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Microbial Ecology of Moving Bed Biofilm Reactors Treating Municipal Wastewater

Kristi Biswas

Abstract

Moving bed biofilm reactor (MBBR) technology was introduced relatively recently to the wastewater industry to help overcome the difficulties faced by conventional treatment processes. The major advantage of this technology is the high treatment capacity due to the majority of the microbial biomass being retained for extended periods within the reactor on suspended polyethylene carriers. However, much remains unknown about the microbial ecology of these biofilm-based systems. This research examined the bacterial and archaeal community structure in full-scale MBBR systems treating municipal wastewater at Moa Point and Karori in Wellington, New Zealand. Molecular characterisation of these communities was based on 16S rRNA gene sequencing and fluorescence in situ hybridisation, together with automated ribosomal intergenic spacer analysis. Bacterial communities within the biofilm were dominated by putative anaerobes such as *Deltaproteobacteria* and *Clostridia*, with minor variations between treatment facilities. In contrast, the suspended fraction of the MBBRs was dominated by fast-growing, putatively aerobic members of *Gamma-, Beta- and Alphaproteobacteria*. The dominant archaea across all biofilm samples were members of the *Methanosarcinaceae*, which represented <5% biovolume.

A subsequent study investigated biofilm succession on K1 carriers (AnoxKaldnes™) from initial seeding to maturation using 16S rRNA gene amplicon pyrosequencing and *dsrAB* gene-based analyses. Comparisons of successional development of bacterial communities were also made between two commonly used carrier types (K1 and K3). The 16S rRNA gene results provided in-depth knowledge of the development of bacterial and archaeal communities within biofilm of MBBR from initial attachment to maturation. The archaeal community structure changed over time from a diverse system, in the younger biofilm, to one that was dominated by *Methanosarcinales*, in the older biofilm. In contrast, the bacterial community increased in diversity over time with the appearance of potential sulfate-reducing bacteria (SRB) in the later stages of biofilm growth. A major motivation of this study was to identify the SRB at Moa Point WWTP, as there are large amounts of hydrogen sulfide emitted at this site. Karori WWTP, with no reported sulfide-related issue, served as a useful comparison. SRB diversity results based on *dsrAB* genes indicated that *Desulfovibrio* and *Desulfomicrobium* were almost exclusively found at Moa Point WWTP. The total SRB
community accounted for <1% of the bacterial community at each treatment plant. Interestingly, the larger carriers (K3) had reduced growth of SRB within the developing biofilm compared with K1 carriers.

The final part of this thesis involved investigations into the microbial communities and nutrient removal efficiency of lab-scale MBBRs that were subjected to changes. Functionally important organisms including nitrifiers, sulfate reducers and phosphate accumulators, were identified by 16S rRNA gene pyrosequencing. The number of sequences detected for each of these functional groups correlated positively with measurements for nutrient removal. However, the biggest effect on nutrient removal rates was from manipulating seeding material, which reiterates the notion that seeding material is an important factor for optimal plant performance as it can also have long term effects on nutrient removal rates.

This is the first report on the microbial ecology of MBBR systems treating municipal wastewater and should provide a basis for optimising MBBR plants in the future.
For my mother and in loving memory of my father, Baba,

who would have been so proud to see me finish.
Acknowledgements

First, I would like to thank my primary supervisor Dr Susan J Turner for all her advice, guidance, and support throughout the years. She has motivated, encouraged, and even visited wastewater treatment sites with me during this project. Thank you for seeing the project through to the end. I would also like to thank Dr Clark Ehlers for all his help with the bioreactors and editing of my chapters. His advice and help have been valuable assets to this project. I do not know what I would have done without the help of Marlene Pillay (the lab manager) on all the administrative jobs. Thank you for being so patient with me and for all your help on numerous occasions! I would also like to thank all the members of the Taylor and Goddard labs for adopting me into their group in the later stages of my project. In particular, Rachel, Melissa and Dave, who have provided immense support and a good team of people to share a lab with. They have also been my friends, and forever ready to drink a cup of tea or visit the gym when things didn’t go according to plan! Special thanks to Peter Tsai - for the bioinformatics advice, Sneaky Pete - for numerous discussions on sulfate reducers, Ruth - for all the autoclaving of the bioreactors, and Liam and Kristine - for their technical support. The ‘aquatic roosters’ from Panapa are an awesome bunch of people that made me laugh a lot in the past two years!

Veolia Limited staff at Moa Point and Karori wastewater treatment plants have been incredibly patient with dealing with a non-engineer, and providing me with samples and operational data when required. I also appreciate the use of the lab facilities and allowing me to set up my fishing line experiments within the MBBRs at these sites. In particular, I would like to thank Valitha Roos (contract manager), Stuart Pearson (operational manager), Peter Pritchard (business developer), Bob, Rob, Nic and Ron. Special thanks to James Feary, our initial contact person at the treatment sites who has since changed jobs, for assisting with securing funding for this project.

As always, I could not have done this without the help and love from my brother (Shambu), mother (Momski) and father (Baba). Momski has been there to support me through everything, from cooking dinner, financially and even reading through my chapters. Baba was a great inspiration and I looked up to him greatly.
Finally, I would like to express my special thanks to my incredibly patient, kind, smart, amazing baker and loving partner - Mike Taylor. Thank you for all your help with reading my chapters, preparing dinners, allowing me to bounce ideas off you and for putting up with me through the highs and lows of this study. It would have been a much less enjoyable experience without you.
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### List of Abbreviations

S.I. (*Système international d'unités*) abbreviations for units and standard notations for chemical elements, formulae, and chemical abbreviations are used in this work. Other abbreviations used in the text are listed below.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA</td>
<td>Ammonia monooxygenase</td>
</tr>
<tr>
<td>ANOSIM</td>
<td>Analysis of similarity</td>
</tr>
<tr>
<td>AOA</td>
<td>Ammonia-oxidising archaea</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonia-oxidising bacteria</td>
</tr>
<tr>
<td>ARISA</td>
<td>Automated ribosomal intergenic spacer analysis</td>
</tr>
<tr>
<td>AS</td>
<td>Activated sludge</td>
</tr>
<tr>
<td>BNR</td>
<td>Biological nutrient removal</td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical oxygen demand</td>
</tr>
<tr>
<td>BR1</td>
<td>Bioreactor 1</td>
</tr>
<tr>
<td>BR2</td>
<td>Bioreactor 2</td>
</tr>
<tr>
<td>CARD-FISH</td>
<td>Catalysed reporter deposition</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscope</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>dsrAB</td>
<td>Dissimilatory bi-sulfite reductase (alpha and beta subunits)</td>
</tr>
<tr>
<td>EBPR</td>
<td>Enhanced biological phosphorus removal</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
</tr>
<tr>
<td>FC</td>
<td>Faecal coliforms</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>HC</td>
<td>High conductivity</td>
</tr>
<tr>
<td>HN</td>
<td>High nutrient</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic retention time</td>
</tr>
<tr>
<td>IFAS</td>
<td>Integrated (bio)film activated sludge</td>
</tr>
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<td>Invasion gene A</td>
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<td>ITS</td>
<td>Intergenic transcribed spacer</td>
</tr>
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<td>LCFA</td>
<td>Long chain fatty acids</td>
</tr>
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<td>LN</td>
<td>Low nutrient</td>
</tr>
<tr>
<td>MBBR</td>
<td>Moving bed biofilm reactor</td>
</tr>
<tr>
<td>MDS</td>
<td>Multi-dimensional scaling</td>
</tr>
<tr>
<td>MID</td>
<td>Multiplex identifier</td>
</tr>
<tr>
<td>nifH</td>
<td>Dinitrogenase reductase</td>
</tr>
<tr>
<td>NOB</td>
<td>Nitrite-oxidising bacteria</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamine gel electrophoresis</td>
</tr>
<tr>
<td>PAO</td>
<td>polyphosphate accumulating organisms</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCoA</td>
<td>Principal co-ordinate analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>Rotating biological contactors</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
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<td>Ribonucleic acid</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Sulfur-oxidising bacteria</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulfate-reducing bacteria</td>
</tr>
<tr>
<td>SRT</td>
<td>Solid retention time</td>
</tr>
<tr>
<td>SS</td>
<td>Suspended solids</td>
</tr>
<tr>
<td>VSS</td>
<td>Volatile suspended solids</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
</tr>
</tbody>
</table>
1. GENERAL INTRODUCTION

It is well recognised that effective wastewater treatment is of fundamental importance to both human and environmental health and, ultimately, economic prosperity. It is therefore unsurprising that the wastewater industry is increasingly becoming one of the largest biotechnology industries in the world. In 2000, the USA spent ~US$30 billion on wastewater management (OECD, 2003), whereas in Europe €16.2 billion (~US$21 billion) was spent in 2009 (Eurostat, 2013). New Zealand, with a much smaller population, still spends a substantial amount each year, with the total spent on wastewater management estimated at NZD$727 million (equivalent to ~US$607 million) in 2011 (Statistics New Zealand, 2013). Despite this expenditure the wastewater treatment industry is still largely reliant on the management of biological processes that are fundamentally simple, yet often difficult to control.

1.1. Fundamental principles of wastewater treatment

Wastewater is mostly (99%) composed of water, along with a range of inorganic and organic compounds, microorganisms, metals, oils, and greases (Henze et al., 2001). The purpose of treatment is to render the wastewater suitable for discharge to the receiving environments without causing unacceptable risk to public health or environmental quality. Though the degree of treatment required will depend on the nature and sensitivity of the receiving environment, most modern treatment systems are configured to effect the reduction of pathogens, organic and inorganic nutrients, chemical contaminants, and heavy metals (Metcalf and Eddy, 2003).

Generally wastewater treatment involves three sequential steps, namely (1) primary, (2) secondary, and (3) tertiary treatment processes. Primary treatment involves mechanical and physical processes such as screening and settling for the removal of heavy particles such as grit, stones and macro particles. Oils, greases, and foam that float on the surface are also extracted at this stage of treatment. Secondary treatment utilises biological processes to achieve the removal of organic and inorganic nutrients – typically through microbial mineralisation or incorporation into the microbial biomass. At the end of this stage,
accumulated microbial biomass is removed, often through gravity settling, leaving a clarified effluent which may be discharged with or without further treatment. Tertiary treatment is performed to remove pathogens, or to reduce the levels of chemical contaminants which are not effectively removed in secondary treatment processes. Disinfection is achieved through chemical or physical treatments including chlorine, ozone, UV light or microfiltration (Metcalf and Eddy, 2003; Seviour and Nielsen, 2010). The latter may also be used for advanced removal of chemical contaminants. This thesis is concerned with the microbial processes involved in biological (secondary) treatment and therefore further discussion is focussed on this aspect of wastewater treatment.

1.2. Biological treatment of wastewater

Biological treatment using microorganisms is the most cost-effective and efficient way of treating complex organic carbon compounds and removing inorganic nutrients from wastewater. Although biological treatment has been utilised for the past century, the microorganisms involved in these processes were, for a long time treated as a ‘black box’ (Rittmann et al., 2006). One reason for this lack of microbiological understanding was the lack of tools to reliably study the occurrence and/or function of the microbes involved in these processes. Advances in molecular techniques that have occurred over the past two decades have enabled a much better understanding of the complex microbial communities and interactions that underpin treatment systems today, especially those based around the activated sludge process (Daims et al., 2006).

1.2.1. Microbial communities of wastewater

The microbial community treating wastewater is a complex ecosystem of many different organisms including eukaryotes, protozoa, bacteria, archaea, fungi and viruses (Metcalf and Eddy, 2003). Bacteria are the most functionally important and numerically dominant microorganisms in biologically treated wastewater facilities; hence, their role at any WWTP is vital. There are believed to be between 1-10 x 10^{12} bacteria /g volatile suspended solids in activated sludge, of which the majority (80%) are metabolically active (Nielsen and Nielsen, 2002). The dynamics and occurrence of viruses, archaea, and fungi in wastewater systems are
less well described due to the limited technologies for studying these organisms. These organisms were also thought to represent a very minor (<1% each) component of wastewater, leading to a perception that they play a minor functional role in wastewater treatment (Seviour, 2010). Protozoa in activated sludge are well documented and are thought to play an important role due to their relatively large abundance (50,000 cells/mL) and their ability to graze and prey on bacteria (Curds, 1982).

As bacteria are the key microbial organisms capable of nutrient removal and organic carbon degradation, most of the ensuing discussion on the microbial community composition of wastewater is focused on bacteria. Activated sludge processes have been the subject of extensive studies on bacterial community composition due to their prevalent use for large-scale municipal wastewater treatment (Seviour and Nielsen, 2010). These studies have used modern molecular techniques, such as 16S rRNA gene pyrosequencing and fluorescence in situ hybridisation (FISH), to determine that the communities are mostly composed of *Proteobacteria*, along with *Actinobacteria*, *Chloroflexi*, *Bacteroidetes* and *Planctomycetes* (Wagner et al., 2002; Seviour, 2010; Ye and Zhang, 2012). The abundance of these individual phyla can vary within and between different treatment systems. For example, a detailed investigation into the bacterial communities of an integrated fixed-film activated sludge (IFAS) system showed differences between the attached biofilm communities and those in the suspension phase. The suspended fraction was dominated by *Proteobacteria* (Beta-: 59.3%, Gamma-: 8.1%, Alpha-: 3.9%), *Bacteroidetes* (5.2%) and *Actinobacteria* (3.2%), whereas the attached biofilm was dominated by *Proteobacteria* (Beta-: 9.9%, Gamma-: 9.2%, Alpha-: 7.4%), *Actinobacteria* (14.6%), *Firmicutes* (13.6%) and *Bacteroidetes* (11.6%) (Kwon et al., 2010). Increases in putative anaerobes, such as members of the *Firmicutes* and *Bacteroidetes*, within the biofilm samples of an IFAS compared with the suspended fraction, and compared to those of activated sludge systems, suggest that plant configuration plays an important role in the selection of residential bacterial communities.

1.3. WWTP processes and the functional role of microorganisms

Wastewater treatment systems capitalise on the metabolic versatility and resilience of microbes, which enables them to utilise a wide range of compounds for growth. Moreover, microbes can be easily maintained in the dynamic treatment environment. The key functional
groups of bacteria and their associated processes in wastewater treatment will be discussed in the next section.

1.3.1. Organic matter removal

Organic matter comprises a range of chemical compounds that contain carbon. These include both inorganic (e.g. carbonates, bicarbonates) and organic (e.g. carbohydrates, amino acids) carbon compounds. Removal of organic matter is measured in terms of reduction of volatile suspended solids (VSS) and Biochemical Oxygen Demand (BOD). VSS provides a measure of the suspended organic carbon present within the system, regardless of the degree of bioavailability. BOD is an indirect measure of organic matter, which quantifies the amount of dissolved oxygen required by microorganisms to aerobically degrade organic matter in a defined time period and volume of water (Metcalf et al., 2010). Typical levels of VSS and BOD of primary effluent in municipal treatment plants are 210 g VSS/m$^3$ and 280 g O$_2$/m$^3$, respectively (Henze et al., 2001). Reduction in the levels of these components is achieved through microbial mineralisation and utilisation for growth. Most WWTPs are configured to support the predominance of aerobic or facultatively anaerobic chemoorganoheterotrophic bacteria (Henze et al., 2001). Activated sludge WWTPs are typically aerated to support aerobic respiration, though manipulation of dissolved oxygen can be undertaken to promote nitrogen or phosphorus removal (discussed in sections 1.3.2 and 1.3.3). However, under anaerobic conditions such as those found at biofilm-based WWTPs, sulfate, nitrate and carbonate, along with other inorganic and organic compounds, can be utilised as electron acceptors (Seviour and Nielsen, 2010).

1.3.2. Nitrogen removal

Nitrogen occurs in wastewater in many different forms including ammonia, nitrate, nitrite, and within organic molecules such as amino acids. The average NH$_4$ –N level found in wastewater is 20 mg/L (Wiesmann, 1994). In wastewater, microbes are able to break down nitrogen compounds for use in growth or for harvesting energy. Nitrification and denitrification are two important processes for the removal of nitrogenous compounds at a WWTP.
1.3.2.1. **Nitrification**

Nitrification is defined as the biological conversion of ammonia to nitrate via nitrite (Daims and Wagner, 2010). Ammonia-oxidising bacteria (AOB) catalyse the oxidation of ammonia to nitrite, which is the first step of nitrification (Fig. 1.1) (Daims et al., 2006; Daims and Wagner, 2010). Our knowledge of the diversity of AOBs has been based on analyses of one of the subunits of the functional gene ammonia monoxygenase (amoA) and 16S rRNA gene sequences (Rotthauwe et al., 1997; Zhang et al., 2011). The major AOBs that have been identified in wastewater are members of the *Proteobacteria* including *Nitrosomonas* (Dionisi et al., 2002), *Nitrosococcus* (Rowan et al., 2003) and *Nitrosospira* (Schramm et al., 1998). In addition, recent studies have found ammonia-oxidising archaea (AOA) in activated sludge treatment plants (Park et al., 2006; Stahl and de la Torre, 2012) that can carry out the same process as AOBs (Könneke et al., 2005; Prosser and Nicol, 2008). Ammonia oxidisers are slow growing and sensitive to changes in pH and temperature within a reactor, which make them unreliable and unpredictable at a WWTP (Bellucci and Curtis, 2011).
The second step of nitrification is carried out by nitrite-oxidising bacteria (NOBs) that convert nitrite to nitrate. *Nitrobacter* were once believed to be the most dominant NOB in WWTPs, based on cultivation methods (Bock and Koops, 1992). More recently, molecular techniques have identified *Nitrospira* as the most important NOB in these systems (Daims et al., 2001). These NOBs belong to two different phyla (*Proteobacteria* and *Nitrospirae*, respectively) and have different strategies for survival. *Nitrospira* are believed to be K-strategists that are slow growing and can survive on low nitrite and oxygen concentrations (Nogueira and Melo, 2006). In contrast, *Nitrobacter* are postulated to be r-strategists that are fast growing and have lower affinities for nitrite and oxygen (Schramm et al., 1999).

Ammonia concentrations in the influent of domestic wastewater range between 12-50 mg/L (Drinan and Spellman, 2012), which is much lower than organic matter concentrations. As a result, chemoorganoheterotrophic bacteria flourish at the inlet of a WWTP, and outcompete the nitrifying bacteria. However, nitrification does occur further away from the inlet, in areas where carbon concentrations are lower (Daims and Wagner, 2010). Nitrite is an intermediate in nitrification and therefore does not accumulate at a WWTP. It is, thus, not surprising that *Nitrospira* are the most dominant NOBs found at WWTPs, due to their physiological advantage of surviving under low nitrite concentrations (Daims et al., 2001).

### 1.3.2.2. Denitrification

Denitrification is defined as the reduction of nitrate to nitrogen via a series of intermediate nitrogenous gaseous compounds (Daims and Wagner, 2010). The nitrogen cycle (Fig.1.1) at a typical WWTP is completed under anaerobic conditions by denitrifying microbes (Seviour, 2010). Denitrifiers are a physiologically diverse group of organisms that can grow using CO$_2$ (autotrophs) or organic compounds (heterotrophs). Their survival has been recorded in oxic and anoxic conditions where they are involved in carbon or nitrogen removal, respectively (Wiesmann, 1994). The most commonly found denitrifiers in nitrogen removal treatment facilities are *Aquaspirillum*, *Azoarcus* and *Thauera*, while at an industrial treatment facility *Azoarcus*, *Zoogloea* spp. and *Thauera* were found to dominate (Juretschko et al., 2002; Thomsen et al., 2007; Morgan-Sagastume et al., 2008).
1.3.3. Phosphorus removal

Biological phosphorus removal capitalises on the ability of certain groups of bacteria to accumulate intracellular phosphorus as polyphosphate during aerobic growth (McMahon et al., 2010). Many organisms are capable of this process but what makes polyphosphate accumulating organisms (PAO) unique from other bacteria is the ability to utilise simple carbon compounds as an energy source without the need for an external electron acceptor. In WWTPs with low levels of electron acceptors such as nitrate or oxygen, PAOs will have a selective advantage over other wastewater organisms (Martín et al., 2006). To achieve enhanced biological phosphorus removal (EBPR), reactors need to be operated under anaerobic-aerobic cycling conditions (Crocetti et al., 2000). PAOs are selectively enriched for in the anaerobic phase due to their ability to outcompete other organisms under conditions with low levels of electron acceptors. Under aerobic conditions, phosphate is accumulated as polyphosphate within the cells which are then removed as biomass in the clarification process. Previously, culture-based studies identified *Acinetobacter* spp. as the dominant PAO in activated sludge (Fuhs and Chen, 1975; Deinema et al., 1980; Streichan et al., 1990); however, since then, more advanced molecular techniques have shown *Rhodocyclus*-related ‘*Candidatus Accumulibacter phosphatis*’ (belonging to the phylum *Proteobacteria*) as the most important PAO in wastewater (Hesselmann et al., 1999; He et al., 2007).

1.3.4. Sulfur removal

Sulfate-reducing bacteria (SRB) undertake anaerobic respiration using sulfate as a terminal electron acceptor and ultimately produce sulfide. The production of excess sulfide has detrimental effects at WWTPs due to corrosion and toxicity of the gaseous sulfide compounds. SRBs have been detected as 0.5–8% of total bacteria at WWTPs (Manz et al., 1998; Nielsen and Nielsen, 2002). The dominant SRBs at a WWTP belong to the families *Desulfovibrionaceae* and *Desulfobacteriaceae* within the *Deltaproteobacteria* class (Manz et al., 1998). Many species of SRB are aero-tolerant and slow growing. These include *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans*, and *Desulfobacter postgatei* (Hardy and Hamilton, 1981; Cypionka et al., 1985). Their ability to survive under both oxic and anoxic conditions makes them highly versatile (Muyzer and Stams, 2008).
Sulfur-oxidising bacteria (SOB) are capable of oxidising sulfide compounds to elemental sulfur for energy in activated sludge plants (Seviour, 2010). This group of organisms is capable of growing heterotrophically or autotrophically, thus giving them a competitive advantage to survive in WWTPs. Commonly identified SOBs in wastewater include *Thiothrix* and *Beggiatoa* (Williams and Unz, 1985; Howarth et al., 1999).

1.4. Treatment systems

Most WWTPs can be classified as belonging to one of two main types, based on whether the functional microbial communities are maintained as planktonic assemblages (floc-based systems) or as surface-associated biofilms (fixed-film systems).

1.4.1. Floc-based treatment systems

Most modern wastewater treatment systems utilise a system known as activated sludge, which was developed in 1914 by Edward Ardern and William Lockett (Seviour, 2010). The system takes its name from the process of seeding influent with the active microbial biomass that is removed in the final effluent clarification step. Activated sludge is a floc-based system in which microbes, extracellular polymeric substances (EPS) and other solids present in wastewater form aggregates that are maintained in suspension within the reactor through aeration. These floc-based communities utilise the available oxygen, organic carbon and inorganic nutrients within the influent to support microbial growth. Microbial biomass is removed from the effluent in a clarification process that typically utilises gravity settlement to separate flocs from the liquid phase. This separation process can be problematic in conventional activated sludge treatment plants due to a range of microbial factors including poor flocculation, proliferation of filamentous organisms or production of excess EPS, all of which impede settlement (Visvanathan et al., 2000).

The settled biomass containing active microorganisms is referred to as sludge and is either returned to the reactors as activated sludge or consolidated and disposed of in landfills (Drinan and Spellman, 2012). Effluent from activated sludge systems may still contain elevated numbers of pathogens and for this reason the treated wastewater will often be subjected to tertiary treatment before discharge into the environment.
Due to increasingly stringent regulations around nutrient discharge, growing scarcity of freshwater and the huge economic burden of treating wastewater, there has been an urgent need to find ways to improve the efficiency and reliability of microbes at a WWTP (Hunter, 1998; OECD, 2003). This has resulted in numerous studies over the past few decades to characterise the microorganisms involved in activated sludge systems (Metcalf et al., 2010; Seviour and Nielsen, 2010). More recently, research has also begun into the metabolism and function of these communities at WWTPs (Neufeld et al., 2007; Yu and Zhang, 2012).

1.4.2. Biofilm-based treatment systems

Biofilms are potentially complex structures that are mostly composed of microbial cells embedded within a polymeric matrix. The use of biofilm-based membrane bioreactors has increased in the last 20 years (Visvanathan et al., 2000). There are several advantages to be gained from the use of a biofilm system in the treatment of wastewater. Surface-associated microbes potentially have a longer residence time within the reactors, thus such systems may support the growth of slow growing microorganisms such as nitrite-oxidising bacteria (NOB) and phosphorus-accumulating organisms (PAO) that are central to nutrient removal in wastewater. In addition, anoxic niches may develop within the biofilm, supporting the growth of anaerobic organisms (Gray et al., 2002). Studies of drinking water systems have also suggested that environmental conditions such as pH, temperature, nutrient concentrations, and toxic substances, have less of an impact on fixed film cultures (Pedersen, 1990). Finally, biofilm-based treatment systems have the potential to be more compact than floc-based systems, as the majority of biomass is retained within the reactor to facilitate nutrient removal, and lower processing times.

The activity of a biofilm is heavily dependent on its thickness, which is a key factor in limiting nutrient diffusion. Thicker biomass leads to reduced diffusion of nutrients, resulting in cell inactivation (Kornegay and Andrews, 1968; La Motta, 1976). Biofilm systems most often need to be coupled with a clarification step in order to remove the suspended solids from the effluent. Overall, biofilm systems produce excellent quality of treated water, smaller footprint, lower net sludge production and greater reliability than traditional activated sludge systems (Visvanathan et al., 2000; Ivanovic and Leiknes, 2012).
A wide range of biofilm-based treatment systems have been developed since the 1960s (Visvanathan et al., 2000; Odegaard, 2006; Rusten et al., 2006). These vary in the type of matrix used for attachment and the method in which wastewater is applied. In fixed film ‘trickling filters’, wastewater is trickled or sprayed over the immobilised carriers, which may include small rocks, wood bark or plastic media. Rotating biological contactors (RBCs) represent another example of a fixed-film system, which uses a spinning drum or plastic discs that are mounted side by side. These discs/drums are slowly rotated within the reactor with approximately 40% of the discs submerged within the wastewater, thus facilitating oxic and anoxic growth of microbes on the attached surface (Drinan and Spellman, 2012).

Biofilm-based systems have also been used in combination with the activated sludge process to capitalise on the advantages of both systems. These treatment plants are known as integrated (bio)film activated sludge reactors and involve the inclusion of some form of biofilm support within the activated sludge reactor (Kwon et al., 2010). In one example, biofilm supports retrofitted to activated sludge reactors were shown to increase the numbers of slow-growing NOB, which resulted in increased nitrification rates (Ivanovic and Leiknes, 2012). However, there are also a number of potential problems associated with these systems including membrane fouling, mechanical failures, the need for backwashing, and uneven distribution of load on carrier surfaces (Rusten et al., 2006). The moving bed biofilm reactor (MBBR) system overcomes many of these problems and is discussed in detail in the subsequent section.

1.5. Moving bed biofilm reactor (MBBR)

The MBBR process (European Patent no. 0575314, US patent no. 5,458,779) was invented in 1989 in Norway by AnoxKaldnes™ (Odegaard et al., 1994). The research and development of the MBBR process was driven by a political agreement between North European countries to reduce nutrient discharge into the North Sea by 50% between 1985 and 1995 (Hem et al., 1994). The existing treatment systems were unable to meet the requirements of this political agreement and required large upgrades. Limited land area near cities was also an important factor that led to the development of a compact and more efficient treatment process, which became known as MBBR systems. There are an increasing number of WWTPs (600 in 2006 (McQuarrie and Boltz, 2011) to ~1200 in 2012 (Pritchard pers. Comm.)), in more than 50
countries, that utilise this technology. The innovation of this technology is in the use of non-clogging suspended (moving-bed) carriers which provide for a high specific biofilm surface area and ultimately increased treatment capacity (Rusten et al., 1994; Odegaard, 2006). A major advantage of this technology over other fixed bed systems is thus low head-loss, thereby minimising pumping costs and tolerance to high loading rates, enabling a small footprint.

A consequence of the comparatively recent introduction of these systems into mainstream use is that the description of microbial communities in MBBRs is limited to a few recent studies focused on specific groups of microorganisms. These include ammonia-oxidising bacteria (AOBs), nitrite-oxidising bacteria (NOBs) and phosphate-accumulating organisms (PAOs) (Xia et al., 2008; Liang et al., 2010; Yang et al., 2010). However, little attention has been paid to the overall community structure of these biofilm-based wastewater treatment systems. A recent fingerprinting analysis of the bacterial community of a lab-scale MBBR system revealed dominance by Betaproteobacteria (46%), Firmicutes (34%), Alphaproteobacteria (14%), and Gammaproteobacteria (9%) (Calderón et al., 2012). At present, there remains a distinct lack of understanding about the microorganisms involved in the biofilm and suspended fraction of a full-scale MBBR WWTP.

### 1.5.1. Design features of MBBR systems

The MBBR system typically utilises plastic carriers with high surface area that are maintained in suspension through either aeration or mechanical stirring (Fig. 1.2). The carriers are made from high-density polyethylene (density of 0.95 g/cm³) and are retained within the reactors with the help of a sieve at the outlet. To maintain free movement of the suspended carriers, a filling fraction below 70% has been recommended (Odegaard et al., 1994). Biomass grows primarily on the inside, protected surface of the carriers (Bjornberg et al., 2009), which is also known as the effective surface area. Biofilm growth on the outer surface is limited due to constant collisions between carriers. The total effective surface areas of different carriers used in the MBBR process are shown in Table 1.1 (McQuarrie and Boltz, 2011). AnoxKaldnes™ K1 carriers are most commonly used for municipal wastewater applications.
Nutrient diffusion within a biofilm is influenced by biofilm thickness. In the MBBR process an ideal biofilm that carries out aerobic reactions is evenly distributed over the surface area, with an optimal thickness of less than 100 μm (Odegaard, 2006). In order to maintain this, turbulence in the reactor (through either aeration or mechanical agitation) is an essential element. On occasions when the biofilm is too thick, anoxic niches develop within the biofilm, supporting anaerobic processes such as sulfate reduction. Such reactions may lead to the increased production of toxic compounds such as hydrogen sulfide, which is problematic in and around a WWTP. A thin biofilm is equally problematic, with reduced microbial biomass leading to reduced treatment efficiency. As indicated previously, one of the disadvantages of MBBR systems is the comparatively high level of suspended solids remaining in the reactor effluents, necessitating the inclusion of a clarification step prior to tertiary treatment or discharge of effluent. There are numerous liquid-solid separation procedures used in conjunction with MBBRs and these include sedimentation basins, dissolved air flotation, deep-bed granular media filters and membrane filters (McQuarrie and Boltz, 2011).
## Table 1.1. Plastic biofilm carrier characteristics as reported by different manufacturers.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Name</th>
<th>Bulk specific surface area</th>
<th>Dimensions (Depth; Diameter)</th>
<th>Images of carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veolia Inc.</td>
<td>AnoxKaldnes™ K1 or K1 Heavy</td>
<td>500 m²/m²</td>
<td>7 mm; 10 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AnoxKaldnes™ K3</td>
<td>500 m²/m²</td>
<td>12 mm; 25 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AnoxKaldnes™ Biotin Chip (M)</td>
<td>1,200 m²/m²</td>
<td>2 mm; 48 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AnoxKaldnes™ Biotin Chip (P)</td>
<td>900 m²/m²</td>
<td>3 mm; 45 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Matrix™ Sol</td>
<td>800 m²/m²</td>
<td>4 mm; 25 mm</td>
<td></td>
</tr>
<tr>
<td>Headworks BIO</td>
<td>ActiveCell™ 450 *</td>
<td>462 m²/m² (450 m²/m² reported by IDI)</td>
<td>15 mm; 22 mm</td>
<td></td>
</tr>
<tr>
<td>(* Licensed by: Intico Degremont, Inc.)</td>
<td>ActiveCell™ 515 *</td>
<td>485 m²/m² (515 m²/m² reported by IDI)</td>
<td>15 mm; 22 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ActiveCell™ 920</td>
<td>680 m²/m²</td>
<td>15 × 15 × 10 mm (L × W × D)</td>
<td></td>
</tr>
<tr>
<td>AqWise</td>
<td>ABC4™</td>
<td>600 m²/m²</td>
<td>14 mm; 14 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABC5™</td>
<td>650 m²/m²</td>
<td>12 mm; 12 mm</td>
<td></td>
</tr>
<tr>
<td>Ettex Technologies, Inc.</td>
<td>Bioprotz™</td>
<td>589 m²/m²</td>
<td>14 mm; 18 mm</td>
<td></td>
</tr>
<tr>
<td>Siemens Water Technologies Corp.</td>
<td>CM-100™</td>
<td>750 m²/m²</td>
<td>9 mm; 13 mm</td>
<td></td>
</tr>
<tr>
<td>Biowater Technology</td>
<td>BWT15™</td>
<td>828 m²/m²</td>
<td>15 × 15 × 5 mm (L × W × D)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BWTX™</td>
<td>640 m²/m²</td>
<td>15 × 15 × 10 mm (L × W × D)</td>
<td></td>
</tr>
</tbody>
</table>

Modified from McQuarrie and Boltz (2011).
1.5.2. Treatment performance

Since its invention, MBBR research has mostly concentrated on nitrogen, phosphorus and organic matter removal (Helness and Odegaard, 1999; Pastorelli et al., 1999; Odegaard, 2006; Rusten et al., 2006). There are many different configurations of the MBBR process depending on the nutrient removal objectives of the treatment plant (Fig. 1.3). MBBR can be used as a pre-treatment to conventional activated sludge systems for enhanced BOD removal. These hybrid plants are often the result of an upgrade to an activated sludge plant.

![Diagram of MBBR configurations](image)

**Figure 1.3. Typical MBBR™ configurations for different applications (Odegaard, 2006).**

1.5.2.1. BOD/COD removal

MBBRs have performed well under high organic loads and high substrate concentrations (Odegaard et al., 2000). The residence time of the carbonaceous material within an MBBR is dependent on organic load and strength of the wastewater, and ranges typically between 15–90 minutes. By contrast, the typical residence time of biological solids in an activated sludge reactor is 4-10 days for SRT and 3-6 hours for HRT (Bisogni and Lawrence, 1971; Clara et
al., 2005; Metcalf and Eddy, 2003). The average organic load entering an MBBR ranges between 15 to 20 g BOD/m²/d at 15°C. At higher loadings (45–60 g BOD/m²/d at 15°C), the separation between the liquid and solid phases becomes problematic (Odegaard, 2006). An explanation for this is that the high organic load stimulates exponential growth of fast-growing organisms in the suspended fraction of the reactor, which creates difficulties in separating out the biomass from the treated liquid. These plants, therefore, use chemicals such as alum, iron chloride or aluminium chloride to assist with biomass flocculation and removal. Turbulence within the reactor, maintained via aeration (3 mg O₂/L), is another important feature that is used to help deal with high organic loads, as this constrains biofilm thickness and prevents the carriers from clogging (McQuarrie and Boltz, 2011).

1.5.2.2. Nitrification

Nitrification within MBBR systems has been researched extensively for both synthetic and municipal wastewater (Hem et al., 1994; Rusten et al., 1995b; Vendramel et al., 2011). Three main factors that determine nitrification rates are organic load, total ammonium concentration in the influent, and dissolved oxygen within the MBBR. An increase in dissolved oxygen within a reactor from 2 to 6 mg/L resulted in greater ammonium removal, as tested in a pilot MBBR plant in Norway (Rusten et al., 1995b). In contrast, increases in organic load (0 to 6 g BOD/m²/d) within a lab-scale and pilot-scale MBBR resulted in lower ammonium removal (Hem et al., 1994), possibly due to the concomitant reduction in DO. Other parameters such as temperature, pH, alkalinity and biomass history, play only a minor role in influencing nitrification processes within MBBRs (Rusten et al., 2006).

1.5.2.3. Denitrification

Biofilms attached to carriers within MBBRs develop aerobic and anoxic niches that provide favourable conditions for simultaneous nitrification and denitrification processes to occur (Leiknes et al., 2006; Yang et al., 2009). The rate of denitrification in an MBBR is influenced by available biodegradable organic matter, nitrate concentration, type of carbon source, oxygen concentration, and wastewater temperature (Odegaard, 2006; McQuarrie and Boltz, 2011). MBBRs can also be utilised to enhance nitrogen removal at an activated sludge treatment plant, either through pre-denitrification or post-denitrification steps. Pre-
denitrification MBBRs are situated upstream of the main process and receive re-circulated nitrified effluent from the treatment facility. A limitation to this process is the primary dependency on soluble organic matter in the influent wastewater as an energy source for denitrifying organisms. Post-denitrification MBBRs are located downstream of treatment facilities, and require the addition of external carbon as a supplementary electron donor because the majority of the soluble carbon is utilised during the primary treatment process. Commonly used external carbon sources are methanol, ethanol, and glycerol, and their use contributes to the overall cost of the treatment process. However, post-denitrification achieves higher nitrogen removal rates (100%) than pre-denitrification from wastewater (60–70%) (Rusten et al., 1995a; Odegaard, 2006).

1.5.2.4. Phosphorus removal

Several studies using lab-scale sequencing batch reactors in combination with MBBRs have shown that simultaneous nitrification, denitrification and phosphorus removal can be achieved (Pastorelli et al., 1999; Helness and Odegaard, 2001; Yang et al., 2010). These studies have shown that up to 84% of total influent phosphorus (12.4 mg/L) can be removed when reactors are operated under alternating anaerobic and aerobic phases, in a process that parallels conventional biological phosphate removal.

1.5.3. Treatment applications

MBBRs have been widely applied for the treatment of domestic and industrial wastewaters (Rusten et al., 2006; McQuarrie and Boltz, 2011). The carriers used in the MBBR process are robust, as observed by a commercially operating plant in Norway which has not replaced its K1 carriers for over 15 years (Rusten et al., 2006). Other applications of MBBRs for the treatment of wastewaters include dairy processing industry (Odegaard et al., 1994), potato chip manufacture (Odegaard et al., 1994), pulp and paper mills (Broch-Due et al., 1994), freshwater and marine fish farming (Rusten et al., 2006), aquaria (Labelle et al., 2005), shipboard wastewater (Sun et al., 2009), swine wastewater (Guanglei et al., 2011) and wineries (Sheli and Moletta, 2010). MBBRs have also been used to enrich for and isolate anaerobic ammonium oxidising (anammox) bacteria from nitrogen-rich wastewater plants (Szatkowska et al., 2006). Moreover, lab-scale MBBRs have since been used to select and
enrich for anammox organisms (López et al., 2007), which has significant commercial implications for biotechnology industries.

1.5.4. **MBBR systems in New Zealand**

There are only two large-scale MBBR plants treating municipal wastewater in New Zealand. Both are located in Wellington within the suburbs of Moa Point and Karori (Pritchard *pers. comm.*). The operation of these treatment facilities is carried out by Veolia Water Limited under contract to Wellington City Council. Both plants are operated as BOD removal facilities and have a 30–50% filling fraction of Kaldnes K1 carriers. At both sites, solids are removed by contact stabilisation and clarification. In addition to these there are several small-scale MBBRs treating specialised wastewater (dairy or winery) within New Zealand.

The Moa Point and Karori MBBR plants are the focus of this research.

1.5.4.1. **Moa Point WWTP**

The Moa Point WWTP was first established in 1899 but was upgraded in 1990 to include secondary and tertiary treatment through the installation of an MBBR plant and UV filtration unit (www.wellington.govt.nz). Due to the close proximity to residential areas and the limited land availability in Wellington, the MBBR process was favoured over conventional wastewater treatment technologies due to its compact nature and minimal effect on the environment (Rusten et al., 1998).

The Moa Point plant was designed to treat an average BOD and suspended solids load of 19,700 kg/day and 19,300 kg/day, respectively. The population equivalence of this site is 200,000 and the influent has an average flow of 822 L/s. There are three moving bed reactors at Moa Point which collectively are designed to take maximum inflow of 3000 L/s. A feature of this system is that it was the first MBBR plant to be combined with a solid contact re-aeration (SCR) process for secondary treatment of municipal wastewater (Rusten et al., 1998). This step enhances biomass flocculation and precipitation of finely divided solids. Effective clarification was necessary to support the tertiary treatment system which is based
on UV disinfection. Treated effluent flows via the UV disinfection system to an 1800 m long outfall that discharges into the Cook Strait.

Trade wastewater, mostly originating from a local abattoir, is also received at Moa Point WWTP. Due to the antiquity of Wellington’s sewer system (over 100 years old) and its close proximity to the sea, infiltration of seawater into this treatment facility is suspected by plant operators. Seawater infiltration and trade wastewater are known to contain high sulfate concentrations and are presumed to be contributing factors to the generation of high levels (31–35 ppm) of gaseous sulfides at this site. Sulfides are pungent, toxic and corrosive, thus in high levels can cause detrimental effects to a WWTP (USEPA, 1991).

1.5.4.2. Karori WWTP

The Wellington City Council commissioned the Karori WWTP in 1997 and it is located in the greater Wellington region (www.gw.govt.nz). For 70 years prior to this, wastewater from this community was disposed of through a gravity pipeline to the South Coast, near the mouth of the Karori stream. It is the smaller of the two treatment plants and treats mainly domestic wastewater with a population equivalence of 20,000. Primary treatment is a two-stage process at this WWTP and involves initial screening, followed by grit traps, to remove dense particles from raw sewage. Secondary treatment occurs within two moving bed reactors at this site, which receives an average inflow into the WWTP of 40 L/s, with a maximum of 180 L/s. The process liquor from the MBBRs enters the re-aeration tanks where further biological treatment occurs using a conventional activated sludge system. The treated water is then passed into a clarifier for settlement, where flocculated solids are separated from the suspended fraction through gravity. The effluent from the clarifier is disinfected with high intensity UV radiation before it is discharged via the original 6.2 km pipeline to the nearest ocean outfall (www.capacity.net.nz).

1.5.5. The big questions of MBBR microbiology

There is increasing national and international interest in the use of MBBR systems, instead of, or in conjunction with, conventional activated sludge systems. As with all biologically-based WWTPs the opportunity exists for process improvement through a better
understanding of the microbiology underpinning these systems. While considerable advances have been made in the understanding of microbial function in conventional activated sludge systems, the knowledge base around MBBR systems is still somewhat rudimentary. Some key questions that arise from preliminary observations of the New Zealand-based systems are:

1. What is the microbial community composition in MBBR systems and does it differ from that of conventional activated sludge systems?
2. How do microbial communities develop and establish within MBBR systems?
3. Does influent composition affect the sulfate-reducing organisms and the rate of sulfide production at a WWTP?
4. Can MBBR microbial communities be manipulated in order to improve the performance of these systems?

1.6. **Objective of this study**

MBBRs are a relatively new innovation for treating wastewater and little is known about the structure and function of the microbial communities within these systems. The main hypothesis of this research project is that the structure of the microbial communities within MBBR systems plays an important role in overall plant performance. The objective of this research project is to address this hypothesis by improving our understanding of microbial community development and dynamics and how these MBBR systems might be better engineered for optimal performance.

1.6.1. **Research strategy**

A range of molecular techniques have been developed in the past two decades that enable a better understanding of the diversity and composition of complex microbial communities in environments such as wastewater (Sanz and Köchling, 2007). The choice of methods is dependent on a number of factors including cost, the nature and number of samples to be analysed, and the timeframe within which data is required. The methods selected for use in this study include a combination of DNA fingerprinting, 16S rRNA and functional gene analyses. These methods are briefly outlined in turn below.
1.6.1.1. **Clone library generation for 16S rRNA gene and functional gene analysis**

Structural genes encoding the 16S rRNA have been the primary molecular target for microbial community analysis due to their conservation across both bacteria and archaea, presence of conserved sequences enabling construction of universal PCR primers, and sequence variability that allows meaningful phylogenetic reconstruction. Other genomic targets, such as 23S rRNA, groEL, rpoB, and atpD have been used for taxonomic discrimination of some genera (Bavykin et al., 2004; Ventura et al., 2004; Martens et al., 2008). However, the reference databases for these sequences have not developed to the point of providing sufficient phylogenetic coverage to enable their use in analysis of complex communities. A number of functional gene targets, such as dsrAB, have been used for assessing diversity of a functional group of organisms within complex microbial samples. There is a database of 97 full-length dsrAB gene sequences (~1800 bp) of described species that has been compiled to provide a reference database for phylogenetic analysis of sulfate-reducing organisms (Zverlov et al., 2005).

A major breakthrough in molecular ecology was the application of cloning techniques to separate mixtures of amplicons into individual fragments for further analysis. Advantages over other molecular techniques included the ability to screen clones by restriction fragment length polymorphism (RFLP) analysis to gain an insight into community structure and to optimise downstream sequencing effort. Cloned fragments can also be sequenced using multiple primers enabling better sequence coverage for culturable and unculturable organisms (Sanz and Köchling, 2007). However, this technique is time-consuming and expensive, making it unsuitable for analysis of large numbers of samples. During this study, clone library techniques were used to obtain full length gene sequences of 16S rRNA (bacteria and archaea) and dsrAB (SRB) communities within MBBRs treating municipal wastewater.

1.6.1.2. **ARISA (Automated Ribosomal Intergenic Spacer Analysis)**

ARISA is a fingerprinting method that can be used to profile the diversity of complex microbial communities from PCR-amplified DNA (Yu and Mohn, 2001). As the name suggests, this method relies on the variable length of intergenic spacer (ITS) regions which generally reflect differences between bacteria at species level. The results of community fingerprinting of samples can be displayed on multivariate plots, which enable the tracking of
community changes over time and in response to environmental variables. The advantage of this molecular technique over clone libraries is that a large number of samples can be analysed in a cost-effective and rapid way, while providing useful information about community dynamics (Muyzer and Yilirim, 2010; Seviour and Nielsen, 2010). The disadvantage of ARISA is that no information is gained on the identity of microbes present. Therefore, ARISA is often used either as a pre-screening method or in conjunction with targeted 16S rRNA sequencing strategies to track community dynamics. In this study, ARISA was selected as the method of choice for investigating changes in bacterial communities at Moa Point and Karori WWTPs over the course of a year.

1.6.1.3. Fluorescence in situ hybridisation (FISH)

The introduction of FISH in the late 1980s provided a tool for microbial identification and quantification, along with a means to visualise the spatial distribution of specific taxa within complex communities (Amann et al., 1990). In this method, DNA oligonucleotides with attached fluorophore labels are designed to hybridise with target-specific sites on the rRNA of an organism of interest. The resulting fluorescence of the targeted organism can be detected by fluorescence microscopy or flow cytometry. Confocal laser scanning microscopy has proven to be particularly useful in this context as it enables resolution of cells within dense assemblages such as biofilms or flocs. This method has been used extensively to identify and quantify target organisms in activated sludge (Amann et al., 1990; Wagner et al., 2003; Amann and Fuchs, 2008). However, this process also presents a number of technical challenges including interference due to autofluorescence from some samples, and difficulties in designing probes that are specific for the target organism(s). The process of quantifying microorganisms within samples by biovolume analysis can also be time-consuming and is dependent on the efficiency of probe hybridisation (Sanz and Köchling, 2007). Some of these limitations are overcome by catalysed reporter deposition (CARD)-FISH, which was introduced to microbial ecology in 2002 (Pernthaler et al., 2002). The use of an enzymatic amplification step in CARD-FISH to enhance the fluorescence signal increased the sensitivity of this technique to low ribosomal content samples. CARD-FISH is 26–41 times more sensitive than conventional FISH techniques (Hoshino et al., 2008).

Because of the limitations outlined above FISH methods tend to be used to compliment and validate fingerprint and sequencing studies, rather than as a first step in community analysis.
In this study FISH has been applied to validate results obtained from clone library analyses and also to investigate the spatial distribution and abundance of specific taxa.

1.6.1.4. **Microbial quantification using real-time PCR**

Accumulation of amplified products during PCR is not linear, and as a consequence endpoint analysis of target concentration does not provide an accurate means for quantification. In 1991, real-time PCR was developed to overcome this problem by utilising internal standards along with the fluorescence detection of target genes that enable accurate measurement of product accumulation at every cycle (Holland et al., 1991). The two most common approaches utilise SYBR green (an intercalating DNA dye) or Taqman DNA probe systems (with attached fluorescent reporter and quencher) (Smith and Osborn, 2009). Real-time PCR differs from regular PCR, by recording the increase in amplicon numbers in ‘real time’ after each PCR cycle. The increase is proportional to the concentration of the specific gene in the DNA sample. This technique allows the absolute quantification of genes in copy numbers, or the abundance of one gene relative to the other genes in a sample (Boon et al., 2003; Harms et al., 2003; He et al., 2007).

Real-time PCR is a sensitive technique that has been used to target organisms representing <1% of the total bacterial community (Fukushima et al., 2007). The advantages of this technique thus include high sensitivity and throughput as well as good reproducibility. Its’ limitations over regular PCR-based methods include cost and the requirement of a standard curve for absolute quantification of genes. Moreover, unknown organisms cannot be quantified by real-time PCR and would require strategies such as clone library or pyrosequencing to be conducted first to obtain sequences of target organisms. Unlike these techniques, real-time PCR is a quantitative approach and should be used in combination with other molecular tools to validate results (Smith and Osborn, 2009). In this study, real-time PCR was used to quantify active sulfate-reducing bacteria within biofilm samples from MBBR plants.
1.6.1.5. Next-generation sequencing technologies

The so-called “next-generation” sequencing technologies, which originated within the past decade, have made comprehensive sequencing of numerous samples more affordable. The first commercial technology of its kind was the 454 GS20 released in 2005 by Roche, which was closely followed by the GS FLX and GS FLX Titanium pyrosequencing platforms. In early 2007, Solexa GA and SOLiD methods by Illumina and Applied Biosystems, respectively, were released (MacLean et al., 2009). Like Sanger sequencing, all three technologies are based on sequencing by synthesis, but each uses different methods for arraying templates and detecting nucleotide addition. 454 pyrosequencing can generate over 400,000 sequence reads per run and, of recent times, has been one of the most widely used approaches for microbial community analysis in wastewater (McLellan et al., 2010; Ye and Zhang, 2012; Zhang et al., 2012). This technology allows samples to be multiplexed by incorporating unique barcodes on primers, thus allowing numerous samples to be analysed in one 454 pyrosequencing run. Compared to other next-generation technologies this technique has longer read lengths (400-600 nucleotides) and short run times (Balzer et al., 2010; Liu et al., 2011; Shokralla et al., 2012). Illumina technologies have also been applied to assessing the microbial diversity of wastewater samples (Degnan and Ochman, 2011). This technology is 1/100 the cost per read of 454 pyrosequencing and allows over 100 samples to be multiplexed in one sequencing run. Over 600 million sequence reads are recovered per run with an average length of ~209 nucleotides, which can be increased by paired-end sequencing of the same amplicon (2 x 209 nucleotides) (Liu et al., 2011). Since 2009, single molecule sequence technologies have been developed by Helicos and Pacific Bioscience to overcome biases encountered during the first step of DNA-template amplification (Trevors and Masson, 2010), but these technologies have not been as widely embraced.

The disadvantage of these ‘next-generation’ sequencing technologies for 16S rRNA gene amplicon-based studies is the relatively short read lengths compared with Sanger sequencing, which limits the taxonomic resolution of these sequences. In addition, these technologies (with the exception of the Illumina platform) encounter errors in sequencing homopolymeric regions that are greater than three bases in length (Hert et al., 2008). The benefits of these technologies include the recovery of large number of reads per sample and the ability to conduct large-scale biodiversity projects, due to multiplexing (Shokralla et al., 2012). In this study, 454 pyrosequencing was used to monitor the microbial community composition of successionaly developing biofilm in full-scale and lab-scale MBBRs.
1.6.2. Thesis structure and specific aims of this project

This research has been structured into five integrated studies that have been prepared and submitted as individual journal publications. Each is presented as a separate chapter in this thesis, together with a concluding chapter which provides a synthesis of the whole study. An overview of each chapter and its current publication status is summarised below.

Chapter 1: General Introduction

Chapter 2: ‘Microbial community composition and dynamics of moving bed biofilm reactor systems treating municipal sewage’.

**Status:** Published (2012)

**Journal:** Applied and Environmental Microbiology

**Specific aims:**

To compare the microbial community structure, and interactions among the constituent microbes, between an MBBR system and a conventional activated sludge WWTP in New Zealand.

To determine whether there is a difference in microbial community structure between biofilm on carriers and those in the suspended fraction of a full-scale MBBR.

Chapter 3: ‘Successional development of biofilms in moving bed biofilm reactor (MBBR) systems treating municipal wastewater’.

**Status:** Published (2014)

**Journal:** Applied Microbiology and Biotechnology

**Specific aims:**

To document the microbial succession within developing biofilm on nascent carriers within a full-scale municipal WWTP.

To monitor the effect of influent composition and season on successional development of biofilms in two MBBR WWTPs.
Chapter 4: ‘**dsrAB-based analysis of sulfate-reducing bacteria in moving bed biofilm reactor (MBBR) wastewater treatment plants**’.

**Status:** In Press  
**Journal:** Applied Microbiology and Biotechnology

**Specific aims:**
To identify the organisms responsible for H$_2$S production at Moa Point WWTP by using dissimilatory (bi)sulfite reductase (dsrAB) gene-based approaches.

To monitor the effect of influent composition and season on SRBs in two MBBR WWTPs.

Chapter 5: ‘**Comparative analysis of bacterial community composition and dsrAB abundance between biofilms of K1 and K3 carriers in moving bed biofilm reactors**’.

**Status:** To be submitted  
**Journal:** Microbial Biotechnology

**Specific aims:**
To compare the successional development process of bacterial communities adhering to two commonly used carrier types in MBBR systems.

To investigate whether the carrier shape and size has an effect on dsrAB gene abundance in two full-scale MBBR plants.

Chapter 6: ‘**Biological nutrient removal and microbial community dynamics in lab-scale moving bed biofilm reactors under different environmental conditions**’.

**Status:** In review  
**Journal:** Water Research

**Specific aim:**
To construct a laboratory-scale MBBR that could be used effectively to monitor the effects of varying operational parameters on the nutrient removal and microbial communities.

Chapter 7: General Discussion
1.7. References


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 1 – General Introduction

*Thiothrix eikelboomii* sp. nov., *Thiothrix unzii* sp. nov., *Thiothrix fructosivorans* sp. nov. and *Thiothrix defluvi* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **49**: 1817-1827.


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 1 – General Introduction


2. Microbial community composition and dynamics of moving bed biofilm reactor systems treating municipal sewage

Publication status: Published

2.1. Abstract

Moving Bed Biofilm Reactor (MBBR) systems are increasingly used for municipal and industrial wastewater treatment, yet in contrast to activated sludge (AS) systems, little is known about their constituent microbial communities. This study investigated the community composition of two municipal MBBR wastewater treatment plants (WWTPs) in Wellington, New Zealand. Monthly samples comprising biofilm and suspended biomass were collected over a 12 month period. Bacterial and archaeal community composition was determined using a full cycle community approach including analysis of 16S rRNA gene libraries, Fluorescence in-situ Hybridization (FISH) and Automated Ribosomal Intergenic Spacer Analysis (ARISA). Differences in microbial community structure and abundance were observed between the two WWTPs and between biofilm and suspended biomass. Biofilms from both plants were dominated by *Clostridia* and sulfate-reducing members of the *Deltaproteobacteria* (SRBs). FISH analyses indicated morphological differences in the *Deltaproteobacteria* detected at the two plants and also revealed distinctive clustering between SRBs and members of the *Methanosarcinales*, which were the only archaea detected and were present in low abundance (< 5%). Biovolume estimates of the SRBs were higher for biofilm samples from one of the WWTPs which receives both domestic and industrial waste, and is influenced by seawater infiltration. The suspended communities from both plants were diverse, and dominated by aerobic members of the *Gammaproteobacteria* and *Betaproteobacteria*. This study represents the first detailed analysis of microbial communities in full-scale MBBR systems and indicates that this process selects for distinctive biofilm and planktonic communities, both of which differ from those found in conventional AS systems.
2.2. Introduction

The Moving Bed Biofilm Reactor (MBBR) system was developed in the late 1980’s for the treatment of domestic and industrial wastewaters. These systems are now operating in over 22 countries (including New Zealand) and range from large to small scale wastewater treatment plants (WWTPs) (Rusten et al., 2006). The MBBR process combines features of both fixed-growth and activated sludge (AS) systems in that the microbial community is largely retained within the reactor as a biofilm on suspended carriers, with a smaller planktonic fraction being present in suspension as free-floating cells or small flocs. MBBR technology offers a number of advantages over conventional technologies for treating waste including a smaller footprint and reactor volume, high effluent quality, and low waste sludge volumes. The high residence time of adherent biofilm biomass also facilitates biological nutrient removal (Yang et al., 2009).

Studies on the microbial community composition of conventional activated sludge systems indicate that the community is typically dominated by aerobic or facultatively anaerobic heterotrophic bacteria belonging to the Betaproteobacteria (Schmid et al., 2003). It is unclear whether similar communities are found in MBBR processes as there have been no microbiological studies on full-scale wastewater systems. Differences in microbial communities might be expected given that MBBR systems support development of microbial biofilms within which microenvironments support the growth of both anaerobic and aerobic organisms within the same ecosystem (Yang et al., 2009). The presence of a reduced oxygen gradient within biofilms has the potential to support the growth of obligate anaerobes such as ammonia-oxidizing bacteria from phylum Planctomycetes (Tal et al., 2003; Fu et al., 2010). Association of these organisms with nitrite-oxidizing bacteria such as Nitrospira sp. confers the added advantage of enabling simultaneous nitrification and denitrification within these systems.

Biofilm development is a key process in the establishment of an effective MBBR process. To maintain effective gas and nutrient transfer the ideal biofilm is relatively thin and evenly distributed over the carrier surface (Odegaard et al., 1999). This can be influenced by turbulence in the reactors which also influences substrate and oxygen transfer. Aside from these few key factors the effects of other operational parameters and influent composition on microbial community structure and function within MBBR systems are poorly understood.
The aim of this study was to investigate the microbial communities in Wellington’s Moa Point (MP) and Karori WWTPs and thus to provide the first comprehensive insight into the key microbial groups in full-scale MBBR systems. Approximately monthly samples, consisting of suspended biomass and biofilm scraped from carriers, were collected over a 12 month period from the two plants. A full cycle community analysis approach including analysis of 16S rRNA gene libraries, Fluorescence in-situ Hybridization (FISH) and Automated Ribosomal Intergenic Spacer Analysis (ARISA), was used to determine bacterial and archaeal community composition and dynamics. Total dissolved sulfides and dry/wet weight of biofilm adhering to carriers were also determined. Microbial community analysis was also performed on a sample from a conventional floc-based activated sludge system for comparison.

2.3. Materials and methods

2.3.1. Sample sites

Sampling was carried out at Wellington’s MP and Karori WWTPs. The MBBR reactors at both plants contained suspended polyethylene carriers (K1 media) comprising 30–50% of reactor volume. Reactor samples, comprising suspended K1 carriers with adherent biofilm, were collected once a month over a year from three MBBR reactors at MP (designated as M1, M2, & M3) and two reactors from Karori (designated K1 & K2) treatment plants. For comparison, mixed liquor from a conventional activated sludge system was collected from a large municipal WWTP in northern New Zealand. Samples were collected in one litre bottles and transported refrigerated overnight to the laboratory.

2.3.2. Physical and Chemical analyses

Dry and wet weight determinations were made on biofilm from five K1 carriers from each sample. To determine the wet weight, carriers with adherent biofilm were blotted dry on tissue paper for 2 min and then weighed. The samples were then transferred to a desiccator and dried for one week, then re-weighed. Control samples comprising five unused carriers were also subjected to desiccation and then weighed. Dry and wet weight determinations
Chapter 2 – Microbial communities of moving bed biofilm reactors

were made following subtraction of the average weight of the control carriers. The statistical significance of differences between samples was determined using a T-Test (unequal variance). To measure the suspended biomass, 1mL of sample was pelleted by centrifugation at 13,000xg for 10 min in a 1.5mL microfuge tube. The supernatant was carefully removed, the wet weight determined, and the opened tube subjected to desiccation, then weighed as described above.

Total dissolved sulfide was measured immediately upon receipt of samples by a colourimetric method as outlined previously (Cord-Ruwisch, 1985). A standard curve was prepared using varying concentrations of sodium sulfide. The absorbance of copper sulfide precipitate for each sample was measured at 460nm in a UV-vis Spectrophotometer.

2.3.3. DNA extraction

Total genomic DNA was extracted from biomass using a phosphate, SDS, chloroform-bead beater method as described previously (Smith et al., 2003). Extracted DNA was eluted in 50 µl of DNase-free water and stored at -20°C until further analysis.

2.3.4. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used as a rapid method to profile bacterial community structure and to make comparisons between monthly samples. The intergenic spacer region between the 16S and 23S rRNA genes was amplified using two universal bacterial primers SDBact and LDBact (Ranjard et al., 2001), in a PCR reaction described previously (Lear and Lewis, 2009). The fluorescently-labelled products were purified using a QIAquick® PCR purification Kit (Qiagen) and analyzed along with an internal LIZ1200 standard on a 3130XL Capillary Genetic Analyzer using a 50 cm capillary (Applied Biosystems Ltd., NZ).

Results from ARISA were analyzed using Genemapper software (v 3.7) to create bacterial community profiles for each sample. Multi-dimensional scaling (MDS) plots were constructed from community profile data using Primer 6 software (v 6.1.6). Manhattan distance was chosen as the measure between samples on the MDS plots.
2.3.5. 16s rRNA gene analysis

2.3.5.1. Clone library analysis

Cloning and RFLP analysis of PCR-amplifed 16S rRNA genes was performed as generally described previously (Ayton et al., 2010). Forward and reverse primers for PCR amplification of bacterial 16S rRNA genes were 5’- AGRGTTTGATCMTGGCTCAG-3’ and 5’- GKTACCTTGTTACGACTT-3’ respectively (Saul et al., 2005). Primers used for analysis of archaeal 16S rRNA gene sequences were 5’-TTCCGGTTGATCCYGCCGA-3’(Arch21F) and 5’-YCCGGCGTTGAMTCCAATT-3’ (Arch958R) (Delong, 1992). Cloned inserts were recovered by PCR amplification using the vector-specific primers PGEM-F (5’-GGCGGTCGCGGAATTGATT-3’) and PGEM-R (5’-GCCGCGAATTCAACTAGTTGATT-3’) (Aislabie et al., 2009).

2.3.5.2. Restriction Fragment Length Polymorphism (RFLP) of clones

Restriction endonuclease Hae III (Invitrogen) digestion was used to generate RFLP profiles for archaeal clones to investigate diversity prior to selection of clones for sequencing as previously described (Ayton et al., 2010). The resulting products were resolved and visualized by Polyacrylamide Gel Electrophoresis (PAGE) through 6% non-denaturing gels as described previously (Sambrook and Russell, 1989). Gels were run at 120 V for 70 min and then stained with ethidium bromide. Unique RFLP profiles were designated as operational taxonomic units (OTUs) and representative clones selected for sequencing.

2.3.5.3. Sequencing and data analysis

PCR-amplified inserts from representative clones were purified and sequenced in one direction using the aforementioned vector-specific (pGEM-F) primer. Purification and sequencing was performed under contract by Macrogen Inc (Kumchun-Ku, Seoul, South Korea) using a 3730 x 1 DNA Analyzer (Applied Biosystems). A total of 96 clones were sequenced from each bacterial clone library, whereas one clone representing each OTU was sequenced from the archaea libraries.
Sequence data was processed using the high-throughput pipeline available through the Ribosomal Database Project II (http://rdp.cme.msu.edu) (Wang et al., 2007) and also compared with the GenBank database sequences (http://www.ncbi.nlm.nih.gov) using nucleotide-nucleotide BLAST (Altschul et al., 1990).

2.3.5.4. Phylogenetic analysis

Sequences were screened for chimeras using Mallard software (version 1.02; School of Biosciences, Cardiff University [http://www.bioinformatics-toolkit.org/Mallard/index.html]) (Ashelford et al., 2006) and unreliable sequences eliminated from further analysis. The remaining partial and full length 16S rRNA sequences were aligned using the SINA web alignment tool, SILVA (www.arb-silva.de/aligner) (Pruesse et al., 2007). These sequences were imported into the ARB software (Ludwig et al., 2004) along with the SSU ref database (SILVA version 106) to construct phylogenetic trees. The SSU ref database contains more than half a million curated full length (>1200 bp) 16S rRNA sequences that have been previously published or uploaded to public databases (such as GenBank). Phylogenetic trees were constructed using Maximum Likelihood methods.

2.3.5.5. Fluorescence In Situ Hybridization (FISH)

FISH was used in combination with confocal laser scanning microscopy to examine samples for the presence of methanogens and SRBs (Manz et al., 1992; Amann et al., 1995). Samples were fixed within 12 h of sample collection using 4% paraformaldehyde as described previously (Manz et al., 1992), and stored at -20°C in a 1:1 PBS and ethanol mixture awaiting further analysis. Slides were prepared from fixed samples by placing 20 µL of sample into 6 mm diameter wells on Teflon ® coated slides (ProSciTech) which were then air dried. Samples were dehydrated by immersion in 50%, 80% and 98% ethanol respectively for 3 min each. Hybridization was carried out in a 50 mL Falcon tube chamber at 46°C for 2 h. Oligonucleotide probes were derived from published sequences with a 5’-fluorescent label (specified in Table 2.1) and synthesized by Thermo Fisher Scientific (Germany). Each well was hybridized with buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, formamide at optimal concentrations for each probe) and 1 µL of 50 ng/µL probe. Following incubation,
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slides were immersed in pre-heated (48°C) wash buffer (20 mM Tris-HCl, 5 mM EDTA, NaCl at optimal concentrations for each probe) and air dried in the dark. Salts were removed from the slides by washing in ice-cold distilled water. DAPI (10 µg/µL) was then applied as a universal DNA stain. Slides were incubated in the dark for 5 min then rinsed with distilled water and air dried.

Table 2.1. Fluorescence in situ hybridisation (FISH) probe sequences.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Target group</th>
<th>Probe sequence</th>
<th>Label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>Eubacteria</td>
<td>GCTGCCTCCCGTAGGAGT</td>
<td>CY3</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>MSMX860</td>
<td>Methanosarcinales</td>
<td>GGCTCGCTTCACGGCTTCC</td>
<td>CY3</td>
<td>(Raskin et al., 1994)</td>
</tr>
<tr>
<td>DELTA 495a</td>
<td>Most Deltaproteobacteria and Gemmatimonadetes</td>
<td>AGTTAGCCCGGTGCTTCTT</td>
<td>CY5</td>
<td>(Loy et al., 2002)</td>
</tr>
<tr>
<td>cDELTA 495a</td>
<td>Competitor for DELTA495a</td>
<td>AGTTAGCCCGGTGCTTCTT</td>
<td>-</td>
<td>(Macalady et al., 2006)</td>
</tr>
</tbody>
</table>

2.3.5.6. Confocal microscopy

FISH slides were viewed using an FV1000 Olympus confocal laser scanning microscope (CLSM) equipped with a 100x oil immersion objective lens. Excitation of FITC, Cy3 and Cy5 was performed at 488 nm (Ar-laser), 543 nm (He-Ne laser), and 635 nm (red diode laser), respectively. Images were viewed by using an FV10-ASW2.0 (Olympus) viewer.

For quantification, at least five z-series of 1 µm depth (6-10 sections) were made for each sludge sample with a universal stain (DAPI) and a group-specific probe (MSMX860 or Delta495a). The percentage biovolume was calculated by using DAIME software (Version 1.2, http://www.microbial-ecology.net/daime) (Daims et al., 2006).
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2.4. Results

Sampling was carried out at Wellington’s MP and Karori WWTPs. Both plants are configured to include primary settling followed by treatment in an aerated moving bed bioreactor. Solids are removed by contact stabilization and clarification. MP receives household and industrial waste, including that from an abattoir, and has an average dry weather flow of 822 L/s with a BOD of 0.23 kg/m³. The Karori WWTP receives only domestic waste with an average dry weather inflow of 20 L/s with a BOD of 0.37 kg/m³. Elevated conductivity levels (yearly average 4.072 mS/cm), indicative of seawater infiltration, are consistently detected at the MP plant while levels in influents reaching the Karori plant are low and typical of domestic effluents. A total of 11 approximately-monthly samples were collected from each of the two treatment plants over a period of 12 months. Samples were taken from the aeration tank of the MBBRs and comprised K1 carriers with adherent biofilm and reactor fluid.

2.4.1. General characteristics of biofilm and suspended biomass

The colour and odour of biofilms attached to K1 media differed between sites. At MP the biofilms were black in colour and had a sulfurous odor. In contrast, biofilm samples from the Karori WWTP were consistently greyish-brown and had no obvious odour. A comparison of biofilm quantity was made by determining the wet and dry weight of biofilms from each MBBR for all time points. No significant differences (p = 0.124) were observed between the dry weight of the biofilm samples from MP (0.029 ± SD 0.033 g, n=8) and Karori (0.013 ± SD 0.006 g, n=8). Similarly, no significant differences were observed between the wet weights of biofilm from the two sites (p = 0.379). P-values < 0.05 were considered significant.

To provide an estimate of the amount of suspended biomass, dry and wet weight determinations were also made on pellets prepared from 1 mL of supernatant fluids. Supernatant dry weight values were low (<0.021 g/mL) for all samples. No significant differences (p = 0.427) were found between samples from the two MBBR systems.
Total dissolved sulfides were detected in all samples from the MP site with values ranging between 1.5 to 13 mM. In contrast sulfide was not detected (<1 mM) in samples collected from the Karori plant.

2.4.2. Bacterial community analysis

2.4.2.1. ARISA

ARISA was used as a rapid method to profile and compare the bacterial community structure in both biofilms and suspended biomass. Multi-dimensional scaling (MDS) was used to investigate differences between ARISA community profiles over time and between treatment plants. These data indicated differences between the two WWTPs and also between biofilm and suspended communities (Fig. 2.1).

![Figure 2.1](image)

**Figure 2.1.** Multidimensional scaling (MDS) plot of ARISA data representing bacterial communities in samples collected over 12 months from MP and Karori WWTPs.

Each symbol within the graph represents a bacterial community for one given time point. Minimum stress = 0.1. Bacterial communities from Moa Point (black) and Karori (grey) samples have formed two distinct clusters. Evidence of biofilm communities (enclosed within the black circles) segregating away from the suspended biomass is also displayed.
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2.4.2.2. 16S rRNA gene analysis

To determine the bacterial community composition, clone libraries of PCR-amplified 16S rRNA genes were prepared and sequenced from biofilm scraped from carriers and from suspended biomass. This analysis was performed on reactor samples collected at three time points (March-2010, August-2010, January-2011). Figure 2.2 presents a summary of phyla or class detected for a representative sample from each plant. Phylogenetic trees constructed using the entire sequence dataset are presented for all phyla (Fig. 2.3), the *Deltaproteobacteria* (Fig. 2.4) and the *Epsilonproteobacteria* (Fig. 2.5). A phylogenetic tree of sequences aligning to the *Firmicutes* is presented in Fig S1.1.

![Figure 2.2](image_url)

*Figure 2.2. Composition of 16S rRNA gene clone libraries in biofilm (A) and suspended biomass (B) from Moa point and Karori reactors.*

Samples were collected in March-2010. For comparison, a clone library prepared from conventional AS mixed liquor (C) is also shown. Each colour on the graph represents the dominant Phyla or Class found within the community.
Figure 2.3. Maximum likelihood tree showing alignment of sequences from clone libraries prepared from biofilm and suspended communities over three time points.

Numbers within brackets indicate percentage of clones from the MP biofilm, Karori biofilm, MP suspended and Karori suspended samples, respectively. The scale bar indicates 10% sequence divergence.
Figure 2.4. Maximum likelihood tree showing alignment of sequences from clone libraries to the sulfate reducing members of the Deltaproteobacteria.

Clone sequences are presented in bold and identified by sample type, date, and clone reference respectively. Sample type identifiers: M1, Moa Point biofilm; MS1 Moa Point suspended biomass; K1, Karori biofilm; KS1, Karori suspended biomass. Numbers in brackets after the sequence identifier indicate number of clones within the cluster. Shaded boxes indicate diversity within a group of clones. Dotted lines indicate partial sequences (450 – 750 bp). Open and filled circles indicate clades supported by bootstrap value of ≥75% and ≥90% respectively. Horizontal bars indicate groups within the Syntrophobacterales (Syn), Desulfobacterales (Dsb) and Desulfovibrionales (Dsv). Outgroup consists of sequences from other bacterial phyla.

Each treatment plant yielded a characteristic community composition that was consistent between reactors and time points (Fig. 2.2(A)). Biofilm communities from both plants showed limited bacterial diversity (Shannon-Wiener index of 0.93 - 1.18) and were dominated by Firmicutes (45–60% of clones). Phylogenetic alignment of sequences indicated a broad diversity within the Firmicutes (Fig. S2.1) although the majority of clones represented members of the Clostridia (27-57% of clones). Strictly anaerobic, sulfate-reducing bacteria (SRB) belonging to the class Deltaproteobacteria were the second most abundant group, comprising 35% of the biofilm clone library at MP and 26% at Karori. Members of Desulfobacterales (9-24 %), Syntrophobacterales (5-15%) and Desulfovibrionales (0-4%) were the most abundant SRBs found within these samples (Fig. 2.4). The biofilms from Karori WWTP differed due to an elevated incidence of Alpha- and Betaproteobacteria (10 and 9%, respectively), which were both in low abundance (<3%) in the biofilm community from MP. A number of other organisms including representatives of the phyla Fusobacteria and Deferribacteres, as well as various unclassified bacteria, were also detected at low abundance (<5% of clones) in the biofilm samples from both plants. Analysis of the archaeal community from the two MBBR systems indicated a very limited diversity, with only one RFLP pattern detected among the 20 clones screened. Sequence analysis indicated that this pattern represented organisms from the methanogen order Methanosarcinales.

In contrast to the biofilm samples, the suspended biomass from both MBBR systems (Fig. 2.2(B)) was dominated by a more diverse group of aerobic organisms from the Alphaproteobacteria (Rhizobiales and Rhodobacterales), Gammaproteobacteria (Pseudomonadales and Aeromonadales) and Betaproteobacteria (Burkholderiales and
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*Rhodocyclales*. Members of the *Clostridia* were also present but in low numbers (10-15%) in both WWTPs. In addition, MP samples had an elevated level of *Epsilonproteobacteria* (43-60% of clones) which were affiliated with the *Campylobacteraceae* and aligned most closely to *Arcobacter* spp (Fig. 2.5). Though a diversity of sequences were observed, the majority aligned most closely to clones obtained from estuarine and river environments or to *Arcobacter nitrofigilis*.

The clone library prepared for comparative purposes from a mixed liquor sample from a conventional AS plant (Fig. 2.2(C)) was more diverse and dominated by members of the *Betaproteobacteria* (28%) and *Bacteroidetes* (25%).

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**Figure 2.5.** Maximum likelihood tree showing alignment of sequences from clone libraries to the *Epsilonproteobacteria*.

Vertical bar with brackets in bold indicates groups within the genus *Arcobacter*. Details are same as those provided in figure 2.3 and 2.4.
2.4.2.3. **FISH analysis**

FISH was used as a PCR-independent approach to validate the 16S rRNA gene library results, and to investigate the spatial distribution and abundance of archaea using a *Methanosarcinales* probe and putative sulfate reducing bacteria using a *Deltaproteobacteria* probe. FISH analysis was performed on samples collected between Mar-2010 to Jun-2010, Sept-2010, Dec-2010 and Jan-2011.

The distinctive black biofilm from MP revealed colloidal clusters of *Deltaproteobacteria* buried within the biofilm structures (Fig. 2.6(A)). Based on the clone library results, these are likely to be sulfate reducing bacteria. Samples from Karori also showed the presence of *Deltaproteobacteria* but these were fewer in abundance and were filamentous (Fig. 2.6(B)). Biovolume analysis confirmed the occurrence of *Deltaproteobacteria* at both treatment plants, with higher volumes recorded in samples from MP WWTP (Fig. 2.6).

* Methanosarcinales were observed in low numbers (<5% biovolume) in biofilms from the two MBBR systems. However, in samples from the MP plant these were observed in distinctive clusters around cells hybridising with the *Deltaproteobacteria* probe, which represent putative SRBs (Fig. 2.6(A)).
### Figure 2.6

Biofilm samples scraped from carriers (as seen in column 1) were hybridized with fluorescently labelled probes targeting methanogenic archaea (MSMX860-red), *Deltaproteobacteria* (DELTA495a-cyan), and eubacteria (EUB338-green).

DAPI (blue) was used as a universal DNA stain. Clusters of *Deltaproteobacteria* (cyan-arrowed ‘s’) were abundant in black biofilm from MP (A). Clusters of *Deltaproteobacteria* from Karori were present as filaments (B). Methanogenic archaea (arrowed ‘m’) are visible in both samples. Biovolume estimates of methanogens and *Deltaproteobacteria* relative to DAPI stained material are shown in graphs for both samples.

<table>
<thead>
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<th>Carriers</th>
<th>FISH image</th>
<th>% biovolume</th>
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<td><img src="image2" alt="Biovolume graph of Moa Point" /></td>
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<tr>
<td>Karori</td>
<td><img src="image3" alt="FISH image of Karori" /></td>
<td><img src="image4" alt="Biovolume graph of Karori" /></td>
</tr>
</tbody>
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2.5. Discussion

Since their invention, MBBR systems have been used to treat both municipal and a wide range of industrial wastes including pulp and paper (Jahren et al., 2002), cheese factory waste (Rusten et al., 1996), and phenolic wastewater (Borghei and Hosseini, 2004). The advantages of MBBR systems over AS derive from the maintenance of a high biofilm biomass enabling capacity for high loading rates, and consequently shorter hydraulic retention times, smaller reactor volume, and no sludge bulking (Chen et al., 2008). Previous studies (Rusten et al., 2006; Yang et al., 2009) have reported on the physical and engineering aspects of MBBR systems while the composition of the microbial communities responsible for the biological activity has received no attention in full-scale systems treating municipal wastewater. This study set out to investigate the prokaryotic community dynamics in a parallel study of two MBBR treatment plants over a 12 month period. The MBBR treatment systems included in this study are situated in the same city and are operated under similar parameters to treat urban municipal wastewater. The major operational difference between the two plants is that the Karori plant receives effluent from a largely residential catchment, while the MP plant services a much larger catchment that includes mixed urban and industrial uses.

2.5.1. Biofilm communities

Differences in bacterial community profiles between the two WWTPs were expected due to the different sources of waste entering these systems. The results of this study indicated no difference in the amount of biomass retained on carriers, but distinct differences were noted in both colouration and dissolved sulfide concentrations. The observation that MP biofilms were black in colour and sulfurous in odour is consistent with the detection of appreciable levels of dissolved sulfides at this site. The detection of sulfides at MP indicates the possible activity of anaerobic SRBs, which are commonly found in anoxic systems (Muyzer and Stams, 2008; Barton and Fauque, 2009). Sulfate is reduced to sulfide by SRBs, resulting in a pungent sulfurous odour and upon reaction with metals yields black precipitates.

Despite differences in the colour of biofilms from the two sites, both yielded bacterial 16S rRNA gene libraries that were dominated by anaerobes, notably Clostridia and members of the Desulfbacterales and Syntrophobacterales – known sulfate-reducing bacteria (Muyzer and Stams, 2008). Both plants also showed similar communities of archaea, which were
dominated by *Methanosarcinales*. Validation of these results was performed by FISH using probes targeted to the *Methanosarcinales* and the *Deltaproteobacteria*. Although the *Deltaproteobacteria* probe is not specific for sulfate-reducing bacteria, these were the only members of the class detected in the clone libraries. It was therefore presumed that cells hybridizing to the *Deltaproteobacteria* probe were SRBs. Biovolume analysis confirmed the presence of SRBs in both sample sets but indicated a higher abundance in the MP site and different cell morphology to that seen in the Karori samples. These differences may go some way towards explaining the higher levels of sulfides detected at the MP site. It is also possible that differences in influent composition influence the community structure and function in these biofilms. In addition to domestic wastewater, the MP plant receives industrial waste from an abattoir, expected to carry large amounts of fats, oils and greases (Mittal, 2006). The presence of long chain fatty acids (LCFA) derived from the degradation of lipids (Sousa et al., 2009) and low water temperatures have been shown to select for SRBs in a wastewater treatment system (Leloup et al., 2005; Ben-Dov et al., 2009). Enhanced SRB activity would also require access to excess sulfate as an electron acceptor. The source of this in the MP plant has not yet been clearly established, although seawater infiltration is suspected in this system, as indicated by elevated conductivity levels. Seawater is known to contain appreciable concentrations of sulfate and high concentrations have been found in an MBBR system treating waste from a marine aquarium (Labelle et al., 2005).

FISH and biovolume analysis confirmed the presence of members of the *Methanosarcinales* in approximately equal abundance at both sites. This analysis also revealed a clear association of these methanogens with *Deltaproteobacteria* in the MP samples. The majority of SRBs described in the past are putatively free-living (Muyzer and Stams, 2008), but recent studies of the marine environment using FISH have revealed consortia of SRBs and methanogens (Boetius et al., 2000; Knittel et al., 2003), suggesting a symbiotic bacterial-archaeal interaction. The physiological basis of this symbiosis is still a matter of conjecture. Although methanogens were detected at the Karori site, these were not observed in association with the filamentous SRBs, further supporting the notion that this community differs from that found in the MP samples. Filamentous SRBs belonging to the genus *Desulfonema* have been detected previously in marine and freshwater sediments (Widdel, 1983; Fukui et al., 1999). Members of this genus exhibit gliding motility which is presumed to confer the ability to move along gradients within dense mats and avoid predation by grazing protozoa. These features would be equally advantageous in both MBBR biofilms, though filamentous SRBs
were only detected in the Karori samples. This further supports the notion that the biofilm communities in these two systems are influenced by external factors which may include differences in influent composition. Indeed, other studies have confirmed that specialized groups of microorganisms develop over time in response to changes in complex organic matter entering the system (Schink, 1997).

2.5.2. Suspended biomass

Clone library analysis indicated that the suspended fraction was dominated by aerobic members of the Alpha-, Beta-, Gamma- and Epsilonproteobacteria. Members of the Clostridia were also present but in much lower abundance than in biofilm samples, possibly resulting from biofilm detachment processes which are known to occur periodically in such systems (Lewandowski et al., 2007). A notable difference between the two plants was the high abundance of sequences in the MP clone library that aligned to Arcobacter spp including Arcobacter nitrofigilis. The genus Arcobacter was proposed in 1991 (Vandamme et al., 1991) to describe a group of aero-tolerant members of the Campylobacteraceae and has been of increasing interest as an emerging zoonotic pathogen. The genus includes species isolated from a diverse range of aquatic and terrestrial environments as well as the faeces and reproductive tracts of domestic livestock, sewage and abattoir effluents. The genus type species, Arcobacter nitrofigilis, is a nitrogen-fixing organism that was isolated from the root zone of a salt-marsh plant although not all species are salt tolerant (Collado and Figueras, 2011). The reason for the abundance of Arcobacter in the MP suspension samples remains unclear although the MP plant receives effluent from a local abattoir and it is possible that they derive from this source.

Consistent differences between the planktonic and biofilm communities are indicated by the MDS analysis of the ARISA results. These differences may be explained by the different conditions that prevail within these structured environments. The relatively short hydraulic retention time within the MBBR system selects for organisms that have a high growth rate in order to withstand wash-out and to be retained in the reactor. The bulk liquid phase is also aerated, supporting aerobic metabolism. While members of the Clostridia were detected in the suspended phase, the majority of taxa identified in the clone library analysis were from
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putatively fast-growing aerobic species including members of the *Burkholderiales*, *Rhodocyclales*, *Aeromonadales*, *Pseudomonadales*, and *Rhodobacterales*.

### 2.5.3. Comparison with activated sludge

Microbial communities in activated sludge systems have been widely discussed in the literature as the majority of modern wastewater treatment facilities utilize this technology (Seviour and Nielsen, 2010). Activated sludge typically comprises free-swimming planktonic cells and aggregated flocs that can be maintained under both aerobic and anoxic conditions. Bacterial communities in AS plants treating industrial and municipal wastewater are typically dominated by *Betaproteobacteria*, followed by *Alpha- and Gammaproteobacteria* (Lee et al., 2002; Schmid et al., 2003). Other groups of bacteria found in low abundance are *Bacteroidetes* and *Firmicutes*. The bacterial community composition results from previous studies correlate with the clone library prepared from a conventional AS mixed liquor sample in this study. The results of the current study also suggest that both the attached biofilm and suspended communities in MBBR systems differ substantially from that found in conventional AS.

### 2.5.4. Concluding remarks

This study provides the first detailed investigation of key microbial groups in full-scale MBBR systems treating municipal wastewater. It also provides insights into the spatial arrangement of key taxa in biofilms. The results indicate that MBBR communities differ substantially from those in conventional AS systems by selecting for two distinct bacterial communities: a biofilm community that is dominated by anaerobes and a suspended community that includes fast-growing aerobic bacteria. Differences were also observed between the bacterial community structure in biofilms from the two treatment plants, possibly reflecting differences in influent composition. The high abundance of sulfate-reducing bacteria, obligate anaerobes and the presence of black biofilm and measurable amounts of sulfide support the notion that microbially-mediated sulfate reduction is occurring within biofilms at the MP plant. It is possible that this is due to the influence of seawater infiltration although further studies are required to confirm this. The observation, from FISH analyses, of
a close association between *Methanosarcinales* and putative SRBs in the MP biofilms implies a functional relationship between these two groups of microorganisms. Further studies are required to elucidate the nature of this relationship and to develop food web models that will underpin manipulation of the system for optimal performance.

### 2.6. Acknowledgements

The authors would like to acknowledge the assistance of Veolia Limited staff from Moa Point and Karori WWTPs for provision of samples and support for this study.
2.7. References


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2.8. Supplementary material
Figure S2.1. Maximum likelihood tree showing alignment of sequences from clone libraries to the *Firmicutes*. Details are same as those provided in figure 2.3 and 2.4.
3. Successional development of biofilms in moving bed biofilm reactor (MBBR) systems treating municipal wastewater

Publication status: Published

Chapter 3 – Biofilm development in MBBRs

3.1. Abstract

Biofilm-based technologies, such as moving bed biofilm reactor (MBBR) systems, are widely used to treat wastewater. Biofilm development is important for MBBR systems as much of the microbial biomass is retained within reactors as biofilm on suspended carriers. Little is known about this process of biofilm development and the microorganisms upon which MBBRs rely. We documented successional changes in microbial communities as biofilms established in two full-scale MBBR systems treating municipal wastewater, over two seasons. 16S rRNA gene–targeted pyrosequencing and clone libraries were used to describe microbial communities. These data indicate a successional process that commences with establishment of an aerobic community dominated by Gammaproteobacteria (up to 52% of sequences). Over time this community shifts towards dominance by putatively anaerobic organisms including Deltaproteobacteria and Clostridiales. Significant differences were observed between the two WWTPs, mostly due to a large number of sequences (up to 55%) representing Epsilonproteobacteria (mostly Arcobacter) at one site. Archaea in young biofilms included several lineages of Euryarchaeota and Crenarchaeota. In contrast, the mature biofilm consisted entirely of Methanosarcinaceae (Euryarchaeota). This study provides new insights into the community structure of developing biofilms at full-scale WWTPs, and provides the basis for optimizing MBBR start-up and operational parameters.

3.2. Keywords

Wastewater; Bacteria; Archaea; Pyrosequencing; Biofilm succession; Microbial communities
3.3. Introduction

The challenges associated with wastewater treatment, such as rising energy costs, increasingly stringent effluent requirements and limited land use for future treatment plants, has led to the increased use of innovative and efficient technologies. The water and wastewater industries alone consume >3% of total electricity in the US (M/J-Industrial-Solutions, 2003) and >1% in the UK (Caffoor, 2008) each year. Biofilm-based technologies for the treatment of municipal and industrial wastewater were developed to overcome several disadvantages faced by conventional floc-based systems and often produce higher effluent quality (Rusten et al., 1997; Andreottola et al., 2000). One such technology, established in 1989 by AnoxKaldnes™, is the moving bed biofilm reactor (MBBR) system (Odegaard et al., 1994). To date, more than 1200 wastewater treatment plants (WWTPs) in >50 countries utilize this biofilm membrane-based technology (Pritchard pers. comm., 2012). These include WWTPs that treat effluent from both industry (including fisheries, wineries, dairy) and domestic sources (Rusten et al., 1992; Odegaard et al., 1994; Rusten et al., 2006).

The establishment and development of an ideal biofilm is integral to the MBBR process, which utilizes microorganisms that are both biofilm-based and floc-based. Suspended carriers are added to the reactors at the treatment plants to retain slow-growing organisms despite the low hydraulic retention time generally incurred at MBBRs. The development of anoxic zones within these biofilms is favorable to the design of MBBRs as it enables the growth of anaerobic organisms that carry out important processes for nutrient degradation such as sulfate reduction, denitrification, phosphorus removal and methanogenesis (Pastorelli et al., 1999; Labelle et al., 2005; Wang et al., 2006). However, excessive anaerobic digestion can also occur at these plants, leading to problems such as high levels of sulfide emissions. Much is known about the mechanical, nutrient removal and technical aspects of the biofilm in MBBR systems (Fu et al., 2010; Cao and Zhao, 2012; Shore et al., 2012), however information on the microbial communities upon which these systems rely is limited.

The microbial ecology of conventional activated sludge wastewater treatment systems has been the subject of a number of recent studies using next-generation sequencing technologies (McLellan et al., 2010; Ye and Zhang, 2011; Hu et al., 2012; Zhang et al., 2012). These studies have identified five core phyla within the bacterial communities, namely Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and, to a lesser extent, Verrucomicrobia. We recently reported the first detailed analysis of the microbial
communities in two full-scale municipal MBBR treatment plants using 16S rRNA gene clone libraries, automated ribosomal intergenic spacer analysis (ARISA) and fluorescence in situ hybridisation (FISH) (Biswas and Turner, 2012). Similar phyla to those reported in activated sludge were present in biofilm and suspended fractions of MBBRs, although their community structure looked very different. Established biofilm communities in the MBBR plants were dominated by presumed anaerobes such as Clostridia and sulfate-reducing members of the Deltaproteobacteria, with methanogens also present albeit at lower abundance (<5% of the total biovolume). By contrast, microbial communities in the surrounding liquid fraction were characterized by a diverse range of putatively aerobic microorganisms including Gammaproteobacteria, Betaproteobacteria, and Alphaproteobacteria (Biswas and Turner, 2012). Similar observations were made previously for integrated fixed-film activated sludge systems treating domestic wastewater (Kwon et al., 2010), which showed major differences in the community composition of the suspended fraction compared with established biofilms.

The key to biofilm formation for successful MBBR operation is considered to be related to microbial community structure and functionality of the organisms that develop on the surface of the carriers. Bacterial community development on surfaces in conventional activated sludge treatment plants (Santegoeds et al., 1998; Fernández et al., 2008) and in lab-scale bioreactors (Connaughton et al., 2006; Miura et al., 2006; Calderón et al., 2012) has been studied using molecular fingerprinting methods and FISH. These studies indicate a successional process for biofilm development, although the interaction between suspended and biofilm communities is still unclear.

The aim of this study was to investigate the establishment and development of biofilm communities in two full-scale municipal WWTPs located in New Zealand (Moa Point and Karori) that utilize MBBR technology but differ in influent composition. As the start-up of any wastewater treatment system is critical to subsequent plant performance, the successional development of biofilms on nascent carriers was investigated in these two MBBR systems. Furthermore, we aimed to determine whether the two WWTPs follow similar patterns of biofilm succession and if, or when, the respective microbial communities diverge. A longitudinal study was performed in which developing biomass was collected periodically from carriers suspended in MBBRs at the two study sites over a period of up to 48 days. 16S rRNA gene-targeted pyrosequencing and Sanger sequencing of 16S rRNA gene clone libraries were used to determine the bacterial and archaeal community composition of samples over time.
3.4. Materials and methods

3.4.1. Study sites and experimental design

Two full-scale MBBR systems, each containing suspended polyethylene carriers (K1 carriers, AnoxKaldnes™) and treating municipal wastewater, were chosen as study sites. Both plants specialize in BOD removal and are located in Wellington, New Zealand. Moa Point WWTP, with a reactor volume of 2760 m³, is the larger of the two sites and receives trade and domestic waste from a larger urban area. The smaller Karori WWTP (350 m³) treats mainly domestic waste from a comparatively small residential catchment. The sludge age of the two sites is between 1 to 2 days with hydraulic retention time within the MBBRs of 1 h. Operational parameters including BOD, suspended solids (SS), dissolved oxygen (DO), fecal counts (FC), H₂S levels, daily flow rate, temperature, rainfall and conductivity of these two WWTPs over the experimental period were recorded onsite on a daily basis, with the exception of conductivity that was measured weekly. In addition, one effluent sample from Moa Point was also measured for pH, NH₃–N, oil and grease during winter and summer periods.

To monitor biofilm development unused K1 carriers were threaded onto a weighted nylon fishing line (0.6 mm thickness) and suspended in the MBBR tanks. Two lines of 40 suspended carriers were established at each WWTP over winter (May-Jun 2011) and repeated in summer (Jan-Feb 2012). Carriers (5 to 7) were collected from each MBBR at various intervals from day 4 (i.e. 4 days after suspension of the fresh carriers in the MBBR) up to day 48. At the commencement of the summer survey, samples were also collected from both the surrounding mixed liquor (suspended fraction; labelled as day 0) and from K1 carriers that had been in operation for several years (labelled as mature). Immediately after collection, samples were stored in RNALater (Ambion) at room temperature for 24 h, then transferred to -20°C for later DNA extraction.

3.4.2. Physical characteristics of the biofilm

The wet weight of the biofilm on K1 carriers was determined by analysing three replicates per sampling time point from each WWTP under study. K1 carriers were blot-dried by placing on tissue paper for 2 min, then weighed. Wet weight measurements were employed in this study due to limited availability of deployed carriers.
3.4.3. DNA extraction

Biofilm was scraped off the carriers, and genomic DNA extracted in a bead-beating approach based on that of Smith et al. (2003). Modification to the published method included use of a shorter lysozyme incubation step of 10 min and a less vigorous bead-beating step (4 m/s for 25 s) that was optimized specifically for developing biofilms on carriers. Extracted DNA was eluted in 30 μl of DNase-free water and stored at -20°C for future analysis.

3.4.4. Pyrosequencing of bacterial 16S rRNA gene amplicons

DNA extracted from biofilm samples scraped from triplicate carriers from Moa Point and Karori WWTPs at each time point (for summer: suspended fraction, days 4, 13, 20, 34, 48 and mature; for winter: days 4, 10, 18, 35) was analysed using 454 pyrosequencing of 16S rRNA gene amplicons. Mature biofilm from resident carriers was collected at a single time point during this study as previous work indicated minimal variation in bacterial community structure over the course of one year (Biswas and Turner, 2012). 16S rRNA gene fragments were amplified using primers 347f and 803r, which have been used previously to characterize the bacterial community of the human foregut (Nossa et al., 2010). The suitability of these primers for wastewater microbial communities was validated in silico by using Probe Match in the RDP database, and empirically, via construction of a 16S rRNA gene clone library of a mature biofilm sample from Moa Point WWTP (data not shown). The results matched the 16S rRNA gene clone library results from our earlier paper (Biswas and Turner, 2012) where generic 16S rRNA gene-targeted primers (PB36 and PB38) were used. The forward primer comprised adaptor A, a 4 nt key, a unique multiplex identifier (MID), and the 16S rRNA gene template-specific primer (347f). The reverse primer consisted of adaptor B, a 4 nt key, and the template-specific primer (803r). Lib-L chemistry (Roche/454 Life Sciences) was used to enable unidirectional sequencing from the adaptor A (forward) end, and amplicons were multiplexed using 24 unique MID tags to enable identification of samples. Genomic DNA was quantified using Picogreen (Quant-iT dsDNA kit, Invitrogen) and standardized quantities (~5-10 ng/μl) were added to a PCR mixture containing equimolar concentrations (0.2 μM) of fusion primers (forward and reverse), dNTPs (0.2 mM), PCR buffer (1X), MgSO₄ (2 mM), 0.5U Platinum Hi-fidelity Taq (Invitrogen) and PCR-certified water to a final volume of 25 μl. PCR amplification was performed in an Applied Biosystems Mastercycle gradient PCR machine with an initial denaturing step of 94°C for 1 min, followed by 30 cycles of
denaturation (94°C for 30 s), annealing (55°C for 30 s), and elongation (68°C for 30 s) and a terminal elongation step at 68°C for 3 min. A sample from each PCR reaction was visualized on a 1% agarose gel to check amplicon quality and yield prior to purification. PCR amplicons were then purified using AMPure Beads (Roche), quantified using Picogreen, and qualitatively checked on Agilent 1200 Bioanalyzer DNA 1000 chips (Agilent Technologies, Santa Clara, CA, USA). Equal concentrations of samples were pooled into a library, resulting in a final concentration of 10 ng/µl and a 260/280 ratio >1.80 in accordance with Macrogen’s instructions. Amplicon libraries were sequenced on the Roche GS FLX Titanium platform by Macrogen Inc. (Seoul, South Korea). Obtained sequences have been deposited in the NCBI SRA database under accession number SAMN02169314 - SAMN02169379. Analysis of all pyrosequencing reads was carried out as described previously (Schmitt et al., 2012; Simister et al., 2012). In brief, a combination of Mothur (Schloss et al., 2009) and custom-made PERL scripts was used to de-noise, filter, trim, and remove contaminant sequences (such as mitochondrial and chloroplast DNA) from the pool of amplicon sequences. Alignment of sequences was carried out using SILVA as the reference database (http://www.mothur.org/wiki/Silva_reference_alignment) and chimeras were removed using UCHIME (Edgar et al., 2011). Operational taxonomic units (OTUs) were assigned at 97% similarity based on an uncorrected pairwise distance matrix. A representative sequence from each OTU was subjected to BLAST (Altschul et al., 1990) against a manually curated SILVA database (Version 108). The 10 best BLAST hits were used to taxonomically assign each sequence at phylum, class, family, and genus level based on sequence similarity thresholds of 75%, 80%, 90%, and 95%, respectively (Webster et al., 2010). The 10 most abundant genera for each sample were selected and their relative abundances visualised using JColorGrid (Joachimiak et al., 2006). Diversity indices were calculated on an equal subsample of gene sequences for all samples at 97% similarity using Mothur. Equal numbers of sequences were subsampled to assess the significance of differences between sample types using Unweighted UniFrac (phylogeny-based) and analysis of similarity (ANOSIM). UniFrac distances obtained in Mothur were plotted in Microsoft Excel using Principal Co-ordinate Analysis (PCoA).
3.4.5. Determination of archaeal community structure by clone library construction

Archaeal diversity was determined for biofilm samples collected from the two study sites over winter (day 2, 4, 6, 7, 10, 18, 35, and mature biofilm) and summer (day 4, 13, 34, and mature biofilm) surveys using 16S rRNA gene clone libraries. Our previous work indicated low archaeal diversity within established MBBR biofilms (Biswas and Turner, 2012), hence the increased sequencing depth offered by pyrosequencing was deemed unnecessary. A ~950 bp fragment from archaeal 16S rRNA genes was amplified from the total genomic DNA extract using primers Arch21F and Arch958R (Delong, 1992). The reaction mixture of 25 µl was composed of 1x Go-Taq Ready mix (Promega), 0.5 µM of each primer, BSA 1%, 1 µl of genomic DNA and PCR-grade water. PCR amplification was performed in an Applied Biosystems Mastercycle gradient PCR machine with an initial denaturing step of 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s), and elongation (72°C for 90 s) and with a final elongation step at 72°C for 7 min. PCR products were then visualized on a 1% agarose gel to check quality and yield.

Archaeal 16S rRNA gene libraries were constructed using the PGEM-T easy Vector System (Promega), following the manufacturer’s instructions. Twenty clones from each sample were digested using the HaeIII restriction enzyme (Invitrogen), and unique banding patterns were designated as separate OTUs. One representative from each OTU per sample was selected for sequencing, which was performed by Macrogen Inc. (Seoul, South Korea) using a 3730 x 1 DNA Analyzer (Applied Biosystems). The resulting sequences were subjected to chimera checks with Mothur using SILVA as the reference database, then aligned using SINA (www.arb-silva.de/aligner) (Pruesse et al., 2007). Phylogenetic analysis of the sequences was performed using the ARB programme package (SILVA version 106) (Ludwig et al., 2004). A maximum-likelihood tree was constructed with 500 bootstrap resamplings (Fig. S3.2). Results were displayed in heat maps constructed using the ‘R’ software package (http://www.r-project.org) (Fig. S3.3).
3.5. Results

3.5.1. Physical and chemical characteristics of Moa Point and Karori WWTPs

Operational data were recorded onsite during the periods of study at both Moa Point and Karori WWTPs (Table 3.1). For each WWTP, average measurements for BOD and inflow were higher in winter compared with summer. In contrast, DO was variable between WWTPs and also between seasons. Yearly average measurements recorded onsite have shown elevated levels of H$_2$S and conductivity at Moa Point, whereas at Karori these values are comparable to standard domestic wastewater (data not shown). Hence, for this study, H$_2$S and conductivity were only recorded at Moa Point WWTP, over winter and summer periods. H$_2$S concentration in the outflow was more than double that of the inflow (Table 3.1).

3.5.2. Physical characteristics of biofilm attached to K1 carriers

Biomass rapidly developed on the inside surface of the deployed carriers, but was very limited on the outer surface, even upon completion of the study. In contrast, the biomass of resident carriers was evenly distributed across the outer and inner surfaces. This suggests that a longer-term study is needed to attain complete maturity of the biofilm on deployed carriers. As expected based on visual observations, biofilm mass (wet weight) associated with the deployed carriers increased with residence time in the reactors but did not reach the biomass levels detected on normal resident carriers (Fig. 3.1). Adherent biomass on deployed carriers decreased initially from day 4 to the next time point at Moa Point WWTP and then again from day 20 to day 34 at both study sites over the summer period. This decrease in biomass at both sites could be related to the heavy rainfall observed during these days in the Wellington region, resulting in large volumes of stormwater entering the MBBRs and flushing loosely attached microbes from the system.
Table 3.1. Operational data over sampling periods at both study sites.

<table>
<thead>
<tr>
<th></th>
<th>Winter (May-Jun 2011)</th>
<th>Summer (Jan-Feb 2012)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moa Point influent</td>
<td>Moa Point effluent</td>
</tr>
<tr>
<td>Average daily BOD (g/m³)</td>
<td>270 ± 79</td>
<td>5 ± 6</td>
</tr>
<tr>
<td>Average daily SS (g/m³)</td>
<td>444 ± 99</td>
<td>16 ± 13</td>
</tr>
<tr>
<td>Average daily flow (m³/h)</td>
<td>3111 ± 708</td>
<td>3116 ± 768</td>
</tr>
<tr>
<td>Average daily H₂S (ppm)</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>Average daily FC (fc/100ml)</td>
<td>123 ± 214</td>
<td>127 ± 311</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Ammonia-Nitrogen (g/m³)</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Oil and grease (g/m³)</td>
<td></td>
<td>&lt;5</td>
</tr>
<tr>
<td>Average DO within MBBR (mg/L)</td>
<td>1.2 (range 0-10)</td>
<td>1.4 (range 0-9.9)</td>
</tr>
<tr>
<td>CONDUCTIVITY within MBBR (mS/cm)</td>
<td>4.1 (range 0-13.7)</td>
<td>4.4 (range 0-13.7)</td>
</tr>
<tr>
<td>Sludge age at WWTP</td>
<td>1-2 days</td>
<td>1-2 days</td>
</tr>
<tr>
<td>Average daily rainfall at WWTP (mm)</td>
<td>3.5 ± 5.4</td>
<td>3.5 ± 6</td>
</tr>
<tr>
<td>HRT within MBBR (h)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Average daily temperature (°C)</td>
<td>18.5</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Values in the table represent means over each survey period ± standard deviation. All measurements were taken daily with the exception of conductivity which was recorded weekly while pH, ammonia-nitrogen, oil and grease was measured once for each time period. Grey boxes indicate that no measurements were made. BOD- biochemical oxygen demand; SS- suspended solids; DO- dissolved oxygen; HRT- hydraulic retention time; FC- fecal counts
Figure 3.1. Progression of biofilm development on surfaces of carriers over time at Moa Point WWTP is shown.

Images of deployed carriers were captured during summer (Jan-Feb 2012) and winter (May-Jun 2011). Average wet weight for each time point at Moa Point and Karori WWTPs is also shown along with the standard deviation (n=3 or 5).

### 3.5.3. Effect of biofilm age on microbial community structure

16S rRNA gene amplicon pyrosequencing was performed on deployed carrier biofilms harvested over a period of up to 48 days. After sequence processing, a total of 170,661 high-quality reads was retained for further analysis, with a range of 998 - 4237 reads per sample and average read length of 375 bp. The number of operational taxonomic units (OTUs), based on 97% sequence identity, ranged from 220 to 884 per sample and totaled 13,541 across the entire data set (Table S3.1). OTU richness increased with increasing residence time in both treatment plants. For example, the number of OTUs increased from 354 (residence time <20 days) to 519 (residence time >20 days) in summer at Moa Point. Other diversity indices also indicated the same general trend for all deployed carriers (Table S3.1).

Three bacterial phyla dominated throughout the time course, namely *Proteobacteria*, which comprised 37-89% of gene sequences for a given sample, *Bacteroidetes* (10-62%) and...
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Firmicutes (<1-17%) (Fig. S3.1a). In all cases, these phyla collectively accounted for >90% of reads across all replicates and time points. The remaining reads were assigned to a broader range of 24 phyla with Fusobacteria, Actinobacteria, Synergistetes and Acidobacteria dominant among these (Fig. S3.1b). Less than 0.08% of sequence data could not be assigned to named phyla.

More substantial differences in bacterial community composition were evident at class level, with Gammaproteobacteria dominating (22-52% of total reads) the suspended fraction samples at both WWTPs, but typically reducing in dominance as the biofilm developed (Fig. 3.2). Many of the Gammaproteobacteria sequences (over 62% in the younger biofilm and over 25% in the older biofilm) were assigned to the genus Acinetobacter (Fig. 3.3). Other major components of the suspended fraction at both WWTPs were Flavobacteria (6-23%), Alphaproteobacteria (3-9%), Betaproteobacteria (2-9%) and Clostridia (4.5-13%). Though uncommon at Karori WWTP, members of the Epsilonproteobacteria made up a significantly larger proportion of the suspended fraction and biofilm communities at Moa Point WWTP (ANOSIM, one-way analysis, p<0.05). More than 90% of Epsilonproteobacteria reads were assigned to the genus Arcobacter (Fig. 3.3). An average of 18.5% (range 3.5-47.6%) of the total sequences across all samples could not be classified to genus level. However, those sequences that could be assigned to genus were spread across 347 bacterial genera, of which 197 were common to both WWTPs, including Arcobacter, Acinetobacter, Flavobacterium, Cloacibacterium and Acidovorax.

The bacterial communities of young (<20 days growth) biofilms were similar to those of the suspended fraction, being dominated by Gammaproteobacteria but with fewer reads assigned to Clostridia, and with more Alpha- and Betaproteobacteria (Fig. 3.2). Sphingobacteria (phylum Bacteroidetes) were abundant in the younger biofilms of Karori WWTP but largely absent from Moa Point. Increasing residence time of biofilm from 20 days up to 48 days (older biofilm) resulted in a gradual increase in putatively anaerobic organisms within the Clostridia and Deltaproteobacteria. Desulfovibrio (<0.3% of the total bacterial community) and Desulfomicrobium (<4.1%) were present at both treatment plants with higher abundance in the older/mature biofilm. The presumed sulfur-oxidizing bacteria Sulfurovum and Thiothrix were observed in older biofilm samples of Moa Point, whereas the facultative anaerobe Cloacibacterium (belonging to Flavobacteria) was observed exclusively within older biofilms at both sites. The bacterial communities in the day 48 samples had a reasonably even distribution of reads across the dominant taxa, which closely resembled
those of the resident biofilms on carriers that had been in functional operation within the MBBRs over two years (mature biofilm). As the two MBBR systems of study function as BOD removal plants, low abundance (<0.05% of the total bacterial community) of nitrifiers such as *Nitrospira*, *Nitrosomonas*, and *Nitrococcus* were found across all samples. Fewer time points were sampled during winter, but overall similar patterns of bacterial community development were observed. The three biological replicates analysed for both winter and summer surveys were relatively consistent for a given time point.

![Graph showing bacterial community development](image)

**Figure 3.2.** Bar graphs representing the bacterial community at class level (of the 3 most dominant phyla) over summer and winter periods at both Moa Point and Karori WWTPs.

Progression of the bacterial community over time was observed in developing biofilm after 4 days of growth (D4) up to 48 days (D48), along with the suspended fraction (sus) and mature biofilm (mature). Three replicates were analysed for each time point and are shown in the figure. Values are presented as % of total bacterial community for a given sample.
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Figure 3.3. Heatmap representing abundance of 10 most abundant genera in all samples collected from Moa Point and Karori WWTPs.

Three biological replicates per time point are shown for suspended fraction (sus), mature biofilm (mature) and developing biofilm on deployed carriers between day 4 (D4) up to day 48 (D48). Sequences that could not be assigned to any genus were grouped under ‘not assigned’. A fraction of the sequences that were assigned at class or family level could not be further classified and are labeled as unclassified (un). Values are presented as % of total bacterial community for a given sample.

In general, the results from both WWTPs exhibit a similar relationship between biofilm age and UniFrac distance over time (Fig. 3.4). As biofilm residence time increased in the MBBRs, UniFrac distances decreased relative to the established biofilm. However, the greatest proportion of variation in the data could be attributed to differences between the two WWTPs, with biofilm age of marginally less importance (Fig. 3.4). Unweighted UniFrac revealed significant differences (p<0.001) between all biofilm samples to the suspended fraction of the same WWTP, with the exception of Karori summer biofilm samples between days 20 and 34. The resemblance of these biofilm samples to the suspended fraction is possibly due to the constant re-establishment of biofilms on carriers that occurs during periods of heavy rainfall that cause loosely attached microbes to be washed away. In addition, comparison between the younger biofilm samples (day 4 - day 20) with older biofilms (day
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34 - day 48) of the same WWTP, revealed significant differences (ANOSIM, one-way analysis, p<0.05 and UniFrac, p<0.001).

Figure 3.4. Principal Co-ordinate Analysis (PCoA) of unweighted UniFrac distances (phylogeny-based) for all samples.

Moa Point samples are represented by open and closed circles representing summer and winter respectively. Karori samples are represented by open and closed triangles also representing summer and winter respectively. The progression of color (cyan to dark blue, or yellow to dark red) represents biofilm age on the carriers. The suspended fraction (green) and mature biofilm (purple) for each treatment plant are also shown on the graph.

Archaeal phylogenetic diversity (as assessed by 16S rRNA gene libraries) decreased as the biofilm developed (Figs. S3.2 and S3.3). This was also observed at OTU level. Biofilms from later time points, including samples from day 18 to mature state, were dominated (23 out of 24 clones) by Methanosarcinaceae, whereas the younger biofilms also included abundant members of the Methanaetaeaceae (ranging from 6/23 to 16/24 clones) and soil Crenarchaeota (3/16 to 11/23 clones). Other families belonging to Crenarchaeota and
Euryarchaeota were also present within the younger biofilm samples, albeit to a lesser extent with individual families representing less than 4 clones out of 24. The overall pattern of archaeal biofilm development was consistent across both season and MBBR system (Fig. S3.3), although the winter young biofilm samples from Karori WWTP did not yield a PCR product for archaea, suggesting a low abundance of archaeal cells.

3.5.4. Between-plant differences in biofilm community structure

At all time points, the microbial communities within both Karori and Moa Point WWTPs were dominated by Proteobacteria, Bacteroidetes and Firmicutes (Fig. S3.1A), while the archaeal assemblage converged towards a low-diversity community dominated by the euryarchaeotal family Methanosarcinaceae (Fig. S3.3). At class level clear differences were observed between Moa Point and Karori samples at all time points (Fig. 3.2). Statistical analysis of microbial communities at different time points and at different plants was performed on both phylogenetic classification (phylum, class, family, genus) and sequence identity (97%-OTU) data. When young samples (<20 days) from Karori and Moa Point were compared there were no significant differences (ANOSIM, p>0.05) in bacterial community structure at phylum level. In contrast, comparison of older biofilm (> 20 days) between the two WWTPs showed significant differences (ANOSIM, one-way analysis, p<0.05). Analysis at other taxonomic levels (including 97%-OTU) revealed significant differences (ANOSIM, one-way analysis, p<0.05) between both WWTPs, for young and mature biofilm samples. Comparison of suspended, young, older, and mature samples between the two MBBRs using unweighted UniFrac revealed significant differences (p<0.05).

Proteobacteria (Alpha, Beta, Gamma classes) were abundant at both WWTPs, while Deltaproteobacteria were more prominent in the latter stages of biofilm development, at least in summer and particularly at Moa Point (Fig. 3.2). Certain bacteria belonging to this class (Desulfo bacter and Desulfosarcina) were found exclusively at Moa Point WWTP but in low abundance (<6% of the total bacterial community). Epsilonproteobacteria, as mentioned above, were found in significantly higher numbers at Moa Point WWTP. Another significant difference at class level related to the Sphingobacteria (within the Bacteroidetes phylum), which were relatively abundant at the Karori plant but largely absent from Moa Point.
3.5.5. Effect of season on biofilm community structure

Seasonal differences were not particularly evident at broader phylogenetic groupings (such as bacterial phyla) but were more marked at class, genus, and OTU levels. For a given WWTP, comparison of bacterial communities between winter and summer samples, for young and older biofilms, revealed significant differences at 97%-OTU, genus, and class levels (ANOSIM, one-way analysis, p<0.05). Significant differences (unweighted UniFrac, p<0.001) were also seen between seasons at the same MBBR for young and mature biofilm samples. The patterns of archaeal community development in biofilms over seasons were consistent, moving from a relatively family-rich community to a low-diversity community as biofilms aged. Major inter-seasonal differences were observed at class level. In particular, Flavobacteria were significantly (ANOSIM, p<0.05) more abundant in winter than summer at Karori WWTP. In contrast, Gammaproteobacteria were significantly (ANOSIM, p<0.05) lower in winter in comparison to summer samples at both WWTPs.
3.6. Discussion

Moving bed biofilm reactor (MBBR) technology is an increasingly popular option for the treatment of wastewater. From ~400 MBBR systems worldwide in 2005 to ~1200 such systems at present, this technology offers an efficient, cost-effective alternative to conventional activated sludge treatment (Rusten et al., 2006). The success of the MBBR process is intimately linked to the establishment of microbial biofilms on the surfaces of suspended carriers, yet until now the pattern of biofilm succession has not been studied. One of the motivating factors for this study was that biofilm processes have been implicated as the cause of excess H₂S levels and associated corrosion of pipes and concrete at Moa Point WWTP. Furthering our understanding of the biofilm development process and the associated microbial communities within MBBRs should assist in resolving such issues and ensuring successful plant operation. Karori WWTP does not generally suffer from excess H₂S problems, thus providing an ideal comparison to Moa Point WWTP. Here we investigated, for the first time, successional changes in bacterial and archaeal community structure in developing biofilms within full-scale MBBR wastewater treatment plants.

3.6.1. Biofilm age influences composition and presumed function of bacterial communities in MBBR plants

Successful MBBR operation employs simultaneous aerobic and anaerobic digestion of nutrients, with much of the latter thought to be taking place within the anoxic zones of a developed biofilm (Rusten et al., 2006). The model for microbial activities within an MBBR system is that, due to the low hydraulic retention time, fast-growing aerobic microorganisms dominate in the suspended fraction, while anaerobic microbes exhibit increased abundance within the biofilms associated with the carriers. Here we consider whether our obtained data fit with this model.

3.6.1.1. Suspended fraction / early biofilm stages

The suspended fraction at both Karori and Moa Point WWTPs was dominated by bacteria belonging to the *Gamma-, Beta- and Alphaproteobacteria*, together with *Clostridia* and *Flavobacteria*. At Moa Point, *Epsilonproteobacteria* within the genus *Arcobacter* were also prevalent. These pyrosequencing-derived results were consistent with our earlier study which examined the suspended fraction from the same WWTPs by using 16S rRNA gene clone
libraries (Biswas and Turner, 2012). In the current study, the nascent biofilm on the polyethylene carriers largely reflected the microbial community composition of the suspended fraction within the MBBR tanks, suggesting that cells are attaching onto the surfaces randomly (Curtis and Sloan, 2006). Although generation time of cells was not measured in this study it can be speculated that the young biofilm is dominated by fast-growing organisms (e.g. *Acinetobacter*, *Pseudomonas* (Favero et al., 1971; Abbott et al., 1973; Sakai et al., 1994)) that are adapted to the aerobic environment present in the suspended fraction of the MBBRs. The presence of certain *Euryarchaeota* and *Crenarchaeota* was also noted within the young biofilm (Biswas and Turner, 2012). These archaeal phyla have been found previously in aerated activated sludge samples but are thought to play a minor role in treatment plant processes (Gray et al., 2002). The obtained data thus support the model described above, whereby the suspended fraction and early biofilm communities are dominated by fast-growing aerobes.

### 3.6.1.2. Later stages / mature biofilm

Consistent with the aforementioned model of microbial function within MBBRs, the early biofilm community is succeeded by one that contains a greater abundance of putatively anaerobic microorganisms including members of the *Deltaproteobacteria* and *Clostridia* (Wang et al., 2003; Pereira et al., 2007). As any biofilm develops, new niches appear (Stoodley et al., 2002); with oxygen typically absent at a biofilm thickness of 200 µm (Santegoeds et al., 1998), there is increasing selection for anaerobic microorganisms and their collective metabolic capabilities.

In this study a “mature” biofilm is defined as biomass on carriers residing in a functional MBBR system for several years. Thus it is assumed that the microbial communities established within these mature biofilms have developed over time to a steady state, as indicated by consistent results from pyrosequencing and 16S rRNA gene clone libraries in our previous paper (Biswas and Turner, 2012). The results of the current study indicate that the process of microbial attachment and development of the biofilm through to a mature state takes longer than 6 weeks. The biomass weight at 6 weeks was ~72% that of the mature biofilm, indicative of a biofilm that is still developing towards a mature state.

In contrast to the increasing diversity of the bacterial community through time, the archaeal community in biofilms was highly restricted and by day 35 was dominated by a single phylotype within the family *Methanosarcinaceae*. This group consists of methanogens that
have been shown previously to co-exist with sulfate-reducing bacteria (SRB) in fixed bed biofilm reactors (Raskin et al., 1996). Although these metabolically diverse groups of organisms compete for the same substrates they often function syntrophically to gain energy sources in low-carbon environments (Boetius et al., 2000; Muyzer and Stams, 2008). Mature biofilms of K1 carriers in the Moa Point and Karori plants were shown previously to contain low abundance (<5% biovolume) of archaea (Biswas and Turner, 2012).

3.6.1.3. Functional implications of changes in biofilm community structure

Although 16S rRNA gene identity does not generally correlate with microbial function, we can nevertheless speculate as to how changes in the bacterial communities of the developing biofilm may impact upon the activities of the relevant bacteria and, ultimately, WWTP performance. While many MBBR systems are operated with an emphasis on removal of nitrogen, both WWTPs in this study are designed for BOD removal and it is therefore not surprising that the number of sequences that could be attributed to known nitrifiers and denitrifiers was low. By contrast, we identified sequences representing a wide range of presumed heterotrophic carbon degraders that have previously been isolated from municipal wastewater (Benedict and Carlson, 1971; Allen et al., 2006). Among the most highly abundant were members of the Bacteroidetes and Proteobacteria such as Cloacibacterium, Flavobacterium, and Acinetobacter (Wiedmann-Al-Ahmad et al., 1994; Aslam et al., 2005; Cao et al., 2010) which have previously been detected in conventional activated sludge or granular wastewater treatment systems. These organisms dominated the bacterial communities in the suspended fraction and at all stages of biofilm development in this study.

One particularly notable group of microorganisms which come to prominence in the later stages of biofilm development are putative SRBs belonging to Deltaproteobacteria and Clostridia (Muyzer and Stams, 2008). These taxa establish only in these later stages presumably due to their slow growth and requirement for anoxic conditions. Evidence of Deltaproteobacteria in biofilms of membrane bioreactors or activated sludge basins treating wastewater has previously been shown (Santegoeds et al., 1998; Miura et al., 2006). SRBs use sulfate as a terminal electron acceptor in anaerobic respiration, and in the process reduce sulfate to hydrogen sulfide (Muyzer and Stams, 2008). The higher levels of hydrogen sulfide detected in the outflow of Moa Point treatment plant are reflective of this chemical reaction occurring within the MBBRs, and a detailed investigation of the organisms involved has been conducted (Biswas et al., unpublished). In that study, we targeted the dissimilatory (bi)sulfite
reductase (dsrAB) gene to directly identify and quantify members of the sulfate-reducing guild. Possible sources of sulfate entering Moa Point are seawater (indicated by elevated conductivity measurements) and trade waste effluent. The sulfide resulting from SRB activity could be further utilized by members of the genera *Sulfurovum* and *Thiothrix* (found largely at Moa Point treatment plant) to produce elemental sulfur. These sulfur-oxidizing, chemolithoautotrophic bacteria are facultative anaerobes and utilize sulfur or thiosulfate compounds as electron donors while also requiring sea salt for growth (Williams and Unz, 1985; Inagaki et al., 2004).

**3.6.2. Differences occur between two MBBR treatment systems**

Significant differences between Moa Point and Karori WWTPs, from initial attachment to maturation over time, were observed at all taxonomic levels except phylum. The suspended fraction and mature biofilm were also consistent with this observation. Differences between the microbial communities of the two WWTPs in this study can be summarized by the consistent presence of high levels of *Arcobacter* at Moa Point (while being virtually absent at Karori), and elevated levels of the classes *Flavobacteria* and *Sphingobacteria* at Karori. Since operational conditions at the two MBBR sites were similar such differences suggest that influent composition could play an important role in determining the microbial community composition of the suspended fraction, as well as colonization of the carriers and subsequent biofilm development. The main difference between the two treatment plants is trade waste and possible seawater infiltration entering Moa Point plant only. Both these components are known to contain high levels of sulfate (Muyzer and Stams, 2008). It can be speculated that in the presence of high sulfate levels, certain organisms flourish and utilize sulfate as a terminal electron acceptor, in turn leading to sulfide production.

**3.6.3. Minor role of seasons on bacterial community development**

Seasonal effects can play an important role in microbial community composition, as previously seen for river biofilms (Lyautey et al., 2005). Winter usually leads to larger amounts of rainfall in the Wellington area (www.gw.govt.nz), thus resulting in selection for microbial communities that are better adapted to low nutrient levels (due to reduced food:microbe ratios in the WWTP influent) (Hu et al., 2012). The elevated levels of
Flavobacterium observed in the winter period at both treatment plants in this study suggest that seasons could play a minor role in microbial community composition of MBBR systems. However, due to the limited number of seasons surveyed, further work is required to test this notion.

To our knowledge, this is the first study of biofilm development in MBBR systems. We employed a combination of 16S rRNA gene-targeted pyrosequencing and clone library construction to document successional changes in the bacterial and archaeal communities within two New Zealand WWTPs utilizing MBBR technology. As biofilms established over time there were significant changes in microbial community structure from nascent to established samples, ultimately resembling mature state. The microbial communities found within both treatment plants are consistent with BOD removal facilities which contain high numbers of bacteria that degrade complex organic compounds. The presence of putative SRBs within Moa Point WWTP correlates with the excess sulfide emissions from this plant compared with Karori WWTP. Seasonal effects and differences between treatment plants were also observed. The data from this study, coupled with ongoing functional analysis of bacterial community genes, will further our understanding of microbes in MBBRs and help to optimize these systems in a more efficient and cost-effective manner.

### 3.7. Acknowledgements

The authors would like to acknowledge staff at Moa Point and Karori WWTPs for their assistance with this study. Funding for this project was made available by Veolia Limited. Additionally, the authors give thanks to R. Simister, D. Waite, and P. Tsai for their input into the bioinformatics aspects of this study.
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3.8. References


moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. *Bioresource Technology* **121**: 119-126.


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3.9. Supplementary material

Figure S3.1. Relative abundance of 3 most abundant bacterial phyla (1A) and the remaining 24 phyla (minus the top 3 most abundant) (1B) in all samples collected over summer and winter periods at Moa Point and Karori WWTPs, as determined by 16S rRNA amplicon sequencing.

‘Others’ in Fig. 3.1A refers to the sum of the remaining groups not indicated in the figure key. Three biological replicate samples were analysed for each time point of biofilm growth on the deployed carriers, suspended fraction (sus) and mature biofilm (mature). Values are presented as % of total bacterial community for a given sample.
Figure S3.2. 16S rRNA-based maximum likelihood tree representing clone-derived archaeal sequences from clone libraries prepared from biofilm samples of Moa Point and Karori WWTPs over summer and winter periods.

One representative sequence per OTU for a given sample is shown on the tree. Names highlighted in red indicate samples taken over the summer period. Vertical bars indicate families within the *Archaea*. Open circle indicates lineage with $\geq 75\%$ parsimony bootstrap support. Scale bar represents 10% sequence divergence.
Figure S3.3. Comparison of the *Archaea* detected in biofilms from the two WWTPs.

Heat maps indicate the relative abundance of the seven family members of the archaeal community detected. Dendrograms were drawn based on correlation similarity metric and average linkage clustering used in the R package. Dashed boxes surrounding sample dates (e.g., [Day 13]) represent biofilm samples collected during the summer survey.
### Table S3.1. Summary of bacterial 16S rRNA gene pyrosequencing reads based on 97% similarity OTUs.

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<th>invsimpson</th>
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The number of high quality gene sequences (nseqs), Good’s coverage, number of unique OTUs, Chao1, invsimpson and Shannon-Wiener diversity indexes per sample has been summarized. Three replicates per time point are shown.

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99
4. *dsrAB*-based analysis of sulfate-reducing bacteria in moving bed biofilm reactor (MBBR) wastewater treatment plants

Publication status: *In Press*

4.1. Abstract

Sulfate-reducing bacteria (SRB) are important members of the sulfur cycle at wastewater treatment plants (WWTPs). In this study we investigate the diversity and activity of SRB within the developing and established biofilm of two full-scale moving bed biofilm reactor (MBBR) systems treating municipal wastewater in New Zealand, over two seasons. Moa Point, the larger of the two WWTPs, faces high levels of sulfide relative to Karori WWTP. Clone libraries of the dissimilatory (bi)sulfite reductase (dsrAB) gene and quantitative real-time PCR targeting dsrA transcripts were used to analyse SRB communities within samples. Desulfobulbus (35-53% of total SRB sequences) and genera belonging to the family Desulfobacteraceae (27-41%) dominated the SRB fraction of the developing biofilm on deployed plastic carriers at both study sites, whereas Desulfovibrio and Desulfomicrobium were exclusively found at Moa Point. In contrast, the established biofilms from resident MBBR carriers were largely dominated (58-100%) by Desulfomonile tiedjei-like organisms. The abundance of active SRB increased with biofilm weight yet remained <1% of the total bacterial community, even in the mature biofilm stage. Our results indicate that SRB are both present and active in the microbial community at both MBBR study sites but their functional role may differ.
4.2. Introduction

Moving bed biofilm reactor (MBBR) technology is increasingly used for the treatment of wastewater due to its comparatively low footprint and efficient treatment performance. A key feature of this process is the extended retention of biomass within the reactors through attachment of microorganisms onto the surface of plastic carriers that are maintained in suspension via aeration or mechanical stirring. Successional development of the attached microbial community leads to the development of an established biofilm that includes both aerobic and anaerobic microenvironments (Odegaard et al., 1994).

Sulfate can play an active role as a terminal electron acceptor in anoxic wastewater environments due to its relatively high concentration (typical wastewater 100 to 1000 µM) when compared with other available electron acceptors such as organic carbon, nitrate or nitrite (Ito et al., 2002). Sulfate reduction is therefore an important process in wastewater treatment plants (WWTPs) and has accounted for more than 50% of organic carbon mineralisation in biofilms at one plant (Kühl and Jørgensen, 1992). Sulfate reduction can also have other beneficial effects at WWTPs including removal of heavy metals through precipitation of metal sulfides, and production of elemental sulfur (which can be used for further applications) by sulfate-reducing bacteria (SRB) in association with sulfur-oxidising bacteria (SOB) (Muyzer and Stams, 2008). However, the process of sulfate reduction can also lead to hydrogen sulfide-associated problems, especially where excess sulfate is present either due to seawater intrusion or septic influents. Hydrogen sulfide is corrosive, odorous and toxic, leading to considerable operational and economic impacts on WWTPs (USEPA, 1991). For example, in Belgium, approximately 10% of total costs of wastewater treatment and collection is spent on repairing damages caused by sulfide corrosion (Vincke, 2002). In addition, this effect has previously been observed in the oil industry, with souring of oil and corrosion of facilities (Hamilton, 1985; Odom, 1990). An understanding of the ecology and dynamics of SRB in WWTPs is therefore required in order to manage both the positive and negative impacts of these organisms in treatment processes.

Recent 16S rRNA-based studies of microbial communities in two MBBR plants in New Zealand (Moa Point and Karori) revealed relatively little difference in overall community composition between the plants, despite Moa Point suffering from problems with elevated H₂S (Biswa and Turner, 2012; Biswas et al., 2013). In both plants established biofilms on resident carriers were dominated by putatively anaerobic members of Clostridia and
Deltaproteobacteria while nascent biofilms (less than 20 days old) were dominated by Gamma-, Beta-, and Alphaproteobacteria. Known genera of sulfur-oxidising bacteria, such as Sulfurovum and Thiothrix, were found in much greater numbers at Moa Point WWTP compared with Karori (Biswa et al., 2013). Methanogens have previously been investigated within established biofilms of MBBRs using fluorescence in situ hybridisation, and were shown to be present at <5% abundance of the biovolume (Biswa and Turner, 2012). Occasionally methanogens were found in close proximity to putative SRB within the biofilm samples.

Oxidants such as oxygen, nitrate, chlorine or hydrogen peroxides are often used to reduce the effects of sulfide at a WWTP (Zhang et al., 2008). These applications have had mixed results due to varying communities of SRB and experimental conditions. The response to these compounds by SRB is highly variable, with some species flourishing while others remain unchanged (Mitchell et al., 1986; Mohanakrishnan et al., 2011). It is therefore important to understand SRB community diversity and activity within a system before any treatment is implemented. This study aims to identify and quantify SRB community members in two full-scale MBBR plants. Karori, the smaller of the two WWTPs, shows no evidence of sulfide-associated problems, thus providing an ideal comparison to Moa Point WWTP. As previous studies of SRB diversity in wastewater have largely focused on the final stages of biomass (i.e. mature state biofilms or granules) (Santegoeds et al., 1998; Okabe et al., 1999; Santegoeds et al., 1999), this study also aims to address how SRB communities develop over time within a complex microbial environment, namely municipal wastewater. Environmental conditions such as seasons are also known to have an effect on the microbial communities in wastewater. Thus, successional development of the biofilms will be investigated in this study over two seasons (winter and summer). For this purpose total RNA (including messenger RNA) and genomic DNA will be extracted from developing biofilm over 48 days, to be analysed using real-time PCR and clone library analysis. SRB are paraphyletic and found within multiple diverse lineages, thus making it unfeasible to use the 16S rRNA gene as a genetic marker (Wagner et al., 2005). However, studies on wastewater biofilm using this approach have identified Desulfobacter, Desulfomicrobium, Desulfobulbus and Desulfovibrio as dominant members of the SRB community (Ito et al., 2002; Mohanakrishnan et al., 2011). Research on SRB diversity has mainly been conducted through the analysis of functional genes, such as dsrAB (Dhillon et al., 2003; Loy et al., 2004; Foti et al., 2007), which will be employed in this study. This gene encodes the dissimilatory (bi)sulfite reductase enzyme
which catalyses the final step of sulfite reduction to sulfide and contains two subunits (alpha and beta). The \textit{dsrAB} gene is conserved in all SRB and has been shown to be constitutively expressed in \textit{Desulbacterium} sp. and \textit{Desulfovibrio} sp. (Brusseau, 1998; Neretin et al., 2003). There are a few exceptions, where organisms, such as \textit{Pelotomaculum}, contain this functional gene but are incapable of reducing sulfate to sulfide (Imachi et al., 2002). However, \textit{dsrAB}-based analyses have been used previously to study SRB communities in industrial wastewater, where \textit{Desulfovibrio} and \textit{Desulfobacter} were identified as dominant members, with \textit{Desulfomicrobium} and \textit{Desulfocella} also present (Ben-Dov et al., 2007; Dar et al., 2007).

### 4.3. Materials and Methods

#### 4.3.1. Experimental setup

Two full-scale municipal WWTPs utilising MBBR technology in Wellington, New Zealand, were chosen as study sites. Moa Point treatment plant, the larger of the two sites (200,000 population equivalent) treats domestic and trade wastewater while Karori treatment facility (20,000 population equivalent) treats only domestic wastewater. The plants are configured for BOD removal. Moa Point was the first MBBR to be combined with the solid contact reaeration process for secondary treatment of municipal wastewater (Rusten et al., 1998). Aerobic conditions are maintained within the MBBR through aeration that also maintains the carriers in suspension. Operational parameters and water quality characteristics that were recorded onsite at the two WWTPs are reported in Table S4.1.

The experimental setup for monitoring SRB communities in developing biofilm of two MBBRs has been described previously for 16S rRNA-based approaches (Biswas et al., 2013). In brief, unused K1 carriers (AnoxKaldnes™) attached to nylon fishing line were suspended into MBBRs at the two study sites. The study was conducted over winter (May-June 2011), then repeated in summer (Jan-Feb 2012). Approximately 6 carriers were sampled from the deployed lines at day 4 (4 days after commencement of the experiment) up to day 48. Resident biofilms were also collected from the MBBRs during these two study periods. In addition, biofilms from two different MBBRs within Moa Point treatment plant were sampled after 10 days of growth on deployed carriers. Samples were preserved in RNAlater at -20°C.
until further analysis. The suspended fraction of the MBBRs was collected during the winter and summer periods of 2011-2012 and stored at -20°C for total dissolved sulfide measurements.

### 4.3.2. Physical and chemical characteristics

Biomass development on the deployed carriers was monitored by wet weight measurements as described previously (Biswas and Turner, 2012). An average of 3-5 carriers was measured per time point.

Total dissolved sulfide present in the suspended fraction of the MBBRs was measured using colorimetric methods as described previously (Cord-Ruwisch, 1985). The results show an average value of 3 representative samples for winter and summer periods of 2010-2011. Suspended samples collected during the 2011-2012 period of this study were destroyed during transportation, hence total dissolved sulfide measurements from the previous year are cited in this study.

### 4.3.3. Nucleic acid extraction

Biomass was scraped from individual deployed carriers for each time point of the study. This process was repeated three times with different carriers to obtain biological replicates. Mature biofilm was obtained in a similar way from carriers that were resident in the MBBRs. Total genomic DNA was extracted from the biomass using a bead-beating method, as previously described (Smith et al., 2003). Extracted DNA was dissolved in 40 μl of DNase-free water and stored at -20°C prior to further analysis.

Total RNA was extracted using the RNeasy minikit (Qiagen, Hilden, Germany) from scraped biomass of three replicate deployed carriers per time point and similarly from three replicate resident carriers. Samples were incubated at 37°C for 10 min with 20 μl of lysozyme (stock concentration of 15 mg/ml) and subjected to further mechanical disruption of cells via bead-beating (4 m/s for 20 s) using acid-washed 0.1 mm glass beads. The remaining procedure for obtaining total extracted RNA was followed as per the manufacturer’s instructions. Genomic DNA was removed from the samples using DNase I treatment as recommended by the manufacturer (Invitrogen). To confirm the absence of genomic DNA, a PCR was performed
on the RNA samples using 16S rRNA-targeted primers (Saul et al., 2005) under previously described conditions (Ayton et al., 2010), but with an increase to 30 PCR cycles. The quality and quantity of extracted total RNA were determined by using RNA 6000 Nano chips (Agilent 2100 BioAnalyzer) and NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific), respectively.

Reverse transcription of extracted RNA into cDNA was performed using a SuperScript III First-Strand kit (Invitrogen). Approximately 300 ng of RNA were used for cDNA synthesis for each sample. Newly synthesised cDNA was stored at -20°C until real-time PCR was performed.

### 4.3.4. Clone library construction

Amplification of the dsrAB gene fragment (~1.9 kb) from the extracted genomic DNA was performed using degenerate primers DSR1Fmix and DSR 4Rmix (Wagner et al., 2005). The reaction mixture consisted of PCR buffer (1X), MgSO₄ (2 mM), each deoxynucleoside triphosphate (dNTP) (0.2 mM), equimolar mixture of primers (0.75 µM of each primer variant), 0.2 µl Platinum Taq Hi-Fidelity DNA polymerase (Invitrogen), 1 µl of genomic DNA and PCR grade water to a total volume of 25 µl. Thermal cycling was performed in an Applied Biosystems Mastercycle gradient PCR machine with initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturing (94°C for 30 s), annealing (50°C for 50 s), and elongation (72°C for 90 s). After a final elongation step at 68°C for 10 min the PCR products were run on a 0.8% agarose gel to check for the band (~1.9 kb) of interest. Negative controls were included in all PCR reactions.

Due to the use of degenerate primers for dsrAB amplification, many shorter length fragments were also observed. Fragments of the correct size (~1.9 kb) were cut from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR product was ligated into the pGEM T-easy vector (Promega) and cloned into Escherichia coli DH5α chemically competent cells (Invitrogen) according to the manufacturer’s protocols. Cloned inserts were recovered by PCR amplification using the vector-specific primers PGEM-F and PGEM-R (Aislabie et al., 2009). Due to the lack of existing dsrAB gene data on MBBR samples, previous 16S rRNA gene data (Biswas and Turner, 2012) were used to infer that established biofilms in MBBR systems contain a low diversity of sulfate reducers.
Consequently 24 to 32 clones were chosen for each sample for sequencing and further analysis. Purification and sequencing were performed by Macrogen Inc. (Seoul, South Korea) using a 3730 x 1 DNA Analyzer (Applied Biosystems) for the dsrAB clones.

Initially, dsrAB clones were sequenced in one direction using the vector-specific primer PGEM-F and imported into an ARB database (Ludwig et al., 2004) containing 97 full-length dsrAB sequences from described species (Zverlov et al., 2005), along with 40 uncultured clone sequences imported from GenBank. These uncultured clones were selected based on their closest match to sequences of this study, using BLAST search (Altschul et al., 1990). Sequences were aligned against the dsrAB database mentioned above using a PT Server and checked manually in ARB EDIT 4. Clones composed of sequences from the alpha-subunit of the dsrAB gene were exclusively used, while the remaining clones (~17%), corresponding to the beta subunit, were removed from this study.

Partial dsrAB sequences were converted to amino acids and assigned to OTUs based on 90% amino acid identity (Leloup et al., 2006; Kjeldsen et al., 2007). In the majority of cases one full-length sequence per OTU was obtained by sequencing in the reverse direction using the PGEM-R vector-specific primer. Full-length sequences were imported into ARB and aligned as mentioned above. Relative abundance of sequences in each OTU was plotted using JColorGrid (Joachimiak et al., 2006).

4.3.5. Nucleotide accession numbers

dsrAB gene sequences from this study have been deposited in GenBank under accession numbers: KC750833-KC750907.

4.3.6. Phylogenetic analysis of dsrAB clones

Phylogenetic trees were constructed from DsrAB amino acid sequences that were deduced from nucleic acid information (Fig. S4.1). To avoid the inclusion of insertions and deletions a suitable alignment mask (indel filter) was applied (Wagner et al., 2005). Full-length sequences were used to construct phylogenetic trees using the PHYLIP package (Felsenstein, 1995) implemented in ARB. Partial sequences (DsrA fragment) were subsequently inserted without influencing tree topology using the Parsimony Interactive tool. Distance
FITCH with global rearrangements and randomised order of input sequences), maximum parsimony, and maximum likelihood (PROML) trees were used to build a consensus tree (Ludwig et al., 1998). Sequences from each OTU were classified to genus, family, class or phylum level based on the constructed phylogenetic tree. The robustness of the tree branches was tested with maximum parsimony bootstrap analysis (500 resamplings). Chimera checks were performed as previously described (Wagner et al., 2005).

Rarefication curves were calculated in the R package based on 90% similarity OTUs for each clone library (Fig. 4.1). The homologous coverage (C) for each library was calculated based on the formula $C = 1 - (n_1/N) \times 100\%$, where $n_1$ is the number of OTUs containing only one dsrAB clone and N is the total number of OTUs in the clone library being analysed (Singleton et al., 2001). Similarities between libraries based on phylogenetic composition were assessed using the Sorensen similarity index, $C_s = 2j/(a+b)$, where j is the number of OTUs common to both libraries being assessed, while a and b are the total number of OTUs in each individual library (Magurran, 1988).

### 4.3.7. Quantification of 16S rRNA genes and dsrA gene transcripts by real-time PCR

For quantifying the total number of bacterial 16S rRNA gene copies in samples, primers 533F (5’-GTGCCAGCAGCYGCGGTMA-3’) and 803R (5’-CTACCRGGGTATCTAATCC-3’) were used. The percentage coverage of these primers with no mismatches, using the RDP Probe Match function for bacteria, was 92.5% (533F) and 86.5% (803R). These primers have previously been used successfully as part of other primer sets for amplicon pyrosequencing (Nossa et al., 2010; Simister et al., 2012). Sulfate-reducing microorganisms were quantified using previously described primers DSR1-F+ and DSR-R (Kondo et al., 2004) targeting the dsrA gene. The specificity of this primer pair was checked using the Probe Match function in ARB against 97 full-length dsrAB sequences from described species (Zverlov et al., 2005). Standards were prepared from cDNA, generated as described above, from a wastewater biofilm sample collected at Moa Point. Preparation of standards has been described previously (Chin et al., 2004). In brief, the prepared cDNA was amplified with gene-specific primers (as used in real-time PCR assays) for dsrA and 16S rRNA genes. The amplicons were purified using QIAquick PCR purification Kit (Qiagen) according to the manufacturer’s instructions then quantified using a PicoGreen double-stranded DNA kit (Molecular Probes). The concentrations of the amplicons were converted to
numbers of target molecules per microliter (assuming average weight of a DNA base pair as 650 Da) and a 10-fold dilution series for each gene of interest was prepared (10^8 to 10^1 target molecules per reaction). Real-time PCR was carried out using an ABI Prism 7900HT cycler (Applied Biosystems). Standard curves were plotted for the 16S rRNA and dsrA genes (Fig. S4.2).

The reaction mix consisted of 5 µl of 1x POWER SYBR Green master mix (Applied Biosystems), 0.5 µl MgCl₂ (50 mM), 0.5 µl of each primer (1 µM), 1 µl of cDNA template or prepared standard and PCR grade water to a final volume of 10 µl. Thermal cycling conditions included an initial incubation at 50°C for 2 min and denaturation step for 10 min at 95°C. This was followed by 40 cycles of denaturation (95°C for 15 s), annealing (59°C for 1 min), and elongation (72°C for 10 s). Non-template control and dilution series of standards were run in triplicate. Three biological replicates of cDNA of MBBR samples and two technical replicates were used to account for any sample variability. Results were analysed using the ABI Prism 7900HT sequence detection system (Version 2.4). It was assumed that 16S rRNA genes are present at an average of 3.6 copies per cell (Klappenbach et al., 2001) and that dsrAB genes are present in only a single copy (Klein et al., 2001). This was accounted for in calculations of % abundance of total bacteria and SRB in samples. Amplification efficiency was calculated based on the respective standard curve using the formula: \( E = 10^{(-1/slope)}-1 \times 100\% \). Melting curve analysis was also performed to verify the specificity of the primer pairs, by using software ‘dissociation curve’ (Applied Biosystems).
4.4. Results and Discussion

4.4.1. MBBR performance

Water quality information collected at Moa Point and Karori WWTPs during summer and winter surveys is reported in Table S4.1. Both BOD and suspended solids reduce substantially from influent to effluent at each site. Typically, wastewater containing BOD of 200-300 g/m$^3$ also contains COD at ranges of 250-1000 g/m$^3$ (Davis and Cornwell, 1991). Although COD was not measured at these sites in this study one can assume based on the BOD values that it was also reduced substantially. Levels of H$_2$S increased from 12-14 ppm entering Moa Point to 31-35 ppm in effluent. At Karori, yields of H$_2$S were similar (1-3 ppm) to those of a conventional activated sludge WWTP (Koe, 1985). Total dissolved sulfides in the suspended fraction were relatively high at Moa Point WWTP (2.83 ± 1.15 mM and 7.37 ± 4.97 mM for winter and summer, respectively) compared to Karori WWTP (0 mM and 0.27 ± 0.46 mM). These results, along with constant corrosion effects observed at the treatment plant, suggest that sulfate reduction reactions are occurring at Moa Point. Elevated conductivity at Moa Point (~4 mS/cm) compared with an activated sludge treatment plant (~1 mS/cm; (Sanin, 2004)) suggests that seawater is infiltrating into this system. Another important constituent of wastewater is ammonia-nitrogen, with typical values ranging between 12-50 g/m$^3$ (Drinan and Spellman, 2012). This suggests that ammonia is being broken down in the MBBR of Moa Point as values of 8.3-8.7 g/m$^3$ were measured in the effluent. These reactors were maintained as aerobic systems with constant aeration throughout this study.

4.4.2. Biomass measurements

Successional development of biofilm on deployed carriers was monitored by measurement of weight increase. Biomass weights on the deployed carriers generally increased slightly during each study period (Table S4.2). However, biomass collected during summer after 34 days of growth had decreased from the previous time point at both study sites. This decrease corresponds to a period of heavy rainfall in the Wellington area at that time, causing a washout of biomass in the MBBR. At day 48 the adherent biomass at Moa Point WWTP was approximately half the weight (0.071 ± 0.027 g) of that of resident MBBR carriers (0.182 ±
0.033 g), with a similar situation occurring at Karori WWTP. These similar trends observed at both treatment plants enabled comparative analysis of successional development of biofilm between the plants. Although during the course of this study the biomass on deployed carriers did not reach the full weight of a resident biofilm, the 16S rRNA gene-based microbial community profiles were similar, suggesting that successional processes were largely complete (Biswas et al., 2013).

### 4.4.3. SRB richness in MBBRs

Clone libraries representing 15 biofilm MBBR samples from the two treatment plants yielded a total of 302 aligned sequences containing the alpha subunit of the *dsrAB* gene (*dsrA*). After translation into amino acids and grouping into operational taxonomic units (OTUs) based on 90% identity, rarefaction and homologous coverage calculations indicated that sufficient clones were picked from each library. An exception was the sample of mature biofilm from Moa Point in summer which showed only 36.8% coverage, indicating a higher SRB richness than that recovered in this study (Fig. 4.1). Interestingly, 16S rRNA gene data (including total bacterial community and potential SRB) of the same samples also indicated that the mature biofilm from Moa Point in summer had the highest species richness (Biswas et al., 2013).

The Sorensen similarity index was used to assess similarities between samples in regards to *dsrA*-based community composition. Values ranged from 0% (indicating no similarity between libraries) to 62.5% (high level of similarity) (Table S4.3). After 10 days of growth in two parallel reactors at Moa Point WWTP, the *dsrAB*-defined biofilm communities exhibited relatively high similarity (44%). A total of 40 distinct OTUs was recorded, with individual samples containing from 1 to 14 OTUs per clone library (Table S4.4). Richness varied amongst the samples, with no distinct pattern developing as biofilm wet weight increased over time. Typically, the mature biofilm sampled from resident carriers exhibited low richness (1-7 distinct OTUs), with the exception of a sample taken from Moa Point MBBR in summer which contained 14 OTUs. However, the remaining 14 clone libraries had coverage values of over 64% with an average of 20 clones (apart from library M1D35: Moa Point day 35) sequenced per library, thus indicating an SRB community of limited richness. Similar SRB richness has been reported from studies in other environments such as marine sediments (4-13 unique OTUs) (Leloup et al., 2006) and corals (11 unique OTUs) (Bourne et al., 2010).
Figure 4.1. Rarefaction analysis of \textit{dsrAB} clone libraries from MBBRs.

Rarefaction curves for 15 \textit{dsrAB} clone libraries from summer (A) and winter (B) surveys. Error bars indicate standard errors. The homologous coverage ‘C’ is also shown for each library. *indicates biofilm sample collected from a parallel MBBR at Moa Point after 10 days of growth on deployed carriers.
4.4.4. Phylogenetic diversity of SRB community in MBBRs

The phylogenetic diversity of SRBs in MBBR systems has not been described previously, yet warrants investigation in order to better understand the potential role(s) of these organisms in MBBR plants and to facilitate development of a suitable remediation strategy for sulfide reduction at Moa Point WWTP. Phylogenetic analyses (Fig. 4.2, Fig. S4.1) revealed that the majority of recovered sequences (>88% of all dsrAB clones) belonged to the class Deltaproteobacteria, while remaining sequences were assigned to the phylum Firmicutes. The dominant genera among the Deltaproteobacteria were Desulfomonile (up to 100% of sequences in one clone library), Desulfomicrobium (up to 83%), Desulfococcus (up to 57%), Desulfobulbus (up to 53%), and Desulforhopalus (up to 36%). In contrast, 16S rRNA gene data of putative SRB within the same samples identified Desulforhopalus (up to 6% of the total bacterial community), Desulfomicrobium (5%) and Desulfobacter (up to 4%) as the prominent members of the community. The family ‘Syntrophaceae’, consisting of the genera Desulfomonile, Desulfobacca and other, non-SRB organisms (DeWeerdt et al., 2005), was absent from the 16S rRNA gene dataset, which reaffirms the importance of dsrAB gene-based analyses to characterise SRB communities. The abundance of clones in each OTU is shown in Table S4.4.

4.4.4.1. Successional development of SRB community

While it is important to describe the SRB community within established biofilms, we also sought to determine how these communities develop over time. Young biofilms, defined as those present after 20 days of growth on the deployed carriers, at Moa Point were composed largely of OTUs that were classified to Desulfobulbus (35-53% of total SRB sequences) and genera belonging to the family Desulfobacteraceae (27-41%), such as Desulfatiferula, Desulfofabia and Desulfobacterium. Other studies on bacterial development in biofilms of aerated activated sludge or industrial wastewater using 16S rRNA gene analysis also identified Desulfobulbus sp. as a dominant member of the SRB community (Santegoeds et al., 1998; Schramm et al., 1999). These species can grow on propionate or sulfate (Widdel and Pfennig, 1982; Sass et al., 2002), but are able to survive under oxic conditions (Dannenberg et al., 1992). Despite repeated attempts, we were unable to amplify dsrAB genes from young Karori biofilm samples, suggesting that the numbers of SRBs within these samples were below PCR detection limits. 16S rRNA gene data of the same samples at
Karori recorded an increase in the putative SRB community from day 4 biofilm (0.3 ± 0.1% of total bacteria) to day 48 biofilm (2.1 ± 0.5%) samples. At Moa Point, putative SRBs increased from 0.2 ± 0.04% at day 4 to 14.2 ± 2.0% at day 48 based on 16S rRNA gene data. Furthermore, there were no significant differences (p>0.05, ANOVA) between dsrAB-defined community compositions of biofilms sampled on day 10 at Moa Point from two different reactors, which suggests negligible variability in biofilm development within an MBBR treatment facility.

As the biofilm developed the SRB community changed, with dominance (>70% of total SRB community) of the older biofilm (>20 days of biofilm growth) by OTUs belonging to Desulfovibrio, Desulfobulbus and Desulfofatarula at Moa Point in summer. The emergence of Desulfovibrio species in the later stages of biofilm development could reflect a competitive advantage due to their higher affinity for sulfate compared with Desulfobulbus and Desulfofobacter (Laanbroek et al., 1984), and their ability to survive under oxic conditions (Marshall et al., 1993), such as those experienced within an MBBR. It could also be indicative of syntrophic relationships developing at Moa Point, such as those documented in other systems between Desulfovibrio and methanogenic archaea (Bryant et al., 1977). Such interactions usually occur in low organic carbon environments that are rich in methane and sulfate, such as hydrothermal vents and anoxic marine sediments (Boetius et al., 2000; Nauhaus et al., 2007). Methanogens, specifically Methanosarcinales, have previously been identified within biofilms on resident carriers at Moa Point and Karori using 16S rRNA clone libraries and FISH (Biswas and Turner, 2012). SRB and methanogens were also observed in close proximity within the biofilm. These results suggest that, as the biofilm ages, anaerobic niches develop at Moa Point, and these support the growth of syntrophic communities. The Moa Point sample taken 35 days after deployment in winter (M1D35) had unusually large numbers of Desulfomicrobium. For this sample, a number of sequences had to be discarded due to poor quality which may have caused biases. Nitrate is often used as a remedy to suppress sulfide emissions from a WWTP (Zhang et al., 2008). However, if the wastewater biofilm contains Desulfovibrio and Desulfomicrobium, then nitrate has very little effect on sulfide emission (Mohanakrishnan et al., 2011). Thus if nitrate is used to suppress sulfide emissions at Moa Point, it should be used with caution, as these two genera were found exclusively at this site.
Figure 4.2. Taxonomic assignment of dsrAB sequences from MBBRs.

Relative abundance of sulfate-reducing communities at OTU-level in Moa Point and Karori MBBR systems over winter and summer periods as determined by dsrAB clone libraries. An assigned genus for each OTU has been identified and labelled in the figure. Values represent the percentage of the total SRB community for a given sample. *indicates biofilm sample collected from a parallel MBBR at Moa Point after 10 days of growth on deployed carriers.
The older biofilms (>20 days of growth) at Karori contained OTUs belonging to *Desulfobulbus*, *Desulfurhopalus* and *Desulfococcus*, with the latter constituting as much as 57% of the SRB community in a given sample (K2D35). Members of this genus, like for *Desulfovibrio*, are known to survive oxic conditions but can only grow under anoxic conditions (Risatti et al., 1994). 16S rRNA gene data do not concur with the *dsrAB* data, as no *Desulfurhopalus*-affiliated 16S rRNA genes were found in Karori samples. In contrast, 15-36% of *dsrAB* sequences obtained from the older Karori biofilm samples were affiliated with *Desulfurhopalus*. Like some *Desulfobulbus* species, *Desulfurhopalus* can also grow on propionate and sulfate (Isaksen and Teske, 1996). However, in the absence of sulfate they can ferment lactate and ethanol. Thus we can speculate that, at Karori, based on the relatively low levels of sulfide detected and the lower numbers of 16S rRNA-affiliated SRB sequences recorded compared with Moa Point, sulfate levels are lower at this site. The SRBs that were detected at Karori could be capable of undergoing fermentation in the absence of sulfate, thus explaining their presence even in a low sulfate environment.

Mature biofilms from resident carriers at both treatment sites were dominated (58-100% of *dsrAB* sequences) by *Desulfomonile tiedjei*-like organisms, with the exception of biofilm from Moa Point collected over summer. Due to the high diversity present within this particular biofilm only 36% of the SRB community diversity was sampled, hence data shown in Figure 4.2 may not be entirely representative for this sample. The dominance of *D. tiedjei*-like organisms in the established biofilm was not unexpected, as they have previously been found to dominate (50-66%) SRB communities in sulfidogenic wastewater and upflow anaerobic sludge bioreactors (DeWeerd et al., 1990; Dar et al., 2007; Kovacik et al., 2010). These anaerobic organisms are capable of growth via dehalogenation or sulfate reduction (DeWeerd et al., 1990). With an increase in proportion of putative SRBs compared with total bacteria as the biofilm develops, with highest numbers (6-23% of total bacterial 16S rRNA sequences) observed in mature biofilms (Biswa et al., 2013), it can be speculated that the mature biofilms within these aerated MBBRs are anoxic. The presence of anaerobic SRBs in aerobic environments is not uncommon, as evidenced by their detection in aerobic layers of a stratified fjord (Teske et al., 1996), freshwater sediments (Sass et al., 1998) and oxic regions of a microbial mat (Krekeler et al., 1997). Our *dsrAB*-based analysis suggests that sulfate-reducing *Firmicutes*, such as *Desulfotomaculum putei*-like organisms, only established within more mature biofilms. In comparison, 16S rRNA gene data suggest that no known SRB-
related *Firmicutes* were present in any samples (Biswas et al., 2013), reaffirming the need to use functional gene analysis to assess SRB community dynamics.

As the two MBBR systems of study function as BOD removal plants, low numbers (<0.05% of the total 16S rRNA gene-defined bacterial community) of sequences belonging to nitrifiers such as *Nitrospira*, *Nitrosomonas*, and *Nitrococcus* were found across all samples. Although the concentration of ammonia within the MBBR reduces, the relatively low numbers of nitrifiers detected within the bacterial community suggest that this process plays a minor role at Moa Point. Putative sulfur-oxidizing bacteria (SOB), important members of the sulfur cycle, comprised 8.2% of the total bacterial community at Moa Point and only 1.4% at Karori. SOBs utilize sulfide or thiosulfate compounds as electron donors for growth, resulting in deposits of elemental sulfur (Williams and Unz, 1985; Inagaki et al., 2004). Based on the results of diversity and sulfide measurements, we speculate that at Moa Point sulfate is being reduced to sulfide within the anoxic biofilms attached to the carriers, which is further utilized by SOB to form elemental sulfur. In contrast, at Karori, the majority of SRBs may be fermenting organic carbon for growth due to the suspected low sulfate environment, thus low levels of sulfide are produced which results in relatively low numbers of SOB within the community.

### 4.4.4.2. Seasonal variations

Most of the differences observed in biofilm SRB community structure between winter and summer were seen in the later stages of biofilm growth, particularly at the Moa Point facility. These differences are possibly due to physical and chemical parameters, such as temperature and dissolved organic carbon concentrations, which have previously been shown to influence SRB occurrence in mudflats (Leloup et al., 2005) and industrial wastewater ponds (Ben-Dov et al., 2007).

### 4.4.5. Activity of SRBs

Quantitative real-time PCR was used to investigate the abundance of active SRB relative to other bacteria in successionally developing biofilms of MBBR systems. We measured the number of *dsrA* gene transcripts in extracts of total RNA, in order to assess the expression of this functional gene. The primers (DSR1F+ and DSR R) used here for measuring *dsrA*
transcript abundance have previously been used with a range of environmental samples including swine manure (Spence et al., 2008), marine sediments (Leloup et al., 2006), and diseased coral (Bourne et al., 2010). 16S rRNA genes were used to quantify total bacterial numbers and also to normalise dsrAB copy numbers to account for different RNA loads (Livak and Schmittgen, 2001; Neretin et al., 2003).

The average number of dsrA gene copies per microliter of cDNA increased markedly from day 4 (31 copies) to day 48 (9907 copies) in summer biofilm samples at Moa Point (Fig. 4.3(B)). A similar trend was observed at Karori WWTP where an increase from 4 to 5032 copies was observed over the same time period. The increase in number of expressed dsrA gene copies correlated with biomass increase on the deployed carriers, suggesting that older biofilm supports greater numbers of active, anaerobic SRB. The dsrA gene expression levels were surprisingly similar at the two WWTPs over the summer period. A possible reason for this unexpectedly high level of gene expression at Karori could be increases in halogenated products at this WWTP over summer, either due to increased use (e.g. of household cleaning products) or reduced rainfall during that period leading to concentration of compounds; this could in turn lead to proliferation of D. tiedjei-like organisms that are able to degrade these compounds. During winter (Fig. 4.3(A)), even though a similar increase was observed during the course of biofilm development, copy numbers were approximately 8-fold lower relative to the same time points in summer (e.g. 147 copies at day 35 in winter compared to 1190 copies at day 34 in summer). This noticeable increase in expression in summer could be due to seasonal fluctuations in sulfate entering the system or changes in optimal growth temperatures for SRB organisms. Unfortunately, sulfate data are not available for the time of this study.

An unusually high abundance of dsrA genes (380 copies) was observed in day 4 winter samples at Moa Point treatment plant. The high expression of this gene in the nascent biofilm is anomalous. More predictable were the large numbers of active dsrA genes in biofilm samples collected from resident MBBR carriers, ranging from 3917 copies in Karori (winter) to 32234 copies in Moa Point (winter). Lower dsrA gene expression was expected at Karori due to negligible levels of sulfide at this site.

Due to biomass variability on individual carriers during successional biofilm growth, SRB communities were determined as a percentage of total bacteria. For all biofilm samples the SRB comprised less than 1% of the total bacterial community (Fig. 4.3(C) and 4.3(D)).
However, there was an overall increase in abundance of SRBs within the bacterial communities over time. In winter, the biofilm after 35 days of growth contained greater proportions of sulfate-reducing organisms at Moa Point (5.3 x 10^-3 %) compared with Karori (1.3 x 10^-3 %). By contrast, in summer there was a 6.5-fold increase in SRB numbers in Karori mature biofilm samples, even though the proportions of SRB were unchanged at Moa Point. We speculate that this increase reflects the proliferation of *D. tiedjei*-like organisms at Karori during this survey period. As expected, proportions of SRB were highest in mature biofilm samples collected at both WWTP and over the two seasons.

![Figure 4.3. Abundance of dsrA gene in MBBR samples.](image)

Quantitative real-time PCR targeting *dsrA* gene transcripts within developing biofilms of two MBBRs (Moa Point and Karori WWTPs) for (A) winter and (B) summer surveys. Mature biofilms from resident carriers from these two systems were also analysed for both survey periods. Values represent *dsrA* gene copies per microlitre of cDNA and are given as mean ± standard deviation of three biological replicates and two technical replicates.

The abundance of SRBs relative to total bacterial numbers was estimated for the same samples as mentioned above for (C) winter and (D) summer periods. Real-time PCR targeting the 16S rRNA gene was used to quantify the total bacterial community within a biofilm sample. Displayed values represent SRBs as percentage of all bacteria.
The combination of the two molecular techniques employed here provides us with the first \textit{dsrAB}-based insights into the role of sulfate-reducing communities within municipal wastewater treatment systems. Other studies of this kind have either used 16S rRNA gene approaches \cite{santegoeds1998, ito2002} lab-scale bioreactors \cite{dar2007, kovacik2010} or analysis of industrial wastewater \cite{ben-dov2007}. The results from this study suggest that the increase in biomass on the carriers corresponds, at least in part, to a greater abundance of sulfate reducers. Thus, we can speculate that the maintenance of a young, thin (<100 µm) biofilm \cite{rusten1994} on the carriers within MBBR systems may help to reduce SRB activity and in turn reduce sulfide emissions. Oxidants such as nitrate that are often used to lower sulfide emissions from WWTPs would likely have a limited effect at Moa Point, due to the presence of \textit{Desulfovibrio} and \textit{Desulfomicrobium} within the biofilm. However, the effect of other oxidants on species of SRB requires further investigation.

To our knowledge this study is the first to describe SRB community diversity and abundance in an MBBR context. We observed changes in SRB community structure as biofilm developed on deployed carriers in two MBBR systems treating wastewater. The higher sulfurous levels at Moa Point compared with Karori may have lead to the greater overall diversity and abundance of SRB within this plant. The findings of this study, coupled with previous 16S rRNA-based analyses, will further our understanding of bacterial community development in high and low sulfate environments treating wastewater. Importantly, this should help enable the optimisation of treatment plants to reduce undesirably high levels of sulfide emissions.

4.5. Acknowledgements

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Chapter 4- Sulfate-reducing communities in MBBRs

4.6. References


Chapter 4- Sulfate-reducing communities in MBBRs


Chapter 4- Sulfate-reducing communities in MBBRs


Chapter 4- Sulfate-reducing communities in MBBRs


4.7. Supplementary material
Chapter 4- Sulfate-reducing communities in MBBRs

**Figure S4.1. Phylogenetic consensus tree based on distance matrix of dsrAB gene sequences.**

Representative clones from each OTU (highlighted in bold), along with a selection of cultured and environmental SRBs, are shown on the tree. 78 full-length sequences were obtained in this study and were used to construct the tree. Vertical bars indicate families of *Deltaproteobacteria* and the phylum *Firmicutes*. Maximum parsimony was used to calculate bootstrap values, depicted on the figure as filled circles (≥90%) or open circles (75-90%). Scale bar represents 10% sequence divergence.

![Phylogenetic consensus tree](image)

**Figure S4.2. Plots of standard curves.**

Standard curves for target amplicons of (A) 16S rRNA and (B) dsrA genes. Values plotted represent mean ± standard deviation of three replicates. The amplification efficiency (E) by real-time PCR of standard curve samples is also shown on the figure.

![Standard curves](image)
### Table S4.1. Operational data over sampling periods at both MBBR study sites.

<table>
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<tr>
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<th>Winter (May-Jun 2011)</th>
<th>Summer (Jan-Feb 2012)</th>
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<tbody>
<tr>
<td></td>
<td>Moa Point influent</td>
<td>Moa Point effluent</td>
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<tr>
<td><strong>Average daily BOD (g/m³)</strong></td>
<td>270 ± 79</td>
<td>5 ± 6</td>
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<tr>
<td><strong>Average daily SS (g/m³)</strong></td>
<td>444 ± 99</td>
<td>16 ± 13</td>
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<tr>
<td><strong>Average daily flow (m³/h)</strong></td>
<td>3111 ± 708</td>
<td>3116 ± 768</td>
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<td><strong>Average daily H₂S (ppm)</strong></td>
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<tr>
<td><strong>Average daily FC (fc/100ml)</strong></td>
<td>123 ± 214</td>
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<tr>
<td><strong>Ammonia-Nitrogen (g/m³)</strong></td>
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<tr>
<td><strong>Oil and grease (g/m³)</strong></td>
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<tr>
<td><strong>Average DO within MBBR (mg/L)</strong></td>
<td>1.2 (range 0-10)</td>
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<td><strong>CONDUCTIVITY within MBBR (mS/cm)</strong></td>
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<td>4.4 (range 0-13.7)</td>
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<td><strong>Average daily rainfall at WWTP (mm)</strong></td>
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<td>3.5 ± 6</td>
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<td><strong>HRT within MBBR (h)</strong></td>
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<td><strong>Average daily temperature (°C)</strong></td>
<td>18.5</td>
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Values in the table represent means over each survey period ± standard deviation. All measurements were taken daily with the exception of conductivity which was recorded weekly while pH, ammonia-nitrogen, oil and grease were measured once for each time period. Grey boxes indicate that no measurements were made. The values in this table have also been presented in a recently published manuscript (Biswas et al., 2013).

*BOD* - biochemical oxygen demand; *SS* - suspended solids; *DO* - dissolved oxygen; *HRT* - hydraulic retention time; *FC* - fecal count
Table S4.2. Biofilm weight on carriers at the MBBR study sites.

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<th>Wet weight (g) (Jan-Feb 2012)</th>
<th>Day 4</th>
<th>Day 10-13</th>
<th>Day 18-20</th>
<th>Day 34-35</th>
<th>Day 48</th>
<th>Mature biofilm</th>
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<td>K: 0.114 ± 0.015</td>
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Wet weight of deployed carriers at Moa Point (MP) and Karori (K) WWTPs for the two study periods. Standard deviation is shown, where n=3. The values in this table have also been presented in a recently published manuscript (Biswas et al., 2013).

Table S4.3. Similarity index of dsrAB clone libraries.

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*Indicates biofilm sample collection at day 10 from a parallel MBBR at Moa Point WWTP.
### Table S4.4. List of OTUs in each sample of this study.

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dsrA sequences from 15 clone libraries were assigned to operational taxonomic units based on 90% amino acid sequence identity. * indicates biofilm sample collected at day 10 from a parallel MBBR at Moa Point WWTP.

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5. Comparative analysis of bacterial community composition and
\textit{dsrA} abundance between biofilms of K1 and K3 carriers in
moving bed biofilm reactors

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5.1. Abstract

The bacterial communities of moving bed biofilm reactor (MBBR) systems play an important role in nutrient degradation and are largely retained on suspended carriers as biofilms. For the first time, bacterial community structure was compared here between two different carrier types (K1 and K3) treating municipal wastewater in MBBR systems. The aim of this study was to determine whether the community of developing K3 biofilms differs from those on K1 carriers. Nascent carriers were attached to nylon lines which were lowered into MBBRs of Moa Point and Karori facilities in Wellington, New Zealand. To monitor the successional development of biofilms on carriers, samples were collected from deployed lines after 4 days of biofilm growth up to 48 days. Molecular methods including 16S rRNA gene pyrosequencing and *dsrA*-based real-time PCR were employed to monitor bacterial community development. Differences in microbial community composition between the two carrier types were evident at class and genus levels, with greater numbers of fast-growing aerobic *Gammaproteobacteria* on K3 carriers (32–60% of the bacterial community) compared with K1 carriers (10–30%). In contrast, greater numbers of anaerobic sulfate-reducing *Deltaproteobacteria* were observed after 20 days of biofilm growth on K1 carriers (<25%) compared with K3 carriers (<4%). However, the abundance of active sulfate reducing bacteria were similar between the two carrier types at Moa Point WWTP, but was much lower in K3 carriers at Karori WWTP.

5.2. Keywords

Wastewater treatment plant; Sulfate-reducing bacteria; 16S rRNA gene; Polyethylene carriers; Biofilm; Bacterial communities
Chapter 5 – Bacterial communities of K3 carriers

5.3. Introduction

Biofilm-based technologies for the treatment of wastewater have become increasingly popular over conventional activated sludge systems due to greater efficiency and stability of these systems. Moving bed biofilm reactors (MBBR) are a biofilm-based technology that was first implemented in 1989, and to date over 1200 industrial and municipal treatment plants operate around the world with applications such as chemical or biochemical oxygen demand (COD/BOD) removal, nitrification and denitrification (McQuarrie and Boltz, 2011). MBBR-based systems retain the majority of the microbial biomass on suspended plastic carriers within the reactors, with a smaller proportion in the suspension as free-floating flocs. These organisms help break down complex molecules present in wastewater, which are further utilised for their development. There are a variety of carrier shapes, sizes and materials used for biofilm-based treatment but the most commonly used is the small K1 carrier (AnoxKaldnes™). Most of the biomass grows within protected surfaces of the carriers; hence surfaces that support biofilm growth (also called effective surface area) can be calculated for different carrier shapes and sizes. For example, K3 carriers (25 mm diameter) are larger than K1 carriers (9.1 mm) yet have the same effective surface area at 60% filling fraction of 300 m²/m³. Filling fraction is defined as the volume of carriers occupying an empty reactor. The maximum recommended filling fraction in MBBRs is 70% to enable free movement of the suspended carriers (Rusten et al., 2006).

Carrier shape is also a key parameter of interest and has been reported to influence functional performance such as levels of nitrification (Bengtsson et al., 2008). Concerns over low nitrification rates have been raised in the literature for K1 carriers with possible reasons such as limitations of oxygen and ammonia concentration within the biofilm and organic loading into the reactors (Odegaard et al., 1994). An ideal biofilm thickness is reported to be less than 100 μm and is usually dependent on factors such as organic loading, shear forces, temperature and oxygen concentration. Biofilm thicker than 100 μm allows only partial diffusion of nutrients such as ammonia and oxygen into the biofilm (Rusten et al., 2006), therefore selecting for anoxic zones that support the growth of anaerobic organisms.

The presence of anaerobic zones can be problematic in circumstances where there are also elevated levels of sulfate present. High sulfate concentrations are often observed in industrial wastewater and can also occur due to sea water infiltration into treatment facilities (Muyzer and Stams, 2008). In anaerobic environments sulfate may be used as a terminal electron
acceptor by sulfate-reducing bacteria (SRB) as they reduce sulfate to sulfide. The toxicity, odorous and corrosive nature of high sulfide levels at a WWTP can lead to detrimental effects such as corrosion of the treatment facilities, with significant operational and economic impacts (USEPA, 1991). A recent study of microbial communities on K1 carriers at two full-scale municipal wastewater treatment plants (WWTP) showed that established biofilms were dominated by putatively anaerobic organisms belonging to the Clostridia and Deltaproteobacteria. In contrast young biofilm, from carriers present for less than 20 days, had higher levels of fast-growing aerobic members of the Gammaproteobacteria, Alphaproteobacteria, and Betaproteobacteria.

Based on these studies it is clear that the K1 carriers support development of biofilms that include anoxic zones. K3 carriers differ from K1 media by being larger and having a more open structure. It was therefore hypothesised that biofilms on K3 carriers could be subjected to greater shear forces, preventing significant biofilm accumulation and thus leading to reduced numbers of anaerobic organisms such as Clostridia and other SRB. Furthermore, the initial colonisation of microbes to surfaces is a critical step to starting-up a biofilm-based WWTP, hence, it is important to understand the successional development of biofilms during this process. However, there is very little understanding of the microbial communities that attach onto K3 carriers for the treatment of wastewater. Although only a small proportion (~7%) of MBBR treatment plants around the world utilise K3 carriers, there have been several studies on the effectiveness of different types of carriers for nutrient removal (Odegaard et al., 2000; Levstek and Plazl, 2009; Kriklavova and Lederer, 2010). One such study compared COD removal between K1 (9.1 mm diameter) and K2 (15 mm) carriers and found no significant difference, although the settleability of biomass leaving the reactor was improved with the use of bigger carriers (Odegaard et al., 2000).

In this study, we aimed to conduct the first detailed analyses of the bacterial community composition of successionaly developing biofilm of K3 carriers treating municipal wastewater. For this purpose two WWTPs were chosen, Moa Point and Karori. Nascent K3 carriers were deployed into aerated MBBRs that contained resident K1 carriers and were sampled between 7 to 42 days of biofilm growth. In addition, we aimed to compare these results with previously published information on K1 carrier biofilms, also grown between days 4 to 48 at the same two WWTPs over the same time period. Due to the high levels of sulfides detected at Moa Point WWTP compared with Karori WWTP, the activity of SRBs was inferred for various growth stages of biofilm on K3 carriers, and compared with
previously reported data on developing biofilms on K1 carriers. Molecular techniques including 16S rRNA gene pyrosequencing and real-time PCR of the alpha subunit of the dissimilatory (bi)sulfite reductase gene (dsrA) were used to determine bacterial community composition and to measure SRB activity, respectively. The results obtained could provide further information to guide the selection of media for optimal operation of biofilm-based systems used to treat municipal wastewater.

5.4. Material and methods

5.4.1. Biofilm Sampling

Two full-scale WWTPs (Moa Point and Karori) employing moving bed bioreactor technology with K1 carriers were used as study sites. These treatment facilities were designed primarily to operate as BOD removal sites with hydraulic retention times of 1 h within the MBBRs. Operational conditions of the two study sites are reported in Table S5.1. Moa Point was the larger of the two sites was exclusively measured for conductivity as seawater infiltration was suspected into this site. Nascent K3 carriers (35-40) were attached onto a weighted nylon fishing line and suspended into the aerated MBBR tanks at both study sites. Weights on the end of the lines were sufficient to prevent lines from floating on the surface, but still allowed lateral movement within the reactors. For this study, two such lines were used per MBBR, during the sampling period of January to February 2012. Between 5 and 6 of the deployed carriers were collected once a week from each MBBR after 7 days of biofilm growth on the deployed carriers, with final samples taken after 42 days and stored in RNAlater Solution (Ambion) at -20°C for further analysis. The wet weight of the carriers for each time point was determined as described previously (Biswas and Turner, 2012).

5.4.2. Nucleic acid extraction

Biofilm was scraped from the carriers and total genomic DNA was extracted using a chloroform-based bead-beating method as described previously (Smith et al., 2003). The extracted DNA was dissolved in 35 μL nuclease-free water and stored at -20°C.

Total RNA from scraped biofilm of three carriers per time point were obtained by breaking open the cells using enzyme (15 mg/ml of lysozyme at 37°C for 10 min) and mechanical
disruption (4 m/s for 20 s on a bead-beater) methods. Acid-washed 0.1 mm glass beads were used for this purpose. The remaining protocol was carried out using the RNeasy Mini Kit (QIAGEN) as per manufacturer’s instructions. Genomic DNA was removed from the extract using DNase I as per the manufacturer’s instructions (Invitrogen). The quantity and quality of the RNA was measured using Nanodrop ND-1000 UV-Vis spectroscopy (Thermo Scientific) and RNA 6000 Nano chips (Agilent 2100 BioAnalyzer). Extracted RNA (300 ng) was converted to cDNA using the First Strand SuperScript III kit (Invitrogen) and stored at -20°C.

5.4.3. Microbial community analysis by 16S rRNA gene pyrosequencing

Genomic DNA was extracted from biofilm from each of three replicate K3 carriers for each time point (days 14, 28, 42 of biofilm growth). The 16S rRNA gene of these samples were amplified using primers 347f and 803r (Nossa et al., 2010) and further analysed by amplicon pyrosequencing as described previously (Biswas et al., 2013). In short, a set of 24 samples with unique multiplex identifiers (MIDs) were amplified using equimolar concentrations (0.2 µM) of fusion primers (forward and reverse), dNTPs (0.2 mM), PCR buffer (1X), MgSO₄ (2 mM), 0.5U Platinum Hi-fidelity Taq (Invitrogen) and PCR-certified water to a final volume of 25 µl. The PCR amplification was performed in an Applied Biosystem Mastercycle gradient PCR machine with the following thermal cycling conditions: 94°C for 1 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and elongation (68°C for 30 s) and a terminal elongation step at 68°C for 3 min. Amplified products were purified using Agencourt AMPure beads (Roche), quantified using PicoGreen and run on Agilent 1200 Bioanalyzer DNA 1000 chips (Agilent Technologies, Santa Clara, CA, USA) for qualitative analysis. Equal concentrations of the 24 amplicons were pooled together and shipped to Macrogen Inc. (Seoul, South Korea) for sequencing on the Roche GS FLX Titanium platform.

Pyrosequencing reads were analysed as described previously (Simister et al., 2012). In short, Mothur (Schloss et al., 2009) was used to sort and retain only high-quality useable reads that were aligned against the SILVA reference database (http://www.mothur.org/wiki/Silva_reference_alignment). Reads were taxonomically assigned using custom-made PERL scripts along with a manually curated SILVA database (version 108). To assess the significance of differences between sample types unweighted
UniFrac (phylogeny-based) analyses were performed. UniFrac distance obtained in Mothur was plotted in Excel using Principal Co-ordinates Analysis (PCoA). Graphical representation of the 10 most abundant genera for each sample was plotted in JColorGrid (Joachimiak et al., 2006).

5.4.4. Real-time PCR to quantify bacterial 16S rRNA and dsrA gene transcripts

The active SRBs and total bacteria within the biofilm of three biological replicate samples scraped from deployed K3 carriers for each time point were quantified using real-time PCR. The resulting cDNA of the biofilm samples was amplified using primer sets 533F/803R, targeting the bacterial 16S rRNA gene, and DSR1-F+/DSR-R (Kondo et al., 2004) targeting the dsrA gene of SRBs, as previously described (Biswas et al., submitted). Standards were prepared for each gene of interest as previously described (Chin et al., 2004). Non-template control and standards for each primer set were loaded on the same real-time PCR run as the K3 biofilm samples. The PCR reaction mix comprised x1 POWER SYBR Green master mix, 2.5 mM MgCl₂, 0.5 µl of each primer (1 µM), 1 µl of cDNA template or prepared standard and PCR grade water to a final volume of 10 µl. Thermal cycling conditions consisted of an initial incubation (50°C for 2 min) and denaturing (95°C for 10 min) step followed by 40 cycles at 95°C for 15 s, 59°C for 1 min, and 72°C for 10 s. All samples, including non-template control and standards, were run in triplicate. The real-time PCR experiment was repeated to account for technical variability between runs. ABI Prism 7900HT sequence detection system (Version 2.4) software was used to analyse the results. For quantification analysis we assumed an average copy number of 3.6 per cell for 16S rRNA genes (Klappenbach et al., 2001) and 1 copy per cell for dsrAB gene (Klein et al., 2001). These values were accounted for when calculating percentage abundance of SRB in samples. Amplification efficiency was calculated based on the respective standard curves using the formula: E = 10^{(-1/slope)}-1 x 100% (Fig. S5.1). Melting curve analysis was also performed to verify the specificity of the primer pairs, using software ‘dissociation curve’ (Applied Biosystems).
5.5. Results and Discussion

The key factor contributing to organic matter removal in MBBR systems is effective surface area on carriers for biomass growth (Odegaard et al., 2000; Levstek and Plazl, 2009). The effective surface area of K1 and K3 carriers is similar and therefore it might be expected that they could be used interchangeably in biofilm-based wastewater treatment processes. In this study, we aimed to provide the first comparative analyses on bacterial communities of developing biofilms between two popular carrier types (K1 and K3) for the treatment of wastewater.

Moa Point, the larger of the two study sites, treats trade wastewater along with domestic wastewater with a population equivalence of 200,000, whereas Karori treats exclusively domestic wastewater from a population of 20,000. Operational data and available water quality analysis of the two study sites have been reported previously (Biswas and Turner, 2012; Biswas et al., 2013) with high levels of H$_2$S (35 ppm) and total dissolved sulfides (7.37 mM) at Moa Point WWTP. Other measurements such as BOD and DO were maintained at fairly similar levels at both treatment facilities. Conductivity was exclusively monitored at Moa Point (average over Jan–Feb 2012 was 4.44 mS/cm) due to suspicions of seawater infiltration.

5.5.1. Biofilm characteristics

The colour and texture of the biofilm largely found on the inner protected surfaces of the K3 carriers differed between the two WWTPs. At Moa Point the biofilms were blackish-brown and compact, whereas at Karori the biofilms appeared light-brown and slimy. Similar differences have been noted between K1 carriers from the two sites (Biswas and Turner, 2012). One difference that was noted was the presence of Tubifex (sludge worm) within the biofilms of the K3 carriers at Karori WWTP. High numbers of these worms have been shown to significantly reduce nitrification rates at a municipal WWTP (Watts et al., 2000).

To monitor biofilm growth and accumulation on the carriers over time, wet weight measurements were made of freshly recovered carriers at each sampling time point. Both K1 and K3 carrier types showed an increase in weight over the course of the study, with the majority of the biomass developing on the inner protected surfaces of the carriers (Fig. 5.1). Biomass on the K3 carriers was evident from day 7 of carrier deployment and generally
increased over the course of the study through to day 42. At Moa Point the increase was gradual, from 0.40 ± 0.25 g at day 7 to 1.16 ± 0.33 g at day 42, however at Karori there was a sudden increase from day 7 (0.55 ± 0.12 g) to day 14 (1.41 ± 0.28 g) and then a gradual decrease to day 42 (1.04 ± 0.24 g). In general, there was also an overall increase of biomass weight on deployed K1 carriers between day 4 to day 48 at both study sites, as reported previously (Biswas et al., 2013). Minor fluctuations observed with biomass weight of these carriers could be due to the occurrence of heavy rainfall observed in the Wellington region, resulting in large volumes of stormwater entering the MBBRs and washing out loosely attached microbes.

Figure 5.1. Image of representative Kaldnes K3 and K1 biofilm carriers used in this study.

The biofilm on the K3 carrier was post 42 days of deployment whereas the biofilm on the K1 carrier had established over several years. Scale bar represents 1 cm.

5.5.2. Bacterial diversity of biofilms attached to K1 and K3 carriers

Pyrosequencing of 16S rRNA gene was undertaken to monitor the successional development of bacterial communities on deployed K3 carriers. A total of 31,563 high-quality reads were obtained across the whole study giving, from 817 to 4141 reads per sample with an average
length of 400 nucleotides. Operational taxonomic units (OTUs) were assigned based on 97% sequence similarity, with values ranging between 218 to 671 OTUs for all biofilm samples adhering to K3 carriers (Table. S5.2). Other diversity indices such as Chao1 richness and Invsimpson were also calculated for samples and showed lower levels of bacterial diversity compared with the diversity of the communities from the smaller K1 carriers. Little change was observed among these species richness estimators as biofilm developed on K3 carriers. In contrast, previously analysed results of K1 carriers showed that diversity increased at both treatment plants (eg. at Moa Point from day 4 (352 OTUs) to day 48 (431 OTUs)) as the biofilm develops. These results suggest that the bacterial community on the K3 carriers stabilises within a shorter time period than the community on the K1 carriers. A longer experimental period would be required to notice substantial changes within the bacterial community of K3 carriers.

5.5.3. Comparative analysis of bacterial community composition

Three phyla (Proteobacteria, Bacteroidetes, Firmicutes) dominated all biofilm samples of both K1 and K3 carriers, along with the suspended fraction, at both study sites (Fig. 5.2(A)). However, at class-level, differences in community structure were observed between the two carrier types at both study sites (Fig. 5.2(B)). At Moa Point, elevated levels of Gammaproteobacteria were observed, particularly in the older biofilms of K3 carriers (post 20 days deployment) where they comprised 32–60% of total bacterial community. In comparison, Gammaproteobacteria comprised only 10–30% of the community on K1 carriers deployed for the same period. The genera Acinetobacter and Pseudomonas (Fig. 5.3) comprised the majority of the Gammaproteobacteria in all samples. These aerobic fast-growing organisms are well known for their importance in biofilm formation and were previously believed to enhance phosphorus removal in activated sludge systems (Wagner et al., 1994; Andersson et al., 2008). Another aerobic member of the bacterial community, the genus Flavobacterium, had similar patterns of development in both carrier types with higher abundance recorded in the initial stages (14 days post deployment) of biofilm growth. The elevated presence of Deltaproteobacteria (up to 25% of the total bacterial community) in the older and mature biofilms of K1 carriers at both study sites was of particular interest due to the putative sulfate-reducing properties of some of these organisms (Fig. 5.2(B)). The emergence of large numbers of Deltaproteobacteria in older biofilms of K1 carriers is a strong indicator for anaerobic niches developing within. In contrast, even after 42 days of
biofilm growth on K3 carriers, negligible levels (<4% of total bacterial community) of sulfate-reducing *Deltaproteobacteria* were observed, suggesting the limitation of anaerobic or other conditions required for the growth of these organisms.

![Figure 5.2](image-url)

Figure 5.2. Comparative analysis of microbial communities from biofilms as determined by 16S rRNA gene pyrosequencing.

(A) Composition of the 3 most abundant phyla for all samples in both carrier types (K1 and K3) at Moa Point and Karori WWTPs. (B) Taxonomic composition by class (of the 4 most dominant phyla) per time point for all samples are also shown. Values are presented as % of total bacterial community for a given sample. Biological replicates for each sample type are displayed on the graph.
Chapter 5 – Bacterial communities of K3 carriers

SRB community composition as inferred from 16S rRNA gene data had similar patterns for both K1 and K3 carrier types. At Moa Point, the carriers were dominated by *Desulfovibacter*, *DesulfHorpalus* and *Desulfomicrobium*. In contrast, Karori biofilm samples were dominated by members of *Desulfovibrio*, *DesulfoBulbus* and *Desulfomicrobium*. However, the overall sequence numbers identified as potential SRBs in all biofilm samples were greater in K1 carriers (0.1–16.2% of the total bacterial community) compared with K3 carriers (0.4–4%). This difference was more pronounced at Moa Point. Furthermore, the genera *DesulfHorpalus* and *DesulfoBacter*, which were found exclusively at Moa Point, have previously been found exclusively in brackish environments (Kuever et al., 2005b, a) suggesting that seawater infiltration is occurring at this facility. The dominant SRB (0.7% of the total bacterial community) belonging to genus *Desulfovibrio* identified at Karori, are able to tolerate oxic conditions (Marschall et al., 1993). Thus, their presence at this site suggests that oxygen is readily available to the microorganisms within the biofilm. The SRB genera identified in MBBRs in this study possess different metabolic pathways, which may explain their variable appearance in developing biofilms. For example, members of the genus *DesulfHorpalus* can reportedly carry out incomplete oxidation of acetate to propionate and CO₂, whereas *DesulfoBacter* are known to completely oxidise a wide variety of substrates to CO₂ (Muyzer and Stams, 2008). In addition, *Desulfomicrobium* has previously been isolated from diverse sources including industrial wastewater (Ben-Dov et al., 2009) hot springs (Thevenieau et al., 2007), and marine sediments (Leloup et al., 2006) and have also been shown to convert nitrite to ammonia under high nitrate environments (Mohanakrishnan et al., 2011). This versatile nature of SRBs allows them to survive in a wide range of environments including wastewater systems (Dar et al., 2007; Ben-Dov et al., 2009). The higher abundance of SRB within the bacterial community of mature biofilms of K1 carriers is most likely due to prevailing anaerobic conditions compared with younger biofilms of K1 carriers and also with mature biofilms of K3 carriers. To complete the sulfur cycle within the MBBR, sulfur oxidising bacteria (SOB) are required to convert sulfide products to elemental sulfur. *Sulfurovum* and *Thiothrix* comprised the SOB population in all biofilm samples in this study. In accordance with a previous study using K1 carriers, greater numbers of SOB were identified in K3 carriers at Moa Point (1.4–4.3% of the total bacterial community) compared with Karori (0.3–0.9%). As expected, negligible numbers of known nitrifiers were identified amongst all samples, as these plants were configured for BOD removal.
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Based on these results we can speculate that the overall function of the communities between the two carrier types within a treatment plant is similar, even though fewer SRB sequences detected for K3 carriers. Moreover, differences between the bacterial community composition of samples was more pronounced between WWTPs. Members of the class Sphingobacteria were exclusively found in biofilms from the Karori WWTP, with slightly elevated levels recorded on K1 carriers (<10% of the total bacterial community) compared with K3 (<4%) carriers. Elevated levels of Clostridia were observed across all time points in the K3 carriers of Karori WWTP (<7% of the total bacterial community) compared with Moa Point WWTP (<2%). Another major difference was in the occurrence of Epsilonproteobacteria, which were largely composed of the genus Arcobacter (Fig. 5.3). Members of this group tended to increase as biomass weight increased on both carrier types but were particularly prevalent at Moa Point WWTP. Microaerophilic Arcobacter are potentially pathogenic, with certain members capable of oxidising sulphides (Collado and Figueras, 2011). A possible source for the elevated levels of this organism at Moa Point WWTP is wastewater discharge from the local abattoir into the treatment facility. This would also explain the high levels of Arcobacter observed in the suspended fraction of Moa Point (34%) relative to Karori (4%) MBBRs (Fig. 5.3). Such observations between the two study sites were expected due to differing influent compositions entering the treatment facilities.

Unweighted UniFrac revealed significant differences (p<0.05) between bacterial communities of K1 and K3 carriers at all stages of biofilm growth at Moa Point WWTP. However, at Karori WWTP significant differences between the two carrier types were observed only after 28 days of biofilm growth. This information suggests that carrier shape and size play a significant role in bacterial community composition of developing biofilms in MBBR systems. Due to the more open structure of the K3 carriers it is possible that greater sloughing of biomass occurs within protected areas of these carriers compared with K1 carriers. This is supported by the observed rapid increase in biomass on the K3 carriers up until day 14 followed by a decrease in biomass by day 42. Relationships between biofilm age and carrier types were determined by phylogenetic information of the community composition of samples using Unifrac distances and are displayed in Fig. S5.2. In brief, as biofilm age increased the UniFrac distance to the mature biofilm of K1 carriers decreased. However, this observation was more evident for K1 carriers compared with K3 carriers at both treatment plants. These data suggest that the biofilm on K3 carriers bear higher resemblance to the bacterial community from younger biofilm or the suspended fraction,
which were dominated by fast-growing, aerobic organisms. However, a longer-term study would be required in order to compare the community structure between biofilms of established K3 and K1 carriers.

Figure 5.3. Heat map of the top 10 genera of biofilm samples from Moa Point and Karori WWTPs along with the suspended fraction of the MBBRs.

Comparisons are made between bacterial communities of developing biofilms of K1 (K1) and K3 carriers (K3). The community composition of mature biofilms of resident K1 carriers is also displayed on the graph. Values are presented as % of total bacterial community for a given sample.
Chapter 5 – Bacterial communities of K3 carriers

5.5.4. Comparative analysis of SRB activity

Quantitative real-time PCR was used to compare the activity and abundance of SRBs in K1 and K3 samples from the two treatment plants. Total numbers of bacterial cells in samples were estimated by measuring the abundance of 16S rRNA gene transcripts. Results shown in Figure 5.4 are an average of three biological replicates and two technical replicates per time point.

The copy number of \( dsrA \) genes had little change between day 7 to day 42 (208 to 394 copies per microliter of cDNA, respectively) for biofilm of K3 carriers at Karori WWTP (Fig. 5.4(B)), suggesting low SRB activity. In contrast, a steep increase from SRB activity was observed from day 4 to day 48 at both study sites for K1 carriers. However, at Moa Point the copy numbers of \( dsrA \) genes in biofilm belonging to K3 carriers decreased between days 7 to 14 and then gradually increased to 6827 copies per microliter at day 42 (Fig. 5.4(A)). The increase in SRB gene expression, as indicated by increasing copy numbers of functional genes for sulfate reduction, supports the notion that as biomass increases on carriers at Moa Point, more anoxic zones develop within the biofilm that support the growth of SRBs. However, at Karori, after the initial increase in \( dsrA \) copy numbers, a fairly stable population of SRBs appear to develop on K3 carriers, which correlates with the wet weight measurements of the biomass. The greater abundance and activity of SRBs at Moa Point, coupled with the comparatively higher levels of hydrogen sulfide in the outlets of this treatment plant, suggest that the SRB communities are functioning differently at the two WWTPs (Biswas et al., submitted). This is possibly due to availability of sulfate within the treatment process though further work is required to confirm this. Similarly, it is possible that, even though SRBs are found on both K1 and K3 carrier types, the structure of the biofilm is sufficiently different to influence sulfate reduction.
Chapter 5 – Bacterial communities of K3 carriers

![Figure 5.4](image)

Figure 5.4. Quantitative real-time PCR targeting *dsrA* gene transcript within developing biofilms of both K1 and K3 carriers at (A) Moa Point and (B) Karori WWTPs.

Established biofilms from K1 resident carriers, identified as ‘mature’, from these two systems were also analysed. Values represent *dsrA* gene copies per microlitre of cDNA and are given as mean ± standard deviation of three biological replicates and two technical replicates. The abundance of SRBs relative to total bacterial numbers was estimated for the same samples as mentioned above for (C) Moa Point and (D) Karori WWTPs. Values are displayed as SRBs as percentage of all bacteria and are given as mean ± standard deviation of three biological replicates and two technical replicates.

The proportion of total bacteria represented by SRBs was also calculated due to biomass variability amongst samples. The bacterial community of biofilms grown on deployed K1 and K3 carriers contained less than 1% of active SRBs at both treatment plants (Fig. 5.4(C), 5.4(D)). There was an overall increase in SRB proportion within the bacterial community as biofilm developed on the carriers at both WWTPs, apart from on K3 carriers at Karori WWTP in which SRB proportion remained fairly constant. Early stages of biofilm growth and attachment produced large differences between the two carrier types, with SRBs representing much greater proportions of the total community developing on K3 carriers. As successional development of the biofilms progressed at both sites, the SRB proportions...
reduced for K3 carriers relative to K1 carriers. Similarly, for the latter stage of biofilm growth, SRB proportions were lower in K3 compared with K1 carriers at Karori WWTP. These results suggest that as long the biofilm remains young (less than 35 days of biofilm growth), K3 carriers support fewer active SRBs within the total bacterial community compared with K1 carriers. This could be a result of greater shear force occurring within the K3 carriers in the aerated MBBR tanks. However, at Moa Point the inverse result was observed for the later time points (day 42 and day 48) of biofilm growth, with higher abundance of SRB proportions in K3 carriers relative to K1 carriers. The mature biofilm of resident K1 carriers at both study sites had the highest abundance of SRBs compared with all other biofilm samples, which is consistent with the theory of a greater number of anoxic zones developing within biofilms over time.

To our knowledge this is the first study of bacterial community structure and composition of biofilms on K3 carriers. Comparative analyses were made to previously published results on bacterial communities within biofilms of K1 carriers. In conclusion, the results from this study suggest that bacterial communities are similar in K1 and K3 carriers. Therefore, K3 carriers could be a suitable replacement for K1 carriers in MBBR systems treating wastewater, with the added advantage of reduced growth of sulfate-reducing microorganisms. Such a modification may help to reduce the production of hydrogen sulfide at WWTPs using biofilm-based treatment systems. The results from this study will further our understanding of biofilm-based systems treating wastewater to help reduce sulfide production.

5.6. Acknowledgements

The authors would like to thank Veolia Limited for funding this project. We would also like to acknowledge the staff at Moa Point and Karori WWTPs for their help with sampling and gathering of the operational data during the study period. Furthermore, the authors are thankful to M. Taylor and P. Mann for their comments on the manuscript and for their assistance in the attachment of nascent carriers to nylon fishing lines.
Chapter 5-Bacterial communities of K3 carriers

5.7. References


Chapter 5- Bacterial communities of K3 carriers


Chapter 5-Bacterial communities of K3 carriers


Chapter 5-Bacterial communities of K3 carriers


5.8. Supplementary material

Figure S5.1. Standard curve for target amplicons of (A) 16S rRNA and (B) dsrA genes.
Values plotted represent mean standard deviation of three replicates. The amplification efficiency (E) by real-time PCR of standard curve samples also shown on the figure.

(A) 16S rRNA gene

(b) dsr gene

$y = -3.3858x + 38.37$

$R^2 = 0.9752$

E = 97.4%

$y = -3.9608x + 39.816$

$R^2 = 0.988$

E = 78.84%
Figure S5.2. Principal Co-ordinate Analysis (PCoA) of unweighted UniFrac distances (phylogeny-based) for all samples.

Moa Point samples are represented by yellow (for K3 samples) and red (for K1 samples). Karori samples are represented by green (for K3 samples) and blue (for K1 samples). The suspended fraction and mature biofilm for each treatment plant are also shown on the graph.
Table S5.1. Operational data over sampling periods at both study sites.

<table>
<thead>
<tr>
<th></th>
<th>Jan-Feb 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moa Point influent</td>
</tr>
<tr>
<td>Average daily BOD (g/m³)</td>
<td>225 ± 92</td>
</tr>
<tr>
<td>Average daily SS (g/m³)</td>
<td>331 ± 89</td>
</tr>
<tr>
<td>Average daily flow (m³/h)</td>
<td>2967 ± 685</td>
</tr>
<tr>
<td>Average daily H₂S (ppm)</td>
<td>14</td>
</tr>
<tr>
<td>Average daily FC (fc/100ml)</td>
<td>230 ± 310</td>
</tr>
<tr>
<td>Average DO within MBBR (mg/l)</td>
<td>2.2 (range 0-10)</td>
</tr>
<tr>
<td>CONDUCTIVITY within MBBR (mS/cm)</td>
<td>4.4 (range 0-13.7)</td>
</tr>
<tr>
<td>Sludge age at WWTP</td>
<td>1-2 days</td>
</tr>
<tr>
<td>Average daily rainfall at WWTP (mm)</td>
<td>3.2 ± 5.6</td>
</tr>
<tr>
<td>HRT within MBBR (h)</td>
<td>1</td>
</tr>
<tr>
<td>Average daily temperature (ºC)</td>
<td>20.4</td>
</tr>
</tbody>
</table>

Values in the table represent means over each survey period ± standard deviation. All measurements were taken daily with the exception of conductivity which was recorded weekly while pH, ammonia-nitrogen, oil and grease was measured once for each time period. Grey boxes indicate that no measurements were made. The values in this table have also been presented in a recently published manuscript (Biswas et al., 2013).
Table S5.2. Summary of bacterial 16S rRNA gene pyrosequencing reads based on 97% OTU similarity.

<table>
<thead>
<tr>
<th>group</th>
<th>nseqs</th>
<th>coverage</th>
<th>OTUs</th>
<th>Chao 1</th>
<th>Invsimpson</th>
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</thead>
<tbody>
<tr>
<td>Moa Point-K1 carrier</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Suspended</td>
<td>3308</td>
<td>0.9</td>
<td>376</td>
<td>723.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Day 13</td>
<td>2778</td>
<td>0.9</td>
<td>402</td>
<td>1039.6</td>
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</tr>
<tr>
<td></td>
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<td>346</td>
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<tr>
<td>Day 34</td>
<td>1069</td>
<td>0.9</td>
<td>223</td>
<td>801.2</td>
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<tr>
<td></td>
<td>1790</td>
<td>0.9</td>
<td>229</td>
<td>608.6</td>
<td>14.0</td>
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<tr>
<td></td>
<td>998</td>
<td>0.9</td>
<td>222</td>
<td>467.9</td>
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<tr>
<td>Day 48</td>
<td>4608</td>
<td>0.9</td>
<td>665</td>
<td>1952.4</td>
<td>16.0</td>
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<tr>
<td></td>
<td>2928</td>
<td>0.9</td>
<td>412</td>
<td>985.2</td>
<td>15.1</td>
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<tr>
<td>Mature</td>
<td>2892</td>
<td>0.9</td>
<td>374</td>
<td>796.0</td>
<td>13.1</td>
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<tr>
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<td>433</td>
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<td>Karori –K1 carrier</td>
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<td>Suspended</td>
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<td>454</td>
<td>1181.5</td>
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<tr>
<td>Day 14</td>
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<td>0.9</td>
<td>397</td>
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<td>577</td>
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<tr>
<td>Day 28</td>
<td>3102</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Day 42</td>
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<td>1483.8</td>
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The number of high quality gene sequences (nseqs), Good’s coverage, number of unique OTUs, Chao 1 and invsimpson diversity indexes per sample has been summarized. Three replicates per time point are shown.
6. Biological nutrient removal and microbial community dynamics in lab-scale MBBRs under different environmental conditions

Publication status: Under Review

6.1. Abstract

This study aims to examine the effects of changes in wastewater composition in high sulfate environment on: (1) key functional groups within the microbial community of biofilms; (2) nutrient removal rates; (3) sulfate-reducing bacteria (SRB) within these systems. To manipulate the influent composition two lab-scale moving bed biofilm reactor (MBBR) systems fed with synthetic wastewater were used. Fresh microbial biomass from an MBBR plant was used to seed the reactors, which led to >85% ammonia removal, even after alterations were made to the concentration of nutrients in the feed. However, changes in seed material to a mixture of 1:1 ratio of fresh and old biomass (stored for 3 months) resulted in ammonia removal efficiency to drop to <40%. Adjustments made to the feed of this reactor (low nutrient in combination with high conductivity) resulted in recovery of ammonia removal (100%). In contrast, high nutrient in combination with high conductivity feed inhibited nitrification processes within the reactor and also had the lowest numbers (0.5 ± 0.5%) of SRB in this study. Sulfate and phosphate removal was <30% during all experimental conditions. Fluctuating nitrite and nitrate levels in the effluent correlated with numbers of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) within the bacterial community, as measured by 16S rRNA gene pyrosequencing. SRBs were most abundant in the control phases and reduced throughout the experimental phases, as measured by real-time PCR and amplicon pyrosequencing. The reduction of SRBs in the experimental phases of this study, under high sulfate conditions, could be of interest to WWTP operators that face sulfide related issues and are looking for alternative solutions to chemical treatment approaches.

6.2. Key words

Moving bed biofilm reactor; Wastewater treatment; Biological nutrient removal; Nitrification; Sulfate reduction; Amplicon pyrosequencing; Bacterial communities
6.3. Introduction

Biological nutrient removal (BNR) is the most cost-effective and efficient way of treating nutrients in wastewater. Due to the limitations of conventional treatment approaches such as activated sludge, newer technologies such as the moving bed biofilm reactor (MBBR) were developed to enhance BNR. Advantages of this biofilm-based technology over conventional activated sludge systems include smaller reactor volumes, increased solids retention time, reduced hydraulic retention time (HRT), and increased robustness to extreme loading conditions (Andreottola et al., 2000). Unlike membrane-based technologies, which also offer advantages over activated sludge for treating wastewater, MBBR systems require very limited cleaning or backwash. The utility of this technology has therefore seen its application to a range of wastewaters in over 50 different countries (Odegaard et al., 1994; Rusten et al., 1995). The short HRT of MBBRs selects for fast growing organisms in the suspended fraction, to prevent being washed out of the system. In contrast the retention of adhering biomass on suspended carriers (such as AnoxKaldnes™ K1 carrier material) within the reactors allows the establishment of slow growing organisms.

MBBRs are generally operated as an aerated system, which facilitate the maintenance of carriers in suspension and enhances aerobic processes within the reactor. However, biofilms attached to these carriers also develop anoxic zones, enabling simultaneous aerobic and anaerobic processes to occur within the same reactor (Baek and Pagilla, 2008). The development of these anoxic zones is integral to the BNR process by enabling the growth of anaerobic microorganisms that are involved in sulfate reduction, denitrification, phosphorus removal and methanogenesis (Pastorelli et al., 1999; Labelle et al., 2005; Wang et al., 2006).

Organic carbon compounds can be broken down in wastewater by organisms that can use electron acceptors such as oxygen, sulfate, nitrate, or carbonate (Seviour, 2010). Majority of the organisms in conventional activated sludge systems utilize oxygen as their primary electron acceptor. However under anaerobic conditions sulfate is often used as an important terminal electron acceptor, which is reduced to sulfide and can account for up to 50% of organic mineralization in biofilms (Kühl and Jørgensen, 1992; Ito et al., 2002). Much of the research on BNR in wastewater systems has centered on nitrogen and phosphorus removal (Rusten et al., 1995; Wagner et al., 2002; Seviour and Nielsen, 2010). Nitrogen removal involves two processes: nitrification and denitrification. The key steps in nitrification involve the oxidation of ammonia to nitrite which is further converted to nitrate. Denitrification is an
anaerobic process that utilizes organic compounds as electron donors to reduce nitrite or nitrate to gaseous nitrogen compounds. High levels of phosphorus have also been observed in wastewater and it can be utilized by microorganisms for cell growth. Specific functional groups of microorganisms for each of these processes can be identified and have been shown to be affected by wastewater composition (Seviour et al., 2003; Haseborg and Frimmel, 2007). These studies showed ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria NOB) to be affected by concentration of nitrogenous ions in wastewater along with pH, temperature, and oxygen concentration levels (Haseborg and Frimmel, 2007). In a recent study examining the effects of varying nitrite concentrations on nitrifying bacteria, NOB Nitrobacter species (r-strategists) out-competed Nitrospira species (K-strategists) under high nitrite (5-10 mg/L NO₂⁻) wastewater concentrations (Haseborg et al., 2010). Nitrosospira and Nitrosomonas (AOB) levels can also be elevated under high ammonia wastewater in lab-scale MBBR systems (Bernet et al., 2004). Additional functional groups include polyphosphate-accumulating organisms (PAO) that are believed to outcompete other groups of bacteria under high phosphate conditions by using internally stored polyphosphates as energy sources. This has been well studied in enhanced biological phosphorus removal (EBPR) for wastewater systems (Seviour et al., 2003). Furthermore, under high sulfate conditions sulfate-reducing bacteria (SRB) flourish, resulting in excessive sulfide production that often leads to detrimental effects such as corrosion of treatment facilities and the emission of pungent, toxic and odorous hydrogen sulfide into the atmosphere. Oxidants such as hydrogen peroxide, nitrate, chlorine or oxygen are often used at WWTPs to reduce sulfide emissions (Zhang et al., 2008). However, these approaches have had mixed reviews and are partly dependent on the composition of the SRB community (Mitchell et al., 1986; Mohanakrishnan et al., 2011).

The composition of wastewater can be highly dynamic as, for example, excess rainfall can lead to nutrient dilution, while drought conditions may concentrate levels of nutrients entering a treatment plant. Seawater infiltration, may also be exacerbated during drought or low flow events leading to elevated salinity (Sun et al., 2010; Hu et al., 2012). Such environmental stresses can have strong impacts on WWTP community composition, nutrient removal and process functionality (Liu and Tay, 2002). Furthermore, reactors at a full-scale MBBR are often drained of any liquid and are turned off to influent during low flow seasons, but still contain carriers with adhering biomass. The duration of the dormant phase can last for up to 3 months (pers. comm. with plant operator). However, when flow into the WWTP
increases, the reactors are restarted by allowing influent to enter into the reactors. This stop-start effect on biomass composition and plant performance has not yet been investigated.

Moa Point WWTP is a large (200,000 population equivalent) facility in Wellington, New Zealand, that utilizes MBBR technology for the treatment of municipal and industrial wastewater. Reports of excess sulfide emission and corrosion at the treatment facility were the initial driver for an investigation into the composition of biofilm communities and in particular, SRB at this and another local MBBR system. The biofilm communities at both plants were dominated by putatively anaerobic *Clostridia* and sulfate-reducing members of *Deltaproteobacteria* although issues with H$_2$S production were only evident at the Moa point plant (Biswas and Turner, 2012; Biswas et al., 2013). In contrast, the suspended community was dominated by typically fast-growing aerobic members of the *Gammaproteobacteria*, *Betaproteobacteria* and *Alphaproteobacteria*. A hypothesis arising from this study was that sulfide production at the Moa Point plant was linked to excess sulfate availability arising from either seawater infiltration or sulfur-rich trade wastes that are known to enter this plant (*pers comm. with plant operator*).

The aims of this study were to (1) evaluate the effects of varying influent composition, including nutrient levels and seawater, on the key bacterial functional groups of two lab-scale MBBR systems; (2) address whether manipulation of these systems could indeed reduce SRBs within the biofilm; (3) monitor the influence of old (stored for 3 months) mixed with fresh seed material on reactor performance and bacterial community composition.

### 6.4. Material and methods

#### 6.4.1. Reactor set-up

Two sterile lab-scale bioreactors (BR1 and BR2) (Bioflo3000, New Brunswick) were filled with 1.5 L of autoclaved synthetic wastewater medium (Table 6.1a) adapted from (Yoo et al., 1999), along with K1 carriers (AnoxKaldnes™) to 30% per reactor volume. Seawater contains 2,700 mg/L of sulfate (Scott, 2003) which was speculated to be diluted by ~13.5x by wastewater at Moa Point WWTP. Thus to simulate conditions resulting from seawater infiltration at the Moa Point plant additional sulfate (to 200 mg/L) was added to each
bioreactor, after the medium was autoclaved. Reactors were operated to achieve an influent soluble chemical oxygen demand (CODs) of 226 mg/L and HRT of 24 h. The process parameters of the lab bioreactors during stabilization and experimental phases, together with those of Moa Point WWTP, are shown in Table 6.2.

Table 6.1. Composition of influent synthetic wastewater under different nutrient conditions. (A) Chemical compound (mg/L); (B) Molar amounts (mM).

(A)

<table>
<thead>
<tr>
<th>Chemical compounds</th>
<th>Standard (phase 1,3) (mg/L)</th>
<th>Low nutrient (mg/L)</th>
<th>High nutrient (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COONH₃</td>
<td>240.88</td>
<td>48.17</td>
<td>1204.40</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>43.94</td>
<td>8.79</td>
<td>175.76</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>125.00</td>
<td>125.00</td>
<td>125.00</td>
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<tr>
<td>CaCl₂</td>
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<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>0.035</td>
<td>0.035</td>
<td>0.035</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>0.035</td>
<td>0.035</td>
<td>0.035</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>50.00</td>
<td>10.00</td>
<td>250.00</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th></th>
<th>Standard (phase 1,3) (mM)</th>
<th>Low nutrient (mM)</th>
<th>High nutrient (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C*</td>
<td>8.35</td>
<td>2.86</td>
<td>35.81</td>
</tr>
<tr>
<td>NH₃-N*</td>
<td>3.82</td>
<td>0.63</td>
<td>16.72</td>
</tr>
<tr>
<td>PO₄-P</td>
<td>0.32</td>
<td>0.06</td>
<td>1.29</td>
</tr>
</tbody>
</table>

*Yeast extract has been included in the calculation and has been shown to compose of 13% carbohydrates and 5% nitrogen (Edens et al., 2002).
The lab bioreactors were initially inoculated with seed biomass adhering to K1 carriers that had been collected from a Moa Point MBBR reactor 24 hours prior. These bioreactors for the first study were run in two distinct phases: 1. stabilization; and 2. high/low nutrient (experimental). Following completion of the first set of bioreactor experiments, the bioreactors were emptied and inoculated with different seeding material that consisted of equal proportions of fresh seed material collected one day before starting the bioreactor experiment and old seed material that had been stored at 4°C for three months. This simulated the stop-start effect of a full-scale MBBR during low influent flow seasons. The second set of bioreactor experiments included three additional phases following phases 1 and 2: 3. stabilization; 4. high/low nutrient (experimental); and 5. high conductivity (experimental) (Fig. 6.1).

During phase 1 the reactors were maintained as a batch system for one week prior to starting continuous operation. A further 7 days of operation under continuous feed allowed the bioreactors to stabilize, based on stable K1 biomass weights. Samples were collected from the bioreactors and phase 2 was then introduced: the administration of high or low nutrient wastewater media. The high or low nutrient wastewater was made by increasing or decreasing the levels of carbon, nitrogen and phosphate in the medium (Table 6.1b). This simulated changing influent wastewater characteristics, such as seasonal variations, in full-
scale WWTP. BR1 was fed low nutrient and BR2 was fed high nutrient wastewater. The high/low nutrient conditions were repeated during phases 2 and 4 of this study (Fig. 6.1).

**Figure 6.1. Schematic of multi-phase bioreactor experiments using either low-nutrient (BR1) or high nutrient (BR2) wastewater feed.**

Two sets of experiments were performed. In the first set the bioreactors were seeded with fresh biomass and run over two Phases (1-2). In the second set of experiments reactors were fed with a combination of fresh and stored biomass and run over three phases (3-5). Operational conditions for each phase were as follows.

**Phase 1 (stabilisation):** reactors seeded with biomass, fed with synthetic wastewater and operated as a batch process for one week, prior to switching to continuous operation.
**Phase 2 (experiment):** reactor feed switch to either low (BR1)- or high (BR2)-nutrient composition
**Phase 3 (stabilisation):** reactor feed reverted to normal synthetic wastewater
**Phase 4 (experiment):** reactor feed switch to either low (BR1)- or high (BR2)-nutrient composition
**Phase 5 (experiment):** reactor feed amended with artificial seawater to increase conductivity in both low (BR1)- and high (BR2)-nutrient reactors.

Each experimental phase was maintained for two weeks prior to sample collection to obtain stable biofilm establishment on K1 carriers and steady community profiles – this was demonstrated by stable biomass levels for K1 carriers. The final phase (phase 5) of the study involved the increase of conductivity of both bioreactors to 1 S/m while still operating under high/low nutrient conditions respectively. The conductivity of seawater is 5 S/m (Chave and
Cox, 1982) and is composed of many electrolytes, which can be measured by their ability to conduct electricity. In this study artificial seawater composed of NaCl (23.9 g/L), KCl (0.7 g/L), NaHCO\(_3\) (0.2 g/L), and 42 mL/L of 1M MgCl\(_2\).6H\(_2\)O and 1M CaCl\(_2\).2H\(_2\)O was used to elevate conductivity within the two bioreactors. Sulfate compounds were not included in artificial seawater as additional sulfate (200 mg/L) was already being administrated in the reactors of this study. Conductivity of the feed was measured using a YSI-Pro30 probe.

### 6.4.2. Sampling

Samples were collected throughout the study to determine reactor performance in terms of nutrient transformation and to investigate bacterial community composition. At the end of each experimental phase the steady state conditions were attained and confirmed by stable dry weight K1 carrier measurements and stable suspended mixed liquor biomass. Samples were collected from the influent and effluent of the bioreactors to quantify nitrate, nitrite, sulfate and phosphate concentrations. These samples were filter sterilized through a sterile 0.20 µm filter and stored at -20°C until analysed by ion chromatography. In addition, biomass from carriers and suspended fraction was collected for dry weight measurements and microbiological analyses. The suspended fraction samples (1 mL) were centrifuged for 5 min at 13,000 rpm and the supernatant discarded. The acquired biomass pellets, along with the collected carriers, were stored in RNALater at -20°C until used in DNA extractions.

### 6.4.3. Analytical methods

#### 6.4.3.1. Ammonium determination

The Phenate method (Clesceri et al., 1998) was used to determine NH\(_4^+\) concentration in the influent and effluent of the bioreactors at the end of each phase. In brief, each assay was performed on 2 mL of sample (diluted 1:20 in water) with the addition of 80 µL of phenol solution, 80 µL of sodium nitroprusside solution and 200 µL of oxidizing solution. Calibration standards were prepared using anhydrous NH\(_4\)Cl dissolved in deionised water to achieve NH\(_4^+\)-N concentrations of 0.1, 1.0, 2.5, 5.0 and 7.5 mg/L. Absorbance of the samples was measured at 640 nm on a spectrophotometer.
6.4.3.2. **Ion chromatography**

Ion chromatography (Dionex DX-100) was used to determine the concentrations of NO$_2^-$, NO$_3^-$, SO$_4^{2-}$ and PO$_4^{3-}$ in samples. Sample blanks comprised of deionised water, and a single mixed calibration standard comprising of 5 ppm of NO$_2^-$, NO$_3^-$, SO$_4^{2-}$, and 10 ppm of PO$_4^{3-}$, was used. Filtered influent and effluent samples from the bioreactors were diluted 1:5 before analysis. Chromeleon software (Dionex) was used to calculate concentrations of ions present in the samples.

6.4.3.3. **Dissolved sulfide**

Total dissolved sulfide present in the suspended fraction of the bioreactors at the end of each experimental phase was measured using colorimetric methods as described previously (Cord-Ruwisch, 1985).

6.4.3.4. **Dry weight**

The dry weight of the biomass attached to the K1 carriers and the suspended fraction of the reactors was quantified separately at the end of each experimental phase. Excess liquid from the collected carriers was absorbed by tissue paper and subjected to further drying within a desiccator for 3 d at room temperature. Dry weight measurements were made for three carriers per time point. Uninoculated carriers subjected to the same drying process were used as blanks. Previously weighed 1.5 mL Eppendorf tubes were filled with 1 mL of suspended fraction and subjected to drying at 65°C for 3 days before being weighed. All biomass measurements were carried out in triplicate with the initial weight of the Eppendorf subtracted from the final weight.

6.4.4. **DNA extraction**

Bioreactor samples consisting of biofilm scraped off carriers and pelleted biomass of the suspended fraction, were subjected to DNA extraction. Total genomic DNA was extracted using a chloroform-based bead-beating method as described previously (Smith et al., 2003). A modification of the method was made by reducing the bead-beating step to 20 s at 4 m/s, to avoid shearing of genomic DNA. The resulting extracted DNA was dissolved in 30 µL of nuclease-free water and stored at -20°C.
6.4.5. Bacterial analysis using 16S rRNA gene pyrosequencing

Genomic DNA extracted from three biological replicates for biofilm and suspended fraction samples at the end of the three experimental phases was used for bacterial community analysis by 16S amplicon pyrosequencing. The method for amplification of the 16S rRNA genes of these samples using primers 347f and 803r (Nossa et al., 2010). The forward primer consisted of the template-specific primer; a unique multiplex identifier (MID); a 4-nucleotide key and adaptor A. The reverse primer consisted of the template-specific primer, a 4-nucleotide key and adaptor B. A set of 24 unique MIDs were used in this study, enabling 24 samples to be multiplexed during one pyrosequencing run. Lib-L chemistry (Roche/ 454 Life Science) was used to enable unidirectional sequencing from the adaptor A (forward) end. A standardized quantity of genomic DNA (~5-10 ng/µL) was added to a PCR mixture consisting of equimolar concentrations (0.2 mM) of fusion primers (forward and reverse), dNTPs (0.2 mM), PCR Buffer (1X), MgSO\(_4\) (2 mM), 0.5 U Platinum Taq High Fidelity (Invitrogen) and PCR-certified water to a final volume of 25 µL. Amplification was performed in an Applied Biosystems Mastercycle gradient PCR machine with an initial denaturing step at 94°C for 1 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 68°C for 30 s. A final elongation step at 68°C for 3 min was performed. PCR-amplified products were visualized for quality on a 1% agarose gel before purification using AMPure Beads (Roche). The purified PCR products were quantified using Picogreen (Quant-iT dsDNA kit, Invitrogen) and qualitatively checked on Agilent 1200 Bioanalyzer DNA 1000 chips (Agilent Technologies, Santa Clara, USA). Equal concentrations of samples were pooled into a library and sequenced on a Roche GS FLX Titanium platform by Macrogen Inc. (Seoul, South Korea).

The results of the pyrosequencing were analysed as described previously (Simister et al., 2012; Biswas et al., 2013) using Mothur (Schloss et al., 2009) and custom-made PERL scripts. Low quality sequences, chimeras (UCHIME (Edgar et al., 2011)) and contaminants were removed from the library of sequences. The remaining high quality sequences were aligned against a SILVA reference database (http://www.mothur.org/wiki/Silva_reference_alignment) and assigned to operational taxonomic units (OTUs) at 97% similarity based on uncorrected pairwise distance matrix. Sequences were assigned taxonomically using custom-made PERL scripts along with a manually curated SILVA database (based on version 108). The 10 most abundant genera for each sample were identified and visualized on a heat map as an percentage abundance of the
total bacterial community using JColorGrid (Joachimiak et al., 2006). Sequences of known AOBs, NOBs, SRBs, and possible PAOs were identified within each sample and grouped together. Unweighted UniFrac (phylogeny-based) was performed to assess significant differences between samples. Values from the Principal Co-ordinate Analysis (PCoA) performed in Mothur using UniFrac distances were plotted in Excel.

### 6.4.6. Quantifying dsrA gene and bacterial 16S rRNA gene using real-time PCR

Total number of bacterial 16S rRNA gene copies in samples were quantified using primers 533F (5’-GTGCCAGCAGCYGCGGTMA-3’) and 803R (5’-CTACCRGGGTATCTAATCC-3’). The percentage coverage of these primers with no mismatches, using Probe Match in RDP database, were 70.7% (533F) and 86.5% (803R). These primers have previously been used as part of other primer sets for amplicon pyrosequencing (Nossa et al., 2010; Simister et al., 2012). Sulfate-reducing microorganisms were quantified using previously described primers DSR1-F+ and DSR-R (Kondo et al., 2004) targeting the dsrA gene. Standards were prepared from genomic DNA from a wastewater biofilm sample collected at Moa Point. DNA was amplified with gene-specific primers for dsrAB gene (DSR1Fmix and DSR 4Rmix (Wagner et al., 2005)) and 16S rRNA gene (PB36 and PB38 (Saul et al., 2005)). These PCR products were cloned into pGEM T-easy vector (Promega) and further transformed into chemically component DH5α Escherichia coli cells (Invitrogen), as per manufacturer’s instructions. Remaining protocol of clone library construction was carried out as stated previously by Sambrook and Russell (2001). Vectors containing inserts were recovered from E. coli cells by using Plasmid Mini Prep kit (Qiagen) and linearised using Sca I restriction enzyme (Invitrogen). Lineraised products were quantified using PicoGreen double-stranded DNA kit (Molecular Probes) and converted to copy numbers of target gene per microliter (assuming average weight of a DNA base pair as 650 Da, size of 16S rRNA gene as 1501 bp (Saul et al., 2005), dsrAB gene as 1942 bp (Wagner et al., 1998) and pGEM T-easy vector as 3015 bp). A ten-fold dilution series for each gene of interest was prepared (10⁶ to 10¹ target gene per reaction). Real-time PCR was carried out on an ABI Prism 7900HT cycler (Applied Biosystems). Standard curves were plotted for the 16S rRNA and dsrA genes (Fig. S6.3).

The reaction mix consisted of 5 µl of 1x POWER SYBR Green master mix (Applied Biosystems), 0.5 µl MgCl₂ (50 mM), 0.5 µl of each primer (1 µM), 1 µl of cDNA template or
prepared standard and PCR grade water to a final volume of 10 µl. Thermal cycling conditions included an initial incubation at 50°C for 2 min and denaturation step for 10 min at 95°C. This was followed by 40 cycles of denaturation (95°C for 15 s), annealing (59°C for 1 min), and elongation (72°C for 10 s). Non-template control and dilution series of standards were run in triplicate. Three biological replicates of gDNA of bioreactor samples and two technical replicates were used to account for any sample variability. Results were analysed using the ABI Prism 7900HT sequence detection system (Version 2.4). It was assumed that 16S rRNA genes are present at an average of 3.6 copies per cell (Klappenbach et al., 2001) and that dsrA genes are present in only a single copy (Klein et al., 2001). This was accounted for in calculations of % abundance of SRB within samples.

Amplification efficiency was calculated based on the respective standard curve using the formula: $E = 10^{(-1/\text{slope})} - 1 \times 100\%$ (Fig. S6.3). Melting curve analysis was also performed to verify the specificity of the primer pairs, by using software ‘dissociation curve’ (Applied Biosystems).
6.5. Results and Discussion

6.5.1. Biomass characteristics in the bioreactors

During all five experimental phases of the study, the majority of the biomass was retained on the inside surface of the suspended carriers within the two bioreactors. In contrast, carriers within the full-scale MBBR system typically retained biofilm on both the inner and exterior surfaces. It is possible that the constant collision between carriers within the lab-scale bioreactor prevented the development of a biofilm on the outer surfaces within the timeframe of the study. Samples were collected from the bioreactors once stable conditions were achieved as demonstrated by stable biomass on K1 carriers and in reactor supernatant mixed liquor. The bioreactor suspended fraction remained clear of planktonic growth for all control and low nutrient phases of this study, as observed visually. In contrast, high nutrient fed bioreactors resulted in the development of a brownish-yellow biomass in the suspended fraction. The dry weight of the biomass in the suspended fraction of BR1 and BR2 measured below 0.0001 g/mL for phases 1 to 4. However, during phase 5, when artificial seawater was administered into the bioreactors, the weight of the suspended biomass increased to 0.005 ± SD 0.0001 g/mL (Table 6.3).

Table 6.3. Dry weight measurements (g) of biomass on carriers and suspended fraction within the bioreactors over the 5 phases of this study.

<table>
<thead>
<tr>
<th>Phase</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control-a</td>
<td>LN1</td>
<td>Control-b</td>
<td>LN2</td>
<td>LN+HC</td>
</tr>
<tr>
<td>BR1</td>
<td>Biomass</td>
<td>0.004 ± 0.008</td>
<td>0.012 ± 0.006</td>
<td>0 ± 0.010</td>
<td>0.006 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>Suspended solids</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>BR2</td>
<td>Control-a</td>
<td>HN1</td>
<td>Control-b</td>
<td>HN2</td>
</tr>
<tr>
<td></td>
<td>Biomass</td>
<td>0.014 ± 0.016</td>
<td>0.015 ± 0.014</td>
<td>0.011 ± 0</td>
<td>0.018 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>Suspended solids</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

± standard deviation. LN: low nutrient; HN: high nutrient; HC: high conductivity.
Amplifiable DNA from the suspended fraction was only obtained for phases 4 and 5 of BR2. This data suggests that the high nutrient conditions within BR2 enhanced the growth of planktonic bacteria in suspension. Bacterial community analysis revealed three dominant phyla belonging to *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, in all samples. Classes belonging to these phyla were present across all samples, although variations were observed in the number of sequences within each class (Fig. 6.2).

Figure 6.2. Bar graphs representing the bacterial community at class-level (of the 3 most dominant phyla) over five experimental phases in two bioreactors.

The bacterial community structure in biofilms of low nutrient (LN) and high nutrient (HN) fed bioreactors along with high conductivity (HC) are shown. Biomass from the suspended fraction of the BR2 (high nutrient) from phase 4 and phase 5 was also analyzed. Standard concentrations of synthetic wastewater was fed into phase 1 (inoculated with fresh seeding material) and phase 3 (inoculated with mixture of fresh and stored seeding material) of this study. * indicates that the same bacterial community data is shown for BR1 and BR2. Replicates of 3 for each time point were analysed and are shown in the figure. Values are presented as % of total bacterial community for a given sample.
Biofilm communities were dominated by *Alphaproteobacteria* (9.7-36.4% of the total bacterial community), *Betaproteobacteria* (6.8-35.1%), *Gammaproteobacteria* (4.5-25.5%), *Deltaproteobacteria* (1.7-26.1%) and *Sphingobacteria* (7.3-31.6%). At class level, the community structure of the bioreactor samples closely resembled that of Moa Point WWTP (Biswas and Turner, 2012) and other lab-scale bioreactor studies treating wastewater (Haseborg et al., 2010; Calderón et al., 2012). In contrast, the suspended fraction of the high nutrient feed (phase 4) was dominated by *Betaproteobacteria* (67-72%), and *Sphingobacteria* (17-20%) to a lesser extent. During phase 5, when artificial seawater was administered into the bioreactors, the composition of the suspended community changed to become dominated by *Gammaproteobacteria* (70-75%) and *Betaproteobacteria* (15-19%). Examination of the sequence data at finer taxonomic resolution indentified *Comamonas* (66% of the total bacterial community) and *Acinetobacter* (71%) as the dominant genera in phase 4 and phase 5 respectively, of the suspended fraction of BR2 (Fig. S6.1). Elevated conductivity within the reactor resulted in an increased dominance of *Acinetobacter*, indicating that this organism was best adapted to the higher salt conditions. Similarities can be drawn between the suspended fraction of the bioreactor samples and Moa Point WWTP as a previous study showed this full-scale MBBR was also dominated by fast-growing aerobic members of *Beta-* and *Gammaproteobacteria* (Biswas and Turner, 2012).

To assess variation among bacterial communities in different samples a principal co-ordinate plot using UniFrac distance values (phylogeny-based) was carried out (Fig. S6.2). Unweighted-UniFrac and PCoA showed that the bacterial communities from the suspended fraction samples were significantly (p<0.001) different to those derived from biofilm samples. Similar observations have been made previously at Moa Point WWTP, where the bacterial community composition was unique to the biomass stage (planktonic and biofilm) within the MBBR (Biswas and Turner, 2012). Resemblances were also observed between the bacterial community compositions of lab bioreactors in this study to that of full-scale MBBR plants that treat municipal wastewater (Biswas and Turner, 2012). Thus the impact of the experimental phases on nutrient removal efficiency and bacterial community structure could be indicative of the effects expected at full-scale MBBR WWTP under similar environmental stresses. In this study bioreactor performance was assessed by the transformation of ammonia, sulfate and phosphate by key microbial groups. For the remainder of the article the effect of varying influent composition under high sulfate levels on key microbial groups that include AOBs, NOBs and a possible PAO will be discussed. The abundance, composition
and diversity of SRBs in all 5 phases of this study will also be reported, as this could provide insights into strategies that could be implemented to reduce sulfide emissions at a WWTP.

6.5.2. Varying influent composition affects key microbial groups and nutrient removal efficiency of bioreactors (phases 1 and 2)

6.5.2.1. AOBs/NOBs

Greater than 94% ammonia removal was achieved in both bioreactors when fed with standard synthetic wastewater medium (Phase 1: Fig. 6.3). The influent ammonia was converted to nitrate (52-70 ppm) and nitrite (27-33 ppm). AOBs, responsible for the first step of the nitrification process, represented 0.65 ± SD 0.19% of the total bacterial community within the biofilm, based on relative abundances of AOB-affiliated 16S rRNA gene pyrosequencing reads. However, known NOBs were not detected within these samples. It is possible that they were present in the suspended bacterial fraction of the bioreactor and were not established as components of the biofilm community on the carriers within the timeframes of the reactor experiments. Unfortunately, the suspended biomass samples yielded insufficient amplifiable DNA for pyrosequencing analysis.

High levels of ammonia removal (100% and 85%, respectively) were also achieved in Phase 2 of the experiment, where BR1 was given a low nutrient feed and BR2 was given a high nutrient feed. However, in BR1 the majority of the ammonia was converted to nitrate, whereas in BR2 nitrite was accumulated (>250 ppm). This difference was reflected within the bacterial community structure. 16S rRNA gene analysis of the bacterial community composition of BR1 indicated the presence of AOBs (0.64 ± SD 0.30% of total bacterial community) and NOBs (0.09 ± SD 0.16%), which facilitate the conversion of ammonia to nitrate with nitrite as an intermediate (Fig. 6.4). In contrast, BR2 had higher levels of AOBs (1.91 ± SD 1.06%) and lower levels of NOBs (0.02 ± SD 0.03%) compared to BR1. Due to the higher amounts of ammonia entering into this reactor (16.72 mM compared with 0.63 mM in BR1) it was not surprising to detect higher levels of nitrite and nitrate within its effluent. These results concur with those of a previous study using lab-scale MBBRs that showed high ammonia levels resulted in increased AOB abundance (Haseborg et al., 2010).
Figure 6.3. Nutrient removal efficiency of biomass and concentration of \( \text{NH}_4^+ \), \( \text{NO}_2^- \) and \( \text{NO}_3^- \) in all five phases of the two bioreactors (BR1 and BR2) is shown.

BR1 was fed low nutrient and BR2 was fed high nutrient synthetic wastewater. The exception was phases 1 and 3, which were fed the standard synthetic wastewater concentrations. Figures 6.3A and 6.3B display \( \text{NH}_4^+ \), \( \text{NO}_2^- \) and \( \text{NO}_3^- \) concentrations (ppm) in the inlet/outlet of the reactors whereas the tables display % nutrient removal efficiency. Samples were collected at the end of a phase following and steady-state conditions.

High levels of ammonia have also been known to have inhibitory effects on the growth of *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) (Wong-Chong and Loehr, 1978; Ford et al., 1980). Furthermore, the build of inhibitory concentrations of nitrous acid within a reactor due to the improper conversion of nitrite to nitrate can inhibit the growth of NOBs, as observed in BR2 with nitrite measurements of 278 mg/L. Levels of 0.22 to 2.8 mg/L of nitrous acid are known to inhibit the nitrification process (Randall and Buth, 1984). Nitrite toxicity has been observed previously in *Pseudomonas* sp. isolated from soil under anaerobic conditions with levels over 200 mg/L (Bollag and Henninger, 1978).
AOB numbers compared with control samples remain unchanged with lower nutrient feed but has a 2.5 fold increase with the higher nutrient feed. In contrast, NOBs develop in biofilms of both BR1 and BR2 in phase 2 compared with negligible levels in control reactors (Phase 1). These results suggest that this process is highly dynamic and is influenced by the wastewater composition and transformation by microbes (i.e. AOB and NOB members) within the biofilm community.

6.5.2.2. Sulfate reducing bacteria

Addition of 200 mg/L of sulfate to the feed in phase 1 (control) resulted in less than 3% sulfate reduction and negligible amounts of total dissolved sulfide measurements in the suspended fraction of the reactors. A change to low level nutrient feed into BR1 (phase 2), resulted in increased sulfate removal (13.1%), indicating increased activity of SRBs. In contrast, BR2 fed with high nutrients showed no sulfate removal. Sulfate reduction is one of the major processes involved in carbon cycling in anoxic environments (Muyzer and Stams, 2008). Even though sulfate reduction was increased in BR1 it is possible that the growth of SRBs was limited due to insufficient available carbon to drive anaerobic respiration or lack of anoxic niches developing within the biofilm of this aerated reactor. The bacterial 16S rRNA gene analysis of the biofilms showed a wide range of SRBs present (Fig. 6.4) across all phases of this study. Of the total bacterial community, phase 1 (control) samples contained 12.7% SRBs while low nutrient (BR1) and high nutrient (BR2) samples were composed of 9.7% and 2.5% SRBs, respectively. This result was unexpected but we can speculate that the high numbers of SRB in phase 1 were remnants from the initial seed biomass from Moa Point WWTP, which has been shown to comprise large numbers (35% of the bacterial community) of putative SRBs (Biswas and Turner, 2012).

The control samples (phase 1) consisted of a diverse group of SRBs including Desulfosarcina (1.8% of the bacterial community), Desulforhabdus (1.3%), Desulfococcus (1.02%), and other members of Desulfobacteraceae (~5%). Desulfococcus was the most abundant SRB (2.1% of the total bacterial community) found in low nutrient samples BR1-phase2, whereas Desulfosarcina was the most abundant (0.5%) in high nutrient samples BR2-phase2. These organisms are strictly anaerobic and nutritionally versatile. Consortia of Desulfosarcina and Desulfococcus with archaea (Methanosarcinales) have been identified previously in anoxic
marine sediments (Orphan et al., 2001). It is possible that such syntrophic interactions are also occurring within the bioreactors in this study, as *Methanosarcinales* have identified previously in biofilms of MBBRs at Moa Point (Biswas and Turner, 2012).

Figure 6.4. Known SRBs, AOBs, NOBs and PAO were identified within the biofilm samples of all five phases of this study and grouped together.

Members of these groups are shown in the figure. BR1 was fed low nutrient and BR2 was fed high nutrient synthetic wastewater. The exception was phases 1 and 3, which were fed the standard synthetic wastewater concentrations. Error bars indicate standard deviation. Values are presented as % of total bacterial community for a given sample.
SRBs were quantified within samples by targeting the alpha subunit of the functional gene (*dsrA*) that codes for the enzyme dissimilatory (bi)sulfite reductase (Fig. 6.5). An increase in SRB abundance was measured within biofilm samples from 0.4% for control (phase 1) to 3% in low nutrient feed BR1 (phase 2) samples. However, negligible amounts (<0.001%) of SRB were recorded for high nutrient feed BR2 (phase 2). Discrepancies between amplicon pyrosequencing and real-time PCR results could be due to different target genes or PCR-biases encountered during amplification steps of pyrosequencing.

These results suggest that under limited carbon and nitrate availability, sulfate is utilized as an electron acceptor within these systems. This has previously been observed in biofilms of wastewater under microaerophilic conditions (Ito et al., 2002). The increase in sulfate removal and abundance of *dsrA* gene in low nutrient feed bioreactor compared with control and high nutrient feed bioreactor (BR2-phase 2) suggest that manipulation of wastewater composition even under high sulfate levels can lead to alterations of SRB communities.

### 6.5.2.3. Phosphate accumulating organisms

*Accumulibacter*, formally known as “*Candidatus Accumulibacter Phosphatis*”, is now recognized as the primary polyphosphate accumulating organism (PAO) in wastewater (Seviour et al., 2003). For many years culture-based studies implicated *Acinetobacter* in this role (Deinema et al., 1980; Streichan et al., 1990). Since the development of molecular techniques studies have shown *Acinetobacter* are present within EBPR but play a minor role (Bond et al., 1998; Seviour et al., 2003). Specific WWTP process configurations and operating conditions are required to drive EBPR by supporting the growth of PAOs within WWTPs (Blackall et al., 2002). Phosphate is taken up by PAOs in aerobic conditions and then released during anaerobic phases. Up to 84% of phosphorus removal was achieved in a sequence batch MBBR under oxic-anoxic phases with 12.4 mg/L of total phosphorus in the influent. (Helness and Odegaard, 2001; Yang et al., 2010). As oxic-anoxic phases were not operated within the bioreactors of this study, it was not surprising that the primary PAO – *Accumulibacter*, was not detected in any of the samples. Under high nutrient conditions (BR2-phase 2) phosphate removal efficiency dropped from 22.4% in the control (phase 1) to 4.2%. In contrast, the low nutrient bioreactor (BR1) measured no phosphate removal. *Acinetobacter* were detected in control samples but reduced from 4.4% in the control (phase
1) to 0% in BR1 (phase 2) and to 1.3% in BR2 (phase 2). These results indicate that the conditions within the bioreactors of this study are not suitable for the growth of PAOs, therefore it is difficult to comment on whether wastewater composition had an effect on this bacterial group.

### 6.5.3. Seed microbial biomass is important to the functioning of lab-scale moving bed biofilm reactors (phases 3 and 4)

Seeding material plays an important role in establishing biofilm or granular sludge reactors (Liu and Tay, 2002) or for the selection of specific organisms such as anaerobic ammonia oxidizing bacteria (anammox) (Tao et al., 2013). During low flow seasons at a WWTP, MBBR tanks are often turned off to influent and remain dormant. These tanks can be restarted 3 months later when flow increases. In this study the influence of dormant seed material on nutrient removal rates was assessed. The two lab-scale bioreactors (BR1 and BR2) were started with new seed material in phase 3. The seed material contained a mixture (1:1) of fresh biomass collected the previous day from Moa Point WWTP in addition to stored biomass that had been collected 3 months prior from the same WWTP. This combination of fresh and dormant biomass was chosen in this study to account for new biomass entering into a full-scale MBBR through the influent after a period of dormancy. Operational conditions and synthetic wastewater for the start-up of the bioreactors (phase 3) was as described for phase 1. Samples were collected for analysis once steady state conditions were reached. The dry weight of biomass on the carriers increased in both reactors from phase 3 to 4 (Table 6.3), indicating biofilm growth. Ammonia removal efficiency in phase 3 (38.4% and 7.8% for BR1 and BR2, respectively) reduced drastically when compared with phase 1 (Fig. 6.3). Nitrate levels were low (<2 ppm) in effluents of both reactors of phase 3 compared with phase 1 (52-69 ppm). These results suggest that nitrification processes were inhibited in phase 3. An alternative explanation could be that nitrate is being efficiently removed by denitrifiers such as Paracoccus or Bradyrhizobium. However, these organisms were detected in less than 0.1% of the sequences of total bacteria in phase 3 samples; therefore it is unlikely to contribute to major nitrate depletion.

Known SRB sequences were identified in 4.7% of the total bacterial community in BR1 and 10.8% in BR2 of phase 3, yet no sulfate removal was detected. Comparing these results to phase 1 it became evident, that even though similar numbers of SRB were detected in
biofilms of these two control phases, the biomass in phase 1 appeared to be more active in sulfate reduction. Real-time PCR was used to quantify SRB communities in samples and revealed greater abundance in phase 1 compared with phase 3 (Fig. 6.5). However, these values were both below 0.5% of total bacterial community, suggesting that SRB have a minor role in the bioreactors of phases 1 and 3 of this study even though 200 mg/L of sulfate was administered. In contrast, phosphate removal efficiency increased in phase 3 in comparison to phase 1. We speculate that the seed material subjected to 3 months dormancy enhances the survival of phosphate utilizing organisms and results in higher phosphate removal efficiency during phase 3.

![Figure 6.5. Quantitative real-time PCR targeting dsrA gene within biofilms of two lab-scale bioreactors (BR1 and BR2) over 5 phases of this study.](image)

The abundance of *dsrA* genes relative to total bacterial numbers (as measured by bacterial 16S rRNA gene abundance) was estimated for the samples and displayed as % with ± standard deviation. Numbers in brackets indicate the phase of the bioreactor. *LN-low nutrient and HN-high nutrient.*
Phase 4 investigated the effects of changes in wastewater composition on nutrient removal efficiency following introduction of new seed material in phase 3, under high and low nutrient conditions. Low nutrient feed (BR1) caused a strong increase in ammonia removal from 38.4% to 95.2%. A 30.5% increase was also observed in BR2 with the high nutrient feed. The influent ammonia was converted to nitrite (BR1: 26 ppm and BR2: 27.5 ppm) but no nitrate was detected. In addition, AOBs were present at <0.2% of the total bacterial community (Fig. 6.4) while NOBs were not detected in either bioreactor. It is highly plausible that the lack of NOBs in the biofilm or suspended fraction could be due to the dilution effects of fresh with dormant biomass and the timeframes of the bioreactor experiment excluded establishment of these nitrifiers within the biomass. Manipulations to influent composition in phase 2 led to the increase of NOBs and nitrate detection, whereas the same manipulations in phase 4 under the same conditions did not provide similar outcomes, thus reiterating the importance of seed material.

Sulfate removal was not recorded in phase 4 of the experiment, therefore it was not surprising to find that the number of SRB sequences, identified by 16S rRNA gene pyrosequencing, within the total bacterial community had reduced dramatically (BR1: 2.5% and BR2: 0.8%) from phase 3 (Fig. 6.4). In addition, SRB abundance in phase 4, as recorded by real-time PCR, constituted a minor proportion of the total bacterial community for low nutrient feed BR1 (0.01%) and high nutrient feed BR2 (<0.001%). The higher numbers of SRB in low nutrient feed is in concordance with phase 2 of this study. Even though biomass weight which is a proxy for biofilm thickness increased during phase 4, it can be speculated that anoxic niches within the biofilm were limited which resulted in inhibition of anaerobic organisms such as SRBs. It is highly possible that SRBs could have been washed out early on in phase 3, as levels of dissolved oxygen (2.25 mg/L) within the reactors were unfavorable to their growth. Another possibility is that due the slow growth of SRBs (Postgate, 1979), they were unable to establish within the biofilm in the timeframe of the experiment. In contrast, possible PAOs increased in phase 4 (1.75% of the total bacterial community) from the previous phase (0.76%) in BR1. BR2 recorded a slight decrease (0.76% to 0.45%) in putative PAO. The efficiency of phosphate removal was supported by the increase or decrease of putative PAO within the bacterial community of both reactors.

The overall results indicate that fresh seeding material inoculated into phase 1 of this study was more efficient at nutrient removal compared to seeding material composed of stored and fresh biomass that was used in phase 3. It is possible that during the dormant stage, many of
the key organisms become unviable and are unable to recover when the reactors are restarted. The viability of these organisms could be tested in future studies. Phosphate removal was the only exception, with higher removal observed in phase 3 than phase 1. This study indicates that addition of new biomass can influence the microbial community structure with potential downstream impacts on reactor performance. Even under altered nutrient conditions the bacterial biomass of the newly seeded bioreactor was unable to perform at the same nutrient removal rates as phases 1 and 2. Thus the results indicate that the selection of seeding material is critical for starting up a WWTP MBBR system. This may impact on nutrient removal efficiencies and timeframes associated with these.

6.5.4. Seawater infiltration leads to changes in nutrient removal efficiency (phase 5)

The effects of salts on aerobic and anaerobic processes in wastewater treatment facilities have been reported previously (Hamoda and Al-Attar, 1995; Uygur and Kargi, 2004; Sun et al., 2010). Infiltration of seawater into treatment facilities results in increased sulfate levels and increased conductivity. As sulfate levels of the influent feed were administrated at 200 mg/L in all phases of this study, artificial seawater was added into the feed of reactors BR1 and BR2 in phase 5 to simulate conductivity levels that were estimated to be found in WWTPs that experience seawater infiltration in order to investigate the effects on microbial biomass and the resulting nutrient removal efficiency. The dry weight carrier biomass in both reactors decreased but suspended biomass increased (Table 6.3). Similar results were observed by Hamoda and Al-Attar (1995), where suspended solids increased as salt concentrations increased in a lab-scale reactor that was fed with synthetic wastewater. Nutrient removal efficiency behaved very differently in both reactors (Fig. 6.3). BR1 containing low nutrient feed had optimal ammonia removal rates (100%) but removal of sulfate or phosphate was negligible (0%). It can be speculated that under low nutrient conditions with high conductivity, nitrification was the primary nutrient removal process. A previous study in sequencing batch reactors has also shown that the removal of phosphate reduces (84 to 22%) when salt concentration increases from 0 to 6% (Uygur and Kargi, 2004). In addition, nitrate in BR1 of phase 5 (16.3 ppm) was elevated compared with phase 4 (0.9 ppm), suggesting that the organisms responsible for nitrification (i.e. AOBs and NOBs) were active, even though they represent less than 0.22% of the total bacterial community. SRB abundance were below detection limit (<0.001% of total bacteria) for BR1 and BR2 samples (Fig. 6.5). As SRBs
were detected previously in BR1 of phase 3 and 4, this result suggests that seawater infiltration effects the growth of SRBs in low nutrient influent.

The effluent of BR2 with high nutrient feed recorded less than 10% ammonia, 2.7% sulfate and no phosphate removal. Nitrite (78.5 ppm) was recorded in high levels within the effluent but no nitrate was detected. A possible explanation for the failure to detect nitrate could be due to the efficient removal of this compound by denitrifiers. *Paracoccus*, a known denitrifer, was detected in <0.2% of the total bacterial community in BR2 samples and <0.05% in BR1. However, this explanation is unlikely due to low numbers of denitrifiers identified and the low conversion rates of ammonia within this reactor (BR2). These results indicate that high conductivity in combination with high nutrients inhibits nitrification, in particular NOB activity, which is responsible for the conversion of nitrite to nitrate. This is yet another example of possible toxicity to the microorganisms (NOB) due to high ammonia concentrations and improper conversion of nitrite to nitrate within the bioreactor (Randall and Buth, 1984). The results of the current study on ammonia and phosphate removal under high salt concentrations concur with those of other studies conducted on bench-scale reactors operated as activated sludge systems under high salt concentrations (Ludzack and Noran, 1965; Panswad and Anan, 1999).

**6.6. Conclusions**

Lab-scale MBBR systems under high sulfate conditions were used successfully in this study to monitor shifts in key bacterial groups and assess nutrient removal efficiency under changes to wastewater composition that simulated environmental conditions in real WWTP. High nutrient and high/low nutrient in combination with seawater feeds, resulted in reduced SRB growth compared with control samples. During low nutrient feed, carbon was limited and readily available sulfate was used as an electron acceptor, leading to elevated levels of SRBs. This study also showed that fresh seed material should be used for optimal nutrient removal efficiency. The results from this study indicate that SRB communities can be altered by changing wastewater composition even under high sulfate conditions. Future studies could aim to investigate the effects of dissolved oxygen or the use of different carrier types to achieve reduced growth of SRB communities within biofilms of MBBRs.
6.7. Acknowledgements

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6.8. References


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### 6.9. Supplementary material

Figure S6.1. Heat map of the top 10 genera of biofilm samples from all five phases of two bioreactors (BR1 and BR2) along with the suspended fraction phases 4 and 5 of BR2.

BR1 was fed low nutrient and BR2 was fed high nutrient synthetic wastewater. The exception was phases 1 and 3, which were fed the standard synthetic wastewater concentrations. Values are presented as % of total bacterial community for a given sample.
Figure S6.2. Principal Co-ordinates Analysis (PCoA) of unweighted UniFrac distances (phylogeny-based) for all samples.

Red circles indicate control biofilm samples. Yellow and blue represents samples from low and high nutrient fed bioreactors, respectively. Biomass of the suspended fraction of phases 4 and 5 of the high nutrient fed bioreactor is also shown in the figure with the same colour coding. Symbols represent the different experimental phases of this study.
Figure S6.3. Standard curves for target amplicons of (A) 16S rRNA and (B) dsrA genes.

Values plotted represent mean ± standard deviation of three replicates. The amplification efficiency (E) by real-time PCR of standard curve samples is also shown on the figure.
7. GENERAL DISCUSSION

The use of MBBR technology for the treatment of assorted wastewaters has risen from 600 plants worldwide in 2006 (McQuarrie and Boltz, 2011) to ~1200 in 2012 (Pritchard pers. comm.). This technology offers many process and cost advantages over conventional wastewater treatment and, as a result, has led to extensive research into the nutrient removal efficiency of these plants (Helness and Odegaard, 1999; Rusten et al., 2006). However, the investigation of the microbial communities and processes within MBBR systems has lagged behind that of activated sludge systems. A greater understanding of the microbial ecology of the MBBR system will underpin its optimisation and further development for a wider range of applications.

7.1. Microbial communities in MBBRs

Prior to this study, there was little information available on the composition of microbial communities in MBBR systems and how they resemble or differ from those found in conventional activated sludge. This study has addressed a number of fundamental questions relating to this and the function of MBBRs. The findings and their relevance to the operation and development of MBBR systems are discussed below.

7.1.1. What is the microbial community composition in MBBR systems and does it differ from that of conventional activated sludge systems?

The first objective of this study was to investigate and compare the resident microbial communities of the biofilm attached to carriers, and the suspended material in full-scale MBBR reactors. It was hypothesised that the communities would differ between these two distinct niches. This is consistent with the findings of a previous study of an integrated (bio)film activated sludge (IFAS) system (Kwon et al., 2010), which showed differences in the microbial community composition between the suspended fraction and fixed biofilm of the reactor.
Using a community fingerprinting method (ARISA) in this study, it was shown that there were clear differences in the microbial community structure between these two MBBR niches, and also between Moa Point and Karori treatment plants. These differences were further investigated by bacterial and archaeal 16S rRNA gene clone library analysis, to provide the first detailed description of MBBR microbial communities. As speculated the bacterial community structure of these two niches within the MBBR differed, with greater numbers of putative anaerobes such as *Deltaproteobacteria* and *Clostridia* found in the mature biofilm compared with fast-growing aerobic species such as *Gamma-* and *Alphaproteobacteria* that dominated in the suspended fraction. Methanogens were identified as the most dominant archaea in biofilms of MBBRs and were quantified at less than 5% of the microbial biomass using biovolume analysis (DAIME: Daims et al. (2006a)) in combination with FISH. The spatial distribution and cell morphology of these archaea and sulfate-reducing bacteria within biofilms were also investigated by using FISH. The close proximity of these cells to each other in a biofilm lead to speculation about the existence of a syntrophic relationship, as observed previously in marine environments (Boetius et al., 2000). These two syntrophic species are known to overcome energy barriers in methanogenic environments by breaking down nutrients that are unable to be digested individually, and transfer electrons between species to sustain growth (Stams and Plugge, 2009).

Conventional activated sludge samples have been shown to be dominated by *Betaproteobacteria* and *Bacteroidetes*, making these communities substantially different to those of the biofilm and suspended fraction of MBBRs. These differences reflect the fact that these two treatment systems function in fundamentally different ways. Therefore, the extensive research that has been carried out on the microbial structure and function of activated sludge is not directly relevant to MBBR systems.

### 7.1.2. How do microbial communities develop and establish within MBBR systems?

The second related objective of this study was to examine the process of biofilm succession within the MBBR from initial seed to mature state biofilm. This aspect of the study benefitted from the use of 454 pyrosequencing technology, which has become more widely available and cost-effective than clone library methods for assessing the microbial diversity of large numbers of samples. 454 pyrosequencing was therefore employed to investigate the bacterial community structure of developing biofilms. Clone library methods were still used to
characterise the archaea, as initial studies indicated that these communities had low diversity compared with bacteria. The results of the 454 pyrosequencing both confirmed and extended upon the bacterial 16S rRNA clone data, and confirmed that a successional development process was occurring. For example, the earliest colonisers were members of the *Gammaproteobacteria* and *Flavobacteria*, followed some 34-48 days later by the *Deltaproteobacteria*. Interestingly, archaeal diversity decreased from two phyla (four families) to one phylum (one family) as the biofilm developed over time and was eventually dominated by members of *Methanosarcinaceae*. This was consistent with observations from the initial studies on resident (mature) biofilms. In conclusion, this study showed that biofilms developed through a successional process that occurred over a period of more than six weeks.

7.1.3. Does influent composition affect the sulfate-reducing organisms and the rate of sulfide production at a WWTP?

This question arose from the observation that the Moa Point treatment plant, used in this study, regularly experiences problems with the production of hydrogen sulfide. Sulfate-reducing bacteria (SRBs) use sulfate as a terminal electron acceptor, reducing it to sulfide in the process. A series of experiments was therefore undertaken to investigate the abundance and diversity of SRBs within both the Moa Point and Karori systems. Higher influent sulfate levels at Moa Point have long been suspected to be due to either seawater infiltration or trade wastewater.

The enzyme dissimilatory (bi)sulfite reductase (DsrAB) is conserved among SRBs and catalyses the final step in the process of sulfite reduction. Active SRBs were quantified by RNA-based real-time PCR (i.e. measuring *dsrAB* transcripts) while diversity of SRBs was examined using DNA-based clone libraries. The results showed that Moa Point and Karori plants were dominated by similar groups of SRBs that included *Desulfobulbus* and members of *Desulfobacteraceae*. However, two genera (*Desulfovibrio* and *Desulfomicrobium*) were exclusively found at the Moa Point site. The reason for this difference remains unclear and requires further investigation into whether concentrations of sulfate in the influent, or in seawater, generally can influence the composition of SRB in this way. Competition for sulfate among the SRB population has previously been investigated in a lab-based environment (Laanbroek et al., 1984; Muyzer and Stams, 2008), where it was shown that
Desulfovibrio spp. had the highest affinity for sulfate followed by Desulfobulbus spp. and Desulfobacter spp. However, to further our understanding of sulfate competition among SRB species pure culture experiments are required.

In summary, the higher sulfide emissions at Moa Point (31-35 ppm) compared with Karori (1-3 ppm) are probably a result of the higher sulfate levels entering into this facility, which supported microbial sulfate reduction.

7.1.4. Can MBBR microbial communities be manipulated in order to improve the performance of these systems?

SRBs are anaerobes, raising the question of whether sulfide production could be reduced by manipulating the density and anaerobic conditions within the MBBR biofilms. It was hypothesised that larger carriers, with more open structure, would be less susceptible to development of dense biofilms and hence functional dominance of SRBs. To address this, a comparative analysis of successional development of biofilms on K1 and K3 carriers was performed using 16S rRNA gene pyrosequencing and RNA-based real-time PCR of the dsrAB gene. Results showed that the small K1 carriers supported greater numbers of SRBs than the more open K3 carriers, offering the potential for limiting the growth of these organisms by changing the support media. Previous studies have also shown that carrier type and shape can influence other performance characteristics such as nitrification rates and COD removal in MBBRs (Odegaard et al., 2000; Levstek and Plazl, 2009). In conclusion, this study supports the hypothesis that carrier type may be manipulated to influence the microbial community structure and functionality in MBBR systems.

New insights have been gained into the overall bacterial community composition and the SRB population of full-scale MBBRs that treat municipal wastewater. How these microbes respond to changes in their environment and how community fluxes impact process efficiency can only be evaluated in manipulation experiments. Due to practical difficulties in conducting these experiments at a full-scale plant, lab-scale bioreactors were used to examine the effects of changing operational conditions on microbial communities in MBBR systems. Parameters that could potentially influence treatment performance, such as nutrient levels, conductivity and the nature of seeding material, were manipulated in these experiments. These parameters were also selected based on the practicality of their manipulation in full-
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scale MBBR systems. Other factors such as temperature, pH or changes in dissolved oxygen levels, will inevitably affect microbial communities and the resulting nutrient removal efficiencies (Haseborg and Frimmel, 2007; Haseborg et al., 2010; Ofiţeru et al., 2010). However, there are major energy and cost implications associated with manipulation of these factors, especially with elevation of dissolved oxygen.

16S rRNA gene pyrosequencing and chemical analytical methods were used to evaluate the effects of selected conditions on the microbial community composition and nutrient removal efficiency in a series of bioreactor studies. Total dissolved sulfide and SRBs were detected at relatively low levels within the bioreactors even though they were inoculated with biomass from Moa Point. This could conceivably be due to practical difficulties encountered in simulating Moa Point WWTP operational conditions in lab-scale bioreactors. However, the bioreactors were stabilised for two weeks prior to experimental phases and provided a good platform to monitor microbial communities based on operational changes. Interestingly, nutrient removal efficiency was drastically reduced when bioreactors were seeded with a mixture of fresh and stored biomass. The use of stored biomass was chosen to simulate the common practice of taking MBBRs offline for periods of time. The results of this study indicate that the seeding material can have significant effects on longer term performance of the treatment system.

7.2. Significance of this research

MBBRs are applied for the treatment of a variety of industrial and municipal wastewaters. This technology is highly adaptable to site constraints, organic load or carrier type (Odegaard, 2006). The insights gained from this project provide for a fundamental understanding of the microbial community structure and microbial dynamics within MBBR systems treating municipal wastewater. Moreover, it is envisaged that the outcomes of the study could directly be translated to optimisation of processes at, and troubleshooting of, the full-scale MBBR plants.

Biofilm communities in wastewater are ideal for studying microbial ecology (Daims et al., 2006b). As many operational parameters are closely monitored and controlled by the plant operators, important theories pertaining to microbial ecology (involving, for example,
competition, predation or niche development) can be tested in biofilms of wastewater systems (Daims et al., 2006b).

Most of our understanding of biofilm development processes has been based on pure cultures or lab-scale environments, under closely monitored conditions (Battin et al., 2003; Kolter, 2005). In this study, it was possible to closely monitor biofilm successional processes in a complex environment, namely a full-scale WWTP. The results from this study provide insights into surface colonisation and the successional development of biofilms which may be relevant in other complex industrial contexts. A good example is that of anammox bacteria, which have been successfully grown in MBBRs and have important commercial applications (Szatkowska et al., 2006).

7.3. Future directions for MBBR research

This study has addressed a number of fundamental questions pertaining to the microbial community in MBBR systems treating municipal wastewater. However, a number of questions remain that could be addressed in future studies as discussed below.

1. Does a “signature” microbial community exist for MBBR systems treating wastewater? If so, could this be used as an indicator to monitor MBBR operation?

The current study was limited to two municipal MBBR systems located in central New Zealand and therefore provides only a snapshot of MBBR communities worldwide. To fully address this question, a worldwide long-term biogeographic study would need to be carried out of MBBR plants to assess the dominant microbial community that is shared between all effectively operating treatment sites. ‘Signature’ microbial communities in activated sludge WWTPs have been previously investigated at a country-wide scale in Denmark (Mielczarek et al., 2012; Mielczarek et al., 2013). These ‘signature’ communities could be used to monitor treatment plant stability and its related performance. For example, deviations in the signature microbial community could indicate pending changes in plant health and relate to downstream plant performance.

Additionally, genomic-based strategies could be employed to enhance our knowledge of microbial taxa unique to, or essential to MBBR performance. For example, the genomes have been sequenced for a number of functionally important organisms in conventional
wastewater treatment systems, such as the nitrite-oxidising bacteria *Nitrobacter winogradskyi* (Starkenburg et al., 2006), the anaerobic ammonia-oxidising bacterium *Kuenenia stuttgartiensis* (Strous et al., 2006), the phosphate-accumulating organism *Candidatus Accumulibacter phosphatis* (Martín et al., 2006), and the ammonia-oxidising bacteria *Nitrosomonas europaea* (Chain et al., 2003). These studies have provided greater in-depth knowledge of functionally important genes within the microbial cells that are responsible for degradation or removal of nutrients including nitrogen or phosphorus, in activated sludge. However, this fundamental research is yet to be translated into practical applications that will help improve treatment performance.

2. **16S rRNA sequencing provides information about composition, but what about the function of these organisms?**

Advances in molecular methods have enabled functional information to be obtained from complex microbial systems such as soil (Urich et al., 2008), sea water (Gilbert et al., 2008), human gut (Qin et al., 2010), and also activated sludge (Wilmes et al., 2008; Yu and Zhang, 2012) by using the ‘omics’ approach. Metagenomics refers to the use of DNA sequencing to identify the composition of genes within a particular sample. This approach can be used to assess the metabolic potential of the sample and to infer functional differences between samples. Such approaches have been used to study the genomes of organisms found in wastewater (Tamaki et al., 2012; Yu and Zhang, 2012). These studies showed that microbial diversity and functional gene expression could be analysed simultaneously at a WWTP, thus matching community with function. Furthermore, this information can be related to operational processes at a treatment site.

Highly diverse microbial ecosystems such as wastewater exhibit functional redundancy whereby multiple organisms perform the same functional role (Curtis and Sloan, 2004). For example, this study has shown that MBBR communities are highly diverse, but to examine the function of these organisms, a broader ‘omics’ approach would need to be implemented. Functional ‘omics’ refers to genome-wide studies of the functional genes or proteins within a sample or samples. Such studies can provide further insight into what component of the metagenome is actually expressed under different conditions. It would be worthwhile to investigate further the presence and relative abundance of functional genes in biofilms of MBBR. Examples of functional genes that have previously been investigated in wastewater
systems include \textit{nifH} (dinitrogenase reductase) to study nitrogen fixation, \textit{amoA} (ammonia monooxygenase) to study nitrification, antibiotic resistance genes (such as \textit{mexXY} in \textit{Pseudomonas}) for medical purposes, and virulence genes (such as \textit{invA} in \textit{Salmonella}) to be tested before treated water is released into the environment (Swamy et al., 1996; Geets et al., 2007; Bowers et al., 2008; Lupo et al., 2012). A better understanding of the function of microorganisms at a MBBR plant could ultimately translate into enhancing nutrient removal or the degradation of complex organic compounds from wastewater.

3. \textit{SRB and methanogen consortia: understanding prokaryotic symbiosis.}

To further our knowledge of prokaryotic symbiosis, MBBR biofilms would provide a perfect study model, as FISH results in this study have shown that SRB and \textit{Methanosarcinales} can occur in close proximity to each other. Consortia of archaea/SRB have been observed in methane-rich environments, such as marine sediments (Boetius et al., 2000). Archaea mediate the anaerobic oxidation of methane (AOM), also known as reverse methanogenesis, while SRBs oxidise the intermediates that are formed (Stams and Plugge, 2009). Such symbiotic interactions are rarely observed in the environment between prokaryotes. Due to the difficulties of culturing and maintaining these organisms involved in AOM within the laboratory, the physiology of these consortia and the role of intermediates is poorly understood (Nauhaus et al., 2007). This raised the question of the functional relationship of these microorganisms and the relevance of this association to treatment performance. Under appropriate growth conditions, AOM consortia could be enhanced within the biofilm of lab-scale MBBRs, thus providing an ideal environment to study their interactions under varying conditions.

4. \textit{What is the role of protozoa and viruses in MBBR systems?}

This study was focused entirely on prokaryotes. Knowledge of the entire community of microorganisms (i.e. prokaryotes and eukaryotes) in MBBRs would provide a more complete picture of the microbial interactions occurring within these systems. Further efforts to link plant performance (such as flocculation) to microbial community composition and function would be of considerable value. Advances in molecular technology have enabled the study of viruses in wastewater using metagenomics (Tamaki et al., 2012). A similar strategy could be applied to characterise viruses and eukaryotes such as protozoa in MBBR systems.
7.4. Concluding remarks

MBBR technology is able to meet the demands of a growing urban population and for this reason is an increasingly widely used technology for the treatment of wastewater. Research on MBBR and other suspended biofilm-based systems has already begun to increase and has thus far focused primarily on nutrient removal. This project provides the foundation for future studies in this field that will lead to a better understanding of the link between microbial communities and system performance. Enhanced collaboration between microbiologists and engineers on research projects in the future will expedite R&D activities and enhance our knowledge of wastewater processes that will, ultimately, translate into better technologies.
7.5. References


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