<table>
<thead>
<tr>
<th>Title</th>
<th>The microbial ecology of novel horizontal flow biofilm reactors (HFBRs) used to treat methane, hydrogen sulphide and ammonia contaminated airstreams at 10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Gerrity, Seán</td>
</tr>
<tr>
<td>Publication Date</td>
<td>2015-05-29</td>
</tr>
<tr>
<td>Item record</td>
<td><a href="http://hdl.handle.net/10379/5214">http://hdl.handle.net/10379/5214</a></td>
</tr>
</tbody>
</table>
The Microbial Ecology of Novel Horizontal Flow Biofilm Reactors (HFBRs) used to treat Methane, Hydrogen Sulphide and Ammonia Contaminated Airstreams at 10°C

Seán Gerrity

Supervisors: Dr. Gavin Collins

Dr. Eoghan Clifford

Department of Microbiology,

School of Natural Science,

National University of Ireland Galway.

May 2015
ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Gavin Collins for offering me the chance to do this PhD and for his help and guidance over the last few years. I would also like to thank my co-supervisor Dr. Eoghan Clifford from the Civil Engineering Department for his help, especially in relation to the engineering aspects of this project. My gratitude must also be extended to Dr. Colm Kennelly who also completed his PhD on this project and was responsible for running the reactors.

Thanks to everyone in the Microbial EcoPhysiology & EcoEngineering Lab, not just for helping out with the science, but also for the general craic we had in the lab. Thanks also to everyone in the Microbial Ecology Laboratory where I spent a good deal of time during my first year. Thanks to everyone else in the department including both staff and the other postgraduate students. I probably spent more time than necessary drinking coffee and socialising in the common room, but on reflection it was more than worth it.

I would also like to thank my family, especially my parents Noel and Mary. They supported me throughout the PhD and are now delighted, after all these years, to be able to say their adult son is no longer a student. Finally, thanks to Fiona for her support and patience, especially during the thesis writing process.

This work has been funded from research conducted with the financial support of a Science Foundation Ireland Grant (08/RFP/ENM1762), European Research Council Starting Grant Award (‘3CBIOTECH’ Grant number 261330) and a Thomas Crawford Hayes Research Bursary.
TABLE OF CONTENTS

ABSTRACT

CHAPTER 1
Introduction to thesis

CHAPTER 2
Literature review

2.1 INTRODUCTION

2.2 GAS TREATMENT
   2.2.1 Biofilter
   2.2.2 Biotrickling filters
   2.2.3 Bioscrubbers
   2.2.4 Horizontal Flow Biofilm Reactors (HFBRs)
   2.2.5 Other biotechnologies used to treat gas emissions
   2.2.6 Future of gas treatment

2.3 AMMONIA- PROBLEMS, EMISSIONS AND MICROBIAL OXIDATION

2.4 HYDROGEN SULPHIDE- EMISSIONS AND MICROBIAL OXIDATION

2.5 METHANE- PROBLEMS, EMISSIONS AND MICROBIAL OXIDATION

2.6 TECHNOQUES IN MICROBIAL ECOLOGY
   2.6.1 Isolation and quantification of microorganisms using culture-dependent methods
   2.6.2 In-vitro activity assays to determine substrate utilisation rates
   2.6.3 Phospholipid ester-linked fatty acids (PLFAs)
   2.6.4 Molecular markers for the study of methanotrophs
   2.6.5 Genetic Fingerprinting
   2.6.6 Fluorescent in-situ Hybridisation (FISH)
   2.6.7 Stable Isotope Probing (SIP)
   2.6.8 Quantification of methanotrophs using real-time PCR
   2.6.9 Genome sequencing
CHAPTER 3
Identification and Quantification of Ammonia Oxidising Bacteria (AOB) and Ammonia Oxidising Archaea (AOA) in Horizontal Flow Biofilm Reactors (HFBs) used to treat an Ammonia Contaminated Airstream at 10 °C.

3.1 ABSTRACT
3.2 INTRODUCTION
3.3 MATERIALS AND METHODS
   3.3.1 HFBR Design and Operation
   3.3.2 Sampling and Analytical Methods
   3.3.3 Biofilm sampling and DNA extraction
   3.3.4 Qualification of bacterial 16S rRNA bacterial amoA and archaeal amoA genes
   3.3.6 Temperature Gradient Gel Electrophoresis (TGGE) fingerprinting of bacterial community in the HFBs
   3.3.7 Statistical analyses of TGGE fingerprinting and environmental variables in HFBs
3.4 RESULTS
   3.4.1 NH₃ removal and nitrogen dynamics of gas and liquid phases
   3.4.2 In-situ rates of NH₃ removal
   3.4.3 Real-time quantification of AOB, AOA and bacterial 16s rRNA genes
   3.4.4 Bacterial community structure of HFBRs
   3.4.5 Community structure of AOA in the HFBRs
   3.4.6 Statistical comparison of TGGE profiles and in-situ HFBR conditions
3.5 DISCUSSION
3.6 CONCLUSIONS
3.7 REFERENCES
CHAPTER 4
The microbial ecology of highly acidic Horizontal Flow Biofilm Reactors (HFBRs) treating hydrogen sulphide at low temperatures

4.1 ABSTRACT
4.2 INTRODUCTION
4.3 MATERIALS AND METHODS
  4.3.1 HFBR set-up and operation
  4.3.2 Sampling and Analytical Methods
  4.3.3 DNA Extraction and Quantitative PCR (qPCR)
  4.3.4 Clonal Library Construction and Gene Sequencing
  4.3.5 Temperature Gradient Gel Electrophoresis (TGGE) Fingerprinting
4.4 RESULTS AND DISCUSSION
  4.4.1 Effect of retention times and gas loading on HFBR performance
  4.4.2 H₂S, sulphate and pH depth profile in HFBRs
  4.4.3 H₂S conversion to sulphate
  4.4.4 Bacterial community composition of the HFBRs
  4.4.5 Biomass distribution and bacterial gene copy concentrations in HFBR
  4.4.6 Statistical comparison of environmental variables and TGGE profiles of the HFBRs
4.5 CONCLUSIONS
4.6 REFERENCES

CHAPTER 5
The optimization of Horizontal Flow Biofilm Reactors (HFBRs) used to treat methane contaminated airstreams at 10 °C and the microbial communities underpinning the process.

5.1 ABSTRACT
5.2 INTRODUCTION
5.3 MATERIALS AND METHODS
  5.3.1 HFBR design and set-up
  5.3.2 Operating Conditions in the HFBRs
  5.3.3 Seeding and Enrichment Cultures
5.3.4 Gas Analyses
5.3.5 Water Analyses
5.3.6 Biofilm sampling
5.3.7 Methane oxidation potential (MOP) biofilm batch assays
5.3.8 DNA extraction of landfill mix biomass and HFBR biofilm
5.3.9 Bacterial clone library of landfill seed mix
5.3.10 TRFLP fingerprinting of bacterial community in the HFBRs
5.3.11 Fluorescence in-situ Hybridization (FISH) of HFBR biofilm

5.4 RESULTS
5.4.1 Phase I (PI) Performance (Day 0-171)
   5.4.1.1 CH$_4$ Removal Efficiency (RE) of HFBRs
   5.4.1.2 In-situ CH$_4$ removal and water chemistry
5.4.2 Phase II (PII) Performance (day 172-233)
   5.4.2.1 CH$_4$ Removal Efficiency (RE)
   5.4.2.2 In-situ CH$_4$ removal and water chemistry
5.4.3 In-situ HFBR methane oxidation rates
5.4.4 Comparison of environmental variables in HFBRs over duration of trial
5.4.5 Methane oxidation potential (MOP) of biofilm from HFBRs
5.4.6 Bacterial clone library of landfill mix inoculum used for enrichment
5.4.6 Bacterial community succession and assembly in HFBRs indicated by TRFLP analysis
5.4.7 Distribution of Type I and Type II methanotrophs observed by FISH

5.5 DISCUSSION
5.6 CONCLUSIONS
5.7 REFERENCES

CHAPTER 6

The Abundance and Diversity of Methanotrophs in Horizontal Flow Biofilm Reactors (HFBRs) treating Methane contaminated Air at 10°C and the Impact of Silicone Oil and Brij35 on Reactor Performance

6.1 ABSTRACT
6.2 INTRODUCTION
6.3 MATERIALS AND METHODS
6.3.1 Seeding of HFBRs
6.3.2 HFBR design and operating conditions
6.3.3 Gas and liquid sampling
6.3.4 Biofilm sampling and DNA extraction
6.3.5 Quantification of bacterial 16S rRNA and pmoA gene copies using quantitative PCR (qPCR)
6.3.6 Temperature Gradient Gel Electrophoresis (TGGE) fingerprinting of the HFBR methanotroph communities
6.3.7 Cloning of TGGE bands
6.3.8 Statistical analyses of the TGGE fingerprinting and clone libraries
6.3.9 Methane oxidation potential (MOP) assays

6.4 RESULTS
6.4.1 HFBR performance
6.4.2 Depth resolved HFBR methane oxidation rates
6.4.3 Water chemistry of HFBRs
6.4.4 Impact of temperature on MOP of HFBR biofilms and seed biomass
6.4.5 Quantification of methanotrophs in HFBRs using real-time PCR
6.4.6 In-situ methane oxidation of biofilm during PIII
6.4.7 TGGE fingerprinting of the methanotroph communities in the seed and HFBR biomass

6.5 DISCUSSION

6.6 CONCLUSIONS

6.7 REFERENCES

CHAPTER 7

Conclusions and future work
7.1 HFBR DESIGN AND FUTURE CONCEPTS

7.2 MICROBIAL ECOLOGY OF HFBRS AND FUTURE INVESTIGATIONS

SUPPLEMENTARY DATA
Ammonia (NH₃), hydrogen sulphide (H₂S) and methane (CH₄) are three problematic gaseous emissions that are regularly encountered in agricultural, industrial and municipal waste sectors. Methane is an important greenhouse gas, while hydrogen sulphide and ammonia are both highly toxic and odorous. It is therefore essential that these three gases are treated before being released into the atmosphere to prevent environmental damage. During this body of work the three gases were biologically treated using a novel Horizontal Flow Biofilm Reactor (HFBR) design at 10°C. The physicochemical parameters and the microbial ecology of the HFBRs were investigated to determine gas removal processes and to link microbial community structure to reactor performance. Four separate studies were conducted over the course of this work; one ammonia, one hydrogen sulphide and two methane trials.

In the ammonia trial, three HFBRs were operated to treat an ammonia-contaminated airstream at 10°C for 90 d. Average removal efficiencies of 99.7% were achieved at loading rates of 4.8 g NH₃ m⁻³ h⁻¹. Biological nitrification of the ammonia to nitrite (NO₂⁻) and nitrate (NO₃⁻) was performed by nitrifying bacterial and archaeal biofilm communities. Ammonia Oxidising Bacteria (AOB) were more abundant than Ammonia Oxidising Archaea (AOA) throughout the depths of the HFBRs. AOB from the *Nitrosomonas* and *Nitrosospira* genera were the dominant bacterial clones in the HFBRs, while an uncultured archaeal clone dominated the AOA community. The only Nitrite Oxidising Bacteria (NOB) species identified in the HFBRs was closely related to *Candidatus nitrotoga arctica*. The overall bacterial community structure between the HBFRs was highly conserved, although variations in community structure occurred between zones in the HFBRs. This study demonstrated that HFBRs are a suitable biotechnology for the treatment of ammonia contaminated airstreams at low temperatures and identified the key nitrifying microorganisms driving the removal process.

During the hydrogen sulphide trial, three HFBRs were tested for the removal of H₂S gas from air streams over a 180-day trial at 10°C. Removal rates of up to 15.1 g H₂S m⁻³ h⁻¹ were achieved during the trial. Bio-oxidation of H₂S in the reactors led to the production of H⁺ and sulphate (SO₄²⁻) ions resulting in acidification of the liquid...
Abstract

phase. Reduced removal efficiency was observed when a loading rate of 15.1 g H₂S m⁻³ h⁻¹ was applied to the HFBRs. The provision of additional NaHCO₃ in the liquid nutrient feed (LNF) during maximum H₂S loading rates, led to decreased liquid phase acidity and improved H₂S removal. The bacterial diversity within the HFBRs was low and the community was dominated by two species from the genus Acidithiobacillus and Thiobacillus. The harsh environmental conditions present in the HFBRs were likely responsible for the lack of bacterial diversity. Depth-resolved genetic fingerprinting of the bacterial communities in the HFBRs using Temperature Gradient Gel Electrophoresis (TGGE), revealed differences in the community structure between zones in the reactors. The variation in bacterial community composition between zones was influenced by alkalinity, pH and SO₄ concentrations. In spite of the low operating temperature, the results of this study indicate that HFBRs have excellent potential to biologically treat H₂S contaminated airstreams.

Two separate methane trials were carried out to treat methane contaminated airstreams using HFBRs at 10°C. In the first methane trial three HFBRs were operated to treat methane contaminated airstreams at low concentrations (1.12%) for 233 d. Removal rates of up to 7.1 g CH₄ m⁻³ h⁻¹ were achieved during the trial, demonstrating that HFBRs are a suitable technology for the treatment of methane contaminated airstreams at low temperatures. Methane removal rates were influenced by temperature, with reduced removal rates observed during a cold period when temperature fluctuated between 1-10°C (Q₁₀ 2.49 ±0.45). The composition of the LNF applied to the HFBRs impacted on methane removal rates. Increased removal was observed when organic carbon was omitted from the LNF, while the impact of nitrogen source and concentration was unclear. Terminal Restriction Fragment Length Polymorphism (TRFLP) fingerprinting of the bacterial communities in the HFBRs identified a diverse and dynamic bacterial population which varied with depth in the reactors and over time. Fluorescent in-situ Hybridisation (FISH) of the methanotrophic 16S rRNA genes indicated that Type II methanotrophs appeared to be more abundant in the HFBR biofilm than Type I methanotrophs, although the microbial community was dominated by other prokaryotes. Results from this study showed that HFBRs were capable of treating
Abstract

methane contaminated airstreams and that the process was facilitated by a diverse and dynamic bacterial population.

In the second methane trial three HFBRs were operated at 10°C for 341 days treating methane-contaminated airstreams at low concentrations (1.6%, v/v). Removal rates of up to 8.2 g CH₄ m⁻³ h⁻¹, with removal efficiencies of 62.1%, were achieved at a loading rate of 13.2 g CH₄ m⁻³ h⁻¹. Maximum removal rates were observed following the addition of silicone oil and Brij 35 to the LNF. Silicone oil addition improved removal efficiencies by an average of 69% across the three HFBRs. The further addition of Brij35 to R1 and R2 resulted in increased methane removal rates of 33%, when compared to operation with silicone oil alone. The methane oxidation potential (MOP) of the HFBR biofilm and seed biomass was assessed in batch incubations at various temperatures. The highest MOP rate (19.0 mg CH₄ g⁻¹ VSS h⁻¹) for the HFBR biofilm was measured at 23°C, whereas highest oxidation rates in the seed biomass were measured at 37°C (33.6 mg CH₄ g⁻¹ VSS h⁻¹). Methanotrophs were present in abundance at all depths in the HFBRs based on the quantification of the functional pmoA gene using qPCR. TGGE fingerprinting and sequencing indicated that overall methanotroph diversity in the HFBRs was low with Type I Methylobacter and Methylomonas species, and Type II Methylocystis species, detected. The methanotroph communities between the three HBFRs was similar but changed over the duration of the trial. Results from this study showed that silicone oil and Brij35 improved methane removal in the HFBRs and that methane oxidation in the reactors was performed by a small group of methanotrophs who were abundant at all depths in the reactors.

Results from across the four trials showed that HFBRs are a suitable biotechnology for the treatment of ammonia, hydrogen sulphide and methane contaminated airstreams at low temperatures. Microbial community structure and function in the HFBRs varied with depth in the HFBRs and was impacted by a number of different environmental parameters. In the future, manipulation of the physical and chemical environment in HFBRs to support the development of desired microbial communities can be implemented to improve reactor performance and operation.
CHAPTER 1

Introduction to thesis
Chapter 1

1 INTRODUCTION TO THESIS

Gas emissions are a major contributor to the greenhouse effect and cause widespread pollution of the atmosphere. Three important gaseous emissions are methane (CH$_4$), hydrogen sulphide (H$_2$S) and ammonia (NH$_3$). They are emitted from a variety of natural environments and from anthropogenic sources including, fossil fuel burning, wastewater treatment facilities, agriculture, industry and biomass burning. Legislation dealing with the release of gaseous emissions into the environment is becoming increasingly stringent and biological treatment technologies are now seen as a cost-effective and practical solution to curb anthropogenic emissions.

During this work three contaminant gases, methane (CH$_4$), ammonia (NH$_3$) and hydrogen sulphide (H$_2$S) were treated separately, using Horizontal Flow Biofilm Reactors (HFBRs) at 10°C. HFBRs had previously been designed and used for the treatment of wastewaters, but this was the first time the technology had been used for gas mitigation. They share a similar design concept with biotrickling filters, where the contaminated gas flows in a co-current direction with a liquid nutrient stream over an inert carrier material, where they come in contact with a biofilm and are biologically broken down. In these studies the HFBRs were operated at 10°C which is typical of conditions found in temperate environments in Ireland and Northern Europe, while most previous biological gas treatment trials have been operated at mesophilic temperatures. Operating the HFBRs at 10°C removes the need to actively heat the reactors, thus reducing the energy input requirement and lowering running costs.

The biological removal processes for the three gases are carried out by distinct groups of microorganisms. The microbial communities involved in the biodegradation of methane, ammonia and H$_2$S can be influenced by numerous environmental factors and providing suitable conditions in the reactors for the microorganisms is paramount. There have been a number of studies that have investigated the microbial populations involved in gas removal in various bioreactor configurations. However, as most previous gas elimination trials have been at mesophilic temperatures there has been very few studies at low temperatures. The microbial ecology of waste treatment bioreactors has regularly been described as a ‘black box’ due to the lack of understanding of the microbial community structure,
dynamics and interactions involved in the removal processes. Understanding the microbiology of HFBRs used to treat waste gases could help researchers to design and operate the reactors to improve removal rates.

For this work four experimental studies were conducted in total; one ammonia, one \( \text{H}_2\text{S} \) and two methane removal trials. The main aims of this work were to determine whether HFBRs are a suitable technology for the treatment of contaminated airstreams and to investigate the microbial ecology underpinning the removal processes. The four experimental chapters are accompanied by a literature review dealing with the microbiology of methane, ammonia and \( \text{H}_2\text{S} \) oxidation in gas treatment technologies, and the microbial techniques used to study methane oxidising organisms.
CHAPTER 2

Literature Review
Chapter 2

2.1 Introduction

Humans are having a major impact on Earth’s atmosphere and the global environment. Since the industrial revolution the global human population has experienced explosive growth and increased human activity has been responsible for pollution of the environment and climate change. The emission of gaseous pollutants into the atmosphere has been the major contributor to the greenhouse effect and has caused widespread pollution of the air. With the current global population of 7.2 billion people expected to reach 9.6 billion by the year 2050 (UN, 2012), it is essential that global gaseous emissions are reduced. Atmospheric pollution, greenhouse gases and malodorous emissions are released into the environment from various natural and anthropogenic sources. Anthropogenic sources include fossil fuel burning, wastewater treatment facilities, agriculture, industry and biomass burning. Three notable gaseous emissions from such facilities are methane (CH_4), ammonia (NH_3) and hydrogen sulphide (H_2S). Methane is an important greenhouse gas, while hydrogen sulphide and ammonia are both highly toxic and odorous. Gaseous emissions are not only harmful to human health and the environment, but the presence of odour emitting facilities, such as wastewater treatment plants, often result in complaints to environmental protection agencies and local authorities from local residents (Bowman et al., 1991; Phillips, 2008). In Finland, a study of housing prices in close proximity to an animal waste treatment facility showed that odour nuisance resulted in a drop of home property values of between 4-8% (Van Broeck et al., 2010) resulting in significant economic hardship for residents. Legislation dealing with the generation and treatment of gas and odours emissions in the EU (Directives 2008/98/EC and S.I 787/2005) (EC, 2005), and the release of greenhouses gases into the environment (Kyoto Protocol, 1998) have become increasingly stringent and therefore the treatment of gaseous emissions is becoming more important.

2.2 Gas treatment

Traditionally the main focus of waste treatment has been on solid and liquid wastes, but recently there has been an increased interest in odour and volatile organic compound (VOC) abatement (Stuetz, 2001). Traditional technologies used to treat
foul or contaminated airstreams have been physical and chemical, including incineration, absorption into a liquid solvent, adsorption onto physical materials such as activated carbon and chemical scrubbing, all of which can be costly, ineffective and result in secondary pollutants (Lopez et al., 2013). The biological treatment of foul airstreams has been growing in popularity, which can be attributed to the ability of the microorganisms to destroy pollutants rather that just transferring them from the gas to liquid phase (Burgess et al., 2001). The biological treatment of waste air is an environmentally friendly mitigation option that has been shown to be effective and economical for the treatment of biodegradable gas streams (Shareefdeen and Singh, 2005). The advantages of biological treatment include moderate installation and operating costs, low maintenance and the ability to treat a wide range of contaminants. However, there are some problems associated with biotechnologies including large space requirements, internal pressure drops and clogging (Delhomenie and Heitz, 2005). There are several bioreactor designs that have been constructed and operated for the treatment of contaminated airstreams and several bioreactor types are described in detail below.

Bioreactors rely on microbial communities to breakdown gaseous pollutants and transform them into less harmful substances. In theory any biodegradable pollutant can be removed by microorganisms in a bioreactor (Kennes et al., 2009), although a specific group of microorganisms may be required for each pollutant, or class of pollutants. For example, the microorganisms required for methane oxidation are completely different to those required for H2S oxidation. Bioreactors can be inoculated with specific pure culture organisms that are capable of metabolising the target pollutant, or with a mixed microbial consortium. Mixed microbial cultures, such as activated sludge, are most commonly used to seed reactors. Mixed cultures from waste water treatment plants (WWTPs) are often readily available and contain a diverse natural microflora that has a broad substrate range. Mixed cultures are more resistant to reactor perturbations and less likely to fail than pure cultures. In complex mixed microbial communities a variety of metabolic pathways may be required to fully treat gaseous pollutants, requiring various groups of microorganisms that often live in close proximity to each other, many of which from symbiotic relationships. Community structures, metabolic pathways and oxidation rates are all impacted by the environmental conditions present in the reactors. By
understanding who the important microorganisms are within a reactor, and what influences their growth, activity and interactions with other species, microbiologist and engineers can design and operate bioreactors to maximise removal efficiencies.

2.2.1 Biofilters

A biofilter is a fixed-bed bioreactor where a humidified airstream is forced through a porous packed bed on which a microbial community is immobilized (Delhomenie and Heitz, 2005). The biofilter is kept constantly moist with the addition of a liquid feed, which flows in either a co/counter-current direction with the contaminated airstream. The gas dissolves into the liquid phase where it becomes accessible to the immobilised microbial community present. Biofilters are the most common bioreactor technology used to treat contaminated airstreams when high gas flow rates are required (Maestre et al., 2005) and they have been shown at bench and pilot scale to be capable of treating up to 60 out of 189 hazardous air pollutants (Devinny et al., 1999; Shareefdeen and Singh, 2005; Mudliar et al., 2010). There are two main biofilter designs that are used for the treatment of odorous and VOCs; (i) the open biofilter with ascending gas flows which are installed outside due to large surface area requirements and are therefore exposed to climate conditions, and (ii) the closed biofilter which can employ either ascending/descending gas flows and are usually installed indoors thus allowing better control of operational parameters (Mudliar et al., 2010).

Choosing a suitable packed bed film material is one of the most important aspects of a biofilter design and Bohn (1992) stated that a good packed bed material must have; (i) high specific surface area, (ii) high porosity, (iii) good water retention capacity, (iv) presence and availability of intrinsic nutrients and (v) contain a dense and diverse indigenous microflora. The biofilter packed bed can be constructed using a variety of organic and inert materials. The most common organic packed bed media used for biofilters include, soil and wood chips (Mudliar et al., 2010). One the main advantages of using these substances is that they are cheap and readily available. Compost is the most widely used biofilter media and has the advantage of having a substantial and diverse indigenous microflora. It also has good water holding capacity, good air permeability and is rich in nutrients (Kumar et al., 2011).
However, there are a number of problems associated with compost beds including the degradation of the bed, which over time leads to pressure drops, channelling and clogging (Morgan-Sagastume et al., 2001).

Other common organic biofilter media include soil, peat and woodchips. Soil contains a rich and diverse microbial community (Mudliar et al., 2010), however, it lacks intrinsic nutrients and is prone to high pressure drops (Swanson and Loehr, 1997). Peat is a low cost filter media with good water retention capacity, high surface area and high organic matter but lacks other essential mineral nutrients and does not have a high microbial population (Champagne and Khalekuzzaman, 2014). Wood chip has been used in several studies (Hong and Park, 2004; Chen et al., 2008a) but as a packed bed media it has a low specific surface area and the low nutrient content. Other examples of organic filter bed materials include coconut fibre (Baquerizo et al., 2009), pine bark (Luo and Lindsey, 2006), sugarcane-baggase (Sene et al., 2002), corn cobs (Rahul et al., 2013) and peat moss (Kalingan et al., 2004).

To overcome the problems associated with bed collapse and pressure drops inert biofilter bed materials have been used. These include, but are not limited to, small stones (Gallastegui et al., 2011; Ménard et al., 2012), expended schist (Romero Hernandez et al., 2013), ceramic spheres (Avalos Ramirez et al., 2008), perlite (Woertz et al., 2002), vermiculite (Pineda et al., 2000), glass beads (Zilli et al., 2000), polyurethane foam (Moe and Irvine, 2000), polystyrene (Arulneyam and Swaminathan, 2000) and lava rock (Chitwood and Devinny, 2001). One of the drawbacks of using inert packing bead materials is that it is necessary to inoculate the biofilter with a microbial community. Many studies mix different filter bed materials to improve reactor performance and alleviate problems associated with pressure drop and channelling, for example mixing compost and wood chips (Nicolai and Janni, 2001; Yang et al., 2014), compost, sludge and plastic media (Taghipour et al., 2008), pine nuggets and lava rock (Akdeniz et al., 2011). The addition of these materials to a biofilter bed can prevent bed crushing and compaction, provide good structure maintenance and rigidity, therefore delaying bed clogging and increasing the lifespan of a filter bed (Delhomenie and Heitz, 2005).
2.2.2 Biotrickling filters

In a biotrickling filter (BTF) gaseous emissions are passed through a reactor packed with a porous inorganic media on which a microbial population is immobilised. The reactor is continuously irrigated with a liquid supply which provides both moisture and essential nutrients for biofilm growth. The gas, when passed through the reactor dissolves into liquid feed where it can be accessed by the microbial biofilm. It has been shown that the choice of a co/counter-current flow regime between the gas and liquid phase does not have an impact on reactor performance (Cox and Deshusses, 1999). The advantage of the continuous addition of the liquid feed is improved control of mass transfer of the gas into the liquid feed, nutrient addition, pH balancing, acid product neutralization, end product removal, and (potentially) temperature (Iranpour et al., 2005). BTFs offer low operating cost, convenient operation, small footprint, and low resistance (Deshusses and Webster, 2000; Iranpour et al., 2005) and due to their constant liquid trickling there are most suitable for the treatment of water soluble VOCs.

As with the biofilter design the choice of the packed bed media for biotrickling filters is important. Some of the major considerations to take into account when choosing a filter bed material are; (i) it must facilitate the passing of gas and liquid streams through the reactor, (ii) provide a large surface area for microbial growth and attachment and (iii) resist crushing and compaction (Delhomenie and Heitz, 2005). Examples of suitable inert or synthetic packing materials that meet these requirements include structured plastic media (Lee et al.), ceramics (Qiang et al., 2006), polyurethane foam (Ramirez et al., 2009b) and lava rock (Namini et al., 2008). As inert or synthetic materials do not possess their own microflora, biotrickling filters must be seeded with a microbial culture. Activated sludge is commonly used to inoculate BTFs for the treatment of gases. However, in some situations a specialised microbial consortium is required to break down a particular pollutant and the BTF must with be seeded with an enriched inoculum.

One of the major drawbacks of the biotrickling filter is its tendency to become clogged with excess biomass. This can lead to performance loss and problems with pressure drop, formation of anaerobic zones and channelling. There are several methods, which can be employed to unclog a biotrickling filter including chemical,
physical and biological means; the most effective of these may often be backwashing the filter bed with water.

### 2.2.3 Bioscrubbers

A bioscrubber unit is a two chamber reactor consisting of a separate absorption tower and suspended growth biofilm reactor. In the absorption tower gas flows in a counter-current direction with a liquid feed where it dissolves from the gaseous to the liquid phase. The absorption tower often contains an inert packing material to help with mass transfer. The washed gas is expelled from the top of the absorption tower and the liquid is then applied to the bioreactor where it comes in contact with a microbial consortium and is biologically consumed. The microbial community is supplemented with a liquid feed that contains essential nutrients required for growth. Most bioscrubbers are inoculated with activated sludge from wastewater treatment plants (Delhomenie and Heitz, 2005) but in some cases are operated using pure culture strains that are capable of treating a specific contaminant. Bioscrubbers offer good process stability and control of environmental variable such as pH, nutrient concentrations, temperature, (Smet and Langenhove, 1998). As the microbial community is suspended in the growth reactor pressure drop is not an issue as in biofilters and biotrickling filters. However, there are some problems associated with bioscrubbers including low solubility of contaminated gas streams, biomass accumulation in the reactor and the effluent liquid stream often requires secondary treatment (Burgess et al., 2001).

### 2.2.4 Horizontal Flow Biofilm Reactors (HFBRs)

HFBRs are a relatively new and novel reactor design that has previously been used to treat domestic-strength wastewater (Rodgers and Clifford, 2009; Clifford et al., 2010) and dairy wastewater (Rodgers et al., 2007; Clifford et al., 2008). A typical HFBR consists of a number of inert plastic sheets, stacked one on top of the other housed in a sealed unit. The wastewater is applied to the first sheet at the top of the reactor where it flows horizontally across the sheet before dropping onto the second sheet below. The wastewater then travels horizontally across the second sheet before
dropping onto the sheet below in a boustrophedonic pattern. The unique flow regime in the HFBR ensures good contact between the wastewater and the biofilm and alleviates problems that can be associated with conventional biofilm reactors, such as clogging, channelling, compaction and pressure drop. As the HFBR design offers excellent contact between the biofilm and the liquid it has the potential to be used as the gas treatment alternative. There are several other potential advantages of using HFBRs rather than the traditional biofilter, bioscrubber or biotrickling filters technologies for the treatment of contaminated airstreams. The design of the HFBR allows for gas and liquid sampling at various depths in the reactor which provides detailed information on various physical and chemical parameters in the gas and liquid phase. From this information it is possible to determine, not only which biological processes are occurring in the HFBRs, but also where in the reactor they occur. Based on these findings it is possible to operate the HFBRs in a specific manner to facilitate desired biological processes. This can allow for greater operational control of the bioreactor than is often possible in other bioreactor configurations. Another advantage of the HFBR design is the ease of access to all sheets in the reactor. This allows for visible inspection of the biofilm and for biomass sampling from different depths in the HFBR. Depth-resolved molecular analysis of microbial populations present in the HFBRs can then be used to generate microbial community profiles. By comparing these molecular profiles to the environmental variables at the different depths in the HFBRs it may be possible to link the microbial ecology to reactor performance. As part of the work undertaken during this PhD, four lab scale trials were undertaken to test the feasibility of using HFBRs to treat H$_2$S, NH$_3$ and CH$_4$ separately, the results of which are described in the experimental chapters of this thesis.

2.2.5 Other biotechnologies used to treat gas emissions

There are several other bioreactor configurations that have successfully been used to treat contaminated airstreams. Although not as commonly used as biofilters, biotrickling filters or bioscrubbers, other biotechnologies including membrane reactors (Kumar et al., 2008; Volckaert et al., 2014), activated sludge diffusion reactors (Æsøy et al., 1998; Bowker, 2000) rotating biological contactors (Vinage
Chapter 2

and Rudolf von Rohr, 2003) and external loop airlift bioreactors (Harding et al., 2003) have successfully been used to treat contaminated airstreams.

2.2.6 Future of gas treatment

This literature review has focused on the treatment of methane, H₂S and ammonia as separate gases, with most attention given to the process of methane oxidation. However, in real-world scenarios, waste gases are often comprised of several different components. There have been many studies that have successfully co-treated two or more gases simultaneously, in the same reactor. Ammonia and H₂S are major components of waste gas from WWTPs (Shareefdeen and Singh, 2005) and several studies have successfully co-treated both in the same reactor (Jiang et al., 2009a; Jiang et al., 2009b; Hernández et al., 2012). These studies have reported good removal efficiencies (>95%) at low to moderate gas loading rates. The production of sulphate and acidification of the liquid phase can inhibit removal but high liquid flow rates and buffering of the biotrickling filter was shown to improve removal (Jiang et al., 2009b). Volatile organic compounds (VOCs), which are also commonly produced from the degradation of wastes, can also be co-treated with gases such as H₂S. For example, H₂S and toluene (Cox and Deshusses, 2002), H₂S and benzene (Oh et al., 2006) and H₂S and a variety of VOCs (Webster et al., 1996), have all been treated simultaneously. For future of gas treatment at waste facilities where the gas effluents are mixed, the improvement of existing bioreactor configurations and development of new technologies will be required. Key to this will be understanding the microbial communities that underpin the removal process and maximising their metabolic potential.

2.3 Ammonia- Emissions and microbial oxidation

Ammonia (NH₃) is a highly toxic and odorous gas produced during organic waste decomposition and is often a by-product of wastewater treatment technologies. Exposure to ammonia vapours can damage the respiratory tract and irritate the eyes, and exposure to high concentrations can be fatal within minutes (Chung et al., 2000). In agriculture, exposure to gaseous ammonia can have detrimental effects on animal
Chapter 2

health and behaviour, in pigs (Wathes et al., 2002) and poultry (Kristensen and Wathes, 2000). Ammonia is the main alkaline component in the atmosphere and is highly reactive forming aerosols (Aneja et al., 2003) which can be deposited onto terrestrial surfaces (Sutton et al., 1993). When ammonia dissolves into water bodies it can cause the acidification and eutrophication of lakes (Skjøth and Geels, 2013), where it is highly toxic to aquatic organisms (Randall and Tsui, 2002). The deposition of ammonia onto terrestrial environments results in increased ammonia concentrations which can have a negative effect on the local environment, altering the microbial, floral and animal populations, causing changes in biodiversity (Stevens et al., 2010).

The major source of global ammonia emissions are from agriculture and global emissions have more than doubled since pre-industrial times (Sutton et al., 2013). This is largely due to growth in intensive agricultural practices and increased fertiliser spreading (Anderson et al., 2003). Ammonia emissions are expected to grow as global temperatures increase due to climate change (Skjøth and Geels, 2013). Sutton et al. (2013) have suggested that 5°C warming would increase ammonia emissions by 42 per cent (28–67%) and when combined with increased anthropogenic activity global NH₃ emissions may increase from 65 (45–85) Tg N in 2008 to 132 (89–179) Tg N by 2100. While some agricultural emissions, such as manure spreading, cannot be treated using biotechnologies such as HFBRs, there is the potential to treat emissions from controlled environments such as livestock storage facilities, wastewater treatment plants (WWTPs) and waste handling facilities.

Ammonia is biologically produced as part of the nitrogen cycle when organic matter is degraded by microorganisms. Nitrification is a two-step process whereby ammonia is converted to nitrate by microorganisms and it occurs naturally in both marine and terrestrial environments (Francis et al., 2007). The first step of nitrification, which is mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), is the oxidization of NH₃ to nitrite (NO₂⁻). In the second step of nitrification nitrite is oxidized to nitrate (NO₃⁻) by nitrite-oxidizing bacteria (NOB). Nitrification converts ammonia into the more soluble forms of nitrate, which is the preferred substrate for plants and aerobic microorganisms and is also the substrate for denitrification which releases N₂ back into the atmosphere.
AOB and AOA are autotrophic organisms that have the unique ability to oxidise ammonia to nitrite as their sole energy source under aerobic conditions (Prosser and Nicol, 2012). Ammonia oxidation is the rate-limiting step in the nitrification process (Martens-Habbena et al., 2009a) and therefore the main focus of this literature review and subsequent experimental chapter (Chapter 3) is on the microbial populations involved in ammonia oxidation.

Ammonia oxidation is catalysed by the ammonia monooxygenase (AMO) enzyme, which is unique to AOB and AOA and encoded for by the amoA gene. The bacterial amoA gene which has been widely studied, is limited to AOB from two narrow clades of the Beta- and Gammaproteobacteria (Martens-Habbena et al., 2009b). It was traditionally believed that AOB were the only group of microorganisms with the ability to convert ammonia to nitrite (Kowalchuk and Stephen, 2001), until recently when the archaeal amoA gene was discovered in marine water columns (Francis et al., 2005). Since then, AOA have been found in a wide range of environments including soils (Leininger et al., 2006), oceans (Wuchter et al., 2006), estuarine environments (Caffrey et al., 2007) and WWTPs (Kayee et al., 2011), highlighting the important ecological role that AOA have in biogeochemical cycles, thus making a substantial contribution to the global nitrogen and carbon cycles (Park et al., 2008).

The abundance and diversity of AOA and AOB are influenced by several environmental conditions including temperature (Han et al., 2013; Zeng et al., 2014), ammonia concentration (Verhamme et al., 2011), oxygen and exposure to light (French et al., 2012). AOB have been shown to grow faster than AOA in the presence of high levels of ammonia (French et al., 2012), whereas AOA growth has been associated with nitrification in soils in the presence of low concentrations of ammonia (Offre et al., 2009). Similarly, AOA are more dominant and active in marine environments where ammonia concentrations are typically low (<10 µM) (Beman et al., 2008). The affinity of AOA and AOB to low and high concentrations of ammonia creates niche separation in the environment (Verhamme et al., 2011), although the presence of AOA and AOB can provide functional redundancy, for example, in soils (Schauss et al., 2009). In the marine environment archaeal amoA genes are generally detected in larger quantities than bacterial amoA genes (Santoro et al., 2011), whereas in terrestrial environments such as soils, the ratio of bacterial to archaeal gene copies can vary considerably (Zeglin et al., 2011). It has been
shown in agricultural soils that although AOA outnumber AOB, ammonia oxidation was carried out by the AOB, indicating that abundance does not reflect activity (Di et al., 2009).

2.4 Hydrogen sulphide- Emissions and microbial oxidation

Hydrogen sulphide (H₂S) is an odorous and highly toxic gas that is biologically produced when organic matter is broken down under anaerobic conditions by sulphate reducing bacteria (SRB). It has a distinctive odour of rotten eggs with very low odour threshold (0.003 ppm). Therefore humans can smell it even at low concentrations, although it becomes odourless to humans at concentrations of 100-200 ppm (Reiffenstein et al., 1992). It is considered a broad spectrum toxicant and concentrations between 0.250–0.300 ppm have been shown to impact on human health causing nausea, headaches and sleep disturbances (Haahtela et al., 1992). Exposure to concentrations between 500-1000 ppm are fatal (Reiffenstein et al., 1992). As well as the impact on human health, hydrogen sulphide can also cause other problems. In the petroleum industry oil wells and natural gas often becomes contaminated with H₂S during the extraction process causing the gas to sour. When natural gas contains H₂S it becomes corrosive and can damage pipes and production equipment. The removal of H₂S from natural gas increases production costs as treatment is required (Tang et al., 2009). H₂S is often produced at anaerobic wastewater treatment facilities as a by-product of the anaerobic digestion process causing corrosion of the bioreactor and associated piping. Gaseous H₂S can react with moisture in the atmosphere forming acid rain and it has the potential to cause damage to the ozone layer (Kump et al., 2005).

Sulphur is an essential element required by all living organisms and is incorporated into all cells for the generation of amino acids and other small organic molecules (Tripp et al., 2008). When organic matter is decomposed the sulphur present in dead cells is recycled by microorganisms as part of the sulphur cycle. Under anaerobic conditions the decomposition of organic matter can result in the generation of H₂S by sulphate reducing bacteria (SRB) who use sulphate as their terminal electron acceptor. Most SRB can also reduce other inorganic sulphur compounds, including sulphite, thiosulphate and elemental sulphur, to produce H₂S. SRB are found in...
almost all anoxic environments including bogs, paddy-fields and marine and freshwater sediments, where they are major players in the sulphur cycle and readily produced H₂S (Muyzer and Stams, 2008). Natural abiotic sources of H₂S include volcanoes, hot spring and other geothermal sources and in total over 90% of global hydrogen sulphide emissions are from natural sources (Driver and Freedman, 1993). Anthropogenic hydrogen sulphide production originates from variety of sources including combustion of fossil fuels, biomass burning, petroleum production, composting and waste treatment facilities. H₂S is commonly encountered in wastewater treatment plants (Devaï and DeLaune, 1999) and can be generated when wastewater is left for excessive periods in sewers or clarifiers becoming septic, during anaerobic treatment or where solid organic matter is decomposed (Oyarzun et al., 2003; Ramirez et al., 2009a). H₂S emissions from WWTPs are generally confined to localised areas, impacting on residents and businesses located in the vicinity of waste treatment facilities (Llavador Colomer et al., 2012).

In the sulphur cycle oxidised and reduced states of sulphur are transformed both biologically and chemically (Tang et al., 2009) and H₂S oxidation can be carried out by both photosynthetic and chemolithotrophic bacteria. Photosynthetic sulphur oxidation carried out by the green and purple sulphur photosynthetic bacteria requires light to drive the oxidation of the H₂S (H₂S → S₀ + 2 H⁺ + 2 e⁻). These bacteria are often found in anoxic marine environments at depths where light penetrates the water and H₂S occurs. A variety of chemolithotrophic bacteria are capable of H₂S oxidation, under either aerobic or anaerobic conditions. The anaerobic oxidation of H₂S can be coupled to denitrification by autotrophic microorganisms (Cardoso et al., 2006) and occurs in anaerobic marine columns where H₂S and nitrate occur in close proximity in substrate mixing zones (Bruckner et al., 2013). Aerobic H₂S oxidation microorganisms utilise oxygen as their terminal electron acceptor transforming the H₂S to elemental sulphur (S₀) or sulphate (SO₄²⁻) (Omri et al., 2011). The biological oxidation of H₂S results in acidification of the surrounding environment due to the production of hydrogen ions. As such aerobic H₂S oxidisers are usually tolerant of low pH conditions, with some Acidithiobacillus species capable of growing at pH 0-1, as observed in hydrogen sulphide-rich caves (Jones et al., 2011). Acidithiobacillus are well known H₂S oxidisers and pure cultures have been used to inoculate H₂S-removing bioreactors (Sercu et al., 2005;
When mixed microbial consortia were used as seed inoculum, in an acidic biotrickling filter (Li et al., 2012) and an acidic biofilter (Charnnok et al., 2013), Acidithiobacillus has been identified as the predominant species. Thiobacillus spp. have also been identified as important H₂S-oxidisers in biological systems and Thiobacillus denitrificans (Ma et al., 2006), Acidithiobacillus thiooxidans (Lee et al., 2006) and Thiobacillus thioparus (Ramirez et al., 2009b) have all been used as immobilised cultures for H₂S removal.

### 2.5 Methane- Emissions and microbial oxidation

Methane is an important greenhouse gas and is a major contributor to the greenhouse effect. As a greenhouse gas it is up to 23 times more potent than CO₂ on a molar basis (IPCC, 2007). It represents approximately 15% of total annual greenhouse gas emissions and 23% of total anthropogenic greenhouse gas emissions (USEPA, 2006). In a balanced global methane cycle, all methane emissions would be oxidized by natural sinks and the concentration of methane in the atmosphere would remain constant. However, in the past three hundred years atmospheric methane concentrations have been steadily increasing (Chistoserdova et al., 2005) and findings from a United States Environmental Protection Agency (USEPA) study showed that in 2010 overall methane emissions were up 23.5% on emissions from 1900. This study has projected that emissions will grow by 32% and 41% by the years 2015 and 2020 respectively (United States, 2006). Methane is released into the atmosphere from variety of natural sources including wetlands, marine and freshwater sediments, soils, terrestrial plants and in the intestines of ruminant animals and termites (Yusuf et al., 2012). The major natural sinks for methane are photo-chemical oxidation in the atmosphere (>40%), diffusion into the stratosphere and by microbial activity in soils (Conrad, 2009). Anthropogenic sources of methane come from landfill, wastewater treatment facilities, rice paddies, cattle ranching, coal mining, oil and petroleum drilling. Yusuf et al. (2012) categorised anthropogenic methane emissions into three groups; agriculture, waste and energy. The breakdown of anthropogenic methane emissions showed that agriculture (53%) was the main contributor, followed by the energy (28%) and waste (19%) sectors. The dividing line between natural and anthropogenic methane sources can sometimes be blurred.
For example, climate change due to human activities has resulted in an increase in global temperatures. This has caused large areas of permafrost and arctic tundra to melt, releasing methane and carbon compounds into the atmosphere. This represents a significant source of global methane emissions, contributing to global warming and releasing methane in a positive feedback cycle (Anisimov, 2007; O’Connor et al., 2010).

One of the most common methods used to treat methane, especially at landfills, has been onsite gas flaring for heat and energy recovery. However, gas flaring is an only effective treatment method once methane concentrations in the biogas are above 35-40% and gas production rates are above 30-50 m$^3$ h$^{-1}$; otherwise this method becomes technically and economically unfeasible (Haubrichs and Widmann, 2006). Biogas collected at landfills often does not contain a sufficient concentration of methane to facilitate flaring, which especially true at older landfills. Other anthropogenic methane emission sources, including coalmines (Karacan et al., 2011) and municipal wastewater treatment plants (Czepiel et al., 1993; Wang et al., 2011; Daelman et al., 2012), are often too low to treat with gas flaring and energy recovery systems. The low concentrations of methane emitted at such facilities make biological treatment a viable option.

Methane is biologically generated as part of the anaerobic digestion process by methanogens. Anaerobic digestion is the successive biochemical breakdown of complex organic molecules to methane and carbon dioxide (biogas) under anaerobic conditions. Methanogenesis is the final step of anaerobic digestion and is carried out by methanogens who convert acetate, H$_2$ and CO$_2$ to methane (Schmidt and Ahring, 1995). Methanogens live in syntrophy with a variety of acidogenic, and hydrogen and acetate forming microorganisms that can breakdown complex multi-carbon substrates to simpler compounds. Methanogens have been cultivated from many anaerobic temperate environments and from extreme environments with varying temperature, pH and salinity and are used in anaerobic digesters to produce methane from organic wastes.

Biological methane oxidation is the conversion of methane to CO$_2$ by methane oxidising organisms. Methane oxidation can occur under aerobic and anaerobic conditions, however the two processes are carried out by two completely different
sets of prokaryotes (McDonald et al., 2008). Methanotrophs are responsible for aerobic methane oxidation and utilise oxygen as their terminal electron acceptor, while the recently discovered anaerobic methane oxidisers use sulphate or nitrate as electron acceptors for methane oxidation (Beotius et al., 2000, Raghoebarsing et al., 2006).

Aerobic methane oxidation is carried out by methanotrophs. They are a group of gram-negative bacteria that can utilize methane as their sole source of carbon and energy and are obligate aerobes. Methanotrophs can be found in a wide range of environments including the tissues of higher organisms, soils, sediments and freshwater and marine columns, and are usually found at oxic-anoxic interfaces where methane and oxygen come in contact (McDonald et al., 2008). Methanotrophs belong to three main groups; Type I, Type II and Type X. In recent years other methane oxidising microorganisms have been discovered that do not belong to any of the three groups. These include two filamentous methane oxidizers, Crenothrix polyspora (Stoecker et al., 2006) and Clonothrix fusca (Vigliotta et al., 2007), and three extremely acidophilic bacteria of the phylum Verrucomicrobia (Op den Camp et al., 2009).

All methanotrophs contain the methane monooxygenase (MMO) enzyme, which is essential for the oxidation of methane. MMO exists as either particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO) and is unique to methanotrophs. All known methanotrophs contain either one or both forms of the enzyme. The majority of Type I methanotrophs do not contain sMMO (Hanson and Hanson, 1996). The pmoA gene, which encodes for pMMO, is present in all know methanotrophs with the exception of Methylocella palustris (Dedysh et al., 2000), while the mmoX gene which encodes for sMMO is only found is a small number of methanotrophs. Copper is known to be a key factor in regulating the expression of the pmoA and mmoX genes in methanotrophs that have the two forms of MMO (Semrau et al., 2010). The pMMO enzyme has lower substrate specificity than sMMO (Burrows et al., 1984), while sMMO has a broader substrate specificity and can utilizes compounds such as alkanes, alkenes, and aromatics (Colby et al., 1977).
Chapter 2

The initial methane oxidation step is the transformation of methane to methanol by the MMO enzyme. Methanol is then further oxidized to formaldehyde, which is either assimilated into biomass or oxidized to carbon dioxide by the enzyme methanol dehydrogenase (MDH) (Semrau *et al.*, 1995). MDH is highly conserved and present in all methylotrophic bacteria (McDonald and Murrell, 1999). The *mxaF* gene encoding for the α-subunit of MDH can be used as a functional gene biomarker (Henckel *et al.*, 1999). A key difference between Type I, Type II and Type X methanotrophs is the carbon assimilation pathway that they possess (Macalady *et al.*, 2002). Type I methanotrophs have stacked internal membranes that contain pMMO and use the ribulose monophosphate (RuMP) cycle, to convert formaldehyde into multi-carbon compounds, for biomass production. Type II methanotrophs have rings of pMMO-harbouring membranes at the edge of their cells and use the serine cycle to convert formaldehyde into biomass, but they may also contain sMMO. The Type X methanotrophs contain pMMO in both stacked internal membranes and membranes at the periphery of the cell, and can utilise both the RuMP and serine cycles as well as the sMMO (Chistoserdova *et al.*, 2005).

Biological methane oxidation can occur under anaerobic conditions and is carried out by anaerobic methanotrophic archaea (ANME). The ANME were only relatively recently discovered by Hinrichs *et al.* (1999), who extracted archaea-specific lipid biomarkers from a hydrate-bearing site on the continental slope off California. Since then ANME have been found in various aquatic environments where sulphate and methane come in contact in sulphate-methane transition zone (SMTZ). SMTZs are found in all anoxic marine environments in which methane travelling upwards comes into contact with sulphate (Knittel and Boetius, 2009). ANME form symbiotic relationships with SRB to facilitate anaerobic oxidation of methane (AOM) coupled to sulphate reduction. ANME have been detected in numerous marine environments including methane seeps, vents and various SMTZs, and in diverse continental habitats including limnic water columns and sediments, soils and aquifers (Knittel and Boetius, 2009).

AOM coupled to nitrate reduction was more recently discovered in anoxic freshwater canal sediment incubations (Raghoebarsing *et al.*, 2006), even though oxidized nitrogenous compounds, such as nitrate, are thermodynamically more favourable electron acceptors than sulphate for the AOM (Strous and Jetten, 2004).
Chapter 2

Since then anaerobic denitrifying bacteria have been detected in a number of studies (Ettwig et al., 2008; Hu et al., 2009). Most interestingly Ettwig et al. (2010) discovered a bacterium, *Methylomirabilis oxyfera*, that is able to produce its own oxygen supply under anaerobic conditions by reducing nitrite and subsequently oxidising methane conditions via a new intra-aerobic pathway.

2.6 Techniques in microbial ecology

Traditional microbiology was focused on the isolation of bacteria from the environment and their subsequent study in culture in the laboratory. This meant that our understanding of microbiology was largely limited to a handful of bacterial isolates (Lasken, 2012), leaving a huge knowledge gap as the vast majority of bacteria (>99%) still remain uncultured (Rappé and Giovannoni, 2003). Whilst the isolation of pure culture organisms allows for the detailed study of bacterial physiology, it is often more beneficial to study the interactions of microbial populations *in-situ* and determine their ecological roles (Wang et al., 2012). This has been made possible with the advent of culture independent techniques that have allowed microbial ecologists investigate the community structure of microbial populations in the environment, and uncover how they interact with each other and their surroundings. Molecular techniques based on the nucleic acid composition of an organism, most notably the 16S rRNA genes, along with the recent advances in high-throughput sequencing and omics techniques have allowed for detailed investigations of the microbial ecology of numerous environments. According to Prosser et al. (2007), a revolution has begun in the field of microbial ecology due the rapid accumulation of molecular data which has uncovered a vast microbial diversity and abundance, and identified a number of novel microbial functions. The majority of microbial techniques available to the modern microbial ecologist are described below, but for the purpose of this literature review the techniques are described with reference to studies investigating the microbial communities involved in methane oxidation only.
2.6.1 Isolation and quantification of microorganisms using culture-dependent methods

Traditional microbiological techniques used to isolate and quantify bacteria dates back to 1881, when Robert Koch first described a technique to isolate bacteria on solid nutrient media. Since then there have been many advances in the methods used to isolate microorganisms in the laboratory, but the principles remain the same. Methanotrophs have been successfully isolated, cultured and characterised from a range of environments and there are number of methanotrophs available in pure cultures. Up until 1970 there had been very few methane oxidizing bacteria isolated in pure cultures and direct isolation of methane utilizing bacteria from the environment was largely unsuccessful. Whittenbury et al. (1970) changed this with the development of an enrichment technique using nitrate mineral salts (NMS) media, which resulted in the isolation of over 100 aerobic methane oxidisers. Since then this method has been widely used and the journal article has been cited over 937 times. However, it is often difficult to isolate methanotrophs for a number of reasons; (i) methanotroph enrichments are often contaminated with other prokaryotes including methylotrophic bacteria that can outcompete or predate methanotrophs, (ii) methanotrophs are relatively slow growing and (iii) many methanotrophs grow poorly or do not grow at all on agar (Dworkin et al., 2006). Currently there is no media available that is uniquely selective for methanotrophs. For these reasons the isolation and quantification of methanotrophs from an environmental sample on nutrient may not accurately represent the population. A critical evaluation of the plate count method to enumerate methanotrophs from the environment concluded that plating is a useful tool to isolate methanotrophs but is unreliable for quantification (Escoffier et al., 1997). However the same study determined that using the most probable number (MPN) method provided stable and reproducible estimates of cultivable methanotrophs.

2.6.2 In-vitro activity assays to determine substrate utilisation rates

The biological activity of a biomass sample can be measured in-vitro in the laboratory under tightly regulated conditions, therefore allowing for the comparison of oxidation rates between samples and under different environmental conditions.
The activity rate for many biological processes can be determined in batch incubations, including methane, ammonia and sulphur oxidation rates. The principle behind an activity test is to measure the rate of substrate utilisation and/or production of a metabolic product over time, and to then express the rate as a function of biomass concentration. When determining the methane oxidation potential of an environmental sample, a known quantity of biomass is incubated in a sealed vessel with a known concentration of methane in the headspace. The methane concentration is then measured over time. Methane oxidation potential measurements are useful for comparing the relative activities of the methanotrophic populations between samples and under different environmental conditions (Kightley et al., 1995) and from numerous environmental samples, including landfill soils (Kightley et al., 1995; He et al., 2008; Ait-Benichou et al., 2009), lake sediments (He et al., 2012b) marine sediments, arctic tundra soils (Wagner et al., 2003; Barbier et al., 2012) and pure cultures (Ren et al., 1997). Using methane oxidation potential assays, temperature (Börjesson et al., 2004), moisture levels (Einola et al., 2007), O₂ concentration (Teh et al., 2005) and nitrogen source and concentration (Bodelier and Laanbroek, 2004) have all been shown to affect the rate of methanotroph in environmental samples.

2.6.3 Phospholipid ester-linked fatty acids (PLFAs)

Biochemical markers, such as PLFAs, have been widely used to study the microbial ecology of environmental samples. PLFAs are the primary lipids in microbial cell membranes and are present in all microorganisms (Kujur and Patel, 2014) and crucially only remain intact in living cells (White et al., 1979). Gas chromatography is used to analyse the PLFA composition of an environmental sample resulting in the generation of a PFLA profile. PLFA profiles provide information on the microbial community structure and relative abundance of various PFLAs within the sample. Individual PFLAs can be compared to a database to identify the microorganism from which it originated. Methanotrophs have distinct patterns of PLFAs that are unique to methanotrophs and can be used to distinguish between Type I and Type II methanotrophs, making them useful biomarkers (Nichols et al., 1985; Bowman et al., 1991). Type I methanotrophs contain mainly 14C and 16C PLFAs, whereas the Type II contain mainly 18C PLFAs, although some overlap of PFLA signatures
occurs between the two groups (Bodelier et al., 2009). This variation is useful for comparing the methanotroph community structure in environmental samples and has been used to investigate the methanotroph populations of landfill soils (Watzinger et al., 2008), soils (Bull et al., 2000; Mills et al., 2013) and freshwater sediments (Costello et al., 2002).

A drawback of PFLA analyses is the lack of deposited methanotrophic PFLA profiles in the databases. Currently it can only be used to identify methanotrophs to group level of Type I or Type II, and sometimes to species level (Bodelier et al., 2009) Even though PFLA analyses is useful for the study of methanotrophic populations, it is considered technically challenging and interpretation of generated results is often difficult (Frostegård et al., 2011).

2.6.4 Molecular markers for the study of methanotrophs

There are a number of genetic markers suitable for use in molecular ecology studies of methanotrophs, including the 16S rRNA genes and some functional genes. Numerous 16S rRNA gene targets have been identified and probes and primers have been developed to target methanotrophs at the genus and group level. Chen et al. (2007) designed primer sets to target type I and type II methanotrophs separately. These primer sets amplify 16S rRNA genes from almost all known methanotrophs, including the genera Methylocaldum, Methylosphaera, Methylocella and Methylocapsa which were non-amplifiable using previous primer sets. These primer sets have been used in a number of studies to access the methanotrophic communities from various locations such as landfill cover soils (Chen et al., 2007; Gebert et al., 2009), deep water coral reef sediments (Jensen et al., 2008), lake sediments (He et al., 2012a) etc. Methanotrophic diversity has also been investigated using universal bacterial primers for sequencing and genetic fingerprinting (Fjellbirkeland et al., 2001).

Most molecular ecology investigations into methanotrophic populations target the MMO functional genes rather that the 16S rRNA genes. Functional gene markers offer two main advantages over housekeeping 16S rRNA genes. Firstly they narrow down the target group thus increasing the specificity and sensitivity of the assay, and
secondly, most uncultivated members of the functional group being targeted can be identified based on the presence of a homologous functional gene sequence (McDonald et al., 2008). They are two main functional gene targets that are used for the study of methanotrophs, the *pmoA* gene which encodes for the pMMO and *mmoX* gene which encodes for sMMO. As pMMO is found in most methanotrophs, with the exception of *Methylocella palustris* (Dedysh et al., 2000), *pmoA* is the most commonly used genetic marker. The *mmoX* gene, which encodes for sMMO, is not as widely used to study methanotrophs as the *pmoA* gene, as it is only found in a subset of methanotrophs. The *mxaF* gene, which encodes for the α-subunit of the MDH, is another useful gene target as it is present in all known methylotrophs and methanotrophs. It is highly conserved and is ubiquitous in methanotrophs, whereas the *pmoA* and *mmoX* genes are absent in some methanotrophic proteobacterial genera (Lau et al., 2013). The major disadvantage of targeting the *mxaF* is that it is also present in methylotrophs. Both 16S rRNA and functional genes are routinely used as genetic targets for a variety of molecular ecology techniques, some of which as described below.

### 2.6.5 Genetic Fingerprinting

Genetic fingerprinting is routinely used to examine microbial communities from the environment and allows for the comparison of population structure, diversity and variability between samples. It is useful for investigating population variability over time or in response to environmental changes such as pH, moisture levels, temperature and nutrient availability. Techniques including TRFLP (Liu et al., 1997), Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993) and Temperature Gradient Gel Electrophoresis (TGGE) (Muyzer and Smalla, 1998) have been widely used for fingerprinting microbial populations from an array of environments. Each method follows a similar pattern, where DNA/RNA is extracted, a target gene is PCR amplified and the resulting PCR amplicon is assessed using the fingerprinting technique. One of the advantages of genetic fingerprinting is the ability to analyse numerous samples simultaneously. Genetic fingerprinting studies of methanotrophic communities have been performed on the 16S rRNA genes of Type I and Type II methanotrophs and on the functional *pmoA* and *mmoX* genes.
DGGE separates mixed PCR-amplified gene fragments, in an acrylamide gel containing a chemical gradient, whereas in TGGE the fragments are separated in a temperate gradient. As the gene fragments travel through the gel under the influence of an electric current they are denatured at a specific denaturant concentration. The point at which they melt is dependent on the GC content of the gene sequence, allowing for the comparison of highly similar sequences. DGGE has been widely used to investigate the methanotrophic community profiles in various environments. The methanotrophic populations in simulated landfill cover soils (Chi et al., 2012), landfill soils (Yang et al., 2011) have been investigated by targeting the 16S rRNA genes. Other studies have focused on the functional pmoA gene, for example in wet fen soils (Yrjälä et al., 2011), steppe grassland soils (Zhou et al., 2008) and wetland soils (Bodelier et al., 2005). Several studies have analysed both the 16S rRNA and functional genes using DGGE fingerprinting, for example in freshwater lake sediments (Tsutsumi et al., 2012) and paddy-field soils (Henckel et al., 1999; Zheng et al., 2008). TGGE is not as widely used as DGGE for genetic fingerprinting and a limited number of studies have used this method to investigate methanotroph communities. DGGE and TGGE fingerprinting have some limitations, as they are only suitable for use with small gene fragments, usually <500 bp, therefore limiting the amount of sequence information available (Myers et al., 1985). In some cases it is not possible to separate 16S rRNA gene fragments obtained from different methane oxidizing bacteria by DGGE, even though they have substantial sequence variation (Vallaey et al., 1997).

TRFLP is a rapid, sensitive and reproducible genetic fingerprinting technique that has been used for the study of methane oxidising communities. In TRFLP, a target gene, usually 16S rRNA, is amplified from a mixed community DNA/RNA sample with fluorescent primers by PCR. The resulting PCR product is digested with a restriction enzyme and the length of the terminal restriction fragments are determined in a suitable electrophoresis platform. A mixed community sample will usually result in a genetic profile containing several fluorescent peaks (gene fragments) of varying fluorescence intensity. The size and intensity of the fragments can then be used to characterise the community profile of the sample. Fluorescence intensity can be used to determine the relative abundance of a fragment within a sample. TRFLP of 16S rRNA genes has been used to assess the methane oxidising
bacterial populations from a number of environments including landfill cover soils (Stralis-Pavese et al., 2006; Sawamura et al., 2010). The functional pmoA gene has also been used as a genetic biomarker in paddy field soils (Krause et al., 2009), littoral lake sediments (Pester et al., 2004) and submerged rice plants (Horz et al., 2001).

2.6.6 Fluorescent in-situ Hybridisation (FISH)

FISH is a technique combining cytogenetics and molecular biology which allows researchers to study microbial populations in-situ, without the need to extract genetic material (Price, 1993). Fluorescently labelled probes, that have specific oligonucleotide sequences, are applied to biological samples where they hybridise with compliantly DNA sequences within the chromosome. The specificity of the probe depends on its nucleotide sequence and probes can be designed to target microorganisms down to the species level. FISH can be used to identify in specific microorganism are present and allows for their quantification. One of the advantages of FISH is the possibility to use several different probes labelled with different fluorescent markers at the same time. This facilitates the simultaneous investigation of different microorganisms in their environment, and their location in relation to each other.

FISH has been widely used for the study of methanotrophic populations from a range of environments. Group specific Type I and Type II methanotroph probes, targeting the 16S rRNA genes, have been used in membrane biofilm reactors (Modin et al., 2010), root samples (Eller et al., 2001), soils and sediments (Carini et al., 2005; Rahalkar et al., 2009). Other studies have used species specific FISH probes to detect methanotrophs in a number of studies including; acidic peatlands (Dedysh et al., 2003) and lake sediments (Kalyuzhnaya et al., 2006).

One disadvantage of FISH is that the 16S rRNA sequence of the methanotrophs must be known. As a large proportion of methanotrophs can only be identified by their pmoA sequence, FISH cannot accurately detect or enumerate these organisms (McDonald et al., 2008). In some cases, when the abundance of the target organism is low, the signal intensities achieved in traditional FISH are too low to detect.
CARD-FISH (catalysed reported deposition–fluorescence in situ hybridization) is a superior hybridisation method that can be used in these situations. CARD-FISH relies on a single oligonucleotide probe containing a horseradish peroxidase (HRP) label, which is radicalised by multiple tyramide molecules resulting in a fluorescent signal (Amann and Fuchs, 2008). It has been used to enumerate methanotrophs in landfill cover soils (Kallistova et al., 2007), the roots of rice plants (Bao et al., 2014) and freshwater lake sediments (Kojima et al., 2012).

2.6.7 Stable Isotope Probing (SIP)

Stable Isotope Probing (SIP) is a technique commonly used in microbial ecology to examine active microbial populations in a culture independent method. In SIP incubations an environmental sample is incubated with a stable isotope labelled substrate, usually containing either ²H-hydrogen, ¹³C-carbon or ¹⁵N-Nitrogen. The labelled substrate can only be incorporated into the cells of organisms with the ability to metabolise the substrate, allowing for identification of active microbial community members. Methanotrophs are ideal candidates for SIP incubations as they can utilise methane as their sole carbon source (Dumont and Murrell, 2005). SIP incubations containing ¹³C-CH₄ have been used in conjunction with DNA, RNA and PFLA analyses to identify active methane oxidising organisms. As the ¹³C-CH₄ is utilised by methane oxidising organisms it becomes incorporated into their DNA, RNA and cellular material. Following incubation and extraction of the DNA and RNA, the ¹³C-labelled genetic material must be separated from the regular ¹²C-labelled genetic material by ultra centrifugation. Both the heavy (¹³C-labelled) and light (¹²C-labelled) DNA/RNA fractions can then be examined using a variety of molecular methods to determine the active methane oxidising members of the community.

SIP incubations have been used to investigate the active methanotroph populations, based on the presence of the 16S rRNA and functional genes in lake sediments (Dumont et al., 2011), rice field soil (Mayumi et al., 2010), landfill soils (Cebron et al., 2007), arctic lake sediment (He et al., 2012b) and paddy field sediments (Qiu et al., 2008). These studies used a variety of molecular techniques to characterise the methanotroph populations including PCR, qPCR, cloning and TRFLP.
In SIP incubations the $^{13}$C-CH$_4$ also becomes incorporated into the phospholipid fatty acids of the cell membrane. Analyses of PFLA profiles allows for the differentiation between active methane oxidising organisms and non methane utilisers. PLFA has previously been used to identify Type I and Type II methanotrophs following SIP incubations and has been used for peatland soil (Chen et al., 2008b), forest soils (Menyailo et al., 2010) and raised bog (Putkinen et al., 2012) samples.

There are a number of technical challenges associated with $^{13}$C-CH$_4$ SIP incubations. The concentration of methane required to produce a detectable $^{13}$C-label from SIP is relatively high in comparison to what is found under natural conditions. As different groups of methanotrophs have varying affinities for methane, this can cause the enrichment of low-affinity methanotrophs, leading to a misrepresentation of the in-situ communities (Martineau et al., 2010). There is also the possibility of cross feeding, where the labelled methane is converted to metabolic products such as methanol which is subsequently taken up by methanol utilising organisms (Dumont and Murrell, 2005). Even so, SIP has proved a powerful molecular tool for the investigation of methanotroph populations.

### 2.6.8 Quantification of methanotrophs using real-time PCR

Since the development of real-time PCR, also known as quantitative PCR (qPCR), by Higuchi et al. (1993) two decade ago, real-time PCR has become an integral tool for the study of microbial communities in environmental samples. Real-time PCR is a quantitative method used to enumerate specific genes in a DNA/RNA sample. Real-time PCR uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction (Bustin, 2005), combining the DNA amplification and detection steps into one homogeneous assay. Real-time PCR assays have been developed to target the 16S rRNA and functional $pmoA$, $mmoX$ and $mxaF$ genes of methanotrophs. The $pmoA$ gene is most commonly used for the quantification of methanotrophs as it is found in all known methanotrophs with the exception of Methylocella palustris (Dedysh et al., 2000). The $pmoA$ gene has been quantified in numerous environments including landfill cover soils (Kong et al., 2013), composts (Sharma et al., 2011), soils (Aronson et al.,
2013) and bogs (Akiyama et al., 2011). Standard real-time PCR allows for the quantification of specific genes from DNA extracts, however it does not provide any information on the activity of the target organisms as genes can also be detected from dead and inactive cells. To quantify the activity of methanotroph cells mRNA can be quantified using real-time reverse transcription polymerase chain reaction (RT-qPCR). This method was applied to wetland tundra (Yun et al., 2012) and peat bogs (Freitag et al., 2010), where gene copies were quantified and linked to in-situ methane fluxes.

### 2.6.9 Genome sequencing

The first bacterial genome was sequenced back in 1995 by Fleischmann et al. (1995) using Sanger sequencing. Traditionally sequencing an organism’s genome had been a costly and time consuming process, limiting the potential for widespread annotation of different genomes. With the development of de novo and next generation genome sequencing techniques there has been a rapid increase in the number of fully sequenced organisms. A number of methanotrophic bacteria had their genomes fully sequenced (Stein et al., 2010; Stein et al., 2011; Svenning et al., 2011; del Cerro et al., 2012). Full genome sequencing of methanotrophic bacteria has helped identify key genes and enzymes involved in various metabolic pathways. With the advent of these new next generation sequencing techniques the number of fully sequenced methanotrophs is set is increase over the next few years helping to further understand the diversity and genetic potential of the methanotrophs.

### 2.6.10 Metagenomics, metaproteomics, metatranscriptomics and metabolomics

Metagenomics is the genetic analysis of genomes from environmental samples and results in the generation of millions of raw metagenome reads. Analyses and assembly of these metagenome reads result in libraries containing thousands of species. The metagenome of an environmental sample gives information on community structure and diversity, and can be screened for functional and 16S rRNA genes (Thomas et al., 2012). Characterisation of the environmental conditions from which the sample was taken, is just as important as the metagenome data.
Information regarding environmental parameters such as date, time, pH, temperature, nutrient concentrations, soluble gases, etc. can be referred to as the metadata (Wooley et al., 2010). Often comparing the metagenome and metadata can lead to biological discoveries due to the presence or absence of certain genes. For example, a metagenome study of a marine sediments from a coal oil point seep area linked methane oxidation measurements and metagenomic data to identify aerobic and anaerobic methane oxidising populations at various depths (Havelsrud et al., 2011). Another metagenomics study on freshwater lake sediments linked the relevant abundances of an aerobic methanotroph and a methylotroph, and their response to methane and nitrate, indicating that they may be engaged in cooperative behaviour (Beck et al. 2013).

Although metagenomics of an environmental sample provides in-depth information on the microbial community structure, it does not identify cellular activity or metabolic pathways at work in the ecosystem. In order to fully understand the complexities and functional diversity of a microbial community it is necessary to combine metagenomics to other omics techniques such as proteomics, transcriptomics and metabolomics. By combining these methods it is possible to identify the microorganisms present and determine their collectively transcribed RNA, translated proteins and metabolites resulting from cellular processes (Siggins et al., 2012). Metaproteomics is the identification of all proteins expressed in a sample at a given time and allows for the identification of metabolic pathways in use. Metaproteomics has been used for the study of microbial communities in a wide range of environments and has helped identify MMO proteins from the root tissues of rice field plants (Bao et al., 2014) and other environments. The metatranscriptome analyses the RNA collectively transcribed by all the microorganisms from an ecosystem and is used to identify the active genes at a given time point, while metabolomics focuses on the detection and identification of metabolic products produced. Metagenomics, metaproteomics, metatranscriptomics and metabolomics, when used in isolation, are all powerful molecular ecology tools that can provide valuable insight into ecosystem biology. However, to provide greater understanding of the complex interaction between microorganisms and their environment it is preferable to combine these techniques in a systems biology approach. As these relatively new, high-throughput, omics techniques become more affordable and
routinely used, they are providing information on thousands of pathways and networks that document millions of interactions between proteins, genes and small molecules (Gehlenborg et al., 2010). Systems biology approaches have lead to the discovery of novel biochemical pathways and genes and have enhanced our understanding of the microbial interactions that occur in many environments. To date there has been limited, if any, omics studies into the microbial populations in gas treatment bioreactors and this is a potential avenue for future research.

### 2.7 Scope of thesis

The optimisation of air-remediation bioreactors has traditionally revolved around the engineering of the reactors, while failing to understand the complex microbial interactions that drive the removal processes. Identifying and understanding the microbial communities that are involved in the breakdown of the waste gases can help scientists and engineers to improve reactor design and operation. It has been shown that diverse and dynamic microbial populations ensure process stability within reactors (Briones and Raskin, 2003) and long-term performance in bioreactors treating nitrogenous wastewater can only be ensured when the microbial community within the sludge functions optimally Geets et al. (2007). Gaining an insight into the microbial populations present in gas treatment bioreactors may help to prevent process failures and improve reactor performance. Verstraete et al. (2007) discussed the concept of Microbial Resource Management (MRM), where microbial communities are manipulated and controlled to perform a required biological function. In the MRM of mixed communities three basic questions are asked; (i) Who is there? (ii) Who is doing what? (iii) Who is doing what with whom? Establishing these facts can allow researchers to determine the operational parameters required in engineered system to facilitate a desired bioprocess. In the future, it could enable researchers, engineers, and policy makers to make decisions based on microbial ecology and environmental microbiology (Read et al., 2011).

The aim of this work was to determine if Horizontal Flow Biofilm Reactors (HFBRs) were a suitable biotechnology for the treatment of contaminated airstreams at 10°C and to investigate the microbial ecology underpinning the removal processes in the reactors. Three contaminated airstreams containing either methane (CH₄),
ammonia (NH₃) or hydrogen sulphide (H₂S) were treated separately using HFBRs at 10°C. Four experimental studies were conducted in total; one ammonia, one H₂S and two methane removal trials and are present in paper format in the following chapters. The HFBRs were operated under controlled conditions and the performance of the reactors was closely monitored over time. Detailed depth-resolved gas and liquid phase analyses was routinely conducted to determine the gas removal pathways. The microbial ecology of the HFBRs was investigated using a range of molecular techniques to determine the identity and quantity of the key microbial community members. Differences in the microbial community composition between locations in the HFBRs, between replicate HFBRs and over time were assessed and the impact of environmental variables on the community structure was also investigated.

2.8 References


Chapter 2


Lee A, Morton R, Tang C-C, Friess P. Removing Hydrogen Sulfide from Digester Gas with Slightly Aerobic Biotrickling Filters.


Llavador Colomer F, Espinos Morato H, Mantilla Iglesias E. 2012. Estimation of hydrogen sulfide emission rates at several wastewater treatment plants through


Chapter 2


CHAPTER 3

Identification and Quantification of Ammonia Oxidising Bacteria (AOB) and Ammonia Oxidising Archaea (AOA) in Horizontal Flow Biofilm Reactors (HFBRs) used to treat an Ammonia Contaminated Airstream at 10 °C.
Chapter 3

This work described in this chapter was collaboration between the Microbiology and Civil Engineering departments in the National University of Ireland Galway. The HFBRs were operated by the Civil Engineering research group but all bioprocess data and molecular work was analysed as part of this PhD programme.
3.1 ABSTRACT

Ammonia is a toxic and odours gas that is routinely produced in agricultural, industrial and municipal waste sectors and requires treatment before release into the environment. Three novel laboratory-scale Horizontal Flow Biofilm Reactors (HFBRs) were tested for the treatment of an ammonia (NH$_3$) contaminated airstream at 10°C over a 90-day trial. Average removal efficiencies of 99.7% were achieved at loading rates of 4.8g NH$_3$ m$^{-3}$ h$^{-1}$. Biological nitrification of the ammonia to nitrite (NO$_2^-$) and nitrate (NO$_3^-$) was performed by nitrifying bacterial and archaeal biofilm communities. Ammonia Oxidising Bacteria (AOB) were more abundant than Ammonia Oxidising Archaea (AOA) throughout the depths of the HFBRs. AOB from the *Nitrosomonas* and *Nitrosospira* genera were the dominant bacterial clones in the HFBRs, while an uncultured archaeal clone dominated the AOA community. The only Nitrite Oxidising Bacteria (NOB) species identified in the HFBRs was closely related to *Candidatus nitrotox arctica*. The overall bacterial community structure between the HBFRs was highly conserved, although variations in community structure occurred between zones in the HFBRs. This study demonstrated that HFBRs are a suitable biotechnology for the treatment of ammonia contaminated airstreams at low temperatures and identified the key nitrifying microorganisms driving the removal process.
3.2 INTRODUCTION

Ammonia (NH$_3$) is a highly toxic and odorous gas produced during organic waste decomposition and is often a by-product of wastewater treatment technologies. Stringent legislation for example in Europe (Directive 2008/98/EC), requires that waste and wastewater treatment facilities minimise emissions. The release of NH$_3$ to the atmosphere is not only harmful to the environment but the emission of odours from wastewater treatment plants makes their presence less acceptable to the general public and results in complaints to Environmental Protection Agencies and local authorities (Phillips, 2008). NH$_3$ is also an irritant to the eyes and the respiratory tract, and exposure to high concentrations can be fatal within minutes (Taghipour et al., 2008). For these reasons it is essential that anthropogenic NH$_3$ emissions are curtailed and that effective methods for the treatment of contaminated airstreams are developed.

Traditional methods for the treatment of waste gases include carbon adsorption, wet scrubbing, and incineration, but these often require high treatment costs and the production of secondary waste streams (Chung, 2007). However, and in recent years, biological treatment of waste gases has been favoured and biofilm reactors have been demonstrated as highly effective in the treatment of NH$_3$ contaminated air (Chen et al., 2005; Taghipour et al., 2008; Ottosen et al., 2011). Horizontal Flow Biofilm Reactors (HFBRs) are a relatively new biotechnology, which employ a boustrophedonic flow regime of liquid and gas, ensuring good contact between the gas and liquid phases. Traditional biofilters and biotrickling filters are prone to channelling, clogging and pressure loss issues (Nanda et al., 2011), while HFBRs have been shown to avoid these problems for wastewater (Clifford et al., 2008; Rodgers et al., 2008; Clifford et al., 2010) and gas treatment (Clifford et al., 2012; Kennelly et al., 2014). In his trial, HFBRs were operated in triplicate to treat ammonia contaminated airstreams at 10°C.

The removal of NH$_3$ from a contaminated airstream in a biological system occurs in two stages. Initially the NH$_3$ is absorbed/dissolved into the liquid phase forming ammonium (NH$_4^+$) which is then, in turn, biologically degraded by a microbial consortium (Jiang et al., 2009). In aerobic bioreactors, such as HFBRs, the NH$_4^+$ is removed in a two-step nitrification process know as (NH$_4^+$ → NO$_2^-$ + NO$_3^-$). The
first step of nitrification, which is mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), is the oxidation of NH$_4^+$ to nitrite (NO$_2^-$). In the second step of nitrification, NO$_2^-$ is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Ammonia oxidation is the rate-limiting step in the nitrification process (Martens-Habbena et al., 2009a), and therefore the main focus of this study was to assess the microbial populations involved in ammonia oxidation.

Ammonia oxidation is catalyzed by the ammonia monooxygenase (AMO) enzyme, which is unique to AOB and AOA and encoded for by the amoA gene. The bacterial amoA gene which has been widely studied, is limited to AOB from two narrow clades of the Beta- and Gammaproteobacteria (Martens-Habbena et al., 2009b). The archaeal amoA gene, which was only recently identified in marine water columns (Francis et al., 2005), but has subsequently been found in a wide range of environments, is only found in AOA, which belong to a newly described phylum of Archaea, the Thaumarchaeota (Brochier-Armanet et al., 2008). Traditionally AOB were thought to be responsible for the majority of ammonia oxidation in natural ecosystems but it has subsequently been shown that AOA are present in higher numbers in many environments, including soils (Leininger et al., 2006), oceans (Wuchter et al., 2006), estuarine environments (Caffrey et al., 2007) and in WWTPs (Kayee et al., 2011). This abundance highlights the important ecological role that AOA have in biogeochemical cycles, thus making a substantial contribution to the global nitrogen and carbon cycles (Park et al., 2008).

To date there has been a limited number of studies (Yasuda et al., 2013; Posmanik et al., 2014) on the structure, diversity and abundance of AOA and AOB communities in bioreactors treating ammonia gas, and to our knowledge there have been no previous investigations of ammonia-treating bioreactors at low temperatures. Geets et al. (2007) concluded that consistent, long-term performance in bioreactors treating nitrogenous wastewater can only be ensured when the microbial community within the sludge functions optimally. Similarly, and for this reason it is important to assess the microbial community structure and identify and quantify key microorganisms present in the HFBRs. Understanding the environmental factors that impact on microbial community structure and function in HFBRs could help improve reactor design and operation for improved NH$_3$ remediation. The objectives of this study were to test the feasibility of HFBRs as a NH$_3$ elimination technology and to
describe the microbial community underpinning the oxidation of the ammonia. Genetic fingerprinting of the bacterial community in the HFBRs using TGGE was used to compare the community structure from different locations in the HFBRs. Key bacteria, AOB and AOA, were identified by clone library analyses and quantified using real-time PCR.

3.3 MATERIALS AND METHODS

3.3.1 HFBR design and operation

Three HFBRs were designed, constructed and operated for the treatment of ammonia-contaminated airstreams. Each HFBR comprised of 60 horizontal plastic sheets, with integrated frustums, which were stacked one above the other (Fig. 1). The sheets were bolted together to form sealed reactors that could be opened for visual assessment and biofilm sampling. The working volume of each reactor was 20 l and the top plan surface area (TPSA) of the plastic sheet media was 0.04 m², giving a total plan surface area (TPSA) of 2.4 m². Six intermediate sample ports were located along the vertical profile of each reactor at sheets 4, 12, 21, 30, 40, 50 and 60 allowing for intermediate samples of gas and water to be taken.
Chapter 3

Figure 1. Schematic of HFBR set-up

Table 1. Composition of Liquid Nutrient Feed.

<table>
<thead>
<tr>
<th>Component</th>
<th>(g/L)</th>
<th>Component</th>
<th>(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃ (Phase I)</td>
<td>1.2</td>
<td>MgSO₄·7H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>NaHCO₃ (Phase II)</td>
<td>1.9</td>
<td>FeSO₄·7H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>0.1</td>
<td>Urea</td>
<td>0.03</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.025</td>
<td>MnSO₄·H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.06</td>
<td>KHCO₃</td>
<td>0.05</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.025</td>
<td>CaCl₂·6H₂O</td>
<td>0.003</td>
</tr>
</tbody>
</table>
NH\textsubscript{3} was mixed with compressed air to desired influent gas concentrations using mass flow controllers (Bronkhorst High Tech BV), flow meters (Key Instruments) and pressure regulators (DruVa) (Fig. 1). An air mixture containing the NH\textsubscript{3} was introduced above Sheet 1, at the top of the reactor and flowed horizontally across each sheet before moving to the sheet below in a boustrophedonic flow regime. Liquid Nutrient Feed (LNF) was applied to the HFBR at Sheet 1 and flowed in a co-current direction with the gas stream. The LNF was delivered intermittently (10 min h\textsuperscript{-1}) via a peristaltic pump in a step feed manner, i.e. 75 \% of the LNF (4.5 l d\textsuperscript{-1}) was dosed onto Sheet 1 and 25 \% of the LNF (1.5 l d\textsuperscript{-1}) onto Sheet 30. The composition of the LNF is given in Table 1. The HFBRs were seeded with activated sludge from a municipal wastewater treatment plant. LNF, with additional NH\textsubscript{4}Cl (25 mg l\textsuperscript{-1}), was applied to the HFBRs for 12 weeks in the absence of NH\textsubscript{3} to facilitate biofilm growth.

The HFBRs were operated in triplicate at 10°C for the duration of the trial, which was divided into two phases; Phase I (PI) and Phase II (PII), lasting 57 and 14 days, respectively. During PI the NH\textsubscript{3} loading rate was 2.44 g m\textsuperscript{3} h\textsuperscript{-1}, which was doubled to 4.88 g m\textsuperscript{3} h\textsuperscript{-1} in PII (Table 2). The concentration of NaHCO\textsubscript{3} was increased during PII to buffer the HFBRs against a reduction in pH. Operation parameters are summarised in Table 2.

Table 2. Operational parameters of the laboratory-scale HFBRs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase I</th>
<th>Phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Mixture Flow Rate (AFR) (m\textsuperscript{3} m\textsuperscript{3} h\textsuperscript{-1})</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Gas Loading Rate (g NH\textsubscript{3} m\textsuperscript{3} h\textsuperscript{-1})</td>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Average Influent Concentration (ppm\textsubscript{v})</td>
<td>140</td>
<td>172</td>
</tr>
<tr>
<td>Empty bed residence time (EBRT) (s)</td>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>Trial Duration (d)</td>
<td>57</td>
<td>14</td>
</tr>
</tbody>
</table>
3.3.2 Sampling and Analytical Methods

For the purpose of gas, liquid and biofilm sampling, each HFBR was divided into seven sample zones (Z1-Z7). Sample zones corresponded with the gas/water sampling ports located along the reactor at sheets 4, 12, 21, 30, 40 and 50 (Fig. 2). Influent, effluent and profile gas and water samples from the HFBRs were analysed throughout the trial.

Concentrations of NH₃ in the gas phase were measured using a GAXT-A2-DL Ammonia Detector (BW Technologies, Calgary, Canada). Liquid samples from influent, effluent and intermediate sampling ports were analysed. Total Organic Carbon (TOC), Total Nitrogen (TN) and Total Phosphorous (TP) concentrations were evaluated using a Biotector TOC TN TP Analyser (Biotector, Cork, Ireland). Ammonium-nitrogen (NH₄-N), nitrite-nitrogen (NO₂-N), nitrate-nitrogen (NO₃-N), sulphate sulphur (SO₄-S) and phosphate-phosphorous (PO₄-P) concentrations were determined using a Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts). Suspended solids were measured in accordance with standard methods (AWWA, 1998).

3.3.3 Biofilm sampling and DNA extraction

Biofilm (1.0 g) samples were removed from Z1-Z7 in the HFBRs on the last day of trial (Day 71) for molecular analyses. The remaining biomass from each zone was then removed and biofilm mass (g), Total Suspended Solids (TSS; g) and Volatile Suspended Solids (VSS; g) for each zone were determined using standard methods (AWWA, 1998).

DNA was extracted from 0.1 g of biofilm samples using a Maxwell 16 Tissue DNA Purification Kit and a Maxwell 16 Research Instrument System (Promega, Wisconsin, USA). All extracts were visualised on a 1% (w/v) agarose gel containing SYBR Gold (0.01% v/v) under UV light, and quantified spectrophotometrically using a Nano-Drop (Thermo Scientific, UK).
3.3.4 Quantification of bacterial 16S rRNA, bacterial amoA and archaeal amoA genes

Bacterial 16S rRNA, bacterial amoA and archaeal amoA genes were quantified by real-time PCR in 20µl reactions using a LightCycler 480 instrument (Roche, Basel, Switzerland). Bacterial 16S rRNA genes were quantified using primers 338f and 805r and TaqMan probe 516f-ROX, using same reaction concentrations and cycling conditions (Table 3) described by Yu et al. (2005). Bacterial amoA gene copies were quantified using primers amoA-1F and amoA-2R (Rotthauwe et al., 1997) and Syber Green Mastermix (Roche, Basel, Switzerland) as described by Geets et al. (2007). Archaeal amoA gene copies were quantified using primer Arch-amoAF and Arch-amoAR (Francis et al., 2005) as described by Rogers and Casciotti (2010). Internal standard curves were generated for each target to ensure accurate gene copy quantification. Real-time PCR was performed on all twenty-one DNA extracts from the three HFBRs and cycling conditions are outlined in Table 3.

Based on the total mass of biofilm in the reactors on the last day of the trial (day 71) and gene copy concentrations, the absolute abundance of AOB and AOA in the
HFBRs was determined, assuming the frequency of two amoA gene copies in each AOB cell (Dionisi et al. 2002) and one copy of amoA in each AOA cell (Trias et al., 2012). Accurate quantification of bacterial cells was not possible as different bacterial species may hold between one and fifteen 16S rRNA gene copies per cell (Kembel et al. 2012). The relative abundance of AOB 16S rRNA:Total Bacterial 16S rRNA gene copies was calculated assuming that each AOB contained one 16S rRNA gene copy (Aakra et al., 1999).

3.3.5 Bacterial 16S rRNA and archaeal amoA clone library construction

A composite HFBR DNA sample was prepared, by combining an equal volume (2 µl) of each of the 21 DNA extracts, for bacterial 16S rRNA and archaeal amoA clone library construction. Bacterial 16S rRNA and archaeal amoA genes were PCR amplified using primers 338f and 805R (Yu et al., 2005), and Arch-amoAF and Arch-amoAR (Francis et al., 2005), respectively. PCR cycling conditions are outlined in Table 3. PCR amplicons were gel purified using a Wizard SV Gel and PCR Clean Up kit (Promega, Madison, WI, USA) and cloned using TOPO TA vector and TOPO-10 chemically competent cells (Invitrogen, CA, USA). Luria broth plates with kanamycin (50 µg ml\(^{-1}\)) were prepared and X-gal (20 µg ml\(^{-1}\)) was spread on the surface of each plate. The ligation mixture was spread on the plates and incubated overnight at 37°C, and following growth standard blue/white screening was performed to confirm transformation had occurred. The plasmid inserts from the resulting white clones were sequenced by Macrogen (Seoul, South Korea). The resulting sequences were first orientated in the 5’-3’ direction and screened for vector contamination using VecScreen (NCBI website). The community sequence data were analysed using the Mothur software package (Schloss et al., 2009). Chimeric sequences were identified and removed. Following screening a total of 75 suitable clones were identified. Sequences were aligned and those that showed >97% similarity were grouped together as one single operational taxonomic unit (OTU). A representative sequence from each OTU was selected and the library coverage (%) was calculated. The representative sequences were aligned with reference sequences from the GenBank sequence database using ClustalX2 (Larkin et al., 2007). Phylogenetic trees were constructed using MEGA5 software (Tamura
et al., 2011), by neighbour joining analyses with bootstrap method for 1000 replications using the Jukes-Cantor model. Clonal sequences from the bacterial 16S rRNA and archaeal amoA libraries were deposited in GenBank with the accession numbers KJ438649-KJ438687.

Five OTUs, three AOB and two NOB, were identified from the clone library and selected for TGGE fingerprinting. The plasmids from each of the five OTUs were extracted using a plasmid DNA purification kit (Invitrogen, CA, USA) according to the manufacturers instructions and stored at -20°C for downstream processes. Details of TGGE fingerprinting are described below.

3.3.6 Temperature Gradient Gel Electrophoresis (TGGE) fingerprinting of bacterial community in the HFBRs

A total of 15 samples, 10 from HFBR samples and 5 from clone library OTUs, were selected for TGGE fingerprinting. The HFBR samples consisted of DNA, from Z1-Z7 in R1 and from three composite biofilm samples from R1, R2 and R3. The composites from R1, R2 and R3 were prepared by adding equal volumes (2 µl) of DNA extract from the seven zones (Z1-Z7) within a given HFBR. The plasmid extracts of the five clone library OTUs from the bacterial clone library were also analysed.

The bacterial 16 rRNA genes of the DNA and plasmid extracts were PCR amplified with primers 338f and 805r (Yu et al., 2005) and visualised on a 1% (w/v) agarose gel containing SYBR Gold (0.01% v/v) under UV light (Fig. 8). A 40bp GC clamp (CGCCCGCGCCGCCCCCGCCCCCGCCCCCGCCCCCGCCCCCG) was attached to the reverse primer, and PCR cycling conditions are described in Table 3. TGGE of the resulting amplicons was performed using a Maxi System (Biometra, Göttingen, Germany). The denaturing gel was composed of acrylamide (6%, w/v), urea (8M), TAE (1x), glycerol (2% v/v), deionized formamide (20%, v/v), with polymerisation agents TEMED (0.09%, v/v) and APS (0.016%, v/v). A preliminary perpendicular TGGE run of the PCR amplicons was performed in a temperature gradient of 40-70°C for 16 h to determine the appropriate temperature gradient for parallel analyses.
The individual PCR amplicons were then run in parallel in a temperature gradient of 54-61°C for 16 h. TGGE gels were stained using silver nitrate (Bassam et al., 1991).

3.3.7 Statistical analyses of TGGE fingerprinting and environmental variables in HFBRs

Genetic fingerprinting profiles, based on the presence and absence of bands from TGGE gels, were analysed using Primer6 software (Clarke, 1993) to determine the similarity of the bacterial communities between samples. A resemblance matrix was generated using Bray-Curtis similarity. Clustering of the samples by similarity using the group average method was performed, and SIMPROF (significance p<0.05) was used to test the statistical validity of the clustering (Clarke, 1993). Two-dimensional, non-metric multi-dimensional scaling (MDS), based on 1000 restarts, was used to visualise the relationship between the different bacterial community profiles.

Physicochemical data from Z1-Z7 in the three HFBRs were normalised and compared using principle component analyses (PCA). A resemblance matrix of the normalised data set was generated using Euclidean distance. Permutational MANOVA of the resemblance matrix determined whether, based on the environmental conditions measured that; (i) the HFBRs were statistically different, and (ii) if the zones in the different reactors were statistically different. Pair-wise testing of the three HFBRs, and separately the seven zones across the three HFBRs, was also performed using Permutational MANOVA of the resemblance matrix.

The impact of the physicochemical parameters on the biological community assembly was determined by BEST analysis method using Primer 6 (Clarke et al., 2006) and was used to determine which, or if any, abiotic factors best explain the bacterial community structure. The agreement between the physicochemical and biological matrices was determined by Spearman’s rank correlation using 999 permutations.
### Table 3. Primers, probes and cycling conditions used for PCR amplification for cloning, qPCR and TRFLP

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers/Probes</th>
<th>Sequence (5’-3’)</th>
<th>Cycling Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clone Library</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S rRNA</td>
<td>338f, 805r</td>
<td>ACTCCTACGGGAGGCAGCAG, GACTACCAGGGTATCTAA</td>
<td>94 °C 2 min; 30 cycles 94°C 1 min, 57°C 1 min, 72°C 1 min, 72°C 10 min</td>
<td>(Yu et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Arch-amoAF, Arch-amoAR</td>
<td>STAATGGTCTTGCTTAGACG, GCGGCCATCCCATCTGTATGT</td>
<td>94 °C 5 min; 35 cycles 94°C 1 min, 56°C 35 sec, 72°C 1 min, 72°C 10 min</td>
<td>(Francis et al., 2005)</td>
</tr>
<tr>
<td><strong>Real-time PCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S rRNA</td>
<td>338f, 805r, 516f-ROX</td>
<td>ACTCCTACGGGAGGCAGCAG, GACTACCAGGGTATCTAA, TGCCAGCAGCCGG TAA TAC</td>
<td>94°C 10 min; 40 cycles 94°C 10 sec 60 °C 30 sec</td>
<td>(Yu et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Arch-amoAF, Arch-amoAR</td>
<td>STAATGGTCTTGCTTAGACG, GCGGCCATCCCATCTGTATGT</td>
<td>95 ºC 10 min; 40 cycles 94°C 1 min, 56°C 1 min, 72°C 1 min, 78°C 15 sec</td>
<td>(Francis et al., 2005)</td>
</tr>
<tr>
<td>Archaeal amoA</td>
<td>amoA-1F, amoA-2R</td>
<td>GGGGTTTTCTACTGGTTGTT, CCCCTCKGSAACGCTCTGCTTCC</td>
<td>50 °C 2 min; 95°C 10 min; 40 cycles 95°C 1 min, 50 °C 1 min, 60°C 1 min</td>
<td>(Rotthauwe et al., 1997)</td>
</tr>
<tr>
<td><strong>TGGE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S rRNA</td>
<td>338f, 805r</td>
<td>ACTCCTACGGGAGGCAGCAG, GACTACCAGGGTATCTAA-(GC clamp)*</td>
<td>94 °C 2 min; 30 cycles 94°C 1 min, 57°C 1 min, 72°C 1 min, 72°C 10 min</td>
<td>(Yu et al., 2005)</td>
</tr>
</tbody>
</table>
3.4 RESULTS

3.4.1 NH₃ removal and nitrogen dynamics of gas and liquid phases

During PI, when a loading rate of 2.43 g NH₃ m⁻³ h⁻¹ was applied to the HFBRs, the average NH₃ removal efficiency across the three HFBRs was 99.7% (±1.6%). During PII, when the loading rate was doubled to 4.86 g NH₃ m⁻³ h⁻¹, the removal efficiency across the three HFBRs was 100% (Table 4).

Table 4. HFBR NH₃ removal results (standard deviation in parenthesis).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase I</th>
<th>Phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas Loading Rate (g NH₃ m⁻³ h⁻¹)</td>
<td>2.4</td>
<td>4.9</td>
</tr>
<tr>
<td>NH₃ Influent Concentration (ppm)</td>
<td>142 (27.1)</td>
<td>172 (2.9)</td>
</tr>
<tr>
<td>R1 Removal Efficiency (%)</td>
<td>99.6 (2.1)</td>
<td>99.7 (0.4)</td>
</tr>
<tr>
<td>R2 Removal Efficiency (%)</td>
<td>99.8 (1.5)</td>
<td>99.7 (0.5)</td>
</tr>
<tr>
<td>R3 Removal Efficiency (%)</td>
<td>99.8 (1.3)</td>
<td>99.8 (0.3)</td>
</tr>
</tbody>
</table>

Depth-resolved analyses of gas phase samples in the HFBRs (Fig. 3) showed that the NH₃ was rapidly removed from the airstream. During PI the majority of the NH₃ had been incorporated into the liquid phase by sheet 10, whereas during PII, when the loading rate was doubled, the NH₃ was completely removed by sheet 30. In-situ gas and liquid measurements showed that the same nitrogen dynamics occurred in the three HFBRs during both phases. NH₃ removal was accompanied by an accumulation of NH₄⁺, NO₂⁻ and NO₃⁻ in the liquid phase, with highest rates of accumulation occurring in the top zones of the HFBRs where the majority of the NH₃ was removed. As concentrations of NH₄⁺ in the liquid stream decreased with depth, there was an increase in NO₂⁻ and NO₃⁻ concentrations, indicating that partial nitrification to NO₂⁻ and complete nitrification to NO₃⁻ occurred. Effluent concentrations of NO₃⁻ in the liquid phase were always higher than NO₂⁻ concentrations. The increased NH₃ loading rate during PII resulted in an increase of both NO₂⁻ and NO₃⁻ concentrations in the effluent. The average nitrogen mass balance across the three HFBRs, which was based on influent and effluent
measurements of NH$_3$ in the gas phase, and NH$_4^+$, NO$_3^-$ and NO$_2^-$ in the liquid phases, showed an overall N loss of 29%.

![Figure 3](image.png)

**Figure 3.** Nitrogen dynamics of the gas and liquid phases in the three HFBs during Phase I in (a) R1, (b) R2, (c) R3 and during Phase II in (d) R1, (e) R2 and (f) R3.

### 3.4.2 In-situ rates of NH$_3$ removal

The average *in-situ* rates of NH$_3$ removal, TON (NO$_3^-$ + NO$_2^-$) production and NH$_4^+$ production/loss varied with location in the HFBs (Table 5). The highest rates of
NH\textsubscript{3} removal were in the top zones of the HFBRs, which coincided with highest TON production rates and highest accumulation rates of NH\textsubscript{4}\textsuperscript{+}. The rate of NH\textsubscript{4}\textsuperscript{+} accumulation in the liquid was highest in Z1 during PI and in Z1 and Z2 during PII, below which accumulation ceased in the HFBRs and NH\textsubscript{4}\textsuperscript{+} was removed.

**Table 4.** Average area specific in-situ NH\textsubscript{3} removal, TON production and NH\textsubscript{4}\textsuperscript{+} balance (+ represents an increase and – a decrease in NH\textsubscript{4}\textsuperscript{+} concentration) of HFBRs during Phase I and II. All rates expressed as mg m\textsuperscript{-2}[TPSA] h\textsuperscript{-1} where TPSA is Top Plan Surface Area.

<table>
<thead>
<tr>
<th>Zone</th>
<th>PI NH\textsubscript{3} removal</th>
<th>PI TON production</th>
<th>PI NH\textsubscript{4}\textsuperscript{+} balance</th>
<th>PII NH\textsubscript{3} removal</th>
<th>PII TON production</th>
<th>PII NH\textsubscript{4}\textsuperscript{+} balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>267.41</td>
<td>35.24</td>
<td>+46.26</td>
<td>384.96</td>
<td>79.85</td>
<td>+62.02</td>
</tr>
<tr>
<td>Z2</td>
<td>11.85</td>
<td>15.63</td>
<td>-11.32</td>
<td>56.02</td>
<td>38.14</td>
<td>+7.67</td>
</tr>
<tr>
<td>Z3</td>
<td>4.18</td>
<td>8.18</td>
<td>-4.74</td>
<td>28.81</td>
<td>35.72</td>
<td>-16.35</td>
</tr>
<tr>
<td>Z4</td>
<td>1.33</td>
<td>14.20</td>
<td>-10.52</td>
<td>16.76</td>
<td>12.15</td>
<td>-9.70</td>
</tr>
<tr>
<td>Z5</td>
<td>0.60</td>
<td>2.94</td>
<td>-2.62</td>
<td>4.19</td>
<td>13.45</td>
<td>-11.09</td>
</tr>
<tr>
<td>Z6</td>
<td>0.00</td>
<td>9.23</td>
<td>-2.32</td>
<td>0.00</td>
<td>10.34</td>
<td>-5.53</td>
</tr>
<tr>
<td>Z7</td>
<td>0.00</td>
<td>0.92</td>
<td>-0.50</td>
<td>0.00</td>
<td>8.19</td>
<td>-0.52</td>
</tr>
</tbody>
</table>

**3.4.3 Real-time quantification of AOB, AOA and bacterial 16s rRNA genes**

The standard curves generated for the three real-time assays were linear (r\textsuperscript{2} > 0.997) and had high efficiency values (Fig. 4). AOB and AOA were detected in all regions of the HFBR in high numbers (Fig. 5). AOB were more abundant than AOA in all samples with an average of 2.6 x10\textsuperscript{3}, 2.0 x10\textsuperscript{3} and 4.0 x10\textsuperscript{3} times more AOB than AOA per gram biofilm in R1, R2 and R3, respectively. The relative abundance of AOB 16S rRNA genes compared to total bacterial 16S rRNA varied between samples but was generally high. Based on Pearson’s coefficient (p=0.84) there was a strong correlation between the abundance of AOB and AOA cells in a given zone (Fig. 6).
3.4.4 Bacterial community structure of HFBRs

The clone library comprised of three phyla; Proteobacteria (72%), Bacteroidetes (23%) and Actinobacteria (5%), with a total library coverage of 49.3% (Fig. 7). The Betaproteobacteria class (phylum Proteobacteria) comprised 39% of the entire library and several clones identified in this class were closely related to known nitrifying bacteria. AOB from the genera *Nitrosomonas* and *Nitrosospira* were detected while the only NOB found was from the *Candidatus nitrotoga* genus. Five OTUs from the betaproteobacteria class that were identified as known nitrifiers, were selected for use in TGE analyses (Table 6, see also section 2.5).
Figure 5. Real-time PCR quantification of bacterial 16S rRNA, bacterial amoA and archaeal amoA gene copy numbers, and the relative abundance of AOB and bacterial 16S rRNA gene copies, from different zones in (a) R1, (b) R2 and (c) R3 on the last day of the trial.

Figure 6. Correlation between AOB and AOA abundance in biofilm samples from the three HFBRs on Day 71. Pearson’s coefficient p=0.84
Figure 7. Neighbour joining tree depicting the bacterial community present in the HFBRs based on Jukes-Cantor distances of the bacterial 16S rRNA genes. The sequences obtained in this study are depicted in bold. Reference sequences were downloaded from the GenBank sequence database.
Table 6. Clones from bacterial clone library selected for TGGE analyses

<table>
<thead>
<tr>
<th>Library Clones</th>
<th>Closest BLAST relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accession Number</td>
<td>Frequency in library (%)</td>
</tr>
<tr>
<td>KJ438655</td>
<td>1.6</td>
</tr>
<tr>
<td>KJ438656</td>
<td>9.4</td>
</tr>
<tr>
<td>KJ438677</td>
<td>11.0</td>
</tr>
<tr>
<td>KJ438678</td>
<td>6.3</td>
</tr>
<tr>
<td>KJ438684</td>
<td>4.7</td>
</tr>
</tbody>
</table>

### 3.4.5 Community structure of AOA in the HFBRs

The AOA community in the HFBRs was investigated by targeting the functional archaeal amoA gene, which is unique to AOA. Overall diversity of AOA was very low with 86% of OTUs showing > 99% homology to the same uncultured archaeal clone AU7 (KF618629.1). The remaining clones also showed high levels of similarity to similar uncultured archaeal clones.

### 3.4.6 Statistical comparison of TGGE profiles and in-situ HFBR conditions

The overall bacterial community structure in the three HFBRs, and from Z1-Z7 in R1, were compared, along with the five isolates from the bacterial clone library, using TGGE fingerprinting (Fig. 8). The bacterial community composition of Z1-Z7 in R1 was compared to determine the similarity between zones. All zones showed >78% similarity, indicating that the bacterial community was well conserved between zones in R1 (Fig. 9). Cluster analyses showed that the bacterial community composition changed with depth in the HFBR and samples from zones closest to each other were most similar. The bacterial community in Z3 and Z4 were identical as were samples from Z6 and Z7. Separate composite samples from each of the three HFBRs (R1, R2 and R3) showed >95% similarity, indicating that the overall
bacterial community structure in the three HFBRs was also conserved. All zones from R1, and the three composite HFBR samples contained bands similar to those seen for the *Nitrosomonas* and *Nitrosospira* clonal isolates. The presence of these bands indicates that *Nitrosomonas* and *Nitrosospira* were found ubiquitously throughout the HFBRs. It is not conclusive from the TGGE banding patterns observed whether the HFBR samples contained *Candidatus nitrotoga arctica* clones.

**Figure 8.** (a) PCR amplicons of isolates from the bacterial clone library (Table 6) and HFBR samples and (b) TGGE profiles of the clones from bacterial clone library and HFBR samples.
Figure 9. a: Cluster analyses showing similarity of bacterial communities in biofilm from Zones 1-7 in R1 based on presence/absence of bands from TGGE fingerprinting. b: MDS of biological bacterial communities in biofilm from Zones 1-7 in R1 based on presence/absence of bands from TGGE fingerprinting compared with physicochemical parameters in HFBR.

Environmental variables in the seven zones from each HFBR were compared to determine similarity between zones and reactors. Comparison of the physio-chemical variables measured in the gas and liquid phases in HFBRs using permutational MANOVA, showed that the environmental conditions in the three HFBRs were significantly different (p=0.001), and therefore the three HFBRs were not statistically replicated. However, pair-wise testing showed that environmental conditions in R2 and R3 were replicated (p=0.209), while R1 was significantly
different than R2 (p=0.012) and R3 (p=0.012). Cluster analyses of the zones from the HBFRs showed that in general, with respect to the environmental variables measures, zones from R1 clustered away from other zones, while zones from R2 and R3 showed higher similarities. As the HFBRs were supplied with the same LNF, the environmental variables measures at Z1 in R1, R2 and R3 were identical (circled in Fig. 10). Environmental variables measured included TON, NO$_2^-$, NO$_3^-$, NH$_4^+$, P, organic carbon, inorganic carbon, SO$_4$ concentrations and alkalinity and pH.

The bacterial community structure was impacted by environmental conditions in the HFBR. In Z1 and Z2 the bacterial consortium was strongly driven by pH and NH$_3$ concentrations (Fig.9). The bacterial populations in Z3-Z5 were mainly impacted by PO$_4$ and TP but also by other environmental parameters. In contrast Z6 and Z7 were relatively unaffected by measured physicochemical variables.

Figure 10. Non-metric multidimensional scaling (MDS) plot the seven samples zones from the three HFBRs, based on environmental variables recorded. Z1 from R1, R2 and R3 are circled.
3.5 DISCUSSION

During the trial the three HFBRs were successfully used to treat ammonia-contaminated airstreams at 10°C indicating the potential of HFBRs for ammonia remediation. Average removal efficiencies of 99.7% were achieved at a loading rate of 4.88 g NH₃ m⁻³ h⁻¹ and although the mass loading rates were conservative in comparison to other studies (Table 7), the consistently high removal efficiencies underscored the process stability in the HFBRs.

During Phase I and II the majority of NH₃ had been removed from the airstream before sheets 12 and 30 respectively, such that the bottom half of the HFBRs were rarely exposed to NH₃. Greater NH₃ removal rates may have been possible if the loading rate was increased to maximise the oxidation potential of the lower sheets. As the HFBRs consistently achieved removal rates of >99% at loading rates of 2.44 and 4.88 g NO₃⁻ m⁻³ h⁻¹, the critical loading rate (defined as the maximum load at which 95% removal occurs) was not determined. Other studies have reported maximum removal rates of between 5 and 65 g NH₃ m⁻³ h⁻¹ at their critical loading rates (Kim et al., 2000; Sakuma et al., 2008; Taghipour et al., 2008). During this trial the HFBRs were operated at 10°C, which is typical of temperate climates, such as in Northern Europe, whereas most other studies have been carried out at higher temperatures (Table 7). Nitrification rates are affected by temperature and it has been well documented that increasing temperature results in higher nitrification rates (Chen et al., 2006). An increased HFBR operating temperature may have resulted in higher in-situ nitrification rates. However, increasing operating temperatures would require a larger energy input, thus increasing HFBR running costs. Further investigation is required to determine the maximum NH₃ removal potential of the HFBRs at increased loading rates and operating temperatures.

As NH₃ and NH₄⁺ removal occurred in the HFBRs, there was an accumulation of NO₂⁻ and NO₃⁻ in the liquid phase, indicating that partial and complete nitrification had occurred. The highest rates of NO₂⁻ and NO₃⁻ production were observed in the upper regions of the HFBRs, where the highest substrate concentrations of NH₃ and NH₄⁺ were detected. As nitrification rates are often limited by substrate concentration and availability (Norton and Stark, 2011), it is unsurprising that the highest nitrification rates were observed in these locations. The reason for NO₂⁻
Table 7. Comparison of HFBR performance from this study with a selection previously reported data from the literature

<table>
<thead>
<tr>
<th>Study</th>
<th>EBRT (s)</th>
<th>Removal Efficiency (%</th>
<th>Elimination Capacity (g NH$_3$ m$^{-3}$ h$^{-1}$)</th>
<th>Operating Temperature (°C)</th>
<th>Reactor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>This Study</td>
<td>60 – 120</td>
<td>95 – 100</td>
<td>Average: 2.4 – 4.9; Maximum: 4.9</td>
<td>10</td>
<td>Horizontal Flow Biofilm Reactor (HFBR)</td>
</tr>
<tr>
<td>Zápotocký and Šváb (2012)</td>
<td>25</td>
<td>60</td>
<td>Maximum: 6.2</td>
<td>20</td>
<td>Biotrickling Filter</td>
</tr>
<tr>
<td>(Baquerizo et al., 2005)</td>
<td>36</td>
<td>55 – 100</td>
<td>Average: 3.2 – 11.5; Maximum: 11.5</td>
<td>22 ± 2</td>
<td>Biofilter packed with coconut fibre</td>
</tr>
<tr>
<td>Pagans et al. (2005)</td>
<td>86</td>
<td>46.7 – 98.8</td>
<td>Average: 0.8 – 21.7; Maximum: 61.3</td>
<td>15 – 26</td>
<td>Biofilter containing mature compost as bed media.</td>
</tr>
<tr>
<td>Taghipour et al. (2008)</td>
<td>30 – 60</td>
<td>97.9 – 99.9</td>
<td>Average: 2.15 – 9.85; Maximum: 9.85</td>
<td>30</td>
<td>Biofilter packed with compost and shredded plastic</td>
</tr>
<tr>
<td>Sakuma et al. (2008)</td>
<td>13.5</td>
<td>92 – 100</td>
<td>Average: 56; Maximum: 120</td>
<td>22 – 25</td>
<td>Biotrickling filter</td>
</tr>
</tbody>
</table>

Accumulation in the HFBRs is unclear but can be influenced by numerous factors including substrate concentration, dissolved oxygen (DO), temperature and pH (Kim et al., 2008). Partial nitrification of NH$_4^+$ to NO$_2^-$ is considered beneficial in some situations. For every 1 mol of NH$_4^+$ oxidised, AOB require 1.5 mol of O$_2$, while NOB require 0.5 mol of O$_2$ for the oxidation of NO$_2^-$ to NO$_3^-$. As such, partial nitrification requires a 25% lower oxygen demand than complete nitrification (Ruiz
et al., 2003). Reducing the oxygen demand on a biological reactor by facilitating partial nitrification could increase the ammonia oxidizing capacity of that reactor. The partial nitrification observed in the HFBRs during this trial may have increased the ammonia oxidising potential of the system. Tertiary treatment of the liquid effluent, which was rich in both \( \text{NO}_2^- \) and \( \text{NO}_3^- \), would be required in real world applications as TN concentrations were well above the discharge limits of 15 mg l\(^{-1}\) that are allowed under the EU Urban Wastewater Treatment Directive (91/271/EEC). Introducing an organic carbon source to the lower zones of the HFBRs could facilitate the development of a denitrifying microbial community, providing a HFBR capable of nitrification and denitrification, with no need for further treatment of the liquid effluent. Denitrification is an anaerobic process in which \( \text{NO}_3^- \) and \( \text{NO}_2^- \) are converted to nitrogen gas (N\(_2\)) by a consortium of denitrifying microbes, and there have been numerous bioreactor trials that have coupled the nitrification and denitrification processes to successfully treat NH\(_4^+\) contaminated wastewaters (Zeng et al., 2003; Chiu et al., 2007; Li et al., 2008; Paetkau and Cicek, 2011). However, adding organic carbon to the liquid effluent can be a costly and difficult process (Boley et al., 2000), and may not be feasible in many situations. Removal of TON from the liquid effluent via autotrophic denitrification, which is an anaerobic, biological process where nitrate conversion to nitrogen is coupled with the oxidation of sulphur compounds (Lampe and Zhang, 1996), could potentially be used to treat the liquid effluent from the HFBRs. Autotrophic denitrifying bacteria, such as \textit{Thiobacillus denitrificans} and \textit{Thiomicrospira denitrificans}, utilise inorganic carbon sources such as CO\(_2\) and HCO\(_3^-\) as energy sources (Lampe and Zhang, 1996) and as such, no additional organic carbon would be required. If partial nitrification to NO\(_2^-\) has occurred, as in this trial, it may also be possible, under anaerobic conditions, to add NH\(_4^+\) to facilitate anammox (NH\(_4^+\) + NO\(_2^-\) \(\rightarrow\) N\(_2\) + 2H\(_2\)O) without the requirement for organic carbon. The anammox process, which is carried out by anammox bacteria (Kuypers et al., 2003), results in metabolic end products (N\(_2\) + H\(_2\)O), that can be safely discharged into the environment. Anammox bioreactors are a relatively new technology but have been used in numerous studies for the treatment of nitrogen rich wastewaters (Molinauevo et al., 2009; Bagchi et al., 2010; Babaie et al., 2013), and offer an attractive alternative to traditional biological nitrogen removal reactors due to the lack of requirement for organic carbon or oxygen (Park et al., 2010).
Nitrogen mass balance of the influent and effluent gas and liquid streams from the HFBRs showed an overall N loss of 29%, which was similar to reported values from other NH$_3$ oxidising biofilm reactors (Sakuma et al., 2008; Jun and Wenfeng, 2009). The disparity in the nitrogen balance was likely due to the conversion of the influent NH$_3$ to species other than NO$_2^-$ and NO$_3^-$ that were not measured in this study, such as particulate ammonium, organic nitrogen and N$_2$ gas (Jun and Wenfeng, 2009). Some nitrogen loss can also be attributed to the nitrogen requirement of microorganisms for protein syntheses and growth.

The environmental conditions that impact on the relative abundance of AOB and AOA is currently of intense scientific interest (Pester et al., 2011). In the HFBRs, abundant AOB and AOA were detected in all zones in the three reactors, with an average of 5.4 x10$^3$ times more AOB that AOA present in the biofilm. Similarly, Jin et al. (2010) reported that AOB were more abundant than AOA in a nitrifying bioreactor, with approximately 60 times more AOB than AOA detected. In contrast, Posmanik et al. (2014) detected approximately 1 log value more AOA than AOB in a biofilter treating ammonia-contaminated air. There are several environmental factors that can influence the relative abundance of the two groups, such as substrate concentration, pH and moisture (He et al., 2007; Hansel et al., 2008; Taylor et al., 2012). AOB have been shown to be more abundant than AOA when high concentrations of substrate are available (Verhamme et al., 2011). The relatively high concentrations of NH$_3$ present in the HFBRs may have influenced the community composition and favoured the proliferation of an AOB-dominated ammonia-oxidising community. It has also been shown in soil incubations that AOB have a preference for inorganic ammonium sources such as NH$_4^+$, while AOA become more abundant when supplied with organic ammonium (Schleper and Nicol, 2010). As NH$_3$ was converted to inorganic NH$_4^+$ in the liquid phase, it was the main substrate for ammonia oxidation, which may also have impacted on the abundance of AOB and AOA.

There was no correlation between the abundance of AOB and AOA cells in HFBRs and the in-situ ammonia oxidation rates with gene copy concentrations. Although real-time PCR is an excellent molecular tool for the quantification of target genes, it targets DNA which does not give any information on gene expression levels or cell activity. To quantify amoA expression levels and cell activity, it would be necessary
to use real-time reverse transcription (RT)-PCR to target genes that have recently been transcribed. Previous studies have successfully quantified the expression of AOB amoA genes in a nitrifying reactor (Aoi et al., 2004), and in marine sponges (López-legendil et al., 2010). Using RT-PCR in future HFBRs studies would further increase the understanding of the relative contribution of AOB and AOA to total ammonia oxidation in the reactors and help link physicochemical parameters to gene expression levels. From this information it may be possible to develop a bioassay based on gene expression levels that could help to manage the microbial communities present to maximise their oxidation potential and give early warnings of potential process failures.

Although the three HFBRs were experimental replicates, they were statistically different when in-situ physio-chemical measurements were compared. Nonetheless, NH₃ removal rates and removal efficiencies in the three HFBRs were similar, and the same nitrogen cycling dynamics were observed. This indicated that functional capacity of the HFBRs to remove NH₃ was unaffected by the in-situ environment. High similarity (>95%) between the bacterial communities in the three HFBRs occurred, indicating that the conditions in the HFBRs were similar enough to support a common bacterial community. Nitrifying bacteria are required for nitrification and it has previously been shown, in wastewater treatment plants, that similar AOB and NOB community populations occur regardless of temperature, solid retention time and input of industrial wastewater (Siripong and Rittmann, 2007). As the bacterial clone library was dominated by nitrifying bacteria, it is possible that common nitrifying populations were present in the three HFBRs, which may account for the high levels of similarity observed. Even though the overall bacterial community structure between the HFBRs was similar, differences in populations were evident between zones in R1. NH₃ concentration and pH had a strong impact on the bacterial population in Z1 and Z2, while other zones were less strongly affected by the environmental parameters measured in this study. Dissolved oxygen (DO) concentration, which was not measured in this study, is another environmental parameter that can impact the structure of microbial populations. As oxygen is required for aerobic ammonia and nitrate oxidation, both of which occurred in the HFBRs, a gradient in DO concentration would have occurred with depth in the HFBR. DO has been shown to affect the bacterial community in nitrifying reactors.
(Mota et al., 2005) and may have impacted on the biofilm communities from different depths in the HFBRs.

AOB from the Nitrosomonas and Nitrosospira genera made up 26.5% of the bacterial clone library and were detected in the TGGE fingerprints from the three HFBRs and from all zones in R1. Clones from the Nitrosomonas genus, which was the most dominant genera in the HFBRs, had previously been identified as the dominant AOB group in many nitrifying bioreactors (Mobarry et al., 1996; Mertoglu et al., 2006; Jin et al., 2010; Zhang et al., 2011). Nitrosomonas eutropha, which was present in the library, is generally associated with activated sludge (Wagner et al., 2002) and its presence can be attributed to the seed sludge used to inoculate the HFBRs. Nitrosospira are also commonly found in nitrifying bioreactors (Wells et al., 2009) but if present are usually detected in lower abundance than Nitrosomonas. Siripong and Rittmann (2007) observed that Nitrosospira became more abundant in wastewater treatment plants during winter months compared to summer months, and Avrahami and Bohannan (2007) found that lower temperatures increased the abundance of Nitrosospira in soil incubations. As the HFBRs were operated at 10°C, temperature may have influenced the bacterial community structure and provided selective pressures that allowed for the proliferation of Nitrosospira sp. in the reactors. Wells et al. (2009) reported that Nitrosospira sp. and Nitrosomonas sp. respond differently to environmental parameters such as dissolved oxygen, nitrite and metals, while Yasuda et al. (2013) proposed that the presence of both Nitrosospira sp. and Nitrosomonas sp. may guarantee functional redundancy in an ammonia biofilter.

Candidatus nitrotoga arctica, which was first described and isolated from Siberian Arctic permafrost affected soils (Alawi et al., 2007), was the only NOB detected in the HFBRs. It has also been found in activated sludge from a municipal wastewater treatment plant, and when the sludge was incubated at 10°C, Candidatus nitrotoga arctica became enriched (Alawi et al., 2009). It was also shown in the same study that other NOB exhibit different growth characteristics. Nitrospira sp. can grow at a range of temperatures (10-28°C) while Nitrobacter species are enriched at temperatures between 17-28°C. The operating temperature of the HFBRs may have allowed for the enrichment of Candidatus nitrotoga arctica and may explain why they were the only NOB detected in the bacterial clone library.
The bacterial nitrifying communities in other low-temperature nitrifying bioreactors were dominated by AOB from genera *Nitrosomonas* and *Nitrosospira* and NOB from genera *Candidatus nitrotoga*, *Nitrospira* and *Nitrobacter* (Ducey et al., 2010; Karkman et al., 2011). The AOB community in this study was similar, whereas the NOB community, which contained *Candidatus nitrotoga arctica*-like clones, did not contain any *Nitrosomonas* or *Nitrosospira* species. Even though *Nitrosomonas* and *Nitrosospira* related organisms were not found in the clone library, it is possible that they were present in low concentrations that may have been detected using next-generation sequencing techniques.

The AOA population was dominated by an uncultured OTU that made up 86% of the clone library. None of the clones in the library showed high levels of similarity (>97%) with any identified AOA in the Genbank database. It was evident the AOA population was highly conserved in the HFBRs and even though there were high numbers of AOA detected by real-time PCR.

### 3.6 CONCLUSIONS

HFBRs were successfully used to treat NH$_3$ contaminated airstreams at 10°C, with average removal efficiencies of 99.7% achieved at loading rates of 4.8g NH$_3$ m$^{-3}$ h$^{-1}$. Partial and complete nitrification of liquid phase NH$_4^+$ to NO$_2^-$ and NO$_3^-$ occurred in the reactors. Nitrification was carried out by nitrifying bacterial and archaeal biofilm communities. AOB vastly outnumbered AOA in the HFBRs and AOB from the *Nitrosomonas* and *Nitrosospira* genera were the most abundant bacterial species, while in an uncultured archaeal OTU was the most common AOA. The overall bacterial biodiversity between the HBFRs was highly conserved although variations in community structure did occur between different zones in the HFBR, partly driven by environmental conditions present.
3.7 REFERENCES


Chapter 3


CHAPTER 4

The microbial ecology of highly acidic Horizontal Flow Biofilm Reactors (HFBRs) treating hydrogen sulphide at low temperatures
Chapter 4

This work described in this chapter was collaboration between the Microbiology and Civil Engineering departments in the National University of Ireland Galway. The HFBRs were operated by the Civil Engineering research group but all bioprocess data and molecular work was analysed as part of this PhD programme.
4.1 ABSTRACT

Hydrogen Sulphide (H\textsubscript{2}S) is an odorous and highly toxic gas commonly encountered in various commercial and municipal sectors. Three novel, laboratory-scale, Horizontal-Flow Biofilm Reactors (HFBRs) were tested for the removal of hydrogen sulphide (H\textsubscript{2}S) gas from air streams over a 180-day trial at 10\textdegree}C. Removal rates of up to 15.1 g [H\textsubscript{2}S] m\textsuperscript{-3} h\textsuperscript{-1} were achieved during the trial, demonstrating that HFBRs are a suitable technology for the treatment of H\textsubscript{2}S-contaminated airstreams at low temperatures. Bio-oxidation of H\textsubscript{2}S in the reactors led to the production of H\textsuperscript{+} and sulphate (SO\textsubscript{4}\textsuperscript{2-}) ions resulting in acidification of the liquid phase. Reduced removal efficiency was observed when a loading rate of 15.1 g [H\textsubscript{2}S] m\textsuperscript{-3} h\textsuperscript{-1} was applied to the HFBRs. Buffering the liquid nutrient feed (LNF) with NaHCO\textsubscript{3} led to stabilisation of the liquid phase acidity, resulting in improved H\textsubscript{2}S removal. The bacterial community in the HFBRs was investigated by sequencing and fingerprinting the 16S rRNA genes. The bacterial diversity was low and the HFBRs were dominated by two species from the genus Acidithiobacillus and Thiobacillus. The harsh environmental conditions present in the HFBRs were likely responsible for the lack of bacterial diversity. Even so, there were differences in the community structure between zones in the reactor which were influenced by alkalinity, pH and SO\textsubscript{4} concentrations. In spite of the low operating temperature, the results of this study indicate that HFBRs have excellent potential to biologically treat H\textsubscript{2}S contaminated airstreams. The simple, low maintenance technology will allow the technology to be easily retrofitted to existing installations to satisfy ever stringent legislation in Europe.
Chapter 4

4.2 INTRODUCTION

Hydrogen sulphide (H$_2$S) is an odorous and highly toxic gas that is commonly encountered in wastewater treatment. It can be generated when standing wastewater in sewers or clarifiers become septic; during anaerobic treatment; or during decomposition of solid organic matter (Oyarzun et al., 2003; Ramirez et al., 2009). The emission of odours from wastewater treatment plants make their presence less acceptable to the general public and results in complaints to environmental protection agencies and to local authorities (Phillips, 2008). Legislation in Europe requires that waste and wastewater treatment facilities avoid excessive emissions of odours (Directive 2006/12/EC). Many gases emitted from wastewater treatment plants, such as hydrogen sulphide (H$_2$S), ammonia (NH$_3$) and volatile organic compounds (VOCs) can cause significant odour nuisance and can be toxic.

Methods for remediating odorous gas emissions from the waste and wastewater sectors include physical, chemical and biological techniques. Physical and chemical techniques include scrubbing, absorption, membrane separation and iron oxide oxidation (Burgess et al., 2001). These approaches are often effective but can be subject to various limitations including high capital costs, generation of secondary pollutants and high maintenance costs (Phillips, 2008; Peu et al., 2012). In recent years, biological technologies, and in particular biofiltration, have been promoted as providing the most effective methods of air pollution control (Dumont et al., 2008; Jun and Wenfeng, 2009). This is due to the inherent advantages which include low capital, and maintenance, costs; energy efficiency; reduced, or eliminated, requirements for chemicals; and good long-term performance (Lee et al., 2005; Moosavi et al., 2005; Park et al., 2009).

The sulphur cycle is a complex biogeochemical cycle in which oxidised and reduced states of sulphur are transformed both biologically and chemically (Tang et al., 2009). In the biological sulphur cycle, H$_2$S is oxidised by bacteria to elemental sulphur (S$^0$) or sulphate (SO$_4^{2-}$) (Tang et al., 2009). Several bacterial species have been identified as effective H$_2$S-oxidizers, and bioreactors with immobilised cultures of *Thiobacillus denitrificans* (Ma et al., 2006), *Thiobacillus thioparus* (Ramirez et al., 2009) and *Acidithiobacillus thiooxidans* (Lee et al., 2006) have all been used for H$_2$S removal. Indeed, an alternative to pure culture inoculation of bioreactors for H$_2$S
Chapter 4

Abatement is to use activated sludge, as described by Gabriel and Deshusses (2003) and Moussavi et al. (2007). H$_2$S-oxidisers isolated from activated sludge have been immobilised on inorganic media by Jiang et al. (2009), Kim et al. (2008) and Duan et al. (2006). The advantage of using activated sludge as a seed biomass is that it is readily available and likely contains a mixed microbial community capable of many biological processes, including H$_2$S oxidation.

H$_2$S is soluble in water, particularly when compared to other problem gases, e.g. methane, and this facilitates more extensive mass transfer of contaminants to the aqueous phase (Metcalf et al., 2004) and H$_2$S solubility increases at lower temperatures (Chen and Szostak, 2013). There are several bioreactor configurations that have been previously used for the treatment of methane contaminated airstreams including, biofilters, bioscrubbers and biotrickling filters. Removal rates for biofilm-based reactors of between 5 and 125 g H$_2$S m$^{-3}$ reactor volume h$^{-1}$ at residence times of less than 1 min have been reported (Oyarzun et al., 2003; Kim et al., 2008; Ramirez et al., 2009; Rattanapan et al., 2009; Namgung et al., 2012).

The novel configuration of the horizontal-flow biofilm reactor (HFBR) facilitates simultaneous flow of liquid and gas, ensuring good contact between the liquid phase (and therefore the biofilm) in the reactor and the odorous compounds in the gas phase. Traditional biofilters, which use combinations of soil, peat or compost as the bed media are prone to channelling, clogging and pressure loss issues (Nanda et al., 2011), which are exacerbated with time due to degradation of the bed media. HFBR technology has previously been demonstrated to avoid these issues for wastewater treatment (Rodgers et al., 2008; Clifford et al., 2010) and this study investigates whether the technology is suitable for the treatment of contaminated airstreams. The unique design of the HFBR can allow for detailed analysis of the liquid and gas phases, and thus lead to improved performance optimisation and control.

As the microbial community underpins bioreactor performance, an insight into the microbial ecology of bio-oxidation systems is necessary to understand, model and manage the reactions, processes and populations involved. The main metabolic end-products of H$_2$S oxidation in a biofilm reactor are sulphate, elemental sulphur (S$^0$) and polysulfur, and oxidation often results in a reduction of pH in the system (Dumont et al., 2008; Goncalves and Govind, 2008; Namgung et al., 2012).
overall microbial community structure may be related to the cascade of different populations prevailing along an acidity gradient as pH is reduced with the production of H\(^+\) and SO\(_4^{2-}\) ions during sulphide oxidation (Jin et al., 2005; Duan et al., 2006). H\(_2\)S oxidation in bioreactors has been recorded at a pH ranging between 1 (Jing, 2010) and 10 (van den Bosch et al., 2007) with some studies reporting an optimum pH of 6 (Jin et al., 2005), while other studies found maximum oxidation rates as low as pH 3 (Jing, 2010). The pH of the system also impacts on the microbial community that develops (Syed et al., 2006). Buffering of the pH can be provided by dissolution of alkaline materials intrinsic in the bed media, such as calcium carbonate. Where inorganic bed media is used, manual addition of alkalinity in the form of CaCO\(_3\) or NaHCO\(_3\) can be employed to buffer the pH (Oyarzun et al., 2003; Jin et al., 2005).

The study was carried out at 10\(^\circ\)C, which is typical of ambient air and wastewater temperatures in Northern Europe, whereas most H\(_2\)S removal trials to date have been carried out at ambient and mesophilic temperatures. Temperature has been shown to affect H\(_2\)S removal rates in both lab scale and full scale bioreactors, with optimum temperature ranging between 30-40\(^\circ\)C (Yang and Allen, 1994; Naegele et al., 2013). This is unsurprising as most known H\(_2\)S oxidisers have an optimum growth temperature between 28-35\(^\circ\)C (Syed et al., 2006), with the notable exception of *Thermothrix azorensis* who’s optimum temperature is between 76-78\(^\circ\)C (Odintsova et al., 1996). To our knowledge this is the first trial to investigate H\(_2\)S removal in a bioreactor operated at 10\(^\circ\)C for a prolonged period. The advantage of low temperature treatment is the reduction in energy requirements needed to heat the HFBRs, therefore reducing operational costs.

In this study, the performance of HFBRs used to treat H\(_2\)S-contaminated air at various loading rates and concentrations was investigated using gas and liquid phase analyses. The bacterial diversity of the HFBRs was investigated, and the community structure and rates of H\(_2\)S oxidation, in different zones of the HFBRs were compared.
4.3 MATERIALS & METHODS

4.3.1 HFBR set-up and operation

Three HFBR units (R1, R2 and R3) were constructed with each HFBR comprising of a stack of 60 horizontal plastic sheets, with integrated frustums positioned vertically one above the other (Fig. 1). The sheet stacks were bolted together to form sealed enclosures that could be opened for visual assessment and biofilm sampling. The working volume of each HFBR was 20 l and the top plan surface area (TPSA) of the plastic media was 0.04 m$^2$, giving a total media plan area of 2.4 m$^2$. Six sample ports were located along the vertical profile of each HFBR, at sheets 4, 12, 21, 30, 40, 50, and at influent and effluent ports, allowing for intermediate sampling of air and water (‘profile samples’), and allowing seven specific reactor zones (Z1-Z7) to be isolated for detailed analysis; for example, Z1 was located between the influent and first sample port; Z2 between the first and second ports; etc. The HFBR units were housed in a temperature-controlled laboratory and maintained at 10°C. Pure H$_2$S was mixed separately with compressed air to compose desired influent gas concentrations. Mass flow controllers (Bronkhorst High Tech BV), flow meters (Key Instruments) and pressure regulators (DruVa) were used to control gas flow rates and gas mix proportions as required. Flow and loading parameters are given in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PI</th>
<th>PII</th>
<th>PIII</th>
<th>PIV</th>
<th>PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Mixture Flow Rate (m$^3$ m$^{-3}$ h$^{-1}$)</td>
<td>15</td>
<td>30</td>
<td>60</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>H$_2$S Loading Rate (g m$^{-3}$ h$^{-1}$)</td>
<td>3.0</td>
<td>5.6</td>
<td>10.5</td>
<td>15.1</td>
<td>14.5</td>
</tr>
<tr>
<td>Average Influent H$_2$S Concentration (ppm$_v$)</td>
<td>150</td>
<td>118</td>
<td>119</td>
<td>144</td>
<td>109</td>
</tr>
<tr>
<td>Empty Bed Residence Time (s)</td>
<td>265</td>
<td>112</td>
<td>60</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>Trial Duration (days)</td>
<td>35</td>
<td>49</td>
<td>42</td>
<td>38</td>
<td>16</td>
</tr>
</tbody>
</table>
An air mixture containing the H₂S gas was introduced above Sheet 1, at the top of the reactor and flowed horizontally across each sheet before moving to the sheet below. Nutrients were added to the liquid phase of each of the reactors in the form of a Liquid Nutrient Feed (LNF) mixture (Table 2). LNF, which did not contain a source of organic carbon, so that autotrophic growth could be favoured, was pumped intermittently (10 min h⁻¹) onto each HFBR using a peristaltic pump. It was added intermittently in a step-feed manner with 75% of the LNF (4.5 l d⁻¹) pumped onto Sheet 1 and the remaining 25% of the LNF (1.5 l d⁻¹) onto Sheet 30. The step-feed facilitated more even distribution of nutrients through the units. As with the air mixture, the liquid phase flowed over each sheet before dropping to the next sheet below; therefore, the HFBRs did not operate as submerged reactors. The HFBRs
were seeded with activated sludge from a local wastewater treatment plant (Mutton Island WWTP, Galway City, Ireland).

Table 2. Composition of the liquid nutrient feed

<table>
<thead>
<tr>
<th>Component</th>
<th>(g l⁻¹)</th>
<th>Component</th>
<th>(g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃ (Days 1-66)</td>
<td>1.2</td>
<td>MgSO₄·7H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>NaHCO₃ (Days 67-152)</td>
<td>2.5</td>
<td>FeSO₄·7H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>NaHCO₃ (Phase 153-177)</td>
<td>5</td>
<td>Urea</td>
<td>0.03</td>
</tr>
<tr>
<td>CaCl₂·6H₂O</td>
<td>0.003</td>
<td>MnSO₄·H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>0.1</td>
<td>NH₄Cl</td>
<td>0.06</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.025</td>
<td>KHCO₃</td>
<td>0.05</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Sampling and Analytical Methods

The study was divided into five phases (Phases I - V lasting 35, 49, 42, 36 and 15 days, respectively) of varying loading rates and gas air mixture flow rates (Table 1). For the purpose of sampling each HFBR was divided into seven sample zones based on the location of the sample ports (Fig. 2).

Influent, effluent and profile samples of the gas mixtures were taken from each reactor. Profile samples were taken from the six ports located along the vertical profile of each HFBR. H₂S concentrations were measured using a BW Technologies GAXT-H-2-DL GasAlert Extreme Hydrogen Sulphide Detector (0-500 ppm detection range). Liquid phase Total Organic Carbon (TOC), Total Nitrogen (TN) and Total Phosphorous (TP) concentrations were evaluated using a Biotector TOC TN TP Analyser (Biotector, Cork, Ireland). Liquid phase ammonium-nitrogen (NH₄-N), nitrite-nitrogen (NO₂-N), nitrate-nitrogen (NO₃-N), sulphate sulphur (SO₄-S), phosphate-phosphorous (PO₄-P) concentrations and alkalinity were determined using a Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts) and pH was measured using a multiple probe (HI9828, HANNA). The biomass was separately removed from each of the zones.
of the HFBRs on day 180 and transferred to sterile containers at -20°C for downstream molecular analyses. The Total Suspended Solids (TSS) and the Volatile Suspended Solids (VSS) of the biomass from each zone was calculated using Standard Methods (AWWA, 1998).

**Figure 2.** Schematic of the gas, liquid and biofilm sample zones in the HFBRs.

### 4.3.3 DNA Extraction and Quantitative PCR (qPCR)

DNA was extracted from biofilm samples (0.1 g) from each of the zones of each HFBR (i.e. 21 samples) using a Maxwell 16 Tissue DNA Purification Kit and a Maxwell 16 Research Instrument System (Promega). All extracts were visualised on a 1% agarose gel containing SYBR Gold under UV light, and quantified spectrophotometrically using a nano-drop (Thermo Scientific, UK).

Bacterial 16S rRNA genes were quantified by qPCR using a LightCycler 480 instrument (Roche) and the same primers (338f and 805r), probe (TaqMan 516f-ROX), reaction concentrations and cycling conditions described by Yu et al. (2005).
Concentrations of gene copies (GC) were expressed as GC m$^{-2}$TPSA, where the TPSA is the top plan surface area of HFBR sheets.

### 4.3.4 Clonal Library Construction and Gene Sequencing

A composite HFBR DNA sample was prepared by combing an equal volume (2 µl) of each of the 21 DNA extractions. Bacterial 16S rRNA genes were PCR-amplified using the 338f and 805r primers in reaction volumes of 50 µl and with the same reaction, and cycling conditions as described by Yu et al. (2005). The resulting PCR amplicon was purified using Wizard SV Gel and PCR Clean-Up System (Promega), cloned using TOPO TA Cloning Kit and transformed into TOP10 cells as per manufactures instructions (Invitrogen). A total of 192 clones were sequenced by Macrogen (Seoul, South Korea).

Mothur software was used to screen for chimeras and identify unique OTUs (at evolutionary distance of 3%) in the library (Schloss et al., 2009). Reference sequences were downloaded from GenBank database and aligned with representative sequences from the library using ClustalX2 (Larkin et al., 2007). Phylogenetic trees were constructed using Mega5 (Tamura et al., 2011) by neighbour joining analyses with bootstrap method for 1000 replications using Jukes-Cantor model.

### 4.3.5 Temperature Gradient Gel Electrophoresis (TGGE) Fingerprinting

Bacterial 16S rRNA genes from each of the 21 DNA samples were amplified using primers 338f (Yu et al., 2005) and 534r (Muyzer et al., 1993), where the reverse primer included a 40-base pair GC clamp attached at the 5’ end (CGCCCGCCGCCTCCGACCGCCCGCCGACCGCCGACCGCCGACCGCCGACCGCCG), according to the PCR conditions described previously. TGGE fingerprinting of the amplicons was performed using a Maxi System (Biometra, Göttingen, Germany). Denaturing gels composed of 6% acrylamide, 20% deionized formamide, 2% glycerol, 8 M urea with with polymerisation agents TEMED (0.09%, v/v) and APS (0.016%, v/v), were run at 130 V for 16 h in Tris-acetate-EDTA buffer in a temperature gradient of 54-61°C. The temperature gradient used was determined by a previous perpendicular TGGE
Chapter 4

run. PCR banding patterns were visualised by silver staining as described by Bassam et al. (1991). Statistical analyses of the banding patterns, and cluster and non-metric Multi-Dimensional Scaling (MDS) analyses, were done using the Primer6 software (Clarke, 1993).

4.4 RESULTS & DISCUSSION

4.4.1 Effect of retention times and gas loading on HFBR performance

The HFBR performance data from each of the five phases (Phase I – V) are summarised in Figure 3 and Table 3. Maximum H$_2$S removal rates of 15.1 g H$_2$S m$^{-3}$ h$^{-1}$ were achieved in the HFBRs during Phase IV. During Phase I each of the HFBRs R1-R3 experienced an acclimation phase of seven days, during which time the H$_2$S removal efficiency increased from 40% to 100%. Similar acclimation periods, usually lasting 6-30 d have previously been observed (Duan et al., 2006; Dumont et al., 2008) in H$_2$S-oxidising systems and generally occur as the microbial community adapts to operating conditions and the H$_2$S availability. Following the acclimation, H$_2$S removal was averaged over 99% (Fig. 3; Table 3). After the loading rate was increased, an adjustment period of one week was observed at the beginning of Phase II, when average removal efficiencies were lower (86%, 95% and 92% for R1, R2 and R3, respectively; Fig. 3) and less consistent (standard deviations were 14%, 6% and 8% for R1, R2 and R3, respectively). However, H$_2$S removal efficiency was consistently $>98\%$ for the remainder of Phase II. Adaptation periods can be expected following increased H$_2$S loading rates before steady state values are observed (Kim et al., 2008; Moghanloo et al., 2010). The HFBRs adapted to the next increment applied to the flow regime at the beginning of Phase III more rapidly than in Phase II (Fig. 3) and H$_2$S removal efficiencies of 100% were consistently observed during the remaining of Phase III. During Phase IV initial removal rates remained high, however after seven days reduced performance was observed (Fig. 3). Variable performance was then observed in the HFBRs for approximately 20 d. The drop in RE may have been due to the low pH experienced during this period (Fig. 3) or limited by carbon availability, coupled with the high H$_2$S loading rates. When the alkalinity in the HFBR was increased on Day 153 by adding additional NaHCO$_3$ to the LNF, the RE recovered to 100% almost immediately. Although 100% RE was
achieved during Phase II and Phase III under similar pH conditions, the H$_2$S loading rates were lower. The optimum pH for biological H$_2$S oxidation can vary depending on the microbial community present (Syed et al., 2006) and in some cases it has been shown to be at pH values between 3-7 (Chung et al., 1996; Jin et al., 2005; Duan et al., 2006). However some H$_2$S oxidising organisms, such as *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, have optimum growth between pH 1.5-3.5 (Nemati et al., 1998) and pH 2.0-2.5 (Jin et al., 1992). Alternatively, the system may have been limited by inorganic carbon availability. Autotrophic H$_2$S oxidising bacteria require an inorganic carbon source for the oxidation of H$_2$S and the addition of NaCO$_3$ provided an extra carbon load into the HFBRs. The exact mechanism of how the NaCO$_3$ improved the RE of the HFBRs is unclear, however performance did stabilise following adjustment of the alkalinity (Fig. 3) and was above 99.7% for the remainder of the trial. Further detailed investigation and mathematic modelling of H$_2$S oxidation in the HFBRs is required to determine how NaCO$_3$ addition to the LNF impacts oxidation rates.
Figure 3. a: H₂S loading rate (represented by solid line) and removal efficiency, b: alkalinity concentration (represented by solid line) in influent liquid nutrient feed and pH of liquid effluent and c: effluent SO₄ concentration in liquid effluent, of the three HFBRs during each of the experimental phases, I-V. R1, R2 and R3 depicted by an open circle, closed square and closed circle respectively.
### Table 3. Average H$_2$S loading rate, concentration and H$_2$S removal efficiencies over the five experimental periods (PI-PIV). Standard deviations shown in parentheses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P I</th>
<th>P II</th>
<th>PIII</th>
<th>PIV</th>
<th>PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$S Loading Rate (g m$^{-3}$ hr$^{-1}$)</td>
<td>3.0</td>
<td>5.6</td>
<td>10.5</td>
<td>15.1</td>
<td>14.5</td>
</tr>
<tr>
<td>Influent Concentration (ppm$_v$)</td>
<td>150</td>
<td>118</td>
<td>119</td>
<td>144</td>
<td>109</td>
</tr>
<tr>
<td>H$_2$S Removal (%) R1</td>
<td>99.1</td>
<td>97.8</td>
<td>99.6</td>
<td>95.3</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td>(2.4)</td>
<td>(7.3)</td>
<td>(1.9)</td>
<td>(7.0)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>H$_2$S Removal (%) R2</td>
<td>98.8</td>
<td>99.4</td>
<td>99.9</td>
<td>98.1</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>(3.5)</td>
<td>(2.5)</td>
<td>(0.3)</td>
<td>(5.5)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>H$_2$S Removal (%) R3</td>
<td>99.9</td>
<td>98.8</td>
<td>99.8</td>
<td>97.7</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(3.9)</td>
<td>(1.1)</td>
<td>(5.8)</td>
<td>(0.4)</td>
</tr>
</tbody>
</table>

#### 4.4.2 H$_2$S, sulphate and pH depth profile in HFBRs

Depth-resolved gas and liquid phase analyses of the HFBRs showed a close relationship between H$_2$S removal from the gas phase and accumulating SO$_4$ in the liquid phase, accompanied by a reducing pH (Fig. 4). During Phases I and II, most H$_2$S removal occurred by Sheet 30 (Fig. 4). Increased H$_2$S loading rates during Phases III-V resulted in higher concentrations of H$_2$S at lower sheets (sheets 30-60). Increased exposure of biofilm in the lower zones of the HFBR to H$_2$S resulted in the development of H$_2$S oxidising activity. The H$_2$S removal rate in specific zones in the HFBRs, which was based on the total TPSA of all of the sheets in respective zone, was up to 0.21 g [H$_2$S] m$^2$ [sheet area] h$^{-1}$, depending on loading rate and location in the reactor. The top zones in the HFBRs showed highest H$_2$S oxidation rates throughout the trial although in the latter phases of the trial the activity of the lower zones increased (Fig. 5) and contributed to overall H$_2$S oxidation in the HFBRs. In a previous biotrickling filter study by Dhussa et al. (2012) it was shown that at high concentrations of H$_2$S, the diffusional transfer rate of H$_2$S from gas to biofilm was high, resulting in high biological removal rates; concomitantly at lower gaseous H$_2$S concentrations the diffusional transfer and biological removal rates were lower. Similarly, in this study, highest H$_2$S removal rates were observed in locations with highest concentrations of H$_2$S.
Concentrations of sulphate in the liquid phase increased with depth in each of the HFBRs in each of the five phases (Fig. 4) and accumulation in the HFBRs coincided with the oxidation of H₂S. Higher concentrations of sulphate were produced in the latter phases with increased H₂S loading rates. In most cases a reduction in sulphate production was observed after Sheet 30, due to lower rates of H₂S oxidation. During Phases IV and V, sulphate concentrations were in the range of 2 – 3 g [SO₄²⁻] l⁻¹. The accumulation of sulphate ions can also impair performance directly by inhibiting sulphide oxidisers in the biofilm (Jin et al., 2005) and this has previously been observed to occur in the range of 2-15 g [SO₄²⁻] l⁻¹, depending on loading regime, bioreactor configuration and operational pH (Jin et al., 2005; Sercu et al., 2005; Ramirez et al., 2009). Long term inhibition was not apparent in R1-R3 as the performance recovered during Phase IV when pH was increased by buffering with NaHCO₃, despite high concentrations of sulphate in the liquid phase. This may also be related to the constant flow of fresh LNF through the reactor (6 l d⁻¹) which limited the accumulation of sulphate ions from accumulating in the reactor. Periodic media washing can also be employed to flush toxic sulphur compounds from the liquid phase (Duan et al., 2006). Sulphate in the liquid effluent can then be precipitated out using CaCO₃ or CaO to form CaSO₄, to be reused for reclamation of, for example alkali soils, rather than being recirculated and eventually converted back to H₂S in a wastewater treatment plant (Jin et al., 2005).
Figure 4. a: Average gas phase concentrations of H$_2$S in R1 during Phases I-V at each of the seven depth-resolved sampling ports. b: Average pH of liquid phase in R1 during Phases I-V in the liquid nutrient feed and at each of the seven depth-resolved sampling ports. c: Average liquid phase concentrations of SO$_4^{2-}$ in R1 during Phases I-V in the liquid nutrient feed and at each of the seven depth-resolved sampling ports. For clarity only data from R1, which was typical of all 3 HFBR units, is shown.
4.4.3 H$_2$S conversion to sulphate

The conversion ratio of H$_2$S to sulphate was calculated based on influent and effluent measurements of H$_2$S and sulphate (Fig. 6). Measurements were recorded several times weekly over the duration of the trial. H$_2$S removal correlated with effluent sulphate (correlation coefficient, 0.92), but there was a large proportion of unaccounted sulphur representing an average of 0.89 g [S] d$^{-1}$ (33% of influent sulphur). The disparity was likely due to the production of sulphur forms other than sulphate, such as elemental sulphur and sulphide, which were not measured in this study. There were visible elemental sulphur deposits on the plastic sheet media in the HFBRs on the last day of the trial (Fig. 7).

**Figure 5.** Average *in situ* H$_2$S oxidation rates of the seven oxidation zones (zones 1-7) from across the three HFBRs during Phases I-V.
Figure 6. Sulphur mass balance of three HFBRs over duration of trial based on the influent and effluent measurements of H$_2$S and sulphate.

The conversion of H$_2$S to sulphate in this trial (67%) was similar to the rates (60%) reported by Jin et al. (2005), while Kim et al. (2008) reported conversion rates of 38%. Other studies have reported much higher conversion rates (100%) dependent on the pH and oxygen availability (Naegele et al., 2013). As the H$_2$S loading rate was increased during the trial and the H$_2$S was removed from the gas phase, the effluent sulphate concentration also increased (Fig. 1). This was evident at all loading rate increases, with the exception of Phase II when effluent sulphate concentrations were similar to those measured during Phase I. This may have been due to the conversion of the H$_2$S to products other than sulphate. Sulphate is considered a desirable end-product with respect to environmental sustainability as it can be easily precipitated from the liquid stream, while the excessive production of alternative end-products, such as elemental sulphur, can lead to problems including reactor clogging (Rodriguez et al., 2014).
Figure 7. Visible elemental sulphur deposits on the plastic sheet media from HFBRs on the last day of the trial.

4.4.4 Bacterial community composition of the HFBRs

Total clone library coverage was 83%, while overall bacterial diversity was low. The library was dominated by two clones from the class Gammaproteobacteria, which were related to the Acidithiobacillus and Thiobacillus, and which constituted 34% and 23% of the library, respectively (Fig. 8). Acidithiobacillus is associated with very acidic environments and has been detected in hydrogen sulphide-rich caves where the organism grows in biofilms at pH 0-1 (Jones et al., 2011). Acidithiobacillus spp. are well known H$_2$S oxidisers and pure cultures have been used to inoculate H$_2$S-removing bioreactors (Sercu et al., 2005; Aroca et al., 2007; Charnnok et al., 2013). In other systems, where mixed microbial consortia were used as seed biomass, Acidithiobacillus has been identified as the predominant species, including in an acidic biotrickling filter (Li et al., 2012) and an acidic biofilter (Charnnok et al., 2013). Thiobacillus spp. have also been identified as important H$_2$S-oxidisers in biological systems and some species, including Thiobacillus denitrificans (Ma et al., 2006) and Thiobacillus thioparus (Ramirez et al., 2009), have been used as immobilised cultures for H$_2$S removal. Critically, both Acidithiobacillus and
Figure 8. Neighbour-joining phylogenetic tree of the 16SrRNA gene sequences from the bacterial clone library. Reference sequences were downloaded from GenBank and their accession numbers are given. The numbers at the branch nodes are bootstrap values derived from 1,000 replicates. The open star represents the sequence SGH2S27 and the solid star represents sequence SGH2S14 which make up 23% and 34% of the clone library respectively.
*Thiobacillus* are capable of H$_2$S oxidation under low pH conditions as found in the HFBRs, and they appeared to be important members of the bacterial community. The bacterial community in the HFBRs was assessed by generating a clone library from composite DNA sample to identify the main species present across the three reactors while TGGE fingerprinting was used to compare the community structure between HFBRs and zones (section 4.4.6).

### 4.4.5 Biomass distribution and bacterial gene copy concentrations in HFBR

Biomass concentrations varied between zones in the HFBRs with zones 5-7 having the lowest density of biofilm in all three HFBRs (Fig. 9). Bacterial gene copy (GC) concentrations (GC m$^{-2}_{TPSA}$) were highest in the top three zones of each of the HFBRs (Fig. 9). Z4 (sheets 21-30) had the lowest density of bacterial gene copies of any zone across the three HFBRs. After the step-feed (sheet 30), the gene copy concentrations (GCC) in Z5-Z7 increased, although the levels detected were always lower than in zones Z1-Z3. The addition of LNF to the HFBRs at the step-feed may have provided additional nutrients, which had already been consumed in the upper zones of the HFBRs, or it may have diluted inhibitory compounds present in the liquid stream, thus stimulating bacterial growth. The increase in bacterial gene copies indicates the benefit of providing a step-feed in the HFBRs. Based on Pearson’s coefficient there was no correlation between bacterial 16S rRNA gene copies and weight of biomass (p=0.38), however there was a stronger correlation between 16S rRNA gene copies and DNA concentration (p=0.80). The lack of correlation between gene copies and biofilm mass may be due to the presence of dead cells, or extra cellular polymeric substances (EPS) which can accumulate in H$_2$S oxidising biofilms (Duan *et al*., 2005). Although the correlation between bacterial gene copies and DNA concentration is stronger, the number of 16S gene copies in a bacterial cell can vary between 1-15, (Kembel *et al*., 2012), indicating that biomass weight is not an accurate proxy for bacterial gene copies.
4.4.6 Statistical comparison of environmental variables and TGGE profiles of the HFBRs

All zones (Z1-Z7) within a given HFBR were compared to determine the similarity of the bacterial communities and the effect of the environmental variables on community structure. Comparison of the environmental variables measured from the gas and liquid phases in the three HFBRs using permutational MANOVA (Euclidean distance) showed that, although the three HFBRs were operated in triplicate, the environmental conditions in the HFBRs were significantly different ($p=0.004$). However, a pair-wise test of the individual HFBRs showed that R2 and R3 were not significantly different ($p=0.42$), while R1 was different to R2 ($p=0.01$) and R3 ($p=0.016$). Variables measured included NO$_2^-$, NO$_3^-$, NH$_4^+$, P, organic carbon, inorganic carbon, SO$_4^{2-}$, H$_2$S concentrations and alkalinity and pH.

Based on the presence/absence of bands from the TGGE fingerprinting and using permutational MANOVA (Bray-Curtis distance), the overall bacterial communities in the three HFBRs were observed not to be significantly different ($p=0.247$). Interestingly, even though the three HFBRs experienced different environmental conditions the same overall bacterial community was present in all three. However there was some differences in the bacterial community structures observed with

Figure 9. Quantification of (a) real-time PCR of bacterial 16S rRNA gene copies, and (b) biomass, from different zones in R1, R2 and R3 on the last day of the trial.
depth in the HFBRs (Fig. 10), indicating that spatial orientation in the reactors impacted on community assembly and development. Maestre et al. (2010) also observed variation in community composition with depth in a biotrickling filter treating $\text{H}_2\text{S}$. The bacterial community structure showed greater than 55% similarity between zones, with some zones showing 100% similarity. The bacterial communities in the HFBRs were influenced by the environmental conditions in the reactors (Fig. 10). In each HFBR, Z1-3 showed highest similarity to each other and community structure was impacted by alkalinity and pH of the LNF. Similarly, the bacterial communities of Z4-Z7 shared similarity but were mainly impacted by sulphate and TOC. Other environmental variables had an impact on the bacterial community structure although none impacted across all three HFBRs. The high level of similarity between the three HFBRs may have been due to the extreme conditions, such as very low pH, high sulphate concentrations and limited organic carbon. These conditions provided an inhospitable environment that required a specialised consortium of bacteria resulting in a constricted community with limited diversity and dominated by *Acidithiobacillus* and *Thiobacillus* spp.
Figure 10. Non-metric multidimensional scaling (MDS) plots of bacterial community structure showing correlation with environmental variables in a: R1, b: R2, c: R3 and d: in the three HFBRs. Bacterial community structure based on presence/absence of bands from TGGE fingerprinting. Direction and length of arrows visualise the correlations of the respective environmental variables. Only environmental variables with correlation of >0.5 are shown.
Chapter 4

4.5 CONCLUSIONS

The HFBRs are a suitable technology for the treatment of H\textsubscript{2}S-contaminated airstreams at 10°C, with removal rates of up to 15.1 g [H\textsubscript{2}S] m\textsuperscript{-3} h\textsuperscript{-1} and removal efficiencies of 99% achieved. Bio-oxidation of H\textsubscript{2}S in the reactors led to acidification of the liquid phase and the production of a sulphate (SO\textsubscript{4}\textsuperscript{2-}) rich liquid effluent. Buffering the HFBRs with NaHCO\textsubscript{3} resulted in improved H\textsubscript{2}S removal at loading rates of 15.1 g [H\textsubscript{2}S] m\textsuperscript{-3} h\textsuperscript{-1}. Bacterial diversity was low and the HFBRs were dominated by *Acidithiobacillus* and *Thiobacillus*. Differences in the community structure between HFBR zones were influenced by alkalinity, pH and SO\textsubscript{4} concentrations.

4.6 REFERENCES


Jiang X, Yan R, Tay JH. 2009. Simultaneous autotrophic biodegradation of \( \text{H}_2\text{S} \) and \( \text{NH}_3 \) in a biotrickling filter. Chemosphere 75(10):1350-1355.


Chapter 4


The optimization of Horizontal Flow Biofilm Reactors (HFBRs) used to treat methane contaminated airstreams at 10 °C and the microbial communities underpinning the process.
Chapter 5

This work described in this chapter was collaboration between the Microbiology and Civil Engineering departments in the National University of Ireland Galway. The HFBRs were operated by the Civil Engineering research group but all bioprocess data and molecular work was analysed as part of this PhD programme.
5.1 ABSTRACT

Methane (CH₄) is an important greenhouse gas that is commonly encountered at various waste-treatment facilities when organic matter is broken down anaerobically. Three novel, laboratory-scale, Horizontal-Flow Biofilm Reactors (HFBRs) treating methane contaminated airstreams at low concentrations (1.12%) were operated for 233 days at 10°C. Removal rates of up to 7.1 g CH₄ m⁻³ h⁻¹ were achieved during the trial, demonstrating that HFBRs are a suitable technology for the treatment of methane contaminated airstreams at low temperatures. Methane removal rates were influenced by temperature, with reduced removal rates observed during a cold period when temperature fluctuated between 1-10°C (Q₁₀ 2.49 ±0.45). The composition of the liquid nutrient feed (LNF) applied to the HFBRs had an effect on methane removal rates. Increased removal was observed when organic carbon was omitted from the LNF, while the impact of nitrogen source and concentration was unclear. The bacterial community in the HFBRs was investigated using Terminal Restriction Fragment Length Polymorphism (TRFLP) fingerprinting of the 16S rRNA genes and Fluorescent in-situ Hybridisation (FISH) of the methanotrophic 16S rRNA genes. The bacterial communities in the HFBRs were very diverse and varied with depth in the reactors and over time. Type II methanotrophs appeared to be more abundant in the HFBR biofilm than Type I methanotrophs, although the microbial community was dominated by other prokaryotes. Results from this study showed that HFBRs were capable of treating methane contaminated airstreams and that the process was facilitated by a diverse and dynamic bacterial population.
5.2 INTRODUCTION

Methane (CH\textsubscript{4}) is an important greenhouse gas which is produced when organic matter is degraded by microorganisms under anaerobic conditions. As a greenhouse gas it is up to 23 times more potent than CO\textsubscript{2} on a molar basis (IPCC, 2007). It represents about 15% of total annual greenhouse gas emissions and makes up 23% of total anthropogenic greenhouse gas emissions (USEPA, 2006). Anthropogenic sources of methane include municipal solid wastes, landfills, rice paddies, coal mining, oil and gas drilling and processing, cattle ranching, manure management, agricultural products, wastewater treatment plants and rising main sewers (Yusuf et al., 2012). In the past three hundred years atmospheric methane concentrations have been steadily increasing (Chistoserdova et al., 2005) and it is predicted that methane emissions will grow by 32% and 41% by the years 2015 and 2020 respectively (USEPA, 2006). In the EU, legislation dealing with the generation and treatment of odours and gases (EC, 2005), is becoming increasingly stringent and under the Kyoto Protocol many countries are required to reduce greenhouse emissions by 2020. As such, treating methane emission before they are released into the environment is becoming increasingly important. Traditional technologies used to treat foul airstreams have been physical and chemical, however the growing popularity of biological treatment can be attributed to the ability of microorganisms to destroy pollutants rather than just transferring them from the gas to liquid phase (Burgess et al., 2001). Various biological technologies have previously been used for the treatment of methane contaminated airstreams including; biofilters (Haubrichs and Widmann, 2006; Josiane and Michèle, 2009; Park et al., 2009; Avalos Ramirez et al., 2012a), biotrickling filters (Avalos Ramirez et al., 2012b; Estrada et al., 2014) and membrane bioreactors (Mudliar et al., 2010). Horizontal Flow Biofilm Reactors (HFBRs) are a relatively new, biotrickling filter reactor design, that have successfully been used to treat domestic-strength wastewater (Rodgers and Clifford, 2009) and dairy wastewater (Rodgers et al., 2007). One of the advantages of the HFBR design is that the unique flow regime employed has been shown, in wastewater treatment trials, to avoid problems such as channelling, clogging and pressure loss, (Clifford et al., 2008; Rodgers et al., 2008; Clifford et al., 2010). These issues often also impact negatively on traditional biofilter and biotrickling filters used to treat contaminated airstreams. Most other biological methane
elimination trials to date have been carried out at ambient or mesophilic temperatures, but in this trial three HFBRs were operated at 10°C, which is typical of temperate climates found in Ireland and Northern Europe. Low temperature operation removes the need to actively heat the systems, therefore reducing energy inputs and lowering operational and capital investment costs.

Biological oxidation of methane is mainly performed by methanotrophs, a group of aerobic bacteria, which are found in a wide variety of environments including soils, sediments, and freshwater and marine columns (McDonald et al., 2008; Jiang et al., 2010). There are three major phylogenetic methanotroph groups, Type I, Type II and Type X, along with a small number of other filamentous and acidophilic bacteria capable of methane oxidation (McDonald et al., 2008). Methanotrophs contain two key enzymes that are required for methane oxidation; methane monooxygenase (MMO) and methanol dehydrogenase (MDH) (Henckel et al., 1999). MMO is unique to methanotrophs and catalyses the conversion of methane to methanol, which is the first step of methane oxidation. MMO occurs in two forms, particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO). The pMMO enzyme is found in all known methanotrophs with the exception of Methylocella palustris (Dedysh et al., 2000), while the sMMO enzyme is found in some but not all methanotrophs (Theisen and Murrell, 2005). Due to their ability to oxidise methane, methanotrophs have been identified as important industrial microorganisms, as they have significant potential for use in engineered systems to mitigate methane greenhouse effects and for the bioremediation of pollutants (Jiang et al., 2010). Methanotroph community structure and methane oxidation rates are influenced by several factors including temperature (Börjesson et al., 2004), nitrogen source and concentration (Bodelier and Laanbroek, 2004), substrate availability (Henckel et al., 2000), moisture levels (Einola et al., 2007) and O₂ concentration (Teh et al., 2005), making them key considerations when designing and operating bioreactors to treat methane contaminated airstreams.

The aims of this study were, (i) to evaluate the potential of HFBRs to treat methane contaminated airstreams at 10°C, (ii) measure in-situ methane removal rates in the HFBRs, (iii) determine the maximum methane oxidation potential of the biofilm, (iv) to investigate the impact of temperature, organic carbon and nitrogen source on methane removal rates, and (v) to investigate the bacterial community present in the
HFBR biofilms using Terminal Restriction Fragment Polymorphism (TRFLP) fingerprinting and Fluorescent in-situ Hybridization (FISH). We tested the hypotheses that HFBRs are capable of methane removal at 10°C, operation conditions affect methane removal rates and that biofilm activity varied between locations in the HFBRs. We also tested the hypotheses that microbial community structure varied with location in the HFBRs, that operational parameters in the reactors would affect the microbial population and that methanotrophs were present in the HFBRs.

5.3 MATERIALS AND METHODS

5.3.1 HFBR design and set-up

Three pilot-scale HFBR units (R1, R2 and R3), each comprising of 52 horizontal plastic sheets stacked one above the other, enclosed in airtight, sealed housings (Fig. 1), were constructed. Each sheet measured 0.2 m x 0.2 m in plan and had integrated frustums 8 mm high and 8 mm diameter at 15 mm centres. The working volume of each reactor was 20 l with a total top plan surface area (TPSA) of 2.08 m$^2$. A methane contaminated airstream was introduced at the top of each HFBR, just above Sheet 1, and flowed horizontally and vertically downwards in a boustrophedonic pattern. A Liquid Nutrient Feed (LNF) was applied to the HFBR and flowed in a co-current direction with the gas stream. The LNF (8 l d$^{-1}$) was delivered intermittently (10 min h$^{-1}$) via a peristaltic pump in a step feed manner, i.e. 75% of the LNF (6 l d$^{-1}$) was applied onto Sheet 1 and 25% of the LNF (2 l d$^{-1}$) onto Sheet 27. The composition of the LNF is given in Table 1. Six sampling ports located vertically along the side of each reactor permitted intermediate gas and liquid sampling (Fig. 2). The HFBRs were each divided into 7 distinct sampling regions for gas and water analyses (Fig. 2), such that removal ‘profiles’ could be established.
Chapter 5

5.3.2 Operating Conditions in the HFBRs

The three HRBRs were operated for 233 days and the trial was divided into two distinct phases, Phase I and Phase II. During Phase I (Day 1-171), the HFBRs were operated in triplicate under the same conditions. During Phase II (Day 172-233) the LNF applied to the three HFBRs was altered and the HFBRs were operated independently. The average CH₄ loading rate for each HFBR was 9.6 g CH₄ m⁻³ h⁻¹ and the average influent CH₄ concentration was 1.12%. The gas loading rate for each HFBR was 1.26 m⁻³ m⁻³ h⁻¹. An intermittent supply of LNF was pumped into the reactors for 10 min h⁻¹. During PI the same LNF (Odegaard and Rusten, 1980) was added to the three HFBRs. During PII the LNF supplied to the HFBRs was altered.

Figure 1. Schematic of HFBR set-up.
(Table 1) to investigate the effect of organic carbon, nitrate (NaNO\textsubscript{3}) and ammonia (NH\textsubscript{4}Cl) on reactor performance.

The HFBRs were operated at 10°C for the duration of the trial, except during a 17 d Cold Perturbation (CP) period, between days 90-107, when air temperature in the laboratory fluctuated between 1°C and 9°C. The HFBRs were also subjected to a 14-d shutdown (SD) period between day 120-133, when the CH\textsubscript{4} supply was turned off. During the SD period, the LNF supply was kept on to hydrate the biofilm.

Table 1. LNF composition

<table>
<thead>
<tr>
<th>Component</th>
<th>LNF (All HFBRs)</th>
<th>LNF I (R1)</th>
<th>LNF II (R2)</th>
<th>LNF III (R3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO\textsubscript{3}</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>MgSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>FeSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Urea</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>MnSO\textsubscript{4}.H\textsubscript{2}O</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>CaCl\textsubscript{2}.6H\textsubscript{2}O</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Na\textsubscript{2}NPO\textsubscript{4}.12H\textsubscript{2}O</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>KHCO\textsubscript{3}</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2</td>
<td>nil</td>
<td>0.2</td>
<td>nil</td>
</tr>
<tr>
<td>Dried milk</td>
<td>0.12</td>
<td>nil</td>
<td>0.12</td>
<td>nil</td>
</tr>
<tr>
<td>NH\textsubscript{4}Cl</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>nil</td>
</tr>
<tr>
<td>NaNO\textsubscript{3}</td>
<td>Nil</td>
<td>nil</td>
<td>1.52</td>
<td>1.52</td>
</tr>
</tbody>
</table>
5.3.3 Seeding and Enrichment Cultures

The HFBRs were initially seeded with activated sludge sourced from a full-scale aerobic wastewater treatment plant. After one month of operation, no methane oxidation was detected in the HFBRs and an enrichment strategy was employed to cultivate a methane oxidising inoculum to be used to re-seed the HFBRs. A composite ‘landfill mix’ was prepared by adding equal volumes of landfill cover soil, landfill leachate, composted organic matter and compost leachate. Several enrichment cultures were prepared by adding 2 ml of the landfill mix into 40-ml glass vials with 10 ml Adapted Whittenbury Medium (AWM) (Whittenbury et al., 1970). The composition of the AWM is outlined in Table 2. The landfill fluid used in the AWM was prepared by adding 250 g landfill soil, 250 ml landfill leachate, 250 ml compost leachate, 500 ml H₂O and 0.5 g yeast extract, and incubating at 37°C on a shaker (120 rpm) for 48h. The mix was allowed to settle for 2 h and the resulting supernatant was collected, autoclaved and stored at 4°C. The vials were sealed with butyl bungs and the headspace was adjusted to a methane concentration of 10% (v/v) at atmospheric pressure. Vials were incubated in the dark at 10°C on a shaker at 80 rpm. The methane concentration in the headspace was monitored twice weekly by gas chromatography (GC; Varian CP-3800 Gas Chromatograph). When the methane concentration was <0.5%, the headspace was flushed with air and a 10% methane headspace was re-instated. Over the course of 4 months, the enrichments were sub-cultured (c. 10% inoculum) five times into fresh AWM and eventually scaled to 2-l cultures. The enriched culture was added to the HFBRs (Day 0), along with the LNF, and re-circulated around the reactors for several days to promote biofilm formation and to bio-augment the biofilm already present, before full LNF feeding was resumed. Biomass samples from the landfill mix, from each of the 5 enrichment sub-culturing stages and from the seed biomass used to reseed the HFBRs, were stored at -80°C for DNA extraction.
Table 2. Composition of Adapted Whittenbury Media (AWA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$\text{4}.7\text{H}_2\text{O}$</td>
<td>1.0 g l$^{-1}$</td>
</tr>
<tr>
<td>KNO$_1$</td>
<td>1.0 g l$^{-1}$</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.272 g l$^{-1}$</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.2842 g l$^{-1}$</td>
</tr>
<tr>
<td>CaCl$_2.2\text{H}_2\text{O}$</td>
<td>0.1342 g l$^{-1}$</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>0.00038 g l$^{-1}$</td>
</tr>
<tr>
<td>Landfill fluid</td>
<td>50 ml l$^{-1}$</td>
</tr>
<tr>
<td>Trace Elements*</td>
<td>0.5 ml l$^{-1}$</td>
</tr>
</tbody>
</table>

* Trace elements solution as described by Shelton and Tiedje (1984)

5.3.4 Gas Analyses

HFBR performance was monitored over the course of the trial based on CH$_4$ removal efficiency (RE, %) and the total mass of CH$_4$ removed (g m$^{-3}$ d$^{-1}$). In-situ HFBR methane removal rates were determined for each sample zone (Fig. 2) by measuring CH$_4$ concentrations at the gas sampling ports located vertically along the reactors. The CH$_4$ removal rate in a given zone was calculated by measuring the mass of CH$_4$ removed between two neighbouring sample ports, and expressing removal over the TPSA in the zone (g CH$_4$ m$^2$ [TPSA] d$^{-1}$).

The impact of the change in LNF composition, on RE during PII was assessed in two ways, (i) the immediate impact was examined by comparing RE of the HFBRs during the last 10 days of ES2 and the first 10 days of PII, and (ii) the long term impact of the LNF was assessed by comparing RE of the HFBRs over the entire duration of ES2 and PII.

All gas samples were taken using a gas-tight syringe and analysed using a Varian CP-3800 Gas Chromatograph (GC) equipped with a flame ionized detector (FID).
5.3.5 Water Analyses

Depth resolved liquid phase analyses of the influent, effluent and the liquid sampled from the intermediate sampling ports was performed on days 28, 48, 64, 90, 151, 224 and 231. Suspended solids (SS), chemical oxygen demand (COD) and total nitrogen (TN) were measured in accordance with standard methods (AWWA, 1998), and ammonium-nitrogen (NH$_4$-N), nitrite-nitrogen (NO$_2$-N), nitrate-nitrogen (NO$_3$-N) and phosphate-phosphorous (PO$_4$-P) concentrations were determined using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts).

![Figure 2](image)

**Figure 2.** Schematic of sampling zones in the HFBRs for (a) the gas and liquid sampling, and (b) the biofilm sample zones.

5.3.6 Biofilm sampling

The HFBR biofilms were sampled six times during the trial: day 0 (immediately prior to HFBR seeding with the enriched methane oxidising biomass), 44, 84, 108, 177 and 233. For sampling purposes, each reactor was notionally divided into six distinct zones (Fig. 2). Sheets from each zone were briefly removed from the HFBRs
and biofilm (c. 4 g) was collected, flash-frozen in liquid nitrogen and stored at -80°C for downstream analyses. A total of 108 biofilm samples were taken from the HFBRs over the course of the trial. Biomass samples from the landfill mix and enrichments were also stored at -80°C, giving a total of 114 samples.

5.3.7 Methane oxidation potential (MOP) biofilm batch assays

The maximum methane oxidation potential (MOP) of biofilm from the six zones in the three HFBRs was calculated on Day 177. The concentration of volatile suspended solids (VSS) of each biofilm sample was calculated, based on standard methods (AWWA, 1998). MOP assays were prepared in triplicate, by adding 5 mg VSS of biofilm to 10 ml AWM in 40-ml hypo-vials. The vials were sealed and the headspace was adjusted to 10% methane concentration at atmospheric pressure. The vials were incubated at 10°C in the dark, on a shaker at 80 rpm. Negative controls containing AWM with no biofilm were also set-up. Headspace methane concentrations in the vials were measured several times daily using GC analyses until <0.5% CH₄ remained.

5.3.8 DNA extraction of landfill mix biomass and HFBR biofilm

Biomass samples were defrosted at room temperature and centrifuged at 4,000 x g for 2 min to remove excess liquid. Biomass from the landfill mix was frozen with liquid nitrogen and crushed to a fine powder using a mortar and pestle, until a fine powder was formed. DNA was extracted from 0.25 g of the resulting powder, using a PowerSoil DNA isolation kit (MoBio, Carlsbad, CA USA). DNA was extracted from all HFBR and enrichment culture biomass samples, using Magtration System GC Plus fully automated DNA/RNA Magtration Extraction and Purification System (PSS, Matsudo, Japan) and a Magtration Genomic DNA Whole Blood Kit 100 (VhBio). Prior to extraction 20 mg biomass was suspended in 80 µl DEPC H₂O and the samples were then homogenised by passing the mixtures through a pipette tip several times and vortexing for 2 min.
All DNA extractions were visualised on 1% (w/v) agarose gels containing SYBR Gold (0.01% v/v) under UV light, and quantified spectrophotometrically using a nano-drop (Thermo Scientific, UK).

5.3.9 Bacterial clone library of landfill seed mix

A bacterial clone library of the landfill seed mix (used for the enrichment culture) was constructed using universal bacterial primers, 27F and 1392R (Lane et al., 1985). An aliquot of 2 µl of the DNA template was added to a PCR mixture of 5 µl PCR buffer, 3 µl MgCl₂ (25 mM), 1 µl dNTPs (10 µM), 1 µl 27F, 1 µl 1392R and 0.5 µl BioTag polymerase (Bioline, Ohio, US). The PCR cycling conditions were: 2 min at 94°C, and 30 cycles consisting of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, followed by 10 min at 72°C. The resulting PCR amplicon was purified in a crystal violet gel using a S.N.A.P. UV-Free Gel Purification Kit (Invitrogen, CA, USA).

The purified PCR product was transformed into a TOPO TA vector and cloned into TOPO-10 chemically competent E. coli cells (Invitrogen, CA, USA). The resulting cloning mixture was spread on LB and kanamycin (50 µg ml⁻¹) plates and grown overnight at 37°C as described in Chapter 3. One-hundred-and-ninety-two clones were grown overnight in 96-well plates containing 200 µl LB broth and kanamycin (50 µg ml⁻¹), at 37°C. To ensure that transformation had been successful the clones were screened by PCR-amplification of the M13 region of the plasmid, using primers M13F (5’-GTTTTCCCAGTCACGAC-3’) and M13R (5’-CAGGAAACAGCTATGAC-3’). Following amplification, M13-PCR products were visualised on agarose gels and clones containing the insert were selected for analyses.

The M13-PCR amplicons were digested with Hae III restriction enzyme and profiled using Amplified Ribosomal DNA Restriction Analysis (ARDRA). Unique operational taxonomic units (OTUs), based on restriction digest banding patterns, were identified, and representative OTUs were selected for sequencing. Plasmids were extracted from the representative clones using an Isolate II Plasmid Mini Kit.
(Bioline) and were sequenced by Eurofins (Ebersberg, Germany). All sequences were deposited in GenBank under the accession numbers KM269199-KM269285.

5.3.10 TRFLP fingerprinting of bacterial community in the HFBRs

PCR amplification of universal bacterial 16S rRNA gene was performed on all DNA extracts using primers 27F (labeled with FAM 6-carboxyfluorescein) and HEX-labeled 1392R (Lane et al., 1985) as described earlier.

The virtual TRFLP restriction profiles of the 16S rRNA clonal sequences from the clone library were determined, and compared in-silico using six different enzymes; Alu I, Hae III, Hhal, Mbo I, Mse I, Msp I using NEBcutter software. Based on the restriction patterns, Alu I was selected for in-vitro TRFLP investigation. PCR products were digested with Alu I restriction enzyme (Fermantas) according to the manufacturer’s instructions. The resulting digested product was precipitated overnight at -20°C in 1/10 volume 3M Na-acetate and 3x volume 100% ethanol. The sample was then centrifuged at 14,000 g for 30 min. The resulting pellet was washed with ice cold ethanol (100%) and centrifuged at 14,000 g for 30 min. This was repeated three times, before the pellet was air dried in the dark.

The restriction digests were analysed by Gene Analyses Service (Berlin, Germany) using an ABI 310 capillary sequencer with a GS2500 TAMRA-labelled internal lane standard.

TRFLP profiles were aligned using T-align programme (Smith et al., 2005) and statistical analyses was performed using Primer6 (Clarke and Warwick, 2001) and PERMANOVA+ software (Anderson, 2005). Permutation multivariate analysis of variance (PERMANOVA) and pair-wise testing on a Bray–Curtis resemblance matrix of square root transformed T-RFLP abundance data, was performed. Similarity percentage (SIMPER) analysis was used to investigate the differences in bacterial community composition, based on the relative abundance of OTUs present, between samples within each HFBR. Species comprising the top 90% most abundant species within the community were analysed. The OTUs that had the most influence on bacterial community similarity were identified using SIMPEP.
5.3.11 Fluorescence in-situ Hybridization (FISH) of HFBR biofilm

On day 177, biofilm samples from various locations in the HFBR were analysed using FISH. Biofilm samples were carefully removed from the HFBRs and incubated in 4% paraformaldehyde (w/v in 1X PBS) at 4°C overnight. The paraformaldehyde was discarded and the biofilm was washed in 1X PBS three times. The samples were stored in ethanol:1X PBS (1:1) at -20°C until required. When required, the samples were removed from -20°C storage and washed in ddH₂O to remove the ethanol/PBS solution. The samples were then immersed in Tissue-Tek OCT freezing medium and incubated at -20°C overnight. Ten-micrometre-thick, vertical cross-sections of the biofilm were cut using a cryomicrotome (Bright 5030 microtome model P143) at -20°C. The biofilm sections were transferred to gelatine-coated, Amann-type slides. Biofilm sections were hybridized using CY5-labelled Type I methanotroph probes; MG84 (5'-CCACTCGTCAGCGCCCGA) and MG705 (5'-CTGGTGTTCCCTTCAGATC), and Cy3-labelled Type II methanotroph probe; MA450 (5'-ATCCAGGTACCGTCATTATC) as described by Eller et al. (2001). The sections were also stained with DAPI as described by (Schramm et al., 1998). Hybridizations were visualised using an epi-fluorescent microscope (Nikon Eclipse E600, Japan).

5.4 RESULTS

5.4.1 Phase I (PI) Performance (Day 0-171)

5.4.1.1 CH₄ Removal Efficiency (RE) of HFBRs

The start-up period at the beginning of PI lasted 44 days (Fig. 3, Table 2), at which point the HFBRs reached Efficient State 1 (ES1). During ES1 (Day 45-89), the average RE across the three HFBRs was 33.1 ±3.0% (198.2 ±18.0 mmol CH₄ m⁻³ h⁻¹). During ES1 removal in the HFBRs was relatively stable and no major fluctuations occurred. Following ES1 removal in the HFBRs was relatively stable and no major fluctuations occurred. Following ES1 a cold perturbation (CP) was applied to the HFBRs, where the air temperature varied between 1-10°C. During the CP period, there was an average drop in reactor performance of 53%, with methane RE in the three HFBRs of 11.7 ±2.5% (108.6 ±15.2 mmol CH₄ m⁻³ h⁻¹). A Q₁₀-value of 2.49 ±0.45 (1-10°C) was observed in HFBRs during this period (the Q₁₀ temperature coefficient is the
measured rate of change in a biological or chemical system resulting from an increase in temperature of 10°C). On day 108, the operating temperature in the HFBRs was returned to 10°C, which resulted in an immediate increase in RE. A recovery period (RP1) of 13 days was observed, where the RE of the HFBRs was continually improving. On day 121 the CH₄ supply to the HFBRs was turned off for 13 days during the shutdown (SD) period. On day 134, when the CH₄ was reintroduced to the HFBRs, immediate CH₄ removal was observed in the three HFBRs. A recovery period (RP2), lasting 17 days, was observed as RE in the HFBRs recovered. During Efficient State 2 (ES 2), between days 148-171, the RE in R1 (47.0%), R2 (39.3%) and R3 (26.7%) varied considerably, with an average RE of 37.7 ±10.3 % (216.3±58.9 mmol CH₄ m⁻³ h⁻¹). Although the HFBRs were operated in triplicate throughout Phase I, differences in RE between the three HFBRs were observed post SD, during RP2 and ES2.

5.4.1.2 In-situ CH₄ removal and water chemistry

Depth resolved gas and liquid phase analyses during PI showed that similar gas removal profiles and liquid phase nutrient dynamics occurred in the three HFBRs (Fig. 4). Methane removal was linear with depth in the three HFBRs. The concentration of NH₄-N in the LNF decreased with depth in the HFBRs, although a spike in NH₄-N concentrations did occur at the step-feed (sheet 27) as additional LNF was applied to the HFBRs. The decrease in NH₄-N concentrations was accompanied by an increase in NO₃-N concentrations, indicating that nitrification occurred in the HFBRs. Low concentrations of NO₂-N were detected in the influent LNF and at all depths in the HFBRs, indicating that partial nitrification to NO₂⁻ had not occurred. Some N loss occurred in the HFBRs, likely due to assimilation in biomass or the production of other forms of nitrogen, such as such as particulate ammonium, organic nitrogen and N₂ gas, which were not measured in this trial. The majority of the COD was rapidly removed from the LNF once it was added to the HFBRs.
5.4.2 Phase II (PII) Performance (day 172-233)

5.4.2.1 CH₄ Removal Efficiency (RE)

During Phase II the LNF of the three HFBRs was changed (Table 1) and the HFBRs were no longer operated in triplicate. During the first 10 days of Phase II, RE of 71.5%, 50.3% and 46.1% was observed in R1, R2 and R3 respectively. In contrast R1, R2 and R3 had a RE of 56.1%, 47.7% and 31.7%, respectively, for the last 10 days of Phase I. This represented an increase in RE of 15.4%, 2.6% and 14.4% in R1, R2 and R3 (Table 3).

Increased RE was observed in all three HFBRs over the duration of Phase II, when compared to RE achieved during ES2 in Phase I. The highest RE was observed in R1 (77.4%), representing an increase in RE of 30.4 % compared to ES2 (47.0%). Overall RE in R2 during PII was 49.1 %, an increase in RE of 9.8% compared to ES2 (39.3%). Overall RE of 45.1 % was observed in R3 during PII which was an increase in RE of 24.7% from ES2 (26.7%). During PII the greatest improvement in RE was observed in R1 and R3, which both had organic carbon removed from the LNF, whereas the LNF applied to R2 still contained organic carbon.

5.4.2.2 In-situ CH₄ removal and water chemistry

Although the LNF applied to the HFBRs was altered during PII (Table 1), similar nitrogen dynamics were observed in PII as in PI and CH₄ removal was linear (Fig. 4). In R1 complete nitrification was observed with the majority ammonia being converted to nitrate. In R2 the influent NO₃⁻ concentration was 207 mg l⁻¹ and it remained consistently high throughout the reactor while the ammonia present in LNF II was converted to nitrate via nitrification. In R2, COD removal was consistent with observations seen during PI, whereby the majority of the COD was rapidly removed from the LNF. In R3 NO₃⁻ concentrations fluctuated in the HFBR but remain high while NH₄⁺ and NO₂⁻ levels were low.
Figure 3. Methane removal efficiency (%) in the three HFBRs during Phase I and Phase II.
Table 2. Particulars of operational periods, including mean performance indicators (CH$_4$ removal and efficiencies) from HFBRs 1-3. Standard deviations from the mean are presented in parentheses.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Period</th>
<th>Details</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duration</td>
<td></td>
<td>CH$_4$ removal</td>
<td>Removal</td>
<td>CH$_4$ removal</td>
</tr>
<tr>
<td></td>
<td>(days)</td>
<td></td>
<td>(g CH$_4$ m$^{-3}$ h$^{-1}$)</td>
<td>efficiency (%)</td>
<td>(g CH$_4$ m$^{-3}$ h$^{-1}$)</td>
</tr>
<tr>
<td>PI</td>
<td>1-44</td>
<td>Start-up (SU)</td>
<td>2.24 (1.11)</td>
<td>23.13 (11.5)</td>
<td>2.47 (1.22)</td>
</tr>
<tr>
<td></td>
<td>45-89</td>
<td>Efficient State 1 (ES1)</td>
<td>2.85 (0.38)</td>
<td>29.56 (3.7)</td>
<td>3.37 (0.61)</td>
</tr>
<tr>
<td></td>
<td>90-107</td>
<td>Cold Perturbation (CP)</td>
<td>1.46 (0.51)</td>
<td>14.88 (5.3)</td>
<td>1.88 (0.60)</td>
</tr>
<tr>
<td></td>
<td>108-120</td>
<td>Recovery 1 (RP1)</td>
<td>2.77 (1.04)</td>
<td>27.20 (10.2)</td>
<td>2.91 (0.54)</td>
</tr>
<tr>
<td></td>
<td>121-133</td>
<td>Shutdown (SD)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>134-147</td>
<td>Recovery 2 (RP2)</td>
<td>3.86 (2.18)</td>
<td>39.87 (23.1)</td>
<td>1.96 (1.51)</td>
</tr>
<tr>
<td></td>
<td>148-171</td>
<td>Efficient State 2 (ES2)</td>
<td>4.33 (1.03)</td>
<td>47.03 (11.2)</td>
<td>3.62 (0.86)</td>
</tr>
<tr>
<td>PII</td>
<td>172-233</td>
<td>Feed Change (FC)</td>
<td>7.13 (0.85)</td>
<td>77.41 (9.2)</td>
<td>4.52 (1.21)</td>
</tr>
</tbody>
</table>
Figure 4. Nitrogen dynamics and percentage of influent methane remaining in R1 (a), R2 (b) and R3 during Phase I, and in R1 (d), R2 (e) and R3 (f) during Phase II.
5.4.3 *In-situ* HFBR methane oxidation rates

The *in-situ* methane oxidation rates in the HFBRs was determined for the seven sample zones in the three HFBRs during ES1, ES2 and FC periods (Fig. 5). There were large variations in the methane oxidation rates between locations in the HBFRs and between the sampling phases. In R1 the highest methane oxidation rates were observed during PII (16 g CH$_4$ m$^{-2}$ [TPSA] h$^{-1}$) and Z1 (sheets 1-2) was the most active zone. In R2 and R3, the variation in methane oxidation rates between zones was not as large although rates did fluctuate between zones and operation periods.

![Graph showing methane removal in HFBRs](image.png)

**Figure 5.** *In-situ* methane removal in HFBRs (a) R1, (b) R2 and (c) R3 during Efficient State 1 (ES1), Efficient State (2) and Phase II, where TPSA is top plan surface area.
5.4.4 Comparison of environmental variables in HFBRs over duration of trial

Depth resolved sampling of the gas and liquid phases in the HFBRs allowed for the comparison of environmental variables at different zones in the HFBRs over time (Fig. 6). Non-metric multidimensional scaling (MDS) plots of the three HFBRs showed that all samples, regardless of zone or day, clustered closely together during Phase I (Fig. 6a). On Days 224 and 231 (Phase II), samples from R2 and R3 clustered separately from the other samples, while samples from R1 clustered together samples from PI. This indicated that the environmental conditions in R2 and R3 had changed considerably during PII.

Individual MDS plots were also constructed for each reactor individually. In R1 samples were widely dispersed throughout the plot, with no obvious clustering of samples from a given zone or day. In R2 and R3 the same clustering pattern was observed, where samples from days 224 and 231 clustered separately from the rest of the samples. There was also some clustering of samples from Day 151. Z1 from days 28, 64, 90 and 151 showed some similarity and were clustered together.

PERMANOVA analyses of the three HFBRs during PI showed that the HFBRs were not significantly different (P=0.058) during this time, although the zones between reactors did differ significantly (P =0.038). During Phase II, PERMANOVA testing showed that the HFBRs (P=0.001) and the zones (P=0.001) were both significantly different
Figure 6. Non-metric multidimensional scaling (MDS) plots of the environmental variables measured in zones 1-7 in the three HFBRs on various sample days. (a) MDS plot of the sample zones from R1, R2 and R3 (numbered 1, 2 and 3 respectively), (b) MDS plot of R1, (c) MDS plot of R2 and (d) MDS plot of R3. Zones Z1-Z7 numbered 1-7 in (d), (c) and (d).
5.4.5 Methane oxidation potential (MOP) of biofilm from HFBRs

The MOP of biofilm samples from the three HFBRs exhibited a similar trend (Fig. 7), where higher oxidation potential occurred in the zones closest to the LNF inlets (at sheet 1 and at the step-feed at sheet 27). In R1 and R3 highest oxidation rates were observed between sheets 1-6, 7-12 and sheets SF 1-6 (sheets 27-32 at the step-feed). Lower oxidation potential was seen in zones between sheets 13-18, 19-24 and SF 7-12 (sheets 33-39 after step feed) which were furthest away from the LNF inlets. The same trend was seen in R2, except that the oxidation potential of the zone between sheets 13-18 was similar to rates seen in the previous zones.

![Figure 7](image)

Figure 7. Methane oxidation potential (MOP) of biofilm samples from different zones in HFBRs. Standard deviation represented by error bars.

5.4.6 Bacterial clone library of landfill mix inoculum used for enrichment

A total of 87 unique OTUs were detected from 140 clones following ARDRA screening (Fig. 8), sequencing and analyses using Mothur software (Schloss et al., 2009) to determine representative OTUs (>97% similarity). The library consisted of *Proteobacteria* (40.0%), *Firmicutes* (23.6%), *Bacteroidetes* (26.4%), *TM7 phylum*
(4.3%), *Verrucomicrobia* (2.1%) and *Planctomycetes* (0.7%) (Fig.9). Total library coverage was 62% and rarefaction curves indicated that a moderate representation of the bacterial community the HFBRs was achieved. No methanotroph sequences were detected in the library.

![Figure 8. Gel images of (a) M13 PCR amplicons from clonal sequences and (b) ARDRA screening of bacterial clone library. Gel images shown are typical of entire library.](image-url)
Chapter 5

Figure 9. (a) Rarefaction curve displaying observed OTU richness versus the number of bacterial 16S rRNA clone from the library. OTUs were defined according to 97% nucleotide sequence similarity. (b) The relative abundance of the seven phyla detected in the bacterial clone library.

5.4.6 Bacterial community succession and assembly in HFBRs indicated by TRFLP analyses

TRFLP analyses were conducted on 108 HFBR samples (six zones from each of the three HFBRs on six sample days) and on the six enrichment cultures. PERMANOVA analyses of the TRFLP relative abundance profiles showed the bacterial communities in the three HFBRs were significantly different over the duration of the trial (P=0.004), during Phase I (P=0.01) and during Phase II (P=0.003), based on the Bray-Curtis resemblance matrix. The bacterial community similarity in the HFBR was also compared for each sample day using PERMANOVA (Table 4). The bacterial communities in the reactors were significantly different on all days with the exception of Day 0 (Table 4).
### Table 4. PERMANOVA analyses of square root transformed Bray-Curtis Similarity resemblance matrix of TRFLP data between HFBRs and sample zones.

<table>
<thead>
<tr>
<th>Sampling Day</th>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Pseudo-F</th>
<th>P(perm)</th>
<th>Perms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>Reactor</td>
<td>2</td>
<td>10063</td>
<td>5031.5</td>
<td>1.5399</td>
<td>0.01</td>
<td>999</td>
</tr>
<tr>
<td>Average</td>
<td>Zone</td>
<td>4</td>
<td>36210</td>
<td>9052.6</td>
<td>2.7706</td>
<td>0.001</td>
<td>995</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td>Reactor</td>
<td>2</td>
<td>6086.7</td>
<td>3043.3</td>
<td>2.6275</td>
<td><strong>0.1203</strong></td>
<td>871</td>
</tr>
<tr>
<td></td>
<td>Zone</td>
<td>5</td>
<td>13138</td>
<td>2627.7</td>
<td>2.2687</td>
<td><strong>0.134</strong></td>
<td>999</td>
</tr>
<tr>
<td><strong>Day 44</strong></td>
<td>Reactor</td>
<td>2</td>
<td>10316</td>
<td>5157.8</td>
<td>1.3829</td>
<td><strong>0.039</strong></td>
<td>998</td>
</tr>
<tr>
<td></td>
<td>Zone</td>
<td>5</td>
<td>21756</td>
<td>4351.2</td>
<td>1.1667</td>
<td><strong>0.116</strong></td>
<td>995</td>
</tr>
<tr>
<td><strong>Day 84</strong></td>
<td>Reactor</td>
<td>2</td>
<td>10316</td>
<td>5157.8</td>
<td>1.3829</td>
<td><strong>0.05</strong></td>
<td>998</td>
</tr>
<tr>
<td></td>
<td>Zone</td>
<td>5</td>
<td>21756</td>
<td>4351.2</td>
<td>1.1667</td>
<td><strong>0.127</strong></td>
<td>999</td>
</tr>
<tr>
<td><strong>Day 108</strong></td>
<td>Reactor</td>
<td>2</td>
<td>10316</td>
<td>5157.8</td>
<td>1.3829</td>
<td><strong>0.049</strong></td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>Zone</td>
<td>5</td>
<td>21756</td>
<td>4351.2</td>
<td>1.1667</td>
<td><strong>0.11</strong></td>
<td>999</td>
</tr>
<tr>
<td><strong>Phase I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 177</td>
<td>Reactor</td>
<td>2</td>
<td>10316</td>
<td>5157.8</td>
<td>1.3829</td>
<td><strong>0.047</strong></td>
<td>998</td>
</tr>
<tr>
<td></td>
<td>Zone</td>
<td>5</td>
<td>21756</td>
<td>4351.2</td>
<td>1.1667</td>
<td><strong>0.113</strong></td>
<td>997</td>
</tr>
<tr>
<td><strong>Phase II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 233</td>
<td>Reactor</td>
<td>2</td>
<td>12095</td>
<td>6047.6</td>
<td>2.0166</td>
<td><strong>0.016</strong></td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>Zone</td>
<td>5</td>
<td>16900</td>
<td>3380</td>
<td>1.1271</td>
<td><strong>0.258</strong></td>
<td>999</td>
</tr>
</tbody>
</table>
Pair-wise testing revealed that there was no significant difference between the bacterial communities in R1 and R2 during Phase I, whereas R3 was significantly different to R1 (0.014) and R2 (0.02) (Table 5). However, by Day 233 the bacterial community composition had changed considerably and R1 and R2 were significantly different, while the bacterial community in R3 was not significantly different to R1 and R2 (Table 5).

**Table 5.** Pair-wise testing of the bacterial community in the HFBRs based Bray-Curtis similarity resemblance matrix of relative abundances of OTUs from TRFLP.

<table>
<thead>
<tr>
<th>Phase</th>
<th>HFBRs</th>
<th>t</th>
<th>P(perm)</th>
<th>Unique Permutations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Entire Trial</strong></td>
<td>R1,R 2</td>
<td>1.1485</td>
<td>0.118</td>
<td>996</td>
</tr>
<tr>
<td></td>
<td>R1,R 3</td>
<td>1.3809</td>
<td><strong>0.006</strong></td>
<td>997</td>
</tr>
<tr>
<td></td>
<td>R2, R3</td>
<td>1.2319</td>
<td><strong>0.038</strong></td>
<td>996</td>
</tr>
<tr>
<td><strong>Phase I</strong></td>
<td>R1,R 2</td>
<td>1.0678</td>
<td><strong>0.269</strong></td>
<td>998</td>
</tr>
<tr>
<td></td>
<td>R1,R 3</td>
<td>1.3493</td>
<td><strong>0.014</strong></td>
<td>998</td>
</tr>
<tr>
<td></td>
<td>R2, R3</td>
<td>1.2927</td>
<td><strong>0.02</strong></td>
<td>999</td>
</tr>
<tr>
<td><strong>Phase II</strong></td>
<td>R1,R 2</td>
<td>1.6358</td>
<td><strong>0.009</strong></td>
<td>408</td>
</tr>
<tr>
<td></td>
<td>R1,R 3</td>
<td>1.2245</td>
<td><strong>0.057</strong></td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>R2, R3</td>
<td>1.2566</td>
<td><strong>0.098</strong></td>
<td>412</td>
</tr>
</tbody>
</table>

Principal coordinate analysis (PCO) plots of the bacterial communities in the HFBRs were constructed for the three HFBRs and the enrichment cultures together, and for R1, R2 and R3 separately (Fig. 10). In the PCO plot comparing the enrichment cultures and the HFBR biofilm samples (Fig. 10a), the enrichment cultures form a distinct cluster as do biofilm samples from Day 0 and Day 233. Most of the
remaining HFBR samples are distributed over a large area in the centre of the plot. There is some clustering of samples in the PCO plots of R1, R2 and R3, but most samples are widely distributed, indicating large variance between samples. The percentage of variation explained by the two axes in the four plots is low due to the high levels of variation between samples.

SIMPER analyses of the TRFLP profiles indicated that the average similarity between samples within R1, R2 and R3 was 14.05%, 12.71% and 13.64% respectively. OTUs that had the most influence on similarity between samples were determined and are presented in Tables S1, S2 and S3 in the supplementary data. Comparison of the TRFLP profiles from the enrichment cultures and the TRFLP profiles from the HFBRs on Day O indicated that there were no unique OTUs present in the enrichments that were not also present in the HFBRs.

It was not possible to compare the biological data generated from the TRFLP analyses to the in-situ environmental variables as the sampling zones were different for biofilm and gas and liquid sampling.
Figure 10. Principal coordinate analysis (PCO) plots of bacterial communities in the enrichment cultures, and from zones 1-6 on sample days 0, 44, 84, 108, 177 and 233 in HFBRs. POC plots of (a) all samples (b) R1, (c) R2 and (d) R3, based on relative abundances of OTUs from TRFLP. Zones Z1-Z7 are numbered 1-7 in PCO plots.
5.4.7 Distribution of Type I and Type II methanotrophs observed by FISH

FISH analyses of biofilm samples taken from the HFBRs on day 177 indicted that both Type I and Type II methanotrophs were present in the biofilm (Fig. 11). Type I methanotrophs were present in low concentrations and clumped together in small, well-defined clusters, whereas Type II methanotrophs occurred in higher numbers and were more widely distributed in larger cell clusters. Although the presence of methanotrophs was apparent in the biofilm, the microbial community was dominated by other unidentified prokaryotes.

Figure 11. Specific detection of Type I (green) and Type II (red) methanotrophs (A, B) and Type I (green) and Type II (red) methanotrophs with all other prokaryotes (blue) (C, D) using FISH. Scale bars 10µm.
5.5 DISCUSSION

HFBRs were shown to offer a simple, low technology bioreactor configuration capable of treating CH₄ contaminated airstreams at 10°C. Maximum average methane removal rates of up to 7.1 g CH₄ m⁻³ h⁻¹ with a RE of 55% were achieved during Phase II.

The HFBRs were initially seeded with activated sludge from an aerobic wastewater treatment facility. Although biofilm formation occurred in the HFBRs, no methane oxidation was recorded after one month of operation, demonstrating that activated sludge is an unsuitable inoculum for bioreactors treating methane contaminated air. The bio-augmentation of the enriched methane oxidising biomass with the activated sludge biofilm already present in the HFBRs, resulted in the successful development of a methane oxidising biofilm within the reactors. Previous studies that have utilized an inorganic packed bed reactor configuration to treat a methane contaminated airstream also used an enriched methane oxidising inoculum to seed their reactors (Nikiema et al., 2005; Rocha-Rios et al., 2009).

Operational parameters in the HFBRs had an effect on methane removal rates throughout the trial. During the cold perturbation period, when temperatures in the HFBRs were reduced, a drop in removal efficiency was observed. As most methanotrophs are mesophilic and have an optimal temperature range between 20-37°C (Humer and Lechner, 1999), it is unsurprising that methanotrophic biological activity was impaired at lower temperatures. A Q₁₀-value of 2.49 (±0.45) was observed in the HFBRs between 1-10°C, which is similar to values seen in other environments, including landfill cover soils Q₁₀ 3.4-4.1 (2 -25°C) (Scheutz and Kjeldsen, 2004). Although the temperature reduction caused a drop in RE during this period, other factors such as substrate concentration (King and Adamsen, 1992) and water content (Einola et al., 2007) can also influence Q₁₀ values. As most methanotrophs are mesophilic, it is possible that operating the HFBRs at 10°C may have prevented the methanotrophic population from achieving its maximum oxidation potential. The seed inoculum was enriched from a ‘landfill mix’ and it has previously been shown that optimum temperature for methane oxidation in landfill cover soils, soils and peatlands is between 20–31°C (Whalen et al., 1990; Scheutz and Kjeldsen, 2004; Spokas and Bogner, 2011). Further investigation is required to
determine if increased operating temperatures would facilitate higher methane removal rates in HFBRs.

When the methane supply was reintroduced into the HFBRs after the shutdown period, immediate methane oxidation was observed, indicating that the methanotrophic community was still present and functional in the HFBRs despite the absence of methane for 14 d. Pure culture methanotrophs have been shown to survive without methane, under oxic conditions, for up to 10 weeks (Roslev and King, 1994), while Kightley et al. (1995) observed that a landfill soil core, which had been exposed to oxic conditions in the absence of methane for over 180 days, retained its methanotrophic community and was capable of methane oxidation even after such a lengthy starvation period. The ability of the HFBRs to remove CH₄ after the cold and shutdown perturbations highlights the robust nature of the technology and the microbial community present. Both perturbations were typical of scenarios that may occur at full scale operations.

The composition of the LNF was altered during PII and had an impact on HFBR performance. The omission of organic carbon from the LNF in R1 and R2 during PII resulted in an increase in methane RE. Visual inspection of the HFBRs revealed that the mass of biofilm in R1 and R3 was reduced during PII after organic carbon was excluded from the LNF. During PI, the density of biofilm was always greatest on sheets closest to the LNF influent ports, which coincided with highest concentrations of COD. The presence of organic carbon in the LNF during PI likely facilitated the development of a heterotrophic microbial community. Although heterotrophs do not compete with methanotrophs for methane, they may have been in competition for oxygen and other nutrients. Aerobic heterotrophs and methanotrophs require oxygen and nitrogen for respiration and cell growth and it has been shown in rice paddy soil incubations, that heterotrophs outcompete methanotrophs for oxygen in the presence of organic carbon (van Bodegom et al., 2001). The absence of organic carbon from the LNF during Phase II may have reduced competition between the methanotrophic community and other heterotrophs, resulting in higher methane removal rates.

Methanotrophs have a relatively high N requirement, with 0.25 moles N needed for every mole of C assimilated (Anthony, 1982). There are conflicting reports in the literature as to which N source is preferable for methane oxidation. Some studies
have shown that $\text{NO}_3^-$ is more preferable than $\text{NH}_4^+$, as the presence of $\text{NH}_4^+$ can divert methanotrophic activity to nitrification (Josiane and Michèle, 2009), while other studies have shown that $\text{NH}_4^+$ aids methane oxidation (Bosse et al., 1993; Cai and Mosier, 2000). As well as improving methane oxidation, inorganic N, such as $\text{NH}_4^+$, $\text{NO}_3^-$ and $\text{NO}_2^-$, can also limit the methane oxidising potential of a methanotrophic population in some situations (Hanson and Hanson, 1996). However their impact, if any, on methane oxidation in the HFBRs was unclear. Although concentrations of $\text{NH}_4^+$, $\text{NO}_2^-$ and $\text{NO}_3^-$ varied with depth in the HFBRs, methane removal remained linear. Nitrification was observed in the HFBRs and it is possible that the various nitrogen sources in the LNF supported an independent nitrifying community that had little impact on methane oxidation. To determine the impact of N source and concentration on methane oxidation rates in future studies, MOP biofilm assays in the presence of varying concentrations of $\text{NH}_4^+$, $\text{NO}_2^-$ and $\text{NO}_3^-$ would be useful.

The MOP of HFBR biofilm varied with location in the HFBRs, but in general, biofilm from zones closest to the LNF inlets (sheet 1 and step-feed at sheet 27) had the highest MOP, indicating that location in the HFBR had an effect on the methanotrophic community. Methanotrophic activity, cell growth rates and community structure are influenced by various parameters, including moisture content, nutrient availability, dissolved oxygen and substrate concentration (Lopez et al., 2013). Due to the design of the HFBR, gradients in these parameters occurred with depth in the reactors, therefore influencing the methanotrophic populations present. As the MOP assays were performed in batch, they were not an accurate representation of in-situ conditions in the HFBRs, making it difficult to directly compare MOP rates to in-situ methane removal rates. Even so, the differences in MOP indicated that geographical location in the HFBRs impacted on the methanotroph community.

The TRFLP profiles from the last three enrichment cultures contained 42 separate TRFs and it is likely that at least some of these TRFs represented methanotroph species. However, identical TRFs for all 42 OTUs were already present in the HFBRs on Day 0. As the bacterial communities in the HFBRs were unable to oxidise methane prior to inoculation with the enriched seed culture, unique bacterial species
including methane oxidising organisms were introduced to the HFBRs from the enriched culture. One of the limitations of TRFLP is that in many cases, unrelated individuals will share a common TRF (Doroghazi and Buckley, 2008). This prevented the identification of methane oxidising bacteria from TRF biomarkers. As such, it was not possible to confirm the presence or relative abundance of methanotrophs from various regions in the HFBRs throughout the trial. In future studies, it would be advisable to study the functional pmoA gene or methanotroph 16S rRNA genes, to assess the methane oxidising communities present. Even so, the TRFLP profiles showed that the bacterial populations in the HFBRs were both diverse and dynamic over the duration of the trial. Due to the complex LNF used to hydrate the HFBRs, several biological processes occurred simultaneously within the reactors, including COD removal and nitrogen cycling. Hector and Bagchi (2007) found that the number of species required to maintain ecosystem functionality increased with the number of biochemical processes occurring. Although environmental conditions were replicated in the three HFBRs and similar nitrogen and carbon removal profiles were observed, the bacterial communities between the HFBRs were statistically different. This indicated the overall bacterial community retained functional redundancy in the HFBRs regardless of species present.

FISH showed that Type II methanotrophs were more abundant than Type I methanotrophs, which may signify that Type II methanotrophs were the main drivers of methane oxidation in the HFBRs. Spatial arrangement of the methanotroph communities in the biofilm was observed, with Type I and Type II methanotrophs clustering together, indicating that methane oxidation may have occurred in localised regions within the biofilm. Interestingly, in most other low temperature environments the opposite is true, where Type I methanotrophs have been observed to outnumber Type II methanotrophs. Examples include Arctic lake sediments (He et al., 2012), permafrost soils (Liebner and Wagner, 2007) and in a laboratory incubation of landfill soil between 5-10°C (Börjesson et al., 2004). Although the HFBRs were operated at 10°C, temperature may not have been the only factor that influenced the composition of the methanotroph population. CH₄, O₂, nitrogen source and nitrogen concentration can also impact on the composition of methanotroph populations (Hanson and Hanson, 1996). The presence of high numbers of other unidentified prokaryotes in the biofilm was likely due to the
presence of organic carbon, nitrogen sources and other nutrients, which sustained other microbial populations involved in carbon and nitrogen cycling. As the overall bacterial population in the HBFRs varied between zones and over time, it is likely that the methanotrophic population was also changing. FISH was only performed on biofilm samples from Day 171, and as such, observations made cannot be considered representative of the methanotrophic community structure over the duration of the trial. A more rigorous sampling regime would be required to determine whether the trends observed on Day 171 occurred throughout the trial.

5.6 CONCLUSIONS

Methane removal rates of up to 7.1 g CH₄ m⁻³ h⁻¹, representing a RE of 77%, were achieved at 10°C in the HFBRs. Reducing the temperature of the HFBRs resulted in a decrease in reactor performance, with lower methane RE recorded during the cold perturbation period. The composition of the LNF affected the methane removal capacity of the HBFRs, with highest removal rates achieved in R1 and R3 when organic carbon was removed from the LNF. The MOP of biofilm varied with location in the HFBR with maximum oxidation rates of 64 mg [CH₄] g VSS⁻¹ d⁻¹ recorded. For successful start-up of a HFBR treating methane contaminated air choosing a seed inoculum capable of methane oxidation is essential. A specialised methane oxidising culture was developed in batch enrichments to seed the HFBRs. FISH of biofilm sampled from the HFBRs indicated that Type II methanotrophs were more abundant than Type I methanotrophs, although the biofilm was dominated by other unidentified prokaryotes. The overall bacterial community in the HFBRs was diverse and variations in community structure were observed between reactors, zones and sampling days.

5.7 REFERENCES


CHAPTER 6

The Abundance and Diversity of Methanotrophs in Horizontal Flow Biofilm Reactors (HFBRs) treating Methane contaminated Air at 10°C and the Impact of Silicone Oil and Brij35 on Reactor Performance
This work described in this chapter was collaboration between the Microbiology and Civil Engineering departments in the National University of Ireland Galway. The HFBRs were operated by the Civil Engineering research group but all bioprocess data and molecular work was analysed as part of this PhD programme.
6.1 ABSTRACT

Methane (CH$_4$) is an important greenhouse gas that is commonly encountered at various waste-treatment and agricultural facilities when organic matter is broken down anaerobically. Three laboratory-scale Horizontal-Flow Biofilm Reactors (HFBRs) treating methane-contaminated airstreams at low concentrations (1.6%, v/v) were operated for 341 days at 10°C. Removal rates of up to 8.2 g CH$_4$ m$^{-3}$ h$^{-1}$, with removal efficiencies of 62.1%, were achieved at a loading rate of 13.2 g CH$_4$ m$^{-3}$ h$^{-1}$. The addition of silicone oil to the HFBRs improved reactor performance by an average of 69%. The further addition of Brij35 to R1 and R2 resulted in increased methane removal rates of 33%, when compared to operation with silicone oil alone. The methane oxidation potential (MOP) of the HFBR biofilm and seed biomass was assessed in batch incubations at various temperatures. The highest MOP rate (19.0 mg CH$_4$ g[VSS] h$^{-1}$) for the HFBR biofilm was measured at 23°C, whereas highest oxidation rates in the seed biomass were measured at 37°C (33.6 mg CH$_4$ g[VSS] h$^{-1}$). Methanotroph abundance from various depths in the HFBRs was determined by qPCR of the functional $pmoA$ gene. Temporal Gradient Gel Electrophoresis (TGGE) fingerprinting indicated that overall methanotroph diversity in the HFBRs was low with Type I $Methylobacter$ and $Methylomonas$ species, and Type II $Methylocystis$ species, detected. The methanotroph communities in the three HBFRs were similar but changed over time. Results from this study showed that silicone oil and Brij35 improved methane removal in the HFBRs and that methane oxidation in the reactors was performed by a small group of methanotrophs who were abundant at all depths in the reactors.
6.2 INTRODUCTION

Methane (CH\(_4\)) is an important greenhouse gas, which is produced when organic matter is degraded by microorganisms under anaerobic conditions. As a greenhouse gas it is up to 23 times more potent than CO\(_2\) on a molar basis (IPCC, 2007). It represents about 15% of total annual greenhouse gas emissions and 23% of total anthropogenic greenhouse gas emissions (USEPA, 2006). To combat climate change and achieve targets set in Kyoto Protocol it is essential to control methane emissions into the atmosphere. Biological treatment technologies, such as biofilters (Gebert and Gröngröft, 2006; Nikiema et al., 2009; Park et al., 2009; Girard et al., 2011), stirred tank bioscrubbers (Rocha-Rios et al., 2010), bio-trickling filters (Avalos Ramirez et al., 2012b) and HFBRs (Chapter 5) have been previously used to treat methane-contaminated airstreams. Methane oxidization in biotrickling filters is often limited by the mass transfer of methane into the liquid phase (Avalos Ramirez et al., 2012b). The low water solubility of methane (42 mg l\(^{-1}\) at 10°C) means that long gas retention times are often required for efficient methane removal in bioreactors. Previous studies have shown that addition of silicone oil (Rocha-Rios et al., 2009) and Brij35, a non-ionic surfactant, (Avalos Ramirez et al., 2012a) can improve mass transfer of methane into the liquid phase, thus improving overall methane removal. Silicone oil acts as a transfer vector and improves mass transfer of methane into the liquid phase where it becomes available for biological methane oxidation (Rocha-Rios et al., 2011), although it has also been hypothesised that bacteria can take up methane from the transfer vector itself (Rocha-Rios et al., 2010). Brij 35 has previously been shown to improve methane removal rates in a biofilter trial (Avalos Ramirez et al., 2012a). Non-ionic surfactants contain hydrophilic and hydrophobic elements that can increase the solubility of low water soluble compounds such as methane. Brij 35 also acts as an oil water emulsifier, helping to solubilise oils in water, which is beneficial as the effectiveness of silicone oil as a transfer vector is dependent on the degree of oil dispersion in the liquid phase (Rocha-Rios et al., 2010). As the solubility of methane increases at lower temperatures, operating the HFBRs at 10°C during this study, rather than at ambient temperature, could potentially improve methane solubility.

The biological oxidation of methane is mainly performed by methanotrophs, a group of aerobic bacteria that are found ubiquitously in nature (Jiang et al., 2010). There
are three major phylogenetic groups, Type I, Type II and Type X methanotrophs, along with a small number of other filamentous and acidophilic bacteria capable of methane oxidation (McDonald et al., 2008). All methanotrophs contain the methane monoxygenase (MMO) enzyme that catalyzes the conversion of methane to methanol (McDonald et al., 2008). The MMO enzyme, which is unique to methanotrophs, has a broad substrate affinity and is also capable of co-metabolizing a variety of aliphatic compounds, including some halogenated hydrocarbons (Bogner et al., 2003). MMO occurs in two forms, a soluble cytoplasmic form (sMMO) and a particulate membrane-associated form (pMMO). All know methanotrophs contain the pMMO enzyme with the exception of the *Methylocella* genus (Theisen et al., 2005), whereas only a select number of groups have sMMO. The *pmoA* gene, which encodes for pMMO, is unique to methanotrophs and has been used as molecular markers for the study of methanotrophic populations in various environments including landfill soils (Henneberger et al., 2012; Kong et al., 2013), lake sediments (Costello and Lidstrom, 1999; Pester et al., 2004) and soils (Fjellbirkeland et al., 2001; Kolb et al., 2003). The *pmoA* gene can be used to quantify methanotrophs in the environments and can be sequenced to identify methanotroph population composition.

There has been increased interest in the microbial ecology, in terms of microbial diversity and community dynamics, of engineered systems and how the microbial ecology effects reactor performance (Curtis et al., 2003). The use of molecular techniques for the study of the microbial consortia in bioreactors and other engineered systems has uncovered evidence linking diversity and dynamics to process stability (Briones and Raskin, 2003). The methanotrophic community has been investigated in several biofilters treating methane (Gebert et al., 2008; Kim et al., 2012; Kim et al., 2013; Kim et al., 2014) but to our knowledge no studies have investigated the methanotroph community structures in biotrickling filters. Biotrickling filters and HFBRs share a similar design concept and this study is the first to examine the methanotrophic populations in this reactor configuration. Methanotroph community structure and oxidation rates are influenced by a range of environmental variables including temperature (Börjesson et al., 2004), nitrogen source and concentration (Bodelier and Laanbroek, 2004), substrate availability (Henckel et al., 2000), substrate concentration (López et al., 2014), moisture levels
(Einola et al., 2007) and O₂ concentration (Teh et al., 2005). Understanding the microbial population dynamics in methane oxidising HFBRs and observing how they respond to environmental pressures, may help to improve reactor design and operation in order to maximise oxidation rates and ensure performance stability.

The aim of this work was to (i) examine the potential of HFBRs to treat methane-contaminated airstreams at 10°C; (ii) assess the impact of the transfer vectors, silicone oil and Brij35 on methane oxidation rates; and (iii) investigate the methanotrophic community underpinning the process. Methanotroph and bacterial cell were quantified using qPCR, and the methanotrophic community structure, and diversity, was assessed using TGGE. Key methanotrophs were identified by sequencing the functional *pmoA* gene. The methane oxidising potential of the HFBR biomass was determined in batch incubations at a range of temperatures.

### 6.3 MATERIALS AND METHODS

#### 6.3.1 Seeding of HFBRs

Biofilm was taken from a previous methane elimination study (Chapter 5) and incubated at 10°C in sealed 60 ml glass vials containing 30 ml Adapted Whittenbury Medium (AWM) (Whittenbury et al., 1970) in a 10% CH₄ headspace. The culture was sustained for five months by replacing the AWM every two weeks and replacing the headspace once CH₄ concentrations were >0.5 %. After five months the cultures were scaled up to 1 l bottles and to generate 3 l of culture. On day 1, the seed culture was re-circulated through the HFBRs with AWM for one day to allow for biofilm attachment in the reactors.

#### 6.3.2 HFBR design and operating conditions

Three HFBRs (R1, R2 and R3) were used to treat methane-contaminated air at 10°C. The HFBRs (Fig. 1) each comprised 52 plastic sheets, with integrated frustums, stacked one on top of another and enclosed in an air-tight housing unit. Gas and liquid sampling ports were situated vertically along the HFBRs at sheets 4, 12, 21, 30, 40 and 50. The working volume of each reactor was 18 l and the top plan surface
area (TPSA) of the plastic sheet media was 0.04 m², giving a total TPSA of 2.28 m². A constant supply of methane-contaminated air and an intermittent supply of liquid nutrient feed (LNF) were applied to the HFBRs.

**Figure 1**- Schematic of HFBR set-up
The composition of the LNF was based on feed used by Nikiema et al. (2009) and is outlined in Table 1. The contaminated airstream and the LNF were introduced to the top of the HFBRs where they flowed in a co-current direction across the top sheet (Sheet 1) before dropping to the sheet below. The waste streams then flowed horizontally in the opposite direction across Sheet 2 before dropping onto Sheet 3. This boustrophedonic flow regime continued through the rest of the HFBR (Fig. 1). LNF was also added to the HFBRs at a step-feed at Sheet 30 to supply additional hydration and nutrients to the reactors. The average influent methane concentration was 1.6 % (v/v) and the average methane loading rate was 13.2 g CH₄ m⁻³ h⁻¹ over the duration of the trial.

Table 1. Composition of the Liquid Nutrient Feed (LNF)

<table>
<thead>
<tr>
<th>Component</th>
<th>(g l⁻¹)</th>
<th>Component</th>
<th>(g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>1.51</td>
<td>MnSO₄·H₂O</td>
<td>0.000466</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.86</td>
<td>CuSO₄·5H₂O</td>
<td>0.000250</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.53</td>
<td>KI</td>
<td>0.000166</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.17</td>
<td>H₃BO₃</td>
<td>0.000124</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.037</td>
<td>NaMoO₄·2H₂O</td>
<td>0.000096</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.007</td>
<td>CoCl₂·6H₂O</td>
<td>0.000096</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.00112</td>
<td>NH₄Cl</td>
<td>0.06*</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.000576</td>
<td>Urea</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

* Added to R3 only on day 64

The trial was divided into three phases (PI, PII and PIII) and lasted 341 days in total. During PI, which lasted 166 days, the three HFBRs were operated in triplicate until day 64 when additional urea and NH₄Cl was added to the LNF of R3. R1 and R2 were operated in duplicate for the remainder of PI. During PII, which lasted 102 days (days 167-269), silicone oil was added to the LNF to a final concentration of 10% (v/v) to aid with mass transfer of the methane into the liquid phase. A 20-day shutdown period (Day 198-218) was applied to the HFBRs during PII when no
methane was supplied to the reactors. During PIII, which lasted 72 days (Day 270-341), Brij 35 was added to R1 and R2 in varying concentrations (0.5 - 2.0 g l⁻¹) to investigate the effect of silicone oil and Brij 35 on the methane removal in the HFBRs and to determine the optimum Brij35 concentration. Brij35 (C₁₂H₂₅(OCH₂CH₂)₃nOH), also known as polyoxyethylene 23 lauryl ether, is a non-ionic surfactant.

6.3.3 Gas and liquid sampling

The HFBRs were divided into seven sampling zones (Z1-Z7) for in-depth gas and liquid analyses (Fig. 2). Influent and effluent methane gas concentrations were determined 3-4 weekly. Depth resolved methane removal profiles were determined weekly by assessing methane gas concentrations at sample ports located vertically along the HFBRs. Methane concentrations were measured using an Agilent 7890A Gas Chromatograph (Agilent Technologies Ireland Ltd, Cork, Ireland). The average HFBR methane removal rate for each Phase was calculated when the HFBRs were deemed to have reached steady state operation. In-situ HFBR methane removal rates were calculated for each of the seven sample zones (Z1-Z7) and removal was expressed as g CH₄ m⁻² [TPSA] h⁻¹, where TPSA was Top Plan Surface Area.

Weekly depth-resolved liquid phase analyses were determined from liquid samples taken from influent, effluent and intermediate sampling ports. Suspended solids (SS), chemical oxygen demand (COD) and total nitrogen (TN) were measured in accordance with standard methods (AWWA, 1998). Ammonium-nitrogen (NH₄-N), nitrite-nitrogen (NO₂⁻N), nitrate-nitrogen (NO₃⁻N) and phosphate-phosphorous (PO₄⁻P) concentrations were determined using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts).
6.3.4 Biofilm sampling and DNA extraction

For the purpose of biofilm sampling the HFBRs were divided into seven sample zones (Z1-Z7) to coincide with the gas and water sample port locations along the side of the reactor (Fig. 2). HFBRs were sampled at the end of PI (Day 166), PII (Day 268) and PIII (Day 340). Biofilm samples for molecular analysis were stored at -20°C in sterile centrifuge tubes. On the last day of the trial, the total biomass from each zone in the HFBRs was collected. The total volume of biomass from each zone was recorded, and the Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) from each zone were calculated using standard methods (AWWA, 1998). Live biofilm was also taken on the last day of the trial for methane oxidation potential assays (MOPs).

DNA was extracted from the HFBR biofilm and seed biomass using a Maxwell 16 Tissue DNA Purification Kit and a Maxwell 16 Research Instrument System.
Samples were defrosted at room temperature and 0.1 g biofilm was used for extraction. All extracts were visualised on a 1% (w/v) agarose gel containing SYBR Gold (0.01% v/v) under UV light, and quantified spectrophotometrically using a Nano-drop (Thermo Scientific, MA, USA).

6.3.5 Quantification of bacterial 16S rRNA and \textit{pmoA} gene copies using quantitative PCR (qPCR)

Bacterial 16S rRNA and archaeal \textit{pmoA} genes from biofilm DNA extracts were quantified by qPCR in 20\mu l reactions using a LightCycler 480 instrument (Roche, Basel, Switzerland). Bacterial 16S rRNA genes were amplified by qPCR, using primers 338f and 805r and TaqMan probe 516f-ROX and the same reaction concentrations and cycling conditions described by Yu \textit{et al.} (2005), details of which are described in Chapter 3. Functional \textit{pmoA} gene copies were quantified using primers mb661r (Costello and Lidstrom, 1999) and A189f (Holmes \textit{et al.}, 1995) in a Syber Green Mastermix (Roche). For both assays 3 \mu l of a 1/10 dilution of DNA template was used as template in the 20 \mu l reactions. Internal standard curves using purified plasmid extracts of respective gene targets were included for both assays. The absolute abundance of \textit{pmoA} and bacterial 16S rRNA gene copies were quantified for each biofilm sample. Methanotroph cell numbers were calculated assuming the frequency of two \textit{pmoA} gene copies per methanotrophic cell (Stolyar \textit{et al.}, 1999). It was not possible to accurately quantify the total bacterial cells in the samples as there can be between 1-15 16S rRNA gene copies per bacterial cell (Klappenbach \textit{et al.}, 2001), however the ratio of \textit{pmoA}:16S rRNA gene copies was calculated.

The number of methanotrophs present in each zone in the HFBRs was determined on the last day of the trial (Day 340). Methanotroph abundance was calculated by comparing the concentration of methanotroph cells in one gram of biofilm and comparing it to the mass of biofilm in a given zone. By comparing the \textit{in-situ} HFBR methane removal rates in each zone and the number of methanotroph cells in that zone, it was possible to express removal as (a) \text{g CH}_4 \text{ g [biofilm]}^{-1} \text{h}^{-1} and (b) \text{g CH}_4 / \text{methanotroph cell} \text{ h}^{-1} for PIII. It was not possible to calculate these rates for PI or PII as the total mass of biofilm in the HFBRs was unknown.
6.3.6 Temperature Gradient Gel Electrophoresis (TGGE) fingerprinting of the HFBR methanotroph communities

The methanotrophic communities in the HFBRs were compared using TGGE fingerprinting on the last day of PI, PII and PIII. Three composite HFBR DNA samples were prepared for each sample day. To generate a composite sample, DNA extracts from the seven zones in a HFBR from one sample day were combined in equal volumes (5 µl each). There was a total of nine composite DNA samples prepared, one for each HFBR on each of the three sample days.

The *pmoA* gene from the nine composite HFBR DNA samples and from the seed DNA was amplified using primers A189f and mb661r. A 40bp GC clamp (CGCCCGCCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGC

TGGE was performed using Maxi System (Biometra, Göttingen, Germany) instrument. Denaturing gels were prepared (Acrylamide (6%), Urea (8M), TAE (1x), Glycerol (2%), deionized formamide (20%), with polymerisation agents TEMED (0.09%, v/v) and APS (0.016%, v/v). An initial perpendicular TGGE run was performed in a temperature gradient of 40-70°C for 16 h to determine the optimum temperature gradient for parallel analyses. The PCR amplicons were then analysed in parallel in a temperature gradient of 54-61 °C. The resulting TGGE gel was silver stained as described by (Bassam et al., 1991). Six bands of interest were excised from the gel and sequenced as outlined below.

6.3.7 Cloning of TGGE bands

Six TGGE bands were carefully excised from the gel using sterile razor blades and incubated in 50 µl TE buffer, in sterile micro-centrifuge tubes at room temperature, overnight. The liquid fractions were then transferred to fresh, sterile tubes. PCR amplification of the liquid fractions, targeting the functional *pmoA* gene, was performed using primers A189f and mb661r, and the same thermocycling conditions.
described earlier. PCR amplicons were visualised on a 1% (w/v) agarose gel containing SYBR Gold (0.01% v/v) under UV light. The six PCR products were then gel purified using Wizard SV Gel and PCR Clean Up kit. The six PCR products were cloned using TOPO TA vector and TOPO-10 chemically competent cells (Invitrogen) and the resulting clones were sequenced by MacroGen (South Korea), as described in Chapter 3.

6.3.8 Statistical analyses of the TGGE fingerprinting and clone libraries

Statistical analysis of the TGGE gels was performed using Primer 6 software (Clarke and Warwick, 2001). A biological presence/absence matrix was constructed based on the banding patterns from the TGGE gel. Bray-Curtis measures of similarity were calculated and the similarity of the HFBR and seed methanotrophic communities were compared using Non Multi-dimensional Scaling (MDS) and cluster analyses. Permutation multivariate analysis of variance (PERMANOVA) of the Bray-Curtis resemblance matrix was conducted using default settings with 999 permutations to determine if there was a significant difference between the methanotrophic communities in the HFBRs or between sampling days.

Phylogenetic analyses of the clone libraries was performed using Mothur (Schloss et al., 2009) as described in Chapter 3. Phylogenetic trees were constructed using MEGA5 (Tamura et al., 2011), using neighbour-joining trees and the Jukes-Cantor method for 1000 repetitions. Relevant reference sequences were downloaded from GenBank and included for comparison in the phylogenetic trees.

6.3.9 Methane oxidation potential (MOP) assays

MOP assays were performed on the seed biomass used to inoculate the HFBRs and on composite biofilm samples from HFBRs on the last day of trial, at 4, 10, 23 and 37°C. Composite HFBR biofilm samples were prepared by adding an equal mass of biofilm (3 g) from Z1-Z7, from respective reactors. The VSS of the seed biomass and the three HFBR biofilm composites was calculated according to Standard Methods (AWWA, 1998). For each assay, biofilm (5 mg VSS) was transferred to
40ml-hypo-vials containing 10 ml AWM. The vials were sealed and the headspace was adjusted to 10% (v/v) methane concentration. CH₄ concentrations in the headspace were measured over time using gas chromatography (GC; Varian CP-3800 Gas Chromatograph) until <0.1% CH₄ (v/v) remained. The MOP of the biofilm was expressed as mg CH₄ g⁻¹ VSSₜₜ (biofilm) h⁻¹. The Q₁₀ value for the seed and the HFBR biofilm was calculated using the formula

\[ Q_{10} = \left( \frac{R_2}{R_1} \right)^{10/(T_2-T_1)} \]

where R is the metabolic rate at temperatures T₁ and T₂ and T₂>T₁.

### 6.4 RESULTS

#### 6.4.1 HFBR performance

Methane removal efficiencies (RE) for the three HFBRs over the duration of the trial are presented in Figure 3. Average HFBR methane removal rates for the three phases, which were determined when the HFBRs reached steady-state operation, are presented in Table 2. During PI, a start-up period of 40 d was observed in R1 and R2, and 54 d in R3, before the reactors reached steady state. Average methane removal rates during PI steady-state were 4.2, 3.1 and 2.3 g CH₄ m⁻³ h⁻¹ in R1, R2 and R3, respectively. The addition of NH₄Cl and urea to the LNF in R3 on Day 64 resulted in a slight increase in HFBR performance. In the 10 days prior to the change to the LNF methane removal in R3 was 1.04 (±0.19) g CH₄ m⁻³ h⁻¹ and in the 10 days following the change methane removal was 1.27 (±0.26) g CH₄ m⁻³ h⁻¹, representing an 22.1% increase in reactor performance.

During PII (Day 167-169) silicone oil was added to the LNF, resulting in improved methane removal rates across the three HBFRs. PII was divided into two periods, before the shutdown (BS) and after the shutdown (AS). HFBR performances during the BS period were 35.7%, 93.5% and 78.2% higher than removal rates achieved
during PI (Table 2), indicating that the addition of silicone oil improved reactor performance. Following the 20 d shutdown period during PII, there was a 31 d recovery period before methane removal efficiency in the HFBRs had recovered and reached steady-state. Methane removal rates during the AS period in R1 were similar to those achieved before the shutdown, whereas removal in R2 had decreased and removal in R3 had increased (Table 2).

**Figure 3.** Methane removal in HFBRs (a) R1, (b) R2 and (c) R3 over duration of trial. Asterisk (c) indicates when urea and NH₄Cl were added to LNF in R3.
Table 2. Average methane removal of HFBRs during PI, PII BS (before shutdown), PII AS (after shutdown) and PIII.

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th></th>
<th>R2</th>
<th></th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RE (%)</td>
<td>Removal (g CH₄ m⁻³ h⁻¹)</td>
<td>RE (%)</td>
<td>Removal (g CH₄ m⁻³ h⁻¹)</td>
<td>RE (%)</td>
</tr>
<tr>
<td>PI</td>
<td>31.7</td>
<td>(13.4)</td>
<td>23.6</td>
<td>(7.2)</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
<td>(1.8)</td>
<td>3.1</td>
<td>(1.0)</td>
<td>2.3</td>
</tr>
<tr>
<td>PII (BS)</td>
<td>42.8</td>
<td>(9.5)</td>
<td>45.5</td>
<td>(10.0)</td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td>5.7</td>
<td>(1.3)</td>
<td>6.0</td>
<td>(1.3)</td>
<td>4.1</td>
</tr>
<tr>
<td>PII (AS)</td>
<td>42.4</td>
<td>(6.7)</td>
<td>40.0</td>
<td>(5.5)</td>
<td>38.3</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>(0.9)</td>
<td>5.3</td>
<td>(0.7)</td>
<td>5.0</td>
</tr>
<tr>
<td>PIII</td>
<td>62.1</td>
<td>(6.2)</td>
<td>63.9</td>
<td>(8.6)</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>(0.8)</td>
<td>7.3</td>
<td>(1.3)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

During PIII, the addition of Brij35 to the LNF in R1 and R2 improved methane removal rates (Table 2). Highest methane removal rates were observed at Brij35 concentrations of 1.0 g l⁻¹ with methane removal rates of 8.2 and 7.3 g CH₄ m⁻³ h⁻¹ observed in R1 and R2, respectively (Fig. 4). This represented an average increase in reactor performance of 33% when compared to removal rates achieved during PII after the shutdown. Brij35 concentrations of 1.0 g l⁻¹ resulted in highest methane removal rates indicating that this was the optimum concentration of methane removal. Lower removal rates were observed at Brij35 concentrations of 0.5 g l⁻¹, 0.75 g l⁻¹ and 2.0 g l⁻¹. During PIII, no Brij35 was added to R3 and methane removal rates remained the same as those seen during PII (AS).
6.4.2 Depth resolved HFBR methane oxidation rates

The methane oxidation rates of the seven sample zones were compared during steady-state operation of the HFBRs during PI, PII and PIII (Fig. 5). The methane oxidation rates varied between zones with highest rates observed in Z1 during PI for all reactors. There was no correlation between methane removal rates and the environmental parameters measured in the HFBR zones based on Pearson’s correlation coefficient (Table 3).

Table 3. Summary of Pearson’s correlation coefficient (r) analyses between the in-situ HFBR methane oxidation rates and the environmental parameters measured

<table>
<thead>
<tr>
<th>Methane Removal</th>
<th>TOC</th>
<th>NH4-N</th>
<th>NO2-N</th>
<th>NO3-N</th>
<th>PO4</th>
<th>TIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.29</td>
<td>-0.23</td>
<td>-0.16</td>
<td>0.21</td>
<td>0.12</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Figure 4. CH₄ removal during Phase III with varying influent concentrations of Brij35.
6.4.3 Water chemistry of HFBRs

Depth-resolved analysis of the liquid phase in the HFBRs was performed during PI, PII and PIII in the three HFBRs (Fig. 6). During PI the water chemistry profiles of the three HFBRs was similar with very little change in the concentration of measured liquid chemical parameters with depth in the reactors. During PII, the TOC and TIC concentrations increased in R1 and R2 due to the addition of the silicone oil. In R3 the increase in TOC and TIC was less, however there was an increase in NH₄-N concentrations due to the addition of NH₄⁺ and urea to the LNF on Day 64. During PIII the TIC concentration in R1 and R2 was much higher than in R3 due to the

**Figure 5.** *In-situ* methane oxidation in zones Z1-Z7 in the three HFBRs during steady-state operating during (a) PI, (b) PII and (c) PIII.

Methane Removal (g CH₄ m²[TPSA] d⁻¹)
addition of Brij35. Over the duration of the trial, in the three HFBRs, NO$_3^-$ and PO$_4$ concentrations did not vary much between influent and effluent samples indicating that they were not consumed or utilised by the microbial populations present. Concentrations of NO$_2^-$ and NH$_4^+$ in R1 and R2 were negligible throughout the trial at all depths. In R3, NO$_2^-$ concentrations were also negligible throughout the reactor, while the concentration of NH$_4^+$ decreased with depth but was not totally removed.

Figure 6. In-situ water chemistry in HFBRs R1 during (a) PI, (b) PII, (c) PIII; R2 during (d) PI, (e) PII, (f) PIII; and R3 during (g) PI, (h) PII, (i) PIII.
6.4.5 Impact of temperature on MOP of HFBR biofilms and seed biomass

Temperature had a significant effect on the MOP of the three HBBR biofilm samples and the seed biomass (Fig 7). The MOP of the three HFBR biofilm samples were similar over the range of temperatures tested, whereas the MOP profile of the seed biomass differed. The HFBR biofilm samples showed a linear increase in MOP between 4-23°C with an average MOP of 19.0 mg CH$_4$ g$^{-1}$ VSS d$^{-1}$ at 23°C. At 37°C the MOP was slightly lower at 17.5 mg CH$_4$ g$^{-1}$ VSS h$^{-1}$. In contrast, seed biomass methane oxidation rates continued to improve with increased temperature between 4-37°C, with a maximum MOP of 33.6 mg CH$_4$ g$^{-1}$ VSS h$^{-1}$ at 37°C. At 10°C, the MOP of the seed biomass was almost twice that of the HFBR biofilms but at 23°C was similar to the HFBR biofilms. Methane oxidation was observed at 4°C in all samples, albeit at very low rates, 1.5 and 1.1 mg CH$_4$ g$^{-1}$ VSS h$^{-1}$, for the HFBR biofilm and seed biomass respectively. The Q$_{10}$ values were determined for the HFBR biofilm and the seed biomass between at a range of temperatures (Table 4).

![Figure 7. MOP assays of the three HFBR biofilms and the seed biomass at 4, 10, 23 and 37°C](image-url)
Table 4. Q_{10} values for methane oxidation of the three HFRB biofilms and the seed biomass.

<table>
<thead>
<tr>
<th></th>
<th>4-10°C</th>
<th>4-23°C</th>
<th>4-37°C</th>
<th>10-23°C</th>
<th>10-37°C</th>
<th>23-37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFBRs*</td>
<td>5.83</td>
<td>3.78</td>
<td>2.10</td>
<td>3.09</td>
<td>1.67</td>
<td>0.94</td>
</tr>
<tr>
<td>Seed</td>
<td>31.76</td>
<td>4.33</td>
<td>2.83</td>
<td>1.73</td>
<td>1.66</td>
<td>1.59</td>
</tr>
</tbody>
</table>

*HFBR values average of the three HFBR biofilm samples.

6.4.6 Quantification of methanotrophs in HFBRs using real-time PCR

Quantification of the functional pmoA gene showed that methanotrophs were abundant in all seven zones of the three HFBRs on the three sample days (Fig 8). The absolute abundance of methanotrophs in the biofilm varied between zones and reactors on given sample days. The relative abundance of pmoA:16S rRNA bacterial genes also varied considerably between zones ranging between 0.3-17.3% (Table 5). There was no correlation between the abundance of methanotroph cells and concentration of TOC, NH_4-N, NO_2-N, NO_3-N, PO_4 or TIC present based on Pearson’s coefficient correlation (Table 6).

6.4.6 In-situ methane oxidation of biofilm during PIII

The in-situ methane oxidation rates of biofilm from each of the seven sample zones in the HFBRs were compared for PIII (Table 7). Oxidation rates were also calculated for each methanotroph present in each zone. Based on Pearson’s correlation coefficient there no correlation between (i) biofilm mass and in-situ methane removal (r=0.11), or (ii) methanotroph cell numbers and in-situ methane removal (r=0.07). The highest rates of oxidation occurred in Z1 for the three HFBRs. Methane oxidation rates varied considerably within each HFBR and there were large differences in methane oxidation rates observed in Z1 when compared to the least active zones in the reactors.
**Figure 8.** Concentration of methanotrophs in zones 1-7 in HFBRs (a) R1, (b) R2 and (c), determined by real-time quantification the functional *pmoA* gene.
Table 5. Relative abundance of *pmoA* genes: bacterial 16S rRNA genes from the seven sample zones in the three HFBRs at the end of Phase I, II and III.

<table>
<thead>
<tr>
<th></th>
<th>PI (%)</th>
<th>PII (%)</th>
<th>PIII (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 Z1</td>
<td>17.30</td>
<td>1.73</td>
<td>1.08</td>
</tr>
<tr>
<td>Z2</td>
<td>13.86</td>
<td>2.39</td>
<td>1.92</td>
</tr>
<tr>
<td>Z3</td>
<td>10.87</td>
<td>2.31</td>
<td>3.24</td>
</tr>
<tr>
<td>Z4</td>
<td>3.64</td>
<td>3.11</td>
<td>4.93</td>
</tr>
<tr>
<td>Z5</td>
<td>6.29</td>
<td>2.43</td>
<td>6.42</td>
</tr>
<tr>
<td>Z6</td>
<td>3.32</td>
<td>2.04</td>
<td>5.55</td>
</tr>
<tr>
<td>Z7</td>
<td>3.37</td>
<td>1.58</td>
<td>5.52</td>
</tr>
<tr>
<td>R2 Z1</td>
<td>20.81</td>
<td>2.44</td>
<td>0.32</td>
</tr>
<tr>
<td>Z2</td>
<td>17.85</td>
<td>3.52</td>
<td>0.33</td>
</tr>
<tr>
<td>Z3</td>
<td>1.63</td>
<td>2.15</td>
<td>1.00</td>
</tr>
<tr>
<td>Z4</td>
<td>4.83</td>
<td>2.49</td>
<td>1.17</td>
</tr>
<tr>
<td>Z5</td>
<td>1.08</td>
<td>1.40</td>
<td>1.64</td>
</tr>
<tr>
<td>Z6</td>
<td>0.54</td>
<td>1.69</td>
<td>1.71</td>
</tr>
<tr>
<td>Z7</td>
<td>0.52</td>
<td>3.58</td>
<td>1.27</td>
</tr>
<tr>
<td>R3 Z1</td>
<td>2.58</td>
<td>3.34</td>
<td>0.78</td>
</tr>
<tr>
<td>Z2</td>
<td>5.61</td>
<td>2.49</td>
<td>2.76</td>
</tr>
<tr>
<td>Z3</td>
<td>6.10</td>
<td>1.92</td>
<td>3.71</td>
</tr>
<tr>
<td>Z4</td>
<td>2.96</td>
<td>0.80</td>
<td>2.93</td>
</tr>
<tr>
<td>Z5</td>
<td>2.01</td>
<td>0.97</td>
<td>2.87</td>
</tr>
<tr>
<td>Z6</td>
<td>1.38</td>
<td>0.67</td>
<td>2.38</td>
</tr>
<tr>
<td>Z7</td>
<td>0.92</td>
<td>0.76</td>
<td>1.31</td>
</tr>
</tbody>
</table>

Table 6. Summary of Pearson’s correlation coefficient (*r*) analyses carried out the (i) the abundance of methanotrophs and (ii) the mass of biofilm, with chemical parameters measured in the HFBRs.

<table>
<thead>
<tr>
<th></th>
<th>TOC</th>
<th>NH$_4$-N</th>
<th>NO$_2$-N</th>
<th>NO$_3$-N</th>
<th>PO$_4$</th>
<th>TIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanotroph Cells</td>
<td>-0.11</td>
<td>0.22</td>
<td>0.12</td>
<td>-0.54</td>
<td>-0.61</td>
<td>-0.18</td>
</tr>
<tr>
<td>Biofilm (g VSS)*</td>
<td>-0.25</td>
<td>0.55</td>
<td>-0.14</td>
<td>0.34</td>
<td>-0.15</td>
<td>-0.34</td>
</tr>
</tbody>
</table>

*Correlation between biofilm mass and chemical parameter calculated for PIII only*
Table 7. *In-situ* methane oxidation rates for PIII. Removal expresses in terms of (a) g CH$_4$/g VSS (biofilm)/h and (b) g CH$_4$/methanotroph cell($10^{-14}$)/h.

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biofilm g CH$_4$ g$^{-1}$ [VSS]</td>
<td>Methanotroph Cells (g CH$_4$/cell) x10$^{-14}$</td>
<td>Biofilm g CH$_4$ g$^{-1}$ [VSS]</td>
</tr>
<tr>
<td>Z1</td>
<td>0.66</td>
<td>7.75</td>
<td>0.70</td>
</tr>
<tr>
<td>Z2</td>
<td>0.03</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>Z3</td>
<td>0.17</td>
<td>4.65</td>
<td>0.06</td>
</tr>
<tr>
<td>Z4</td>
<td>0.09</td>
<td>1.80</td>
<td>0.13</td>
</tr>
<tr>
<td>Z5</td>
<td>0.04</td>
<td>1.00</td>
<td>0.02</td>
</tr>
<tr>
<td>Z6</td>
<td>0.05</td>
<td>1.40</td>
<td>0.04</td>
</tr>
<tr>
<td>Z7</td>
<td>0.09</td>
<td>2.46</td>
<td>0.13</td>
</tr>
</tbody>
</table>
6.4.7 TGGE fingerprinting of the methanotroph communities in the seed and HFBR biomass

The methanotroph communities in the seed biomass and in the composite HFBR biofilm samples from the end of each phase were compared using TGGE fingerprinting of the functional pmoA gene (Fig. 9). TGGE banding patterns showed that the methanotrophic community differed between samples and changed over time. In total, six distinct bands were visible across all ten samples. MDS plots, based on the presence/absence of bands in the TGGE gel, indicated high levels of similarity between the HFBR samples (>80%). The seed culture had the lowest similarity to any other sample, but still showed >60% with the HFBR samples. The methanotroph community in the HFBRs on Day 1 clustered together, separately from HFBR samples from Day 2 and Day 3 which also clustered together (Fig. 10). Sequencing of bands 1-6 revealed that the methanotroph population comprised of Type I *Methylobacter* and *Methylomonas* species and Type II *Methylocystis* species (Fig. 11). *Methylobacter* and *Methylomonas* were present in all samples while *Methylocystis*, which was represented by bands 4 and 5 was not. Bands 1, 4 and 6 represented single species, while bands 2, 3 and 5 represented mixed methanotrophic communities (Fig. 11).

Based on the presence/absence of bands from TGGE fingerprinting and using permutational MANOVA (Bray-Curtis distance), the overall methanotrophic communities in the three HFBRs were shown not to be significantly different (p=0.603), whereas there was a significant difference between the community structure on different sampling days (p=0.019) (Table 8).
Figure 9. TGGE gel of methanotrophic communities of seed and nine HFBR biofilm samples. Samples labels; D represent sample day and R represented reactor number. For example D1R1 refers to sample Day 1 Reactor 1.

Table 8. PERMANOVA of TGGE banding pattern of methanotrophic community in HFBRs

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>SS</th>
<th>MS</th>
<th>Pseudo-F</th>
<th>P(perm)</th>
<th>Perms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor</td>
<td>2</td>
<td>27.435</td>
<td>13.717</td>
<td>1</td>
<td>0.603</td>
<td>11</td>
</tr>
<tr>
<td>Day</td>
<td>2</td>
<td>560.77</td>
<td>280.38</td>
<td>20.44</td>
<td>0.019</td>
<td>558</td>
</tr>
</tbody>
</table>
Figure 10. Non Multi-Dimensional Scaling (MDS) of Bray-Curtis similarity matrix of methanotroph communities based on presence/absence of bands from TGGE fingerprinting. Samples labels; D represent sample day and R represented reactor number. For example D1R1 refers to sample Day 1 Reactor 1.
**Figure 11.** Neighbour joining tree depicting the methanotrophs sequenced from the TGGE bands 1-6, based on Jukes-Cantor distances of the *pmoA* genes. The sequences obtained in this study are depicted in bold and the band from which they were sequenced is in red. Reference sequences were downloaded from the GenBank sequence database.
6.5 DISCUSSION

HFBRs were successfully used to treat a methane-contaminated airstream, with average maximum removal rates of 8.2 g CH\(_4\) m\(^3\) h\(^{-1}\) and removal efficiencies of 62% achieved. Biological treatment of methane in biotrickling filters can often be limited by mass transfer of methane from the gas to liquid phase, requiring long gas retention times and reducing methane elimination capacities. During PII and PIII the addition of silicone oil and Brij35 to the LNF improved methane removal in the HFBRs. The addition of silicone oil resulted in an average increase in methane removal rates of 69% across all three HFBRs during PII before the shutdown period. It was previously shown that silicone oil, when used as a mass transfer vector for methane, resulted in increased methane removal efficiencies in a stirred tank reactor by 41% and in a trickling bed reactor by 131% (Rocha-Rios et al., 2009). Brij35 is a non-ionic surfactant that increases the solubility of methane in water (King Jr, 1992). It has been shown to act as transfer vector in a methane oxidising biofilm (Ramirez et al., 2012) and the addition of Brij35 to R1 and R2 resulted in an increase in reactor performance of 46% and 60%, respectively, whereas there was no improvement in R3 which had no Brij35. Although Brij 35 has previously been shown to be both biodegradable and non-toxic to methanotrophic biomass at concentrations of < 5 g l\(^{-1}\) (Avalos Ramirez et al., 2012b), the optimum concentration of Brij35 in this trial was 1.0 g l\(^{-1}\), above which methane oxidation rates were inhibited.

Rocha-Rios et al. (2013) found that the effectiveness of silicone oil as a transfer vector is dependent on the degree of oil dispersion in the liquid phase. Previous studies have used mechanical turbulence to disperse silicone oil within bioreactors (Arriaga et al., 2006; Rocha-Rios et al., 2009), however as Brij 35 has a hydrophilic-lipophilic balance (HLB) which solubilises oils into water, it may have improved the degree of silicone oil dispersion within the HFBR without the need for mechanical agitation. Further investigation is required to determine if the increases HFBR methane removal rates post Brij35 addition, were due to the Brij35 acting as a vector for mass transfer, or if it was due to improved silicone oil dispersal. From these results it is evident that the addition of silicone oil and Brij35 improved the methane elimination capacity of the HFBRs. However in real-world applications the cost of these materials may be prohibitory. To reduce costs it may be possible to separate
the silicone oil from the liquid effluent and recycle it back into the HFBR. The feasibility of this approach would have to be investigated further.

The composition of the LNF used during PI was identical to a LNF optimised in a biofilter study by Nikiema et al. (2009), rather than the LNF used in Chapter 5. This LNF contained no organic carbon, as it was previously shown in Chapter 5 that methane oxidation rates were highest when organic carbon was omitted from the LNF. However, the addition of Brij35 and silicone oil to the LNF during PI and PII increased in the organic carbon concentration in the HFBRs. Although the organic carbon concentration in the liquid phase increased during the latter phases of the trial the addition of the silicone oil and Brij35 still had a positive impact on methane removal rates. Silicone oil is a non-biodegradable synthetic organosilicon (Rocha-Rios et al., 2009) whereas Brij35, which cannot be directly utilised by methanotrophs, can be metabolised by other heterotrophic microbes. Introducing Brij35 into the HFBRs may have facilitated the development of a heterotrophic population that was able to utilise the extra carbon in the reactors. As the composition of the bacterial community in the HFBRs was not assessed during this trial, the impact of Brij35 and silicone oil on the microbial community structure is unknown. It would be useful, in future studies, to perform HFBR biofilm batch incubations with silicone oil and Brij35, to determine their impact on methane oxidation rates, and assess the biodegradability of Brij35.

The LNF contained 1.51 g l\(^{-1}\) NaNO\(_3\), which was deemed to be the optimum nitrogen load for methane oxidation (Nikiema et al., 2009). During the first 64 days of PI, the removal efficiency of R3 was lower than in R1 and R2. It is unclear why methane removal rates in R3 were lower as the HFBRs were operated in triplicate, seeded with the same inoculum and the environmental conditions between reactors during PI were not statistically different. As the presence of inorganic nitrogen such as NH\(_4^+\), NO\(_3^-\) and NO\(_2^-\) can either enhance or limit the methane-oxidising potential of a methanotrophic population (Hanson and Hanson, 1996), NH\(_4\)Cl and urea were added to the LNF of R3 on Day 64 to investigate their effect on methane removal. Following the amendment to the LNF for R3, increased methane removal was observed. Similar effects have been recorded in other studies where increased methane oxidation rates were observed when NH\(_4^+\) was added to landfill cover soil (De Visscher et al., 2001) and littoral sediments (Bosse et al., 1993), and when urea
was added to rice field soil (Yang and Chang, 1998). The concentration of NH$_4^+$ in
the liquid phase decreased with depth in the HFBR, however the fate of the NH$_4^+$
was unclear. It is possible that the NH$_4^+$ may have been reduced to NO$_2^-$ or NO$_3^-$ as
part of the biological nitrification process but as the average influent concentrations
of NO$_3^-$ (247 mg l$^{-1}$) were over thirteen times higher than NH$_4^+$ (18.5 mg l$^{-1}$)
concentrations, any contribution of NH$_4^+$ to the total NO$_3^-$ concentrations were
difficult to assess.

A shutdown period of 20 days was applied to the HFBR during PII. When the
methane supply was reintroduced after the shutdown period immediate methane
oxidation was detected, however it took 31 d before the HFBRs reached steady-state.
Although the HFBRs did recover, they recovered with different methane removal
capacities, indicating that the impact of the shutdown had a varying effect on the
reactors.

*In-situ* methane oxidation rates varied between zones in the HFBRs indicating that
location in the reactor was important with respect to biological activity. There are
several factors that can affect the methane oxidation rate of methanotrophs including
temperature (Börjesson *et al*., 2004); nitrogen source and concentration (Bodelier
and Laanbroek, 2004); substrate availability (Henckel *et al*., 2000); substrate
concentration (López *et al*., 2014); moisture levels (Einola *et al*., 2007), and O$_2$
concentration (Teh *et al*., 2005). As the HFBR is a biotrickling system, gradients in
these parameters existed with depth in the reactor and it was shown in Chapter 5 that
the MOP of biofilm varied with location in the HFBR. As there was no correlation
between *in-situ* removal rates and CH$_4$, NO$_3^-$, NO$_2^-$, NH$_4^+$, TOC, TIC or PO$_4$
concentrations it is possible that other unmeasured chemical or physical parameters
were responsible for the variations in activity. Throughout the trial there was no
correlation between methanotroph abundance and the rate of *in-situ* methane
oxidation in the HFRBs, and during PIII there was no correlation between biofilm
density and methanotroph abundance. This indicated that the functional *pmoA* gene
concentration or the mass of biofilm were not accurate proxies for methane oxidation
activity. It was not possible to determine these rates for PI or PII, as the mass of
biofilm in each zone was unknown during these phases.
Although real-time PCR is an excellent molecular tool for the quantification of target genes, it does not give any information on gene expression levels. In contrast, reverse transcriptase-PCR (RT-PCR) only targets actively-expressed genes, and in a previous study by Chen et al. (2008) RT-PCR was used to quantify pmoA gene expression levels in peatland soil. RT-PCR could be applied to HFBRs in future studies to link gene expression levels and methane oxidation rates and to determine if they are correlated. In contrast to this study, Kong et al. (2013) successfully correlated pmoA gene copies to methane oxidation rates in landfill cover soils using qPCR rather than RT-PCR.

It has been repeatedly shown that temperature impacts on biological methane oxidation rates (Whalen et al., 1990; Gebert et al., 2003; Börjesson et al., 2004). In the batch MOP assays the highest methane oxidation rates in the HFBR biofilm was observed at 23°C whereas in the seed biomass the highest oxidation rates occurred at 37°C. The optimum temperature for methane oxidation can range from as low as 4°C in permafrost soils (Liebner and Wagner, 2007) up to 60°C in a thermophilic methanotroph strain isolated from a thermal spring in New Zealand (Dunfield et al., 2007), however most methanotrophs available in pure cultures are mesophilic (He et al., 2012). The shift in the methanotrophic community in the HFBRs over time, as observed from the TGGE fingerprinting, likely affected the methane oxidising potential of the biofilm. The seed biomass was incubated in batch cultures for four months. Although this facilitated the development of a methane oxidising community it did not accurately represent the conditions in the HFBRs, which may explain why the MOP of the HBFR biofilm differed from the seed biomass. The HFBRs were operated for the duration of the trial at 10°C so the effect of temperature on the methane elimination capacity was not investigated in-situ. However, the MOP of the HFBR biofilm increased with temperature in the batch assays it is likely that an increase in temperature in the HFBRs would result in higher methane removal efficiencies. Temperature was previously shown to impact on HFBR removal efficiencies in Chapter 5.

Quantification of the functional pmoA gene and the bacterial 16S rRNA genes indicated that methanotrophs were present in large numbers in the HFBRs and that their abundance varied between zones. As methanotrophs contain between 1-3
copies of the 16S rRNA gene (Auman et al., 2000), a large proportion of the 16S rRNA genes detected can be attributed to other unidentified bacterial species. In addition to supporting a methane-oxidising microbial community, environmental conditions in the HFBRs likely facilitated the development of other bacterial trophic groups involved in biological processes such as carbon, nitrogen and phosphorus cycling. As the focus of this trial was on the methanotroph communities present in the HFBRs, the composition of the non-methane oxidising bacterial community was not investigated.

Genetic fingerprinting of the methanotrophic community in the HFBRs and in the seed biomass, using TGGE, showed that the overall methanotrophic diversity was low and that Type I methanotrophs Methylobacter and Methylomonas, and Type II Methylocystis were present. Type I methanotrophs occurred more often than Type II methanotrophs in the TGGE profiles, however as TGGE is not a quantitative technique it is not possible to conclude whether Type I methanotrophs were more abundant. To accurately quantify Type I and Type II methanotrophs, qPCR would be required of the 16S rRNA genes would be required. Numerous studies have quantified both groups using group specific 16S rRNA primers from various environments including, constructed wetlands (DeJournett et al., 2007) and soils (Zheng et al., 2012). The methanotrophic community in the seed biomass showed the least similarity to any of the HFBR samples, indicating that there was a shift in the methanotrophic community in the HFBRs over time. Conditions in the enrichment cultures did not accurately replicate conditions in the HFBRs. This resulted in changes in the methanotroph community composition. Methane concentrations have been shown to strongly influence microbial community structure (López et al., 2014). Methane concentrations in the batch enrichments (0.5-10%) were much higher than influent concentrations in the HFBRs (1.6%), which may have impacted on community composition. Other variations that may have impacted on community structures include differences between the moisture content in the cultures and in the HFBRs, and difference in the composition of the AWM and the LNF. As the HFBRs were operated at 10°C for the duration of the trial, temperature likely affected the composition of the methanotrophic community. The methanotrophic community in the HFBRs also changed over the duration of the trial. The communities present at the end of PI, differed from the communities present on later days. The changes in
community structure may have been due to the addition of silicone oil during PII. TGGE fingerprinting was successfully used to highlight differences in methanotroph community structure between the HFBRs. Sequencing of the TGGE bands revealed that some bands represented more than one methanotroph species, indicating that TGGE failed to separate the pmoA gene of some species. Overall the methanotroph population was quite conserved with only 16 OTUs detected from the six TGGE bands, however based on the qPCR results it was evident that a large proportion of the bacterial community comprised of other unidentified species.

6.6 CONCLUSIONS

This trial demonstrated that the addition of silicone oil and Brij35 to the LNF increased the methane elimination capacity of the HFBRs. There was no correlation between in-situ methane oxidation rates in the HFBR and methanotroph numbers, biofilm density or NO₃⁻, NO₂⁻, NH₄⁺, TOC, TIC or PO₄ concentrations. The bacterial community largely constituted of unidentified bacterial species but methanotrophs were abundant in all zones of the HFBRs. Based on genetic fingerprinting of the methanotrophic community, using TGGE, the overall community composition was highly conserved, although there was some variation in community structure between reactors and sampling days. Type I Methylobacter and Methylomonas species and Type II Methylocystis species were the only methanotrophs detected during the trial.

6.6 REFERENCES


Hanson RS, Hanson TE. 1996. Methanotrophic bacteria. Microbiological Reviews 60(2):439-+


CHAPTER 7

Conclusions and future work
7 CONCLUSIONS AND FUTURE WORK

7.1 HFBR design and future concepts

HFBRs are a novel bioreactor design that were shown to be capable of treating contaminated airstreams at low temperatures. During this work, HFBRs were successfully used to treat ammonia, H₂S and methane contaminated airstreams, separately, at 10°C. The configuration of the HFBR allowed for depth resolved biological, gas and liquid phase analyses.

During the ammonia trial, average HFBR removal efficiencies of 99.7% were achieved at loading rates of 4.8g NH₃ m⁻³ h⁻¹. The ammonia gas was dissolved into the liquid phase and converted to NO₂⁻ and NO₃⁻ by biological nitrification under aerobic conditions. Highest NH₃ removal rates were observed in the upper regions of the HFBRs and all of the influent NH₃ was removed from the gas stream by sheet 30. The ammonia loading rates applied to the HFBRs during this trial were not high enough to reach the critical loading rate (defined as the maximum load at which 95% removal occurs). Higher ammonia removal rates would likely have been achieved at increased loading rates. Further studies would be required to determine the maximum ammonia removal potential of HFBRs.

H₂S removal rates of up to 15.1 g H₂S m⁻³ h⁻¹ with removal efficiencies of 99% were achieved in the HFBRs during the 180 day hydrogen sulphide trial. Highest H₂S oxidation rates were achieved in the upper regions of the HFBR. Bio-oxidation of H₂S in the reactors led to severe acidification of the liquid phase and the production of a sulphate (SO₄²⁻) rich liquid effluent. Buffering the HFBRs with NaHCO₃ at loading rates of 15.1 g H₂S m⁻³ h⁻¹ resulted in improved H₂S removal rates. Similarly to the ammonia trial, the loading rates applied to the HFBRs during this trial were not high enough to determine the critical loading rate. Further studies at higher loading rates would be required to determine the maximum H₂S removal potential of HFBRs.

The maximum methane removal rate achieved by the HFBRs over the course of the two methane trials was 8.2 g CH₄ m⁻³ h⁻¹. The composition of the LNF had an impact on HFBR performance and the omission of organic carbon from the LNF during the first trial improved methane removal rates. To improve mass transfer of methane into
the liquid phase, two transfer vectors, silicone oil and Brij35, were added to the LNF during the second methane trial. The addition of silicone oil improved methane removal rates by 69%, while the addition of Brij35 further improved removal rates by 39%, showing that transfer vectors are an effective method to improve the methane elimination capacity of HFBRs. Temperature impacted on the methane oxidation rates of the HFBR biofilms in situ and in MOP assays. During a cold-shock perturbation applied to the HFBRs during the first methane trial, methane removal rates decreased when the temperature was reduced. MOP assays of HFBR biofilms from the second methane trial showed that methane oxidation rates were higher at 23°C, when compared to rates observed at 4, 10 and 37°C.

Although HFBRs were shown to be capable of treating ammonia, H₂S and methane contaminated airstreams, there are a number of outstanding research questions that could be addressed in future studies. As well as determining the critical loading rates for H₂S and NH₃, operating the HFBRs at increased temperatures would be advisable. Temperature is a key factor that influences biological activity and most microorganisms involved in ammonia, H₂S and methane oxidation are mesophilic. Most other bioreactor trials treating contaminated airstreams are operated at mesophilic temperatures and it is possible that operating the HFBRs at increased temperatures may have improved removal rates. However, to demonstrate this further investigation is required.

Other potential variables that may impact on gas removal rates that were not assessed during this work, but could be of interest in the future, include the LNF loading rate and design of the plastic sheet media. The LNF is required to firstly transfer the pollutant gas into the liquid phase where it becomes available to the microorganisms growing in the biofilm. The second major role of the LNF is to provide essential nutrients to the microorganisms. The loading rate and flow rate of the LNF in the HFBR may have had an impact on the mass transfer of the gas into the liquid phase. This would be especially important for the methane trials as the solubility of methane is very low. The impact of LNF loading rates and flow rates were not investigated in any of the trials undertaken during this study. Varying these parameters would offer a potential mechanism to improve gas removal rates in HBFRs. Another possible opportunity to increase removal rates would be to redesign the plastic sheets used in the construction of the HFBRs. The plastic sheets were
designed with elevated frustums to increase surface area and allow for biofilm growth and attachment. It is possible that these plastic sheets could be redesigned to further increase surface area available for biofilm colonisation and to limit biomass washout in the liquid effluent. By providing more surface area for biofilm growth and preventing biofilm washout more active biomass would be retained in the HFBRs which may in turn lead to higher gas removal rates.

During this work the three gases were treated separately, whereas some other biotechnologies have been used to treat different gases simultaneously. In real world applications waste gases often contain more than one type of gas and it is likely that HFBRs could also be used in this scenario. Future studies would be required to test the feasibility of co-treating different gases using HFBRs.

One of the advantages of the HFBR design is the ability to monitor numerous physicochemical parameters at various depths in the reactor. This detailed environmental data set could be used to provide mathematical modelling of the HFBRs when treating various gases. Mathematical modelling was beyond the scope of this project but could be used in future studies to predict the removal potential of a HFBR when operated under certain conditions. Modelling of the system could be used to maximise removal as efficiently as possible. Mathematical models are also used for designing reactors when scaling up from lab-scale to either pilot or full-scale operation.

This was the first time that HFBRs were used to treat contaminated airstreams and this work has proved that they are a suitable biotechnology for gas treatment. However, there is scope to optimise the HFBR design and operating conditions to maximise and improve removal rates and efficiencies. With future work it is conceivable that HFBRs could become the favoured biological treatment method to treat contaminated airstreams.

7.2 Microbial ecology of HFBRs and future investigations

The microbial populations in the HFBRs were assessed using a variety of microbial ecology techniques throughout the four trials. In the ammonia trial biological nitrification was carried out by a mix of bacterial and archaeal nitrifying
communities. AOB and AOA were responsible for the oxidation of ammonia to nitrite, where the AOB vastly outnumbered AOA at all depths in the HFBRs. AOB from the Nitrosomonas and Nitrosospira genera were the most abundant bacterial species, while an uncultured archaea clone was the most common AOA. NOB from the Candidatus nitrotoga genera were also detected in the HFBRs, while conditions in the reactors also supported a diverse bacterial community that was not involved in the nitrification process. The overall bacterial diversity between the three HBFRs was highly conserved, although variations in community structure did occur between different zones in the HFBR, partly driven by environmental variables.

The bacterial diversity in the three HFBRs treating H₂S contaminated air was low and the community was dominated by two known H₂S oxidisers, Acidithiobacillus and Thiobacillus, that have previously been shown to grow well under acidic conditions. Even though the bacterial diversity was low there were distinct differences between the bacterial community structure between zones in the HFBRs. These differences were strongly influenced by H₂S concentrations in the gas phase and alkalinity, pH and SO₄ concentrations in the liquid phase.

During both methane trials, Type I and Type II methanotrophs were detected in the HFBRs, although the bacterial communities were dominated by other unidentified prokaryotes. In the first methane trial, TRFLP fingerprinting of the bacterial 16S rRNA gene showed that there was a diverse and dynamic bacterial population that varied over time and between zones in the HFBRs. Methanotrophs were abundant in all HBFR zones throughout the second trial and the methanotrophic community composed of Type I Methylobacter and Methylomonas species and Type II Methylocystis species.

Key microorganisms present in the HFBRs were quantified using qPCR. Although qPCR is an excellent molecular tool for the quantification of target genes, it only amplifies genes from cellular DNA which does not give any information on gene expression levels or cell activity. Therefore it was not possible to link gene abundance to activity rates measured in the HFBRs. Future work focused on RT-PCR of target genes, which would only quantify actively expressed genes, may allow researchers to determine the oxidation rates of active cells. To get a better understanding of the microbial ecology of the HFBRs, a combination of omics
techniques could be employed in a systems biology approach. Simultaneous use of metagenomics and either proteomics, transcriptomics or metabolomics, would allow researchers to investigate the microorganisms present and the pathways utilised in the oxidation processes in more detail. With the costs of these techniques becoming less inhibitory, HFBR systems are an ideal technology to investigate the complexities of biological gas remediation. From this information mathematic modelling of the microbial community dynamics in the HFBR system could be performed. Linking community structure to environmental measurements in the HFBRs could result in the development of a mathematical model that could be used to predict the microbial community development in future studies. The model may also be used to determine the operating conditions required to facilitate the development of a desired microbial consortium in a HBFR. Although mathematic modelling of the microbial community dynamics in complex ecosystems is often difficult it can be useful in making decisions on how best to operate HFBRs in the future.

In summary, HFBRs were shown to be capable of treating ammonia, H\textsubscript{2}S and methane contaminated airstreams. The microbial populations present were investigated with a focus on the key active members. With future work, HFBR performances could be further improved and a more comprehensive understanding of the molecular ecology of the reactors could be achieved.
**SUPPLEMENTARY DATA**

**Table S1.** Results from SIMPER analyses of TRFLP data from R1. The top five TRFs for each sample day (D1-D5) are shown

<table>
<thead>
<tr>
<th>R1</th>
<th>Species</th>
<th>Av.Abund</th>
<th>Av.Sim</th>
<th>Sim/SD</th>
<th>Contrib%</th>
<th>Cum.%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D1</strong></td>
<td>59.45</td>
<td>6.48</td>
<td>18.52</td>
<td>0.93</td>
<td>64.16</td>
<td>64.16</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>0.75</td>
<td>1.53</td>
<td>0.61</td>
<td>5.31</td>
<td>69.47</td>
</tr>
<tr>
<td></td>
<td>70.74</td>
<td>0.71</td>
<td>1.15</td>
<td>0.61</td>
<td>3.98</td>
<td>73.46</td>
</tr>
<tr>
<td></td>
<td>200.92</td>
<td>0.63</td>
<td>1.09</td>
<td>0.61</td>
<td>3.78</td>
<td>77.23</td>
</tr>
<tr>
<td></td>
<td>238.55</td>
<td>0.68</td>
<td>1.05</td>
<td>0.58</td>
<td>3.63</td>
<td>80.87</td>
</tr>
<tr>
<td><strong>D2</strong></td>
<td>233.43</td>
<td>2.18</td>
<td>2.57</td>
<td>0.77</td>
<td>20.37</td>
<td>20.37</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>1.46</td>
<td>1.77</td>
<td>0.75</td>
<td>14.05</td>
<td>34.42</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1.4</td>
<td>1.16</td>
<td>0.47</td>
<td>9.19</td>
<td>43.61</td>
</tr>
<tr>
<td></td>
<td>69.92</td>
<td>1.36</td>
<td>1.12</td>
<td>0.48</td>
<td>8.86</td>
<td>52.47</td>
</tr>
<tr>
<td></td>
<td>70.74</td>
<td>1.82</td>
<td>1.06</td>
<td>0.26</td>
<td>8.45</td>
<td>60.93</td>
</tr>
<tr>
<td><strong>D3</strong></td>
<td>458.95</td>
<td>1.41</td>
<td>2.04</td>
<td>0.78</td>
<td>15.16</td>
<td>15.16</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>1.04</td>
<td>1.39</td>
<td>0.78</td>
<td>10.33</td>
<td>25.49</td>
</tr>
<tr>
<td></td>
<td>137.69</td>
<td>1.01</td>
<td>1.22</td>
<td>0.63</td>
<td>9.03</td>
<td>34.52</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1.19</td>
<td>1.01</td>
<td>0.48</td>
<td>7.49</td>
<td>42.01</td>
</tr>
<tr>
<td></td>
<td>59.45</td>
<td>2.26</td>
<td>0.95</td>
<td>0.26</td>
<td>7.09</td>
<td>49.1</td>
</tr>
<tr>
<td><strong>D4</strong></td>
<td>59.45</td>
<td>4.34</td>
<td>6.26</td>
<td>0.48</td>
<td>22.87</td>
<td>22.87</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>1.17</td>
<td>2.76</td>
<td>5.77</td>
<td>10.07</td>
<td>32.95</td>
</tr>
<tr>
<td></td>
<td>276.68</td>
<td>1.03</td>
<td>2.36</td>
<td>1.31</td>
<td>8.61</td>
<td>41.56</td>
</tr>
<tr>
<td></td>
<td>339.38</td>
<td>0.81</td>
<td>1.98</td>
<td>1.35</td>
<td>7.21</td>
<td>48.77</td>
</tr>
<tr>
<td></td>
<td>249.16</td>
<td>0.8</td>
<td>1.9</td>
<td>1.36</td>
<td>6.93</td>
<td>55.7</td>
</tr>
<tr>
<td><strong>D5</strong></td>
<td>233.43</td>
<td>1.53</td>
<td>3.91</td>
<td>7.18</td>
<td>18.15</td>
<td>18.15</td>
</tr>
<tr>
<td></td>
<td>70.74</td>
<td>2.17</td>
<td>3.26</td>
<td>1.28</td>
<td>15.15</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>53.21</td>
<td>2.94</td>
<td>2.41</td>
<td>0.47</td>
<td>11.21</td>
<td>44.51</td>
</tr>
<tr>
<td></td>
<td>138.74</td>
<td>1.22</td>
<td>2.29</td>
<td>1.29</td>
<td>10.64</td>
<td>55.15</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1.22</td>
<td>2.21</td>
<td>0.77</td>
<td>10.25</td>
<td>65.4</td>
</tr>
<tr>
<td><strong>D6</strong></td>
<td>235.56</td>
<td>3.12</td>
<td>5.26</td>
<td>1.27</td>
<td>22.21</td>
<td>22.21</td>
</tr>
<tr>
<td></td>
<td>200.92</td>
<td>2.8</td>
<td>4.74</td>
<td>1.15</td>
<td>20.05</td>
<td>42.26</td>
</tr>
<tr>
<td></td>
<td>137.69</td>
<td>1.87</td>
<td>3.44</td>
<td>1.18</td>
<td>14.53</td>
<td>56.79</td>
</tr>
<tr>
<td></td>
<td>127.45</td>
<td>1.65</td>
<td>2.11</td>
<td>0.78</td>
<td>8.91</td>
<td>65.7</td>
</tr>
<tr>
<td></td>
<td>183.43</td>
<td>1.54</td>
<td>2.11</td>
<td>0.72</td>
<td>8.9</td>
<td>74.6</td>
</tr>
</tbody>
</table>
Table S2. Results from SIMPER analyses of TRFLP data from R2. The top five TRFs for each sample day (D1-D5) are shown.

<table>
<thead>
<tr>
<th>R2</th>
<th>Species</th>
<th>Av.Abund</th>
<th>Av.Sim</th>
<th>Sim/SD</th>
<th>Contrib%</th>
<th>Cum.%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D1</strong></td>
<td>59.45</td>
<td>7.15</td>
<td>23.04</td>
<td>1.15</td>
<td>66.11</td>
<td>66.11</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>1.25</td>
<td>3.51</td>
<td>1.06</td>
<td>10.08</td>
<td>76.19</td>
</tr>
<tr>
<td></td>
<td>238.55</td>
<td>0.82</td>
<td>1.42</td>
<td>0.59</td>
<td>4.09</td>
<td>80.27</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1.24</td>
<td>1.29</td>
<td>0.6</td>
<td>3.7</td>
<td>83.97</td>
</tr>
<tr>
<td></td>
<td>249.16</td>
<td>0.72</td>
<td>1.19</td>
<td>0.61</td>
<td>3.4</td>
<td>87.37</td>
</tr>
<tr>
<td><strong>D2</strong></td>
<td>59.45</td>
<td>3.34</td>
<td>2.77</td>
<td>0.46</td>
<td>14.24</td>
<td>14.24</td>
</tr>
<tr>
<td></td>
<td>231.43</td>
<td>1.66</td>
<td>2.15</td>
<td>0.78</td>
<td>11.07</td>
<td>25.31</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>1.05</td>
<td>1.36</td>
<td>1.23</td>
<td>6.98</td>
<td>32.29</td>
</tr>
<tr>
<td></td>
<td>205.6</td>
<td>1.37</td>
<td>1.34</td>
<td>0.71</td>
<td>6.9</td>
<td>39.19</td>
</tr>
<tr>
<td></td>
<td>339.38</td>
<td>0.91</td>
<td>1.34</td>
<td>1.31</td>
<td>6.87</td>
<td>46.06</td>
</tr>
<tr>
<td><strong>D3</strong></td>
<td>184.55</td>
<td>1.06</td>
<td>1.11</td>
<td>0.77</td>
<td>10.09</td>
<td>10.09</td>
</tr>
<tr>
<td></td>
<td>238.55</td>
<td>0.96</td>
<td>0.78</td>
<td>0.48</td>
<td>7.15</td>
<td>17.25</td>
</tr>
<tr>
<td></td>
<td>275.44</td>
<td>0.8</td>
<td>0.76</td>
<td>0.7</td>
<td>6.94</td>
<td>24.18</td>
</tr>
<tr>
<td></td>
<td>59.45</td>
<td>1.94</td>
<td>0.65</td>
<td>0.26</td>
<td>5.92</td>
<td>30.11</td>
</tr>
<tr>
<td></td>
<td>123.05</td>
<td>0.62</td>
<td>0.53</td>
<td>0.67</td>
<td>4.86</td>
<td>34.97</td>
</tr>
<tr>
<td><strong>D4</strong></td>
<td>56.05</td>
<td>4.38</td>
<td>6.73</td>
<td>0.48</td>
<td>37.59</td>
<td>37.59</td>
</tr>
<tr>
<td></td>
<td>234.56</td>
<td>1.27</td>
<td>1.9</td>
<td>0.48</td>
<td>10.61</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td>70.74</td>
<td>1.13</td>
<td>1.71</td>
<td>0.68</td>
<td>9.58</td>
<td>57.78</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1</td>
<td>1.09</td>
<td>0.47</td>
<td>6.07</td>
<td>63.85</td>
</tr>
<tr>
<td></td>
<td>275.44</td>
<td>0.61</td>
<td>0.76</td>
<td>0.48</td>
<td>4.26</td>
<td>68.11</td>
</tr>
<tr>
<td><strong>D5</strong></td>
<td>73.72</td>
<td>2.62</td>
<td>3.9</td>
<td>1.06</td>
<td>24.26</td>
<td>24.26</td>
</tr>
<tr>
<td></td>
<td>55.01</td>
<td>2.91</td>
<td>2.5</td>
<td>0.32</td>
<td>15.52</td>
<td>39.78</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>1.12</td>
<td>1.98</td>
<td>0.91</td>
<td>12.34</td>
<td>52.12</td>
</tr>
<tr>
<td></td>
<td>138.74</td>
<td>0.85</td>
<td>1.24</td>
<td>1.01</td>
<td>7.69</td>
<td>59.81</td>
</tr>
<tr>
<td></td>
<td>200.92</td>
<td>0.91</td>
<td>1.18</td>
<td>0.61</td>
<td>7.33</td>
<td>67.14</td>
</tr>
<tr>
<td><strong>D6</strong></td>
<td>38.52</td>
<td>3.46</td>
<td>4.8</td>
<td>0.48</td>
<td>27.37</td>
<td>27.37</td>
</tr>
<tr>
<td></td>
<td>138.74</td>
<td>1.51</td>
<td>3.39</td>
<td>0.77</td>
<td>19.31</td>
<td>46.67</td>
</tr>
<tr>
<td></td>
<td>69.92</td>
<td>1.88</td>
<td>2.66</td>
<td>0.48</td>
<td>15.15</td>
<td>61.82</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>1.8</td>
<td>2.5</td>
<td>0.63</td>
<td>14.25</td>
<td>76.08</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1.45</td>
<td>1.42</td>
<td>0.46</td>
<td>8.08</td>
<td>84.15</td>
</tr>
</tbody>
</table>
Table S3. Results from SIMPER analyses of TRFLP data from R3. The top five TRFs for each sample day (D1-D5) are shown

<table>
<thead>
<tr>
<th>R3</th>
<th>Species</th>
<th>Av.Abund</th>
<th>Av.Sim</th>
<th>Sim/SD</th>
<th>Contrib%</th>
<th>Cum.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>59.45</td>
<td>4.96</td>
<td>9</td>
<td>0.62</td>
<td>27.81</td>
<td>27.81</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>2.68</td>
<td>6.07</td>
<td>1.16</td>
<td>18.75</td>
<td>46.56</td>
</tr>
<tr>
<td></td>
<td>238.55</td>
<td>1.89</td>
<td>4.87</td>
<td>8.28</td>
<td>15.05</td>
<td>61.61</td>
</tr>
<tr>
<td></td>
<td>282.01</td>
<td>1.76</td>
<td>4.5</td>
<td>6.83</td>
<td>13.91</td>
<td>75.51</td>
</tr>
<tr>
<td></td>
<td>73.72</td>
<td>1.54</td>
<td>2.44</td>
<td>0.62</td>
<td>7.53</td>
<td>83.04</td>
</tr>
<tr>
<td>D2</td>
<td>233.43</td>
<td>2.1</td>
<td>3.74</td>
<td>1.31</td>
<td>20.33</td>
<td>20.33</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>1.33</td>
<td>2.35</td>
<td>3.03</td>
<td>12.79</td>
<td>33.12</td>
</tr>
<tr>
<td></td>
<td>205.60</td>
<td>1.36</td>
<td>2.2</td>
<td>1.3</td>
<td>11.97</td>
<td>45.09</td>
</tr>
<tr>
<td></td>
<td>235.56</td>
<td>1.9</td>
<td>1.58</td>
<td>0.48</td>
<td>8.6</td>
<td>53.69</td>
</tr>
<tr>
<td></td>
<td>199.76</td>
<td>0.99</td>
<td>0.82</td>
<td>0.67</td>
<td>4.47</td>
<td>58.17</td>
</tr>
<tr>
<td>D3</td>
<td>73.72</td>
<td>1.26</td>
<td>1.76</td>
<td>0.76</td>
<td>17.73</td>
<td>17.73</td>
</tr>
<tr>
<td></td>
<td>59.45</td>
<td>2.7</td>
<td>1.6</td>
<td>0.34</td>
<td>16.15</td>
<td>33.88</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>1.39</td>
<td>1.6</td>
<td>0.76</td>
<td>16.1</td>
<td>49.98</td>
</tr>
<tr>
<td></td>
<td>205.60</td>
<td>0.95</td>
<td>1.08</td>
<td>0.78</td>
<td>10.87</td>
<td>60.85</td>
</tr>
<tr>
<td></td>
<td>70.74</td>
<td>0.95</td>
<td>0.59</td>
<td>0.48</td>
<td>5.99</td>
<td>66.84</td>
</tr>
<tr>
<td>D4</td>
<td>52.22</td>
<td>5.81</td>
<td>13.17</td>
<td>0.78</td>
<td>42.55</td>
<td>42.55</td>
</tr>
<tr>
<td></td>
<td>275.44</td>
<td>1.37</td>
<td>4.36</td>
<td>6.32</td>
<td>14.09</td>
<td>56.65</td>
</tr>
<tr>
<td></td>
<td>70.74</td>
<td>1.53</td>
<td>2.99</td>
<td>1.25</td>
<td>9.67</td>
<td>66.32</td>
</tr>
<tr>
<td></td>
<td>184.55</td>
<td>0.86</td>
<td>1.52</td>
<td>0.77</td>
<td>4.92</td>
<td>71.23</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>1.15</td>
<td>1.43</td>
<td>0.48</td>
<td>4.61</td>
<td>75.85</td>
</tr>
<tr>
<td>D5</td>
<td>56.05</td>
<td>4.17</td>
<td>5.41</td>
<td>0.48</td>
<td>24.5</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>45.93</td>
<td>1.15</td>
<td>2.99</td>
<td>1.29</td>
<td>13.53</td>
<td>38.04</td>
</tr>
<tr>
<td></td>
<td>233.43</td>
<td>1.26</td>
<td>2.51</td>
<td>0.78</td>
<td>11.38</td>
<td>49.42</td>
</tr>
<tr>
<td></td>
<td>238.55</td>
<td>0.94</td>
<td>1.84</td>
<td>0.77</td>
<td>8.33</td>
<td>57.74</td>
</tr>
<tr>
<td></td>
<td>282.01</td>
<td>0.76</td>
<td>1.43</td>
<td>0.78</td>
<td>6.47</td>
<td>64.21</td>
</tr>
<tr>
<td>D6</td>
<td>127.45</td>
<td>2.31</td>
<td>6.04</td>
<td>5.63</td>
<td>24.1</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td>343.3</td>
<td>2.32</td>
<td>4.24</td>
<td>1.11</td>
<td>16.93</td>
<td>41.03</td>
</tr>
<tr>
<td></td>
<td>41.14</td>
<td>2.51</td>
<td>2.51</td>
<td>0.56</td>
<td>10</td>
<td>51.03</td>
</tr>
<tr>
<td></td>
<td>68.34</td>
<td>2.12</td>
<td>2.06</td>
<td>0.48</td>
<td>8.22</td>
<td>59.25</td>
</tr>
<tr>
<td></td>
<td>137.69</td>
<td>1.37</td>
<td>2.06</td>
<td>0.77</td>
<td>8.22</td>
<td>67.47</td>
</tr>
</tbody>
</table>