KEY MICROBIAL GROUPS OF MBBR SYSTEMS AT WELLINGTON'S SEWAGE TREATMENT PLANTS

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ABSTRACT

Moving Bed Biofilm Reactor (MBBR) systems are used in more than 400 wastewater treatment plants (WWTP) worldwide but little is known about the microbial communities on which these systems rely. This study aimed to address this issue by characterizing the microbial community composition at Wellington's Moa Point (MP) and Western WWTPs. Monthly samples comprising biofilm carriers and mixed liquor were collected from MBBR reactors at each plant. DNA-based molecular methods were used to determine the bacterial and archaeal community composition of biofilms scraped from the carriers and from suspended material. Differences in microbial community structure and abundance were observed between WWTPs and between biofilm versus suspended fractions Biofilms generally showed a dominance of anaerobic sulphate-reducing bacteria (SRBs) including *Desulfobacteriales* and *Clostridia*. In contrast, the suspended communities were more diverse, including aerobes. *Archaea* were found in low diversity and abundance (<5%). These results suggest that the MBBR process differs from a conventional AS system by selecting for two distinctive bacterial communities. A higher abundance of SRBs in the MP biofilms raises the question of whether sea water infiltration influences this microbial community and requires further investigation.

KEYWORDS

Moving bed biofilm reactor (MBBR), Sulphate-reducing bacteria (SRB), Archaea, wastewater treatment plants (WWTP), 16S gene analysis, automated ribosomal intergenic spacer analysis (ARISA)

1 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 (WWTP) (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. This suspended fraction is subsequently removed through a process of contact stabilization, which induces stable floc formation, followed by gravity settling to produce a clarified effluent. MBBR technology offers a number of advantages over conventional AS including smaller foot print and reactor volume, high effluent quality, and low waste sludge volumes (Yang et al., 2009).

Studies on the microbial community composition from conventional AS systems indicate that the community is typically dominated by aerobic or facultatively anaerobic heterotrophic bacteria belonging to the beta *Proteobacteria* (Schmid et al., 2003). It is unclear whether similar communities are found MBBR processes as

there have been few studies on full scale systems. Differences in microbial communities might be expected on the basis that MBBR systems support development of microbial biofilms within which microenvironments support the growth of both anoxic and aerobic microorganisms within the same ecosystem (Yang et al., 2009). The presence of a reduced oxygen gradient within these biofilms has potential to support growth of anaerobic ammonia oxidation bacteria such as *Planctomycetes sp*., *Rhizobium sp.,* and *Agrobacter sp*. (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 de-nitrification 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 (Ødegaard 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 on microbial community structure and function within MBBR systems are poorly understood. The effects of variability in influent composition and volume on the microbial population also require further investigation. Some of the factors influencing this are industrial effluent composition, seasonal variability, sea water infiltration, and variability within the contributing community.

The aim of this study was to investigate the microbial communities in Wellington's Moa Point (MP) and Western WWTPs and thus to provide the first comprehensive insight into the key functional microbial groups in full-scale MBBR systems. DNA-based molecular methods were used to characterize the microbial community and to investigate the abundance and distribution of key organisms. The effects of seasonal and influent variability on the bacterial composition the two WWTPs were also examined.

2 METHODS

2.1 SAMPLING SITES

Sampling was carried out at Wellington's MP and Western WWTPs. MP has an average dry weather flow of 822 l/sec which includes household and industrial waste. In contrast Western WWTP receives only domestic waste with an average inflow of 20 l/sec. The MBBR reactors at both plants contained suspended polyethylene carriers (K1 media) comprising 30 – 50% of reactor volume. Reactor samples, comprising suspended K1 media with adherent biofilm, were collected once a month over a 5 month period from MBBR reactors at MP (designated as M1, M2, & M3) and Western (designated K1 & K2) treatment plants.

2.2 BIOMASS MEASUREMENTS

Excess liquid was drained from the carriers before drying in a desiccation chamber for 48 hours. The dry weight of the biofilms was measured in replicates of 5 per month for each reactor.

2.3 RAINFALL AND TEMPERATURE DATA

Rainfall data for the Wellington area was acquired through the National Institute of Water & Atmospheric research website (www. niwa.co.nz). Influent temperature at MP WWTP was recorded every week by plant operators.

2.4 TOTAL SULPHIDE MEASUREMENT

Total sulfide was measured by a colourometric method as outlined previously by 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 460 nm in a UV-vis Spectrophotometer.

2.5 DNA EXTRACTION

Total genomic DNA was extracted from biomass using a phosphate, SDS, chloroform-bead beater method as described by Miller et al. (1999). Extracted DNA was eluted in 50 µl of DNase free water and stored at -20 ^{o}C until further analysis.

2.6 ARISA

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

Results from ARSIA were analyzed though 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.

2.7 16S GENE ANALYSIS

2.7.1 CLONE LIBRARY ANALYSIS

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

2.7.1.1 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) OF CLONES

Restriction endonuclease *Hae* III (Invitrogen) disgestion 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 by Sambrook and Russell, 2001. 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.

2.7.1.2 SEQUENCING AND DATA ANALYSIS

PCR-amplified inserts from representative clones were purified and sequenced in one direction using the 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 of96 clones were sequenced from each bacterial clone library, whereas one clone representing each OTU was sequenced from the *Archaea* libraries. Sequence data was processed on 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) as an alignment search tool.

2.7.2 FLUORESCENT IN SITU HYBRIDISATION (FISH)

Fluorescence in-situ hybridization methods were used in combination with confocal miscroscopy to examine samples for the presence of methanogens and SRBs (Amann et al., 1995; Manz et al., 1992). Samples were fixed within 12 hours 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 sampleinto 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 50 ml Falcon tube chamber at 46 °C for 2 hours. Oligonucleotide probes were derived from published sequences with a 5'-fluorescent label (specified in Table 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 optimizing concentrations for probes) and 1 µl of 50 ng/ µl probe. Following incubation, slides were immersed in pre-heated (48 °C) wash buffer (20mM Tris-HCl, 5mM EDTA, NaCl optimizing 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 used as a universal DNA stain by incubating the slides for 5 min in the dark and then rinsed.

Table 1: Oligonucleotide sequence used for fluorescent in situ hybridization (FISH)

Probe name	Target group	Probe sequence 5'-3'	Label	Reference
MSMX860	Methanosarcinales	GGCTCGCTTCACGGCTTCCCT	CY3	Raskin et al., 1994
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	CY3	Amann et al., 1990
DELTA495a	Deltaproteobacteria and Gemmatimonadetes	AGTTAGCCGGTGCTTCCT	CY5	Loy et al., 2002
cDELTA495a	Competitor for DELTA495a	AGTTAGCCGGTGCTTCTT	-	Macalady et al., 2006

2.7.2.1 CONFOCAL MICROSCOPY

FISH slides were viewed using FV1000 Olympus Confocal laser scanning microscope (CLSM) equipped with 100x oil immersion objective. 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 FV10-ASW2.0 (Olympus) viewer.

3 RESULTS AND DISCUSSION

3.1 GENERAL SLUDGE CHARACTERISTICS

Biofilms attached to K1 media differed between sites. At MP the biofilms were dense, typically filling the internal sectors of the media, black in colour and had a sulfurous odour. However, variations were noted in samples collected following large inflows into the plant (due to a storm event). Biofilm samples from the Western WWTP were consistently greyish-brown and lacked the sulfurous odor. T-Test (unequal variance) indicated Significant differences were observed between between the dry weight of the black and brown biofilms at MP (p-value = 0.0145) and between black biofilms at MP and biofilms at the Western WWTP (p-value = 0.004). P-values < 0.05 were considered significant (Fig. 1).

Measurements of sulfide in sample from MP reactors ranged between 1.5 to 3.5 mM per litre. In contrast sulfide was not detected in samples collected from the Western plant.

Daily rainfall in the Wellington area and influent temperature (recorded only at MP WWTP) were variable factors over the months of March to July 2010 (as seen in Fig. 2).

Figure 1: Dry weight of biofilms from K1 carriers of the two MBBR systems Moa Point (MP) and Western are displayed as a bar graph. The inlaid images represent typical biofilms that were observed for each phenotypic group.

Error bars = standard deviation; n = number of samples per group.

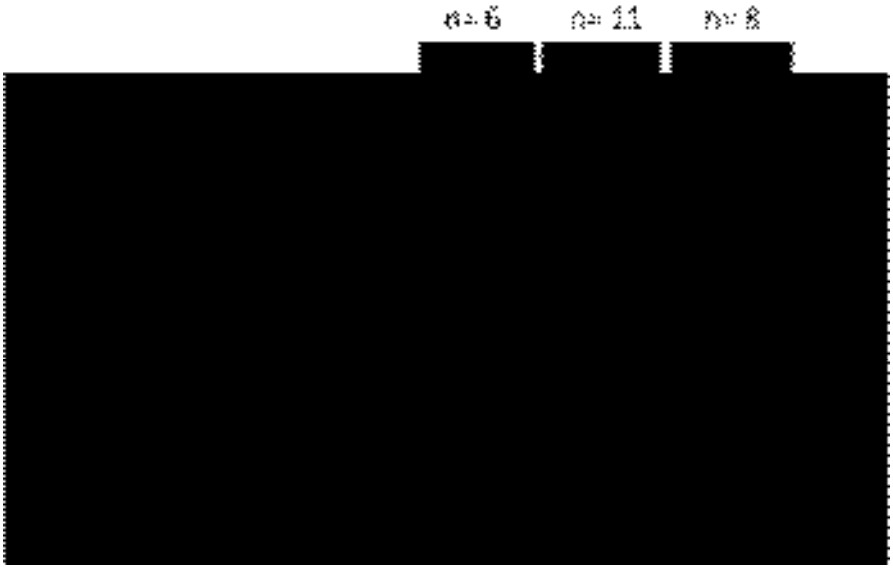
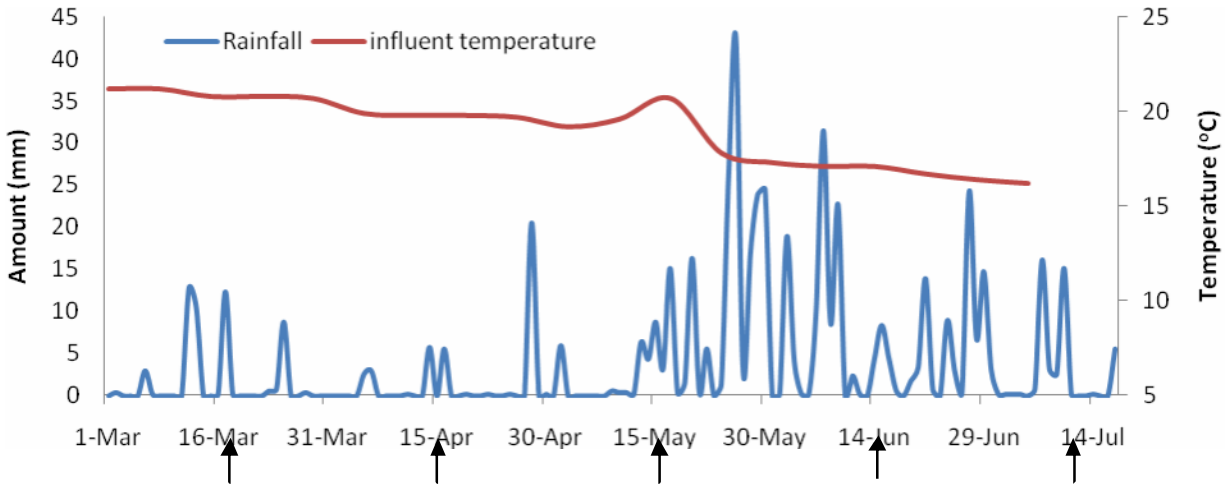


Figure 2: Daily rainfall in the Wellington harbor and influent temperature at MP WWTP. The black arrows indicate sampling times.



The higher biomass of the black biofilm may simply indicate that these communities are older, having developed over time into a denser biofilm. In contrast, the brown biofilm at MP with a lower biomass could be a reasonably young community that has only recently attached onto the surface (K1 carriers) and is still developing. The occurrence of both black and brown biofilms in the MP reactors may be due to the variable effects of sheer occurring within the reactors or changes in influent concentrations associated with rainfall events that induce bacterial detachment. Future studies will attempt to address this question by investigating the process of biofilm establishment and dissociation within these systems.

The Western MBBR WWTP had significantly lower biomass compared with MP. This difference is possibly due to the composition of the influent although further studies are required to understand the effects of influent composition on bacterial communities and their development.

The detection of sulfide at MP indicates the possible activity of anaerobic SRB, which are commonly found in anaerobic digestors (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 coloured precipitates. Elevated levels of activity by SRB could explain the differences in colouration of biofilms observed in MP and Western plants. Enhanced SRB activity would require access to excess sulfate as an electron acceptor. The source of this in the MP plant has not yet been clearly established. However seawater is known to contain appreciable concentrations of sulfate and high concentrations have been found in an MBBR system treating waste from marine aquarium (Labelle et al., 2005). The presence of long chain fatty acids (LCFA) derived from the degradation of lipids (Sousa et al., 2009a) and low water temperatures (Ben-Dov et al., 2009; Leloup et al., 2005) have also been shown to select for SRBs in a wastewater treatment system.

3.2 BACTERIAL COMMUNITY ANALYSIS

3.2.1 ARISA

Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used as a rapid method to profile bacterial community structure and to investigate differences between samples over time. Multi-dimensional scaling (MDS) plots, constructed from community profile data (Fig. 3), indicate differences between the two WWTP and between biofilm and suspended communities. At MP a clear separation of black versus brown biofilm community structure was observed. The impact of seasonal variations on the community composition was also noted.Differences between the two WWTP (as indicated in Fig. 3, Graph A) were expected due to varying sources of waste entering these systems. Complex organic matter entering the system through the influent is degraded by specialized groups of microorganisms that develop over time (Schink, 1997). MP receives industrial waste from an abattoir, expected to carry large amounts of fats, oils and greases (Mittal, 2006). Other factors that could also influence bacterial community structure are operational parameters at a WWTP.

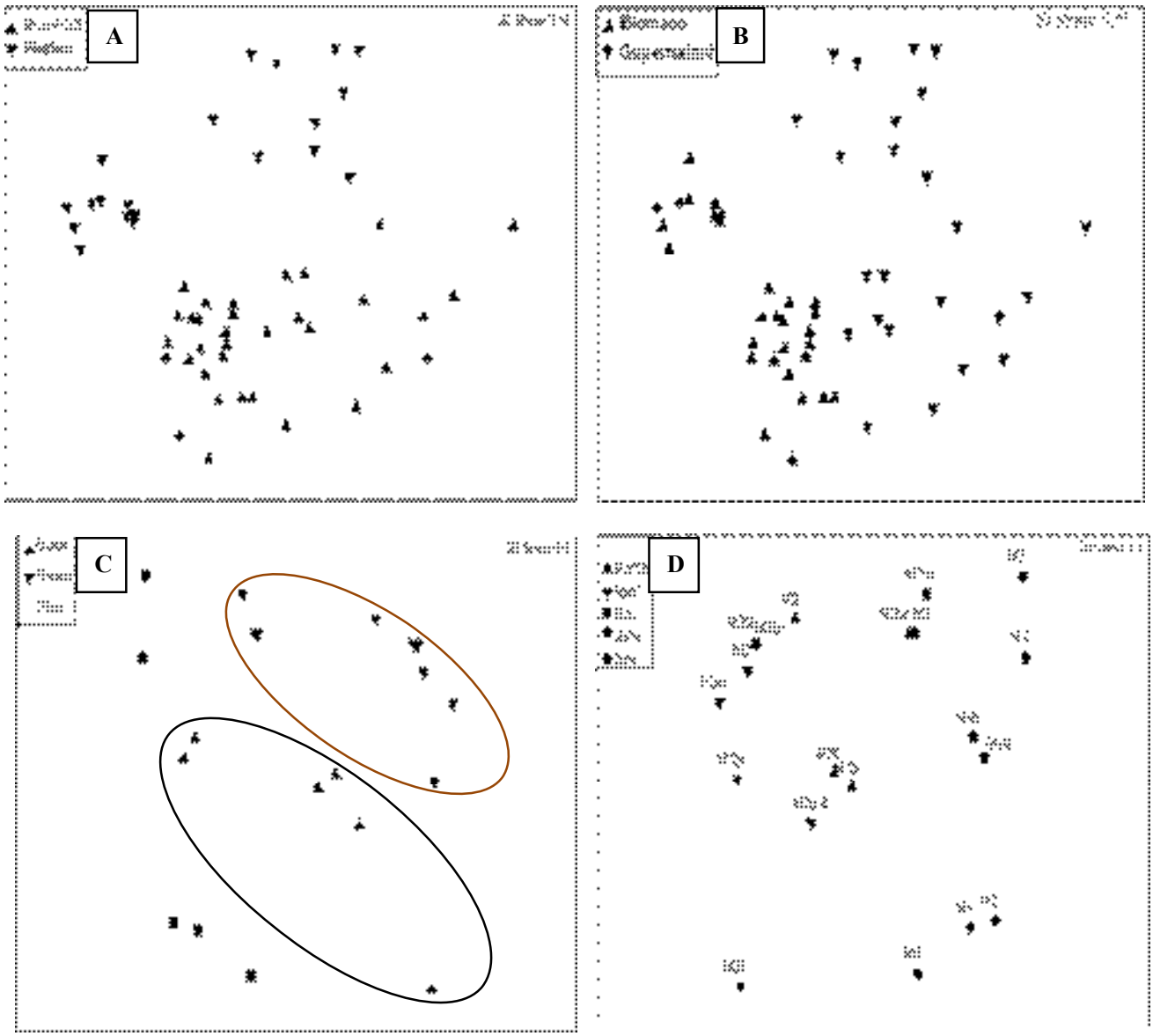
Differences between planktonic and biofilm samples (Fig. 3, Graph B) were also observed and may be explained by the different conditions that previal within these structured environments. The biofilm structure is known to have select advantages over a suspended floc, such as increased porosity and a 5 to 13 times higher density (Xiao and Ganczarczyk, 2006). The K1 carriers in the MBBR system also provide a protective environment for biofilms to establish. Microorganisms within establishing biofilms form syntophic relationships for the degradation of nutrients. In contrast the microbial community in the suspended fraction most likely enters the system through the influent with a short retention time in the MBBR. Rapid growth is required to enable communities to avoid washout of the system.

Differences in the colour of biofilms on carriers from MP (Fig. 3, Graph C) may reflect stages of the successional development of the biofilms. Contemporary models of biofilm development have been laregly developed from studies of single species organisms in a lab environemnt (Stoodley et al., 2002), therefore very little is known about how a biofilm develops into a mature structure in a complex environment such as wastewater treatment systems. From the results of this study it is hypothesized that the brown biofilm seen in

the MP reactors are newly established and possibly still maturing, while the black biofilm is a older more mature community that includes SRBs. The ARISA results also suggest that seasonal variations can also play a role in bacterial community composition (Fig. 3, Graph D). Although further sampling across a longer time period is required to confirm these observations other studies have shown that increased rainfall along with the decrease in temperature in winter months favours the growth of certain groups of organisms (Leloup et al., 2005). Storm events and their associated impacts on food to microbe ratios have also been shown to impact on the bacterial community (Wilen et al., 2008).

Figure 3: Multidimensional scale (MDS) plots of ARISA data representing bacterial community structures of samples collected over five months from MP and Western WWTPs. Each symbol within the graph represents a bacterial community for one given sample. Minimum stress = 0.1

***Graph A**: Bacterial communities between the two WWTPs has been displayed and differentiated by the two colours: purple and orange. **Graph B**: Bacterial communities between the biofilm and suspended fraction has been plotted and differentiated by the two colours: green and blue. **Graph C**: Black and brown biofilm at MP's MBBR were plotted on the MDS graph. Grey represents biofilms that were neither brown nor black. **Graph D**: Biofilm community structure over five months at MP has been displayed and represented by the five colours. Monthly variations were also observed for the suspended fraction at MP (data not shown).*



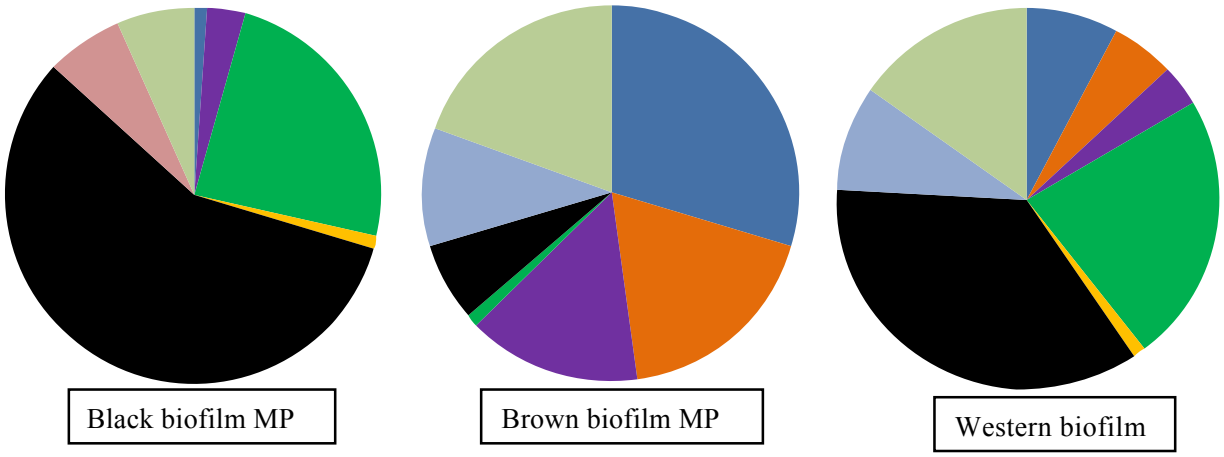
3.2.2 16S GENE ANALYSIS

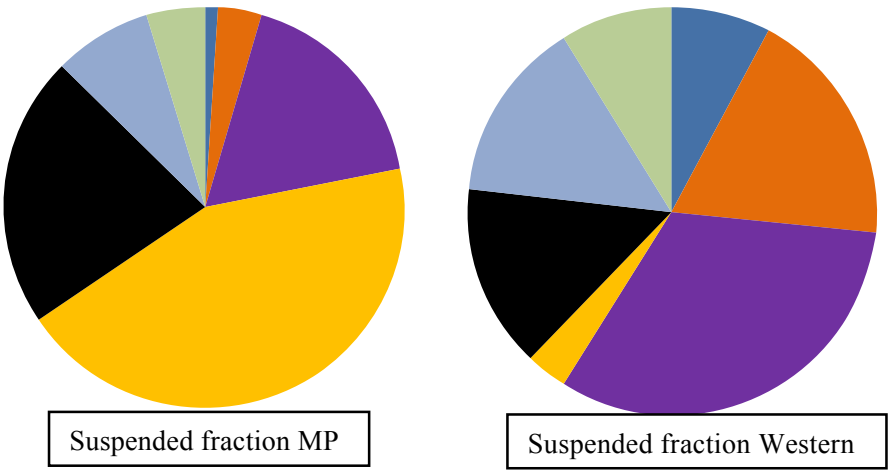
Biofilm communities from the MBBR systems (except for the brown biofilm) showed limited bacterial diversity with dominance of anaerobic *Clostridia* (32-43% of the bacterial community) (Fig. 4). Strictly anaerobic, SRBs belonging to the class *δ-proteobacteria* were the second most abundant group comprising 35% of the black biofilm community at MP and 26% at Western. Members of *Desulfobacterales*, *Syntrophobacterales* and *Desulfovibrionales* were the major contributors of SRBs found within these samples. The biofilms from Western WWTP differed due to the elevated incidence of *α-* and *β- proteobacteria* (10% & 9% respectively) when compared to <3% of the black biofilm community from MP. A collective low abundance (<5%) of *Fusobacteria*, *Deferribacteres* and unclassified bacteria were also detected in the biofilm samples.

In contrast, the suspended fraction from both MBBR systems and the brown biofilm from MP reactors were dominated by a more diverse group of aerobic organisms from the class *α-proteobacteria* (*Rhizobiales* and *Rhodobacterales*) *γ-proteobacteria* (*Pseudomonadales* and *Aeromonodales*) and *β-proteobacteria*., (*Burholderiales* and *Rhodocyclales*). Members of the clostridia were also present but in low numbers (10-15%). In addition, MP samples had an elevated level of the potentially pathogenic *Campylobacteraceae* belonging to the class *ε-proteobacteria*.

Currently there is limited information avaliable in the literature on the microbial community composition in MBBR systems although the microbial community of conventional AS flocs have been well studied (Klausen et al., 2004; Snaidr et al., 1997). Bacterial communities in AS plants treating industrial and municipal wastewater were shown to be dominated by *β-proteobacteria,* followed by *α-* and *γ-proteobacteria* (Lee et al., 2002; Schmid et al., 2003). Other groups of bacteria found in low abundance are *Bacteroidetes* and *Firmicutes*. The results of the current study suggest that the attached biofilm community differs substantially from that found in conventional AS systems.

Figure 4: Bacterial community of biofilm and suspended fraction from Moa point and Western reactors are presented for samples collected in March 2010. Similar community structures have been observed for other sampling dates.





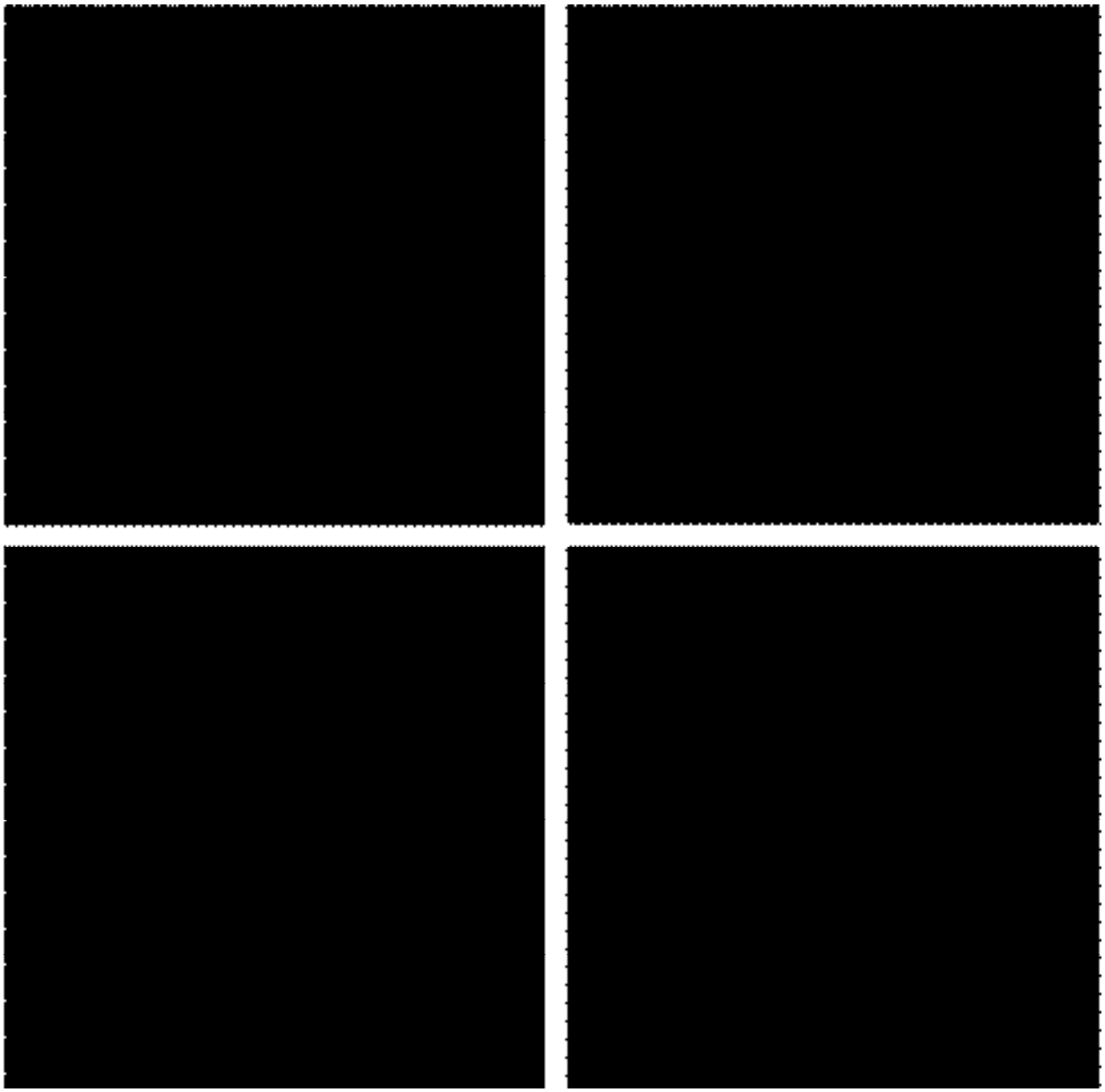
The results from this study reveal the low diversity of bacterial phyla found within the black and Western biofilm at the MBBR plants that were dominated with anaerobes. These results indicate that an anoxic microenvironment exists within these carriers. This phenomena has been suggested previously by Gray et al., (2002), while other studies have also indicated that the outer surface of a K1 carreir is aerobic (Elenter et al., 2007; Yang et al., 2009). The brown biofilm at MP reactors showed clear indications of a young developing community dominated by aerobic, fast growing members of α-, *β-,* and *γ- proteobacteria*. The suspended fraction were also dominated by this group of fast growing aerobic organisms. Based on contemporary biofilm models it is proposed that the planktonic bacteria are the early colonists of the K1 carriers. Over time the community diversifies and develops via a successional process into a mature structured biofilm that may include both aerobic and anaerobic microenvironments.

The high abundance of members of the *Campylobacteraceae* was an unexpected result. However Koenraad et al., 1995, isolated several species of *Campylobacter* from an sewage plant in The Netherlands treating abattoir waste suggesting that such effluents may be a signficant source of *Campylobacter.*

Analysis of the archaeal community indicated a very limited diversity with only one RFLP pattern detected in the 20 clones screened. Sequence analysis indicated that this represented organisms from the methanogenic order *Methanosarcinales*. The spatial distribution of *Methanosarcinales* and sulphate reducing *δ-proteobacteria* was examined for all samples using FISH along with confocal microscopy (Fig. 5). The black biofilm revealed clumps of *δ-proteobacteria* buried within the biofilm structures. Similar clumps were not evident in the brown biofilm of MP. Samples from Western also showed *δ-proteobacteria* but in a filamentous form.

Methanogens were observed in low numbers in biofilms from the two MBBR systems studied (Fig. 5). Clusters of SRBs were also seen within the black biofilm of MP (Fig. 5A). Based on the clone library results these are most likely to be *Desulfococcus* or *Desulfosarcinales*. The FISH images also support the results from the clone library data by showing abundance of SRBs at both WWTP. Images from the black biofilm from MP captured SRBs and methanogens in close proximation to one another (Fig. 5B). Previous studies in the marine environment have also shown FISH images of a consortia of SRBs and Methanogens (Boetius et al., 2000; Knittel et al., 2003) suggesting a bacterial-archaeal symbiosis interaction.
SRBs were also detected at Western WWTP but in lower numbers and in a filamentous form (Fig. 5C & 5D). Filamentous SRBs have been detected previously in marine and freshwater sediments (Fukui et al., 1999) but their exact role at Western WWTP requires further investigation.

Figure 5: MP (M1-black biofilm) and Western (K1) MBBR biofilm samples hybridized with fluorescently labeled probes MSMX860-red (methanogenic Archaea), DELTA495a-cyan (δ-proteobacteria), EUB338-green (all bacteria) and DAPI-blue (universal DNA stain). The black biofilm from M1 contained clusters of δ-proteobacteria as seen in (A) & (B).Biofilm from K1 comprised filamentous δ-proteobacteria as seen in images (C), & (D). Arrows labeled 'm'or's' are indicative of methanogenic Archaea or sulfate reducing δ-proteobacteria.



4 CONCLUSIONS

This study provides the first comprehensive insight into the key functional microbial groups in full-scale MBBR systems and the effects of seasonal and influent variability on the bacterial composition. The result indicate that communities differ substantially from conventional AS systems by selecting for two distinct bacterial communities; an anaerobic biofilm community and a more aerated fast growing suspended community. Differences were 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 suport the notion that the microbially mediated sufate reduction is occuring with biofilms at the MP plant. It is possible that this is due to the influence of seawater infiltration although further studies are required to conform this. A possible archaeal-bacterial relationship was also observed between *Methanosarcinales* and SRBs in the biofilm of K1 carriers. The identification of key functional microbial groups at the two MBBR systems studied will lead to further development of food web models that will underpin manipulation of the system for improved performance.

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