014. Assessing the influence of CH_4 concentration during culture enrichment on the biodegradation kinetics and population structure. Journal of Environmental Management 146, 116-123.

Lu, W.-J., Chi, Z.-F., Mou, Z.-S., Long, Y.-Y., Wang, H.-T., Zhu, Y., 2011. Can a breathing biocover system enhance methane emission reduction from landfill? Journal of Hazardous Materials 191, 228-233.

Meinhardt, 2000. North Queensland regional waste management strategy, http://www.soe-townsville.org/data/HESROC-NQ_regional_waste_management_strategy/regional_waste_management.pdf. Accessed: Novemmber 11, 2016.

Merkx, M., Kopp, D.A., Sazinsky, M.H., Blazyk, J.L., Müller, J., Lippard, S.J., 2001. Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: a tale of two irons and three proteins. Angewandte Chemie International Edition 40, 2782-2807.

Merkx, M., Lippard, S.J., 2002. Why OrfY? Characterization of MMOD, a long overlooked component of the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). The Journal of Biological Chemistry 277, 5858-5865.

Morton, J.D., Hayes, K.F., Semrau, J.D., 2000. Effect of copper speciation on whole-cell soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b. Applied and Environmental Microbiology 66, 1730-1733.

Murrell, J.C., Gilbert, B., McDonald, I.R., 2000. Molecular biology and regulation of methane monooxygenase. Archives of Microbiology 173, 325-332.

Murrell, J.C., Smith, T.J., 2010. Biochemistry and molecular biology of methane monooxygenase, in: TIMMIS, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer, pp. 1045-1055.

Nakajima, T., Hiroo, U.-I., Yagi, T.O., Nakahara, T., 1992. Purification and properties of a soluble methane monooxygenase from *Methylocystis* sp. M. Bioscience, Biotechnology, and Biochemistry 56, 736-740.

Neubauer, S.C., 2014. On the challenges of modeling the net radiative forcing of wetlands: reconsidering Mitsch et al. 2013. Landscape Ecology 29, 571-577.

Nikiema, J., Brzezinski, R., Heitz, M., 2007. Elimination of methane generated from

landfills by biofiltration: a review. Reviews in Environmental Science and Bioechnology 6, 261-284.

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J.W., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philosophical Transactions of the Royal Society B 364, 2047-2062.

Ojumu, T.V., Yu, J., Solomon, B.O., 2004. Production of polyhydroxyalkanoates, a bacterial biodegradable polymers. African Journal of Biotechnology 3, 18-24.

Oni, O.E., Friedrich, M.W., 2016. Metal Oxide Reduction Linked to Anaerobic Methane Oxidation. Trends in Microbiology 25, 88-90.

Op den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S., Jetten, M.S.M., Birkeland, N.K., Pol, A., Dunfield, P.F., 2009. Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. Environmental Microbiology Reports 1, 293-306.

Oshkin, I.Y., Beck, D.A.C., Lamb, A.E., Tchesnokova, V., Benuska, G., McTaggart, T.L., Kalyuzhnaya, M.G., Dedysh, S.N., Lidstrom, M.E., Chistoserdova, L., 2015. Methane-fed microbial microcosms show differential community dynamics and pinpoint taxa involved in communal response. The ISME Journal 9, 1119-1129.

Pariatamby, A., Cheah, W.Y., Shrizal, R., Thamlarson, N., Lim, B.T., Barasarathi, J., 2015. Enhancement of landfill methane oxidation using different types of organic wastes. Environmental Earth Sciences 73, 2489-2496.

Park, S., Hanna, L., Taylor, R.T., Droege, M.W., 1991. Batch cultivation of Methylosinus trichosporium OB3b. I: Production of soluble methane monooxygenase. Biotechnology and Bioengineering 38, 423-433.

Peck, J., 2007. Landfill gas generation at open dump landfills in developing countries, In:10th Annual Landfill Methane Outreach Program, Conference and project expo, January, January 23–24, Baltimore, Maryland.

Pei, L., Schmidt, M., Wei, W., 2011. Conversion of biomass into bioplastics and their potential environmental impacts, in: Elnashar M (Ed.), Biotechnology of Biopolymers. InTech, Rijeka, Croatia, pp. 57-75.

Pfluger, A.R., Wu, W.-M., Pieja, A.J., Wan, J., Rostkowski, K.H., Criddle, C.S., 2011. Selection of Type I and Type II methanotrophic proteobacteria in a fluidized bed reactor under non-sterile conditions. Bioresource Technology 102, 9919-9926.

Philp, J., 2014a. OECD Policies for Bioplastics in the Context of a Bioeconomy, 2013. Industrial Biotechnology 10, 19-21.

Philp, J.C., 2014b. Biobased Chemicals and Bioplastics: Finding the Right Policy Balance. Industrial Biotechnology 10, 379-383.

Pieja, A.J., Rostkowski, K.H., Criddle, C.S., 2011a. Distribution and selection of poly-3-hydroxybutyrate production capacity in methanotrophic proteobacteria. Microbial Ecology 62, 564-573.

Pieja, A.J., Sundstrom, E.R., Criddle, C.S., 2011b. Poly-3-hydroxybutyrate metabolism in the type II methanotroph *Methylocystis parvus* OBBP. Applied and Environmental Microbiology 77, 6012-6019.

Pieja, A.J., Sundstrom, E.R., Criddle, C.S., 2012. Cyclic, alternating methane and nitrogen limitation increases PHB production in a methanotrophic community. Bioresource Technology 107, 385-392.

Plastic the Facts, 2015. An analysis of European plastics production, demand and waste data., http://issuu.com/plasticseuropeebook/docs/final_plastics_the_facts_2014_19122, Accessed: April 20, 2015.

Poindexter, J.S., 2006. Dimorphic prosthecate bacteria: the genera *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and *Thiodendron*, in: Martin Dworkin, S.F., Eugene Rosenberg, Karl-Heinz Schleifer, Erko Stackebrandt (Ed.), The Prokaryotes. Springer, New York, USA, pp. 72-90.

Pol, A., Heijmans, K., Harhangi, H.R., Tedesco, D., Jetten, M.S.M., den Camp, H.O., 2007. Methanotrophy below pH 1 by a new Verrucomicrobia species. Nature 450, 874-878.

Puri, A.W., Schaefer, A.L., Fu, Y., Beck, D.A.C., Greenberg, E.P., Lidstrom, M.E., 2016. Quorum sensing in a methane-oxidizing bacterium. Journal of Bacteriology, JB. 00773-00716.

Quan, X., Zhang, L., Zhao, T., Zhao, Y., Deng, Y., 2012. *Chryseobacterium* Sp. capable of using methane and application thereof, in: Technology, C.I.o. (Ed.), Google Patents, China.

Reddy, C.S.K., Ghai, R., Kalia, V.C., 2003. Polyhydroxyalkanoates: an overview. Bioresource Technology 87, 137-146.

Roland-Holst, D., Triolo, R., Heft-Neal, S., Bayrami, B., 2013. Bioplastics in California,

Economic assessment of market conditions for PHA/PHB bioplastics produced from waste methane., University of California, Berkeley, pp. 1-76.

Rosenzweig, A.C., Frederick, C.A., Lippard, S.J., 1993. Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. *Nature* 366, 537-543.

Rostkowski, K.H., Criddle, C.S., Lepech, M.D., 2012. Cradle-to-gate life cycle assessment for a cradle-to-cradle cycle: biogas-to-bioplastic (and back). Environmental Science and Technology 46, 9822-9829.

Samarajeewa, A.D., Hammad, A., Masson, L., Khan, I.U.H., Scroggins, R., Beaudette, L.A., 2015. Comparative assessment of next-generation sequencing, denaturing gradient gel electrophoresis, clonal restriction fragment length polymorphism and cloning-sequencing as methods for characterizing commercial microbial consortia. Journal of Microbiological Methods 108, 103-111.

Scheutz, C., Bogner, J., De Visscher, A., Gebert, J., Hilger, H., Huber-Humer, M., Kjeldsen, P., Spokas, K., 2009. Microbial methane oxidation processes and technologies for mitigation of landfill gas emissions. Waste Management & Research 27, 409-455.

Seghers, D., Top, E.M., Reheul, D., Bulcke, R., Boeckx, P., Verstraete, W., Siciliano, S.D., 2003. Long-term effects of mineral versus organic fertilizers on activity and structure of the methanotrophic community in agricultural soils. Environmental Microbiology 5, 867-877.

Semrau, J.D., 2011. Bioremediation via methanotrophy: overview of recent findings and suggestions for future research. Frontiers in Microbiology 2.

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

Semrau, J.D., Jagadevan, S., DiSpirito, A.A., Khalifa, A., Scanlan, J., Bergman, B.H., Freemeier, B.C., Baral, B.S., Bandow, N.L., Vorobev, A., 2013. Methanobactin and MmoD work in concert to act as the ‘copper-switch’in methanotrophs. Environmental Microbiology 15, 3077-3086.

Shalini, S.S., Karthikeyan, O.P., Joseph, K., 2010. Biological stability of municipal solid waste from simulated landfills under tropical environment. Bioresource Technology 101, 845-852.

Shendure, J., Ji, H., 2008. Next-generation DNA sequencing. Nature Biotechnology

26, 1135-1145.

Slobodova, N.V., Kolganova, T.V., Boulygina, E.S., Kuznetsov, B.B., Tourova, T.P., Kravchenko, I.K., 2006. Comparative characterization of methanotrophic enrichments by serological and molecular methods. Microbiology 75, 336-342.

Smith, S.M., Rawat, S., Telser, J., Hoffman, B.M., Stemmler, T.L., Rosenzweig, A.C., 2011. Crystal structure and characterization of particulate methane monooxygenase from *Methylocystis* species strain M. Biochemistry 50, 10231-10240.

Smith, T.J., Murrell, J.C., 2008. Methanotrophs, in: Flickenger, M.C. (Ed.), Encyclopedia of Industrial Biotechnology. John Wiley and Sons, pp. 1-13

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L., 2007. Climate change 2007, The physical science basis: *Working Group I contribution to the fourth assessment report of the Intergovernmental Panel on Climate Change*. Cambridge University press, Cambridge, UK and New York, USA.

Spokas, K.A., Bogner, J.E., 2011. Limits and dynamics of methane oxidation in landfill cover soils. Waste Management 31, 823-832.

Staley, B.F., Barlaz, M.A., 2009. Composition of municipal solid waste in the United States and implications for carbon sequestration and methane yield. Journal of Environmental Mangement 135, 901-909.

Stanley, S.H., Prior, S.D., Leak, D.J., Dalton, H., 1983. Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. Biotechnology Letters 5, 487-492.

Steinbüchel, A., Füchtenbusch, B., 1998. Bacterial and other biological systems for polyester production. Trends in Biotechnololgy 16, 419-427.

Stock, M., Hoefman, S., Kerckhof, F.-M., Boon, N., De Vos, P., De Baets, B., Heylen, K., Waegeman, W., 2013. Exploration and prediction of interactions between methanotrophs and heterotrophs. Research in Microbiology 164, 1045-1054.

Stoecker, K., Bendinger, B., Schöning, B., Nielsen, P.H., Nielsen, J.L., Baranyi, C., Toenshoff, E.R., Daims, H., Wagner, M., 2006. Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. Proceedings of the National Academy of Sciences 103, 2363-2367.

Stralis-Pavese, N., Bodrossy, L., Reichenauer, T.G., Weilharter, A., Sessitsch, A., 2006. 16S rRNA based T-RFLP analysis of methane oxidising bacteria—Assessment,

critical evaluation of methodology performance and application for landfill site cover soils. Applied Soil Ecology 31, 251-266.

Stralis-Pavese, N., Sessitsch, A., Weilharter, A., Reichenauer, T., Riesing, J., Csontos, J., Murrell, J.C., Bodrossy, L., 2004. Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. Environmental Microbiology 6, 347-363.

Strong, P.J., Kalyuzhnaya, M., Silverman, J., Clarke, W.P., 2016. A methanotroph-based biorefinery: Potential scenarios for generating multiple products from a single fermentation. Bioresource Technology 215, 314-323.

Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource: can the methanotrophs add value? Environmental Science and Technology 49, 4001-4018.

Su, Y., Zhang, X., Xia, F.-F., Zhang, Q.-Q., Kong, J.-Y., Wang, J., He, R., 2014. Diversity and activity of methanotrophs in landfill cover soils with and without landfill gas recovery systems. Systematic and Applied Microbiology 37, 200-207.

Sullivan, J.P., Dickinson, D., Chase, H.A., 1998. Methanotrophs, *Methylosinus trichosporium* OB3b, sMMO, and their application to bioremediation. Critical Reviews in Microbiology 24, 335-373.

Syamsul Arif, M.A., Houwen, F., Verstraete, W., 1996. Agricultural factors affecting methane oxidation in arable soil. Biology and Fertility of Soils 21, 95-102.

Takeguchi, M., Ohashi, M., Okura, I., 1999. Role of iron in particulate methane monooxygenase from *Methylosinus trichosporium* OB3b. BioMetals 12, 123-129.

Tate, K.R., Walcroft, A.S., Pratt, C., 2012. Varying atmospheric methane concentrations affect soil methane oxidation rates and methanotroph populations in pasture, an adjacent pine forest, and a landfill. Soil Biology and Biochemistry 52, 75-81.

Teuten, E.L., Rowland, S.J., Galloway, T.S., Thompson, R.C., 2007. Potential for plastics to transport hydrophobic contaminants. Environmental Science & Technology 41, 7759-7764.

Thompson, R.C., Moore, C.J., Vom Saal, F.S., Swan, S.H., 2009. Plastics, the environment and human health: current consensus and future trends. Philosophical Transactions of the Royal Society B 364, 2153-2166.

Torsvik, V., Daae, F.L., Sandaa, R.-A., Øvreås, L., 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. Journal of

Biotechnology 64, 53-62.

Trevors, J.T., Cotter, C.M., 1990. Copper toxicity and uptake in microorganisms. J. Ind. Microbiol. 6, 77-84.

Trotsenko, Y.A., Murrell, J.C., 2008. Metabolic Aspects of Aerobic Obligate Methanotrophy. Advances in Applied Microbiology 63, 183-229.

Van Bodegom, P., Stams, F., Mollema, L., Boeke, S., Leffelaar, P., 2001. Methane oxidation and the competition for oxygen in the rice rhizosphere. Applied and Environmental Microbiology 67, 3586-3597.

van der Ha, D., Hoefman, S., Boeckx, P., Verstraete, W., Boon, N., 2010. Copper enhances the activity and salt resistance of mixed methane-oxidizing communities. Applied Microbiology and Biotechnology 87, 2355-2363.

van der Ha, D., Vanwonterghem, I., Hoefman, S., De Vos, P., Boon, N., 2013. Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations. Antonie Van Leeuwenhoek 103, 527-537.

van Teeseling, M.C.F., Pol, A., Harhangi, H.R., van der Zwart, S., Jetten, M.S.M., den Camp, H.J.O., van Niftrik, L., 2014. Expanding the verrucomicrobial methanotrophic world: description of three novel species of *Methylacidimicrobium* gen. nov. Applied and Environmental Microbiology 80, 6782-6791. 6710.1128/AEM.01838-01814.

Vandamme, P., Bernardet, J.-F., Segers, P., Kersters, K., Holmes, B., 1994. NOTES: New Perspectives in the Classification of the Flavobacteria: Description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. International Journal of Systematic and Evolutionary Microbiology 44, 827-831.

von Alvensleben, N., Stookey, K., Magnusson, M., Heimann, K., 2013. Salinity tolerance of *Picochlorum atomus* and the use of salinity for contamination control by the freshwater cyanobacterium *Pseudanabaena limnetica*. Plos One 8.

Vorholt, J.A., 2002. Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. Archives of Microbiology 178, 239-249.

Webb, H.K., Ng, H.J., Ivanova, E.P., 2014. The Family Methylocystaceae. Springer, Verlag Berlin Heidelberg.

Wei, X.M., Su, Y., Zhang, H.T., Chen, M., He, R., 2015. Responses of methanotrophic activity, community and EPS production to CH_4 and O_2 concentrations in waste biocover soils. Waste Management 42, 118-127.

Wendlandt, K.-D., Geyer, W., Mirschel, G., Hemidi, F.A.H., 2005. Possibilities for

controlling a PHB accumulation process using various analytical methods. Journal of Biotechnology 117, 119-129.

Wendlandt, K.-D., Stottmeister, U., Helm, J., Soltmann, B., Jechorek, M., Beck, M., 2010. The potential of methane-oxidizing bacteria for applications in environmental biotechnology. Engineering in Life Sciences 10, 87-102.

Whalen, S.C., Reeburgh, W.S., Sandbeck, K.A., 1990. Rapid methane oxidation in a landfill cover soil. Applied and Environmental Microbiology 56, 3405-3411.

Whittenbury, R., Dalton, H., 1981. The methylotrophic bacteria, in: Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G. (Eds.), The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Springer, Springer Berlin Heidelberg, pp. 894-902.

Whittenbury, R., Phillips, K.C., Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. Journal of Genreal Microbiology 61, 205-218.

Wiles, D.M., Scott, G., 2006. Polyolefins with controlled environmental degradability. Polymer Degradation and Stability 91, 1581-1592.

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

Wise, M.G., McArthur, J.V., Shimkets, L.J., 2001. *Methylosarcina fibrata* gen. nov., sp. nov. and *Methylosarcina quisquiliarum* sp. nov., novel type 1 methanotrophs. International Journal of Systematic and Evolutionary Microbiology 51, 611-621.

Wu, M.L., Ettwig, K.F., Jetten, M.S.M., Strous, M., Keltjens, J.T., van Niftrik, L., 2011. A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium Candidatus '*Methylomirabilis oxyfera*'. Biochemical Society Transactions 39, 243-248.

Xuerning, L., Ruoqian, Y., Shizhong, L., 1999. The relationship between optical density and dry weight of Chlorella vulgaris in batch heterotrophic culture. Wei Sheng wu xue Tong bao 26, 339-341.

Yang, S., Matsen, J.B., Kunopka, M., Green-Saxena, A., Clubb, J., Sadilek, M., Orphan, V.J., Beck, D., Kalyuzhnaya, M.G., 2013. Global molecular analyses of methane metabolism in methanotrophic Alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part II. Metabolomics and 13C-labeling study. Frontiers in

Microbiology 4, Art. No. 70.

Yao, H., Chapman, S.J., Thornton, B., Paterson, E., 2015. ^{13}C PLFAs: a key to open the soil microbial black box? Plant and Soil 392, 3-15.

Yu, S.S.F., Chen, K.H.C., Tseng, M.Y.H., Wang, Y.S., Tseng, C.F., Chen, Y.J., Huang, D.S., Chan, S.I., 2003. Production of high-quality particulate methane monooxygenase in high yields from *Methylococcus capsulatus* (Bath) with a hollow-fiber membrane bioreactor. Journal of Bacteriology 185, 5915-5924.

Zhang, Y., Xin, J., Chen, L., Song, H., Xia, C., 2008. Biosynthesis of poly-3-hydroxybutyrate with a high molecular weight by methanotroph from methane and methanol. Journal of Natural Gas Chemistry 17, 103-109.

Zhang, Y., Xin, J., Chen, L., Xia, C., 2009. The methane monooxygenase intrinsic activity of kinds of methanotrophs. Applied Biochemistry and Biotechnology 157, 431-441.