Mapping the Distribution of the EPS Matrix Within Mixed Microbial Flocs

by

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The efficacy of biological wastewater treatment processes is largely dependent on the formation of microbial flocs and settleability before the water is released into the environment. Settleability and flocculation are reliant upon stable physicochemical parameters. Extracellular polymeric substance constituents dictate physicochemical parameters of flocs. The fluctuation of these constituents within mixed microbial flocs is poorly studied. A novel aspect of this research was the use of CLSM data to get a semi-quantitative assessment of the constituents within mixed microbial flocs.

Wastewater treatment flocs were characterized for eubacterial ecology, physicochemical properties, and they were visualized through correlative microscopy. It was observed that the microbial communities from the three sampling sites exhibited significant variability in numerous physicochemical properties. Overall, these results provide a first step to examine micro-localization of physicochemical properties, architecture and processes within flocs that may help better understand the causes of floc-related inefficiencies in biological wastewater treatment.
ACKNOWLEDGMENTS

Hereby I would like to thank every effort put forward by the following people, without whom this project would have been infeasible.

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<td>AMU</td>
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<tr>
<td>Adenosine-5'-triphosphate</td>
<td>ATP</td>
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<td>Analysis of variance</td>
<td>ANOVA</td>
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<td>Bovine serum albumin</td>
<td>BSA</td>
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<td>Cation exchange resin</td>
<td>CER</td>
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<td>Chemical oxygen demand</td>
<td>COD</td>
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<td>Conventional optical microscopy</td>
<td>COM</td>
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<td>Concanavalin A</td>
<td>ConA</td>
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<td>Confocal laser scanning microscopy</td>
<td>CLSM</td>
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<td>Cytidine triphosphate</td>
<td>CTP</td>
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<td>4’-6-Diamidino-2-phenylindole</td>
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<td>Denaturing gradient gel electrophoresis</td>
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<td>Deoxyribonucleotide triphosphate</td>
<td>DNTP’s</td>
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<td>Derjaguin and Landau, Verwey and Overbeek</td>
<td>DLVO</td>
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<td>Extracellular polymeric substance</td>
<td>EPS</td>
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<tr>
<td>EPS carbohydrate</td>
<td>EPS_C</td>
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<tr>
<td>EPS protein</td>
<td>EPS_P</td>
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<td>Fluorescence in situ hybridization</td>
<td>FISH</td>
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<td>Fluorescein isothiocyanate</td>
<td>FITC</td>
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<td>Folin and Ciocalteu’s Phenol</td>
<td>FCP</td>
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<td>Guanidine and cytosine</td>
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<td>Guanosine-5’-triphosphate</td>
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<td>Guelph wastewater treatment plants</td>
<td>GWWTP</td>
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<td>Membrane bioreactor</td>
<td>MBR</td>
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<td>Mixed liquor suspended solids</td>
<td>MLSS</td>
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<tr>
<td>Polyphosphate accumulating organisms</td>
<td>PAO</td>
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<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
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<td>Polymerase chain reaction</td>
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<td>Polymerase chain reaction-denaturing gradient gel electrophoresis</td>
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<td>Photo multiplier tube</td>
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<td>Propidium iodide</td>
<td>PPI</td>
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<td>Rothsay wastewater treatment plants</td>
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<td>Sludge retention time</td>
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<td>Sludge volume index</td>
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<td>Small Angle x-ray scattering</td>
<td>SAXS</td>
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<tr>
<td>Term</td>
<td>Abbreviation</td>
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<td>----------------------------------</td>
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<td>Transmission electron microscopy</td>
<td>TEM</td>
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<td>Tetramethylethylenediamine</td>
<td>TMED</td>
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<tr>
<td>Thymidine triphosphate</td>
<td>TTP</td>
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<tr>
<td>Trichloroacetic acid</td>
<td>TCA</td>
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<tr>
<td>Ultra violet</td>
<td>UV</td>
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Chapter 1

Literature Review
CHAPTER 1. LITERATURE REVIEW

1.1. CHARACTERISTICS OF MIXED MICROBIAL FLOCS IN BIOLOGICAL WASTEWATER TREATMENT

1.1.1. Physical properties of activated sludge flocs

Since the 1930s activated sludge has been widely used for wastewater treatment processes, including sewage and industrial waste treatment. Activated sludge employs air and biological flocs composed of microorganisms to treat wastewater. While the overall concept of activated sludge has not changed drastically over time, the state of knowledge on specific processes such as floc formation or flocculation has advanced. Activated sludge flocs are composed of bacteria, fungi, protozoa, extracellular polymeric substance (EPS), and organic and inorganic particles. The size of these flocs can range anywhere from 25 µm to 1 mm in diameter. Flocculation of microorganisms in wastewater is determined by three major factors: the physiology of the microorganisms, the environmental conditions, and nutritional factors.

Microorganisms are capable of planktonic or free-swimming survival. They preferentially will colonize surfaces forming biofilms. They may also co-aggregate and form suspensions of various structures floating in liquid called flocs. The efficacy of the wastewater treatment process is partially dependent upon the floc formation process and the separation of microorganisms from the water column. The separation of microorganisms from the water column is dependent upon the physicochemical properties (chemical composition of EPS, surface charge, hydrophobicity and water binding capacity) of the flocs and the floc physiology (size and shape) as dictated by the microbial composition.
Although various microbial genera and species can comprise the floc microbial community, from a structural perspective they are classified into two categories: floc formers and filamentous microorganisms. Examples of floc formers and filamentous microorganisms are listed in Table 1.1. These two major populations of microorganisms influence settleability directly and indirectly. The microbial ecology may directly affect settleability through filamentous bacteria producing EPS structures such as flagella, fimbria and pili, which extend outward and add to the hydrodynamic drag force and would thus have a negative influence on settleability \(^{28,49,53,103}\). Microbial ecology (i.e. types of bacteria, microalgae and fungi) may significantly influence settleability indirectly by influencing the protein to carbohydrate ratio \(^{49}\). The protein to carbohydrate ratio and how it influences settleability is discussed later.

Filamentous bacteria and floc formers occupy different locations of the floc and they compete for the same resources, i.e. electron donors and electron acceptors. It is therefore believed that filamentous bacteria with an outward growth have a competitive advantage when resources become scarce \(^{12,35,115,139}\). Better access to nutrients and oxygen means faster growth of filamentous microorganisms \(^{140}\). The competitive advantage to filamentous microorganisms are, however, not advantageous to the process of wastewater treatment as it causes sludge bulking and inefficiencies in settleability \(^{80}\). However, filamentous microorganisms do act as a backbone for floc formers to grow around and define the overall floc structure by growing solely in one or two directions \(^{109,142}\). An illustration of this backbone phenomenon is shown in Figure 1.1.

The floc formers on the other hand grow in multiple directions and, therefore, create a strong compact structure. The dense structure is beneficial to settleability of the
Table 1.1: Microbial species within mixed microbial floc from wastewater treatment.

<table>
<thead>
<tr>
<th>Microbial species</th>
<th>Role within mixed microbial flocs</th>
<th>References</th>
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<tr>
<td><em>Nostocoida limicola, Microthrix parvicella and Thiothrix sp</em></td>
<td>filamentous microorganisms</td>
<td>36,45,87,118,156</td>
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<tr>
<td><em>Azotobacter sp. and Pseudomonas aeruginosa</em></td>
<td>Alginate production</td>
<td>146</td>
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<td><em>Aeromonas, Pseudomonas, Alcaligenes, Comamonas- Pseudomonas group, Flavobacterium-Cytophaga group, Moraxella, Xanthomonas, Paracoccus, Bacillus, Corynebacterium Pseudomonas, Alcaligenes, Flavobacterium, Paracoccus and Bacillus</em></td>
<td>Phosphate accumulating organisms, floc formers</td>
<td>68,75,147,170</td>
</tr>
<tr>
<td><em>Alcaligenes, Pseudomonas, Methylobacterium, Bacillus, Paracoccus and Hyphomicrobium and member of genus Paracoccus spp. and Hyphomicrobium spp.</em></td>
<td>Nitrifying bacteria, floc formers</td>
<td>8,95,99,121,154</td>
</tr>
</tbody>
</table>
flocs. The compactness of the structure on the other hand may lead to a decreased nutrient diffusion and a lower concentration of oxygen at the core of the flocs \textsuperscript{110}. The lower concentration of oxygen could be beneficial to some floc formers. The gradient of oxygen and nutrients on the inside and outside would lead to different microbial communities occupying different zones within flocs \textsuperscript{110,122}. Anaerobic nitrifying and phosphate accumulating organisms are floc formers, which may benefit from living in low oxygen zones \textsuperscript{76,140,151}. Please refer to Table 1.1. for list of microorganisms known to play a role in nitrification and phosphorus accumulation.

For example the nitrogen cycle involves two classes of bacteria: nitrifying bacteria and denitrifying bacteria. Nitrifying bacteria in wastewater treatment can further be sub-classified into two groups namely: (1) ammonia oxidizing bacteria which catalyze oxidation of ammonia to nitrite and (2) nitrite oxidizing bacteria which catalyze oxidation of nitrite to nitrate \textsuperscript{55}. Nitrifying bacteria in WWTP are very sensitive to shifts in pH and temperature and therefore WWTPs frequently report the breakdown of the nitrification process after a temperature or pH shock \textsuperscript{157}.

Nutritional factors and environmental conditions will be discussed together in this thesis. The environmental conditions are a determining factor for characteristics of flocs such as microbial ecology and surface charge. These two characteristics could further impact hydrophobicity, floc size distribution and bound water \textsuperscript{97}. Environmental conditions include: hydrodynamics, pH, temperature, food to microorganism ratio, sludge retention time (SRT), nutrient ratio [i.e. ratio of chemical oxygen demand to nitrogen to phosphorus (COD:N:P)] \textsuperscript{1,10,80,105}. Hydrodynamic shear force may play a role in floc size, while pH, SRT and temperature influence surface charge and hydrophobicity and finally
the food to microorganism ratio may indirectly influence hydrophobicity and surface charge by varying the EPS constituents\textsuperscript{1,10,69}.

Nutrient content can be classified into macronutrients (C, H, O, N, P, S) and micronutrients (Fe, Na, Ca, K, Mg and trace elements). Carbon, nitrogen and phosphorus are the major elements controlling the metabolism and growth of microorganisms. The oxygen uptake by microorganisms when they metabolize the organic nutrients is known as biological oxygen demand (BOD). Chemical oxygen demand (COD) measures all the oxidizable material in the wastewater and it is easier to measure than BOD. In untreated domestic wastewater the COD values usually are in the range of 250 to 1000 mg/L\textsuperscript{112}. Lack or limited amounts of the essential nutrients, N and P, can lead to changes in the microbial community with a decrease in the biological activity\textsuperscript{136}. Therefore, these macronutrients are frequently added to activated sludge. Addition of nutrients to the biological treatment of wastewater has to be closely monitored in order to provide the microbial community with optimal nutrient levels while still ensuring that no excessive discharge of residual nutrients occurs. Optimized addition of nutrient in accurate ratio, influence hydrophobicity and surface charge and therefore lead to better settleability. Nitrogen and phosphorus in the effluent wastewater lead to eutrophication of the receiving water bodies\textsuperscript{161}.

1.1.2. Mechanisms of floc formation

A floc is defined as an aggregate of particles created by collisions from smaller particles that adhere to one another by short and long range bonding forces such as van der Waals and electrostatic repulsion and attraction forces\textsuperscript{73}. The shapes of flocs are usually ill-defined, i.e. amorphous or fractal\textsuperscript{73}. Flocs in wastewater treatment plants, like
surface-associated biofilms, consist of microorganisms that grow/reproduce and secrete EPS. Multidisciplinary approaches have helped improve our understanding of flocculation and the state of knowledge regarding processes involved in flocculation. For example, determining how the EPS matrix and its composition may impact the physicochemical and microscopic properties of flocs and hence affect flocculation. The increase of knowledge in all the above properties of flocs has led to the implementation of more optimized systems that allow better settleability of microorganisms in WWTP.

Settleability is defined as the tendency of suspended solids to settle. In the context of wastewater treatment processes, this includes settling of flocculated microorganisms along with other suspended particulates. Careful control of settleability is costly and if not optimized, may lead to a negative impact on the wastewater treatment processes. These impacts include sludge bulking and in severe cases pathogen runoff. Settleability is highly dependent upon flocculation, which is in turn dependent upon physicochemical properties of mixed microbial flocs.

Three major theories have attempted to explain the forces involved in cell bridging and cohesion in mixed microbial communities. The theories attempted to explain forces that lead to floc formation at the nanometer scale\textsuperscript{130}. The first, the Derjaguin and Landau, Verwey and Overbeek (DLVO) theory, assumes that charged particles have a double layer of counter-ions that forms around them. The first layer is known as the Stern layer, and is comprised of tightly associated counter ions. The second layer is known as the diffuse layer, which is comprised of less tightly bound counter ions\textsuperscript{2,145}. DLVO was first theorized to explain laws governing charged colloidal particles and was later adopted by floc researchers to explain how flocs form. Charged particles are
either secreted by microorganisms as part of the EPS or found as suspended particles that are entrapped by microorganisms and incorporated in the flocs. These charged particles form two configurations, i.e. the tightly bound and loosely bound. The EPS has also been sub-classified into two kinds: loosely bound EPS at the exterior similar to diffuse layer and tightly bound EPS in the core that is similar to Stern layer, as in DLVO theory. Furthermore the distribution of charged particles at the diffuse external layer may contribute to the surface charge of the floc and the optimized charge may assist in microbial and particle adhesion to the floc. DLVO theory is believed to be the predominant theory of forces governing floc stability and floc formation. In this thesis, an attempt will be made to test the distribution of EPS constituents at various depths to further examine closely packed EPS constituents at the core versus loosely packed EPS at exterior of flocs.

The second mechanism is known as the alginate theory. Alginate is a negatively charged polysaccharide polymer made up of mannuronic and glucuronic acid subunits. This polymer is stabilized by the presence of calcium and not magnesium. For a list of microorganisms that are responsible for alginate production, please refer to appendix 5.2. It is known that, in the presence of calcium, alginate forms a gel-like structure that stabilizes the EPS structure. Therefore, in some WWTPs, where flocculation is challenging due to environmental conditions, plant operators add significant amounts of calcium to induce flocculation. This is a costly intervention, but necessary in some plants such as the Rothsay wastewater treatment plant (RWWTP), one of the sampling sites for this thesis.
The final mechanism, known as the divalent cation theory, states that neither calcium nor magnesium specifically make a difference in the stability of polysaccharide polymers, but that cations generally act to stabilize and bridge the charge hindrance induced by charged polymers\textsuperscript{145,150}. Floc formation requires nucleation, which may be achieved by the use of divalent cations or the presence of pieces of flocs that may serve as a nucleus for floc formation. Though it is expensive, many wastewater treatment plants across the world use divalent cations to induce flocculation and stabilize the charges on EPS and form stronger flocs for better settleability. Floc researchers tend to focus on DLVO and divalent cation theories, which are more applicable to day-to-day process analysis. A direct application of divalent cation theory could be the addition of divalent cations for troubleshooting when proper flocculation is not observed\textsuperscript{80}. DLVO theory assists studying structure of mixed microbial flocs and how the adhesion and cohesion of microorganisms to floc particles occur\textsuperscript{146}.

1.1.3. Extracellular polymeric substances (EPS) of mixed microbial flocs

The Extracellular polymeric substances (EPS) backbone is made up of biomaterial secreted by microorganisms\textsuperscript{49}. To establish strong microconsortia, microorganisms rely on the production of an EPS matrix, which is responsible for adhesion and cohesion of floc particles, act as a protective barrier and serve as a structural backbone.
Figure 1.1: Architecture illustration of filamentous microorganisms and floc formers. A. Representative phase contrast conventional optical microscopy image of MBR floc showing filamentous and floc formers’ relationship (scale bar = 100 µm). B. Floc model illustration showing the distribution of floc formers around backbone structure formed by filamentous microorganisms.
The extracellular polymeric substance (EPS) matrix is responsible for the structural and functional integrity of flocs and is a major determinant of their physicochemical and biological properties. EPS forms a hydrated three-dimensional gel, which often carries a net negative charge. It is a highly dynamic system in which structures are constantly being formed by biopolymers and digested by enzymes, which are constantly being secreted by microorganisms in flocs. The production of EPS can utilize up to 80% of the cellular energy of bacteria within the flocs. EPS is composed of polysaccharides, proteins, lipids, DNA and humic acids, where protein and polysaccharide make up the largest portion (60%-85%) of EPS. The EPS matrix may serve as a source of nutrients during periods of starvation and therefore plays a vital role in nutrient dynamics within the flocs. Microorganisms in activated sludge produce EPS. Bacteria are responsible for EPS production, but the most abundant producers are microalgae, particularly diatoms. Fungi, which include yeasts and molds, also produce EPS. Filamentous proteins secreted by bacteria provide structural stability for the matrix. These proteins or protein complexes may include flagella, fimbria and pili. Table 1.1 summarizes the composition of EPS.

Microorganisms make up 10% of the dry weight and EPS make up the remaining 90% dry weight of flocs. Protein and carbohydrates, the major constituents of EPS, are together responsible for many functions of EPS including: adhesion, aggregation of bacterial cells, water retention, protection barrier and binding of enzymes.
**Table 1.2:** General constituents of the EPS matrix (Modified from Neu et al. (1999)).

<table>
<thead>
<tr>
<th>EPS Component</th>
<th>Subunits</th>
<th>Substituents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polysaccharides</strong></td>
<td>monosaccharaides, uronic acids, amino sugars</td>
<td>Organic: O-acetyl, N-acetyl, succinyl, pyruvyl Inorganic: sulfate, phosphate</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td>amino acids</td>
<td>Oligosaccharides (glycoproteins), fatty acids (lipoproteins)</td>
</tr>
<tr>
<td><strong>Nucleic acids</strong></td>
<td>nucleotides</td>
<td>-</td>
</tr>
<tr>
<td><strong>(Phospho)lipids</strong></td>
<td>fatty acids, glycerol, phosphate, ethanolamine, serine, choline, sugars</td>
<td>-</td>
</tr>
<tr>
<td><strong>Humic substances</strong></td>
<td>phenolic compounds, simple sugars, amino acids</td>
<td>-</td>
</tr>
</tbody>
</table>
The larger proportionality of protein and carbohydrates compared to other constituents means that the overall properties of floc such as charge, hydrophobicity, amount of nutrients adsorbed and amount of water retained are dictated by EPS protein and carbohydrate. Generally, proteins are known to contribute to a net negative charge in mixed microbial flocs, that is stabilized with positively charged carbohydrates and an appropriate ratio leads to stable flocs with good separation of the liquid-solid interface. The appropriate ratio may lead to optimized hydrophobicity and surface charge and ultimately lead to added stability within the flocs by optimizing the distribution of charged EPS constituents. The added stability would result in better flocculation and enhanced settleability in WWTPs. Therefore it is important to study the protein to carbohydrate ratio at various depths of a floc to understand how each region within the floc contributes to settleability.

It has been established that the EPS protein and EPS carbohydrate (EPS\(_p\):EPS\(_c\)) ratios for the flocs varies greatly among systems, such as conventional treatment plants receiving water from diverse sources and MBRs. Correlation between various physicochemical parameters has been tested against the EPS\(_p\):EPS\(_c\) ratios and it has been observed that parameters such as hydrophobicity and surface charge are strongly dependent upon the EPS\(_p\):EPS\(_c\) ratio. However, all of these studies were based on bulk EPS content analyzed from extracted EPS using various extraction methods. Bulk extraction methods only provide information about overall floc composition. To date, no detailed studies of EPS distribution within flocs of one system have been conducted. Therefore, there is little information about the localized chemical composition, architecture and physicochemical properties of EPS within flocs. The goal of this thesis is
to study EPS content distribution within flocs from various sites and to profile the EPSp:EPSc ratio at various depths of a floc using CLSM.

Although EPS is not solely composed of proteins and carbohydrates, the other constituents such as uronic acids, DNA, lipid and humic acids are generally found in significantly lower quantities. Probing for each of these constituents may reveal significant insight into reactions that occur within the flocs, such as biodegradation of contaminants by the flocs and adhesion of contaminant to the flocs. For instance, humics are naturally found in wastewater and the higher humic content in WWTP flocs may reveal important information. Humic substances are non-settleable organic waste that gives the characteristic yellow color to water. Humics are known to react with chlorine and cause cancer if not properly accumulated or adsorbed to the EPS of microorganisms in secondary treatment. As well, probing for nucleic acids reveals information about cell viability within the flocs. Dead bacteria within WWTP flocs will have their cell walls and cell membranes compromised, with the eventual release of their genetic material to the outside of the cell and becoming part of the EPS matrix. Furthermore, bacteria may utilize DNA in the EPS as a source of phosphorus during periods of starvation. Finally, uronic acids are primary subunits of the microbial cell wall and therefore may be probed as a signature of bacteria. Uronic acids also contribute to the hydrophobicity of flocs and, therefore, are important for settleability.

The dual hydrophobic/hydrophilic nature of the EPS constituents makes the interaction of water with flocs complex. Although all EPS constituents may play a role in hydrophobicity, proteins and carbohydrates play a greater role. The EPS is a complex mixture of hydrophobic and hydrophilic functional groups. The net hydrophobicity
however, could be attributed to proteins. On the other hand, the total polysaccharide content would negatively contribute to hydrophobicity. Polysaccharides linked to methyl and acetyl groups would lead to increased hydrophobicity and proteins could also have hydrophilic functional groups and lead to decreased hydrophobicity. The overall ratio of EPSp:EPSc would be a greater determining factor in overall hydrophobicity. The hydrophilic portion of the EPS can directly interact with the surrounding water molecules, while the hydrophobic regions become chemically and physically bound to other hydrophobic EPS constituents and repel water molecules. Hydrophobicity dictates the amount of water that may bind to the EPS and floc. The higher hydrophobicity would enhance flocculation and also yield better settleability.

Water is classified as bound or free water. Free water separates from flocs with simple mechanical treatments such as centrifugation and thermodynamically acts like pure water. Bound water, on the other hand, remains trapped within the floc matrix and between particles. It will not separate with mechanical treatment. Higher amounts of bound water relate to better settleability and therefore the determination of the amount of bound water is important to the understanding of the floc-water relationship. Research has shown that EPS is capable of binding 15-20 grams of water per gram of EPS. It has also been shown that the interaction of water and EPS is far more complicated than just EPS constituent and quantity of bound water. The structure of EPS along with the quantity of EPS may impact the balance of bound water and free water and as a result influence mass transport in flocs. The hydrophobicity within the EPS matrix determines the porosity of the matrix and, therefore, how much water binds to a floc. Note that DLVO theory defines a core structure of which ions would be closely packed.
Therefore, this suggests all EPS constituents would be closely packed at the core of the flocs. As a result, one would expect a high density of closely packed EPS matrix with minimal pores. The exterior would be loosely packed and be characterized by a porous structure. The higher porosity leads to increased amount of bound water and higher bound water may negatively impact settleability.

1.2. METHODS TO EVALUATE MICROBIAL FLOCS

Floc formation and settling are the processes required after the goal of organic waste removal is completed. The evaluation of settleability and flocculation following the completion of the organic waste removal process can be determined at various scales. At the wastewater treatment plant scale, parameters such as sludge volume index (SVI), mixed liquor suspended solid (MLSS), sludge retention time (SRT) and quantitative assays of organic waste matter content for the influent and effluent waters are important. At the floc scale, the composition of the microbial community, physicochemical parameters and microscopic features are the focus. Although the internal architecture of the floc can be revealed at the nanometer scale using transmission electron microscopy (TEM), it will be listed in the floc scale evaluation section in this thesis. Finally, at the sub-floc level EPS composition and the distribution of EPS constituents become significant. Understanding the distribution of EPS constituents at various depths of floc, is the focus of this research.

1.2.1. Evaluation at the wastewater treatment plant scale

Floc formation and settling in activated sludge can be assessed using two measurements, namely the MLSS and SVI. MLSS and SVI are routine tests at the macro scale to assess performance of the reactor. MLSS is a measure of suspended solids in the
sample. Although flocculation is not greatly affected by the concentration of suspended solids, there are reports in the literature describing the negative influence of high MLSS on effluent quality $^{24,158}$. MLSS is a measure of mixed liquor suspended solids and this measure includes the total weight of microorganisms, EPS, organic waste, suspended waste and any other particulate in wastewater $^{64}$.

SVI is a measure of sludge settleability. SVI is defined as the volume in millimeters occupied by one gram of suspension after 30 min $^5$. It indirectly measures morphology of flocs, and is a physical characteristic of activated sludge $^{99,137}$. SVI is measured at the macro level and it tracks the settling of a sludge sample rather than the settling of one single particle. SVI needs to be measured as a function of MLSS. The MLSS consideration is only accurate for sludge samples up to 4000 mg/L, MLSS values higher than 4000 mg/L would introduce errors in the SVI measurement $^{40}$. This makes SVI theoretically not supported, but it is a useful assessment of process control. Furthermore, since it is simple, inexpensive, and fast this test is still considered to be a routine test $^{40,48}$.

SRT is not a test but an operational parameter that states, how long the sludge has been retained; in other words, it is the cell residence time in a reactor. SRT may influence many other characteristics of activated sludge, including: hydrophobicity, surface charge, surface irregularity and EPS $^{98,99}$.

In addition to SRT, other carefully controlled operational parameters are essential to microbial well-being. Microbial cells could be considered an ongoing progress of evolution and as a result, they demand certain optimized conditions for their survival. These conditions include: pH, temperature, food to microorganism ratio and ratio of
different nutrients\textsuperscript{1,10,80,102}. The above conditions are all necessary for the survival of microorganisms in their niche. In WWTP, the above conditions are not easy to maintain optimally at all times due to parameters such as variability of influent water or weather conditions.

When the above conditions are not optimized, the microbial community may change\textsuperscript{17,31}. Changes in the community may cause inefficiencies in reactor performance along with changes in settleability and/or formation of solid/liquid interfaces\textsuperscript{20,82,119}. Formation of solid/liquid interfaces is dependent on the stabilization of physicochemical properties in a floc\textsuperscript{92,98,104}.

1.2.2. Evaluation at the floc scale

A floc is a complicated structure in which microscopic features such as the population of floc formers versus filamentous microorganisms may influence the drag force and, therefore, affect settleability of flocs. Furthermore physicochemical properties such as surface charge and hydrophobicity may also play a role in settleability as discussed earlier. Finally the internal architecture, i.e. compactness of the floc structure may influence the quantity of bound water and free water and impact settleability\textsuperscript{74}.

Mixed microbial flocs are thick, dense structures and have a complicated architecture, such that various microscopic techniques are utilized to examine the floc structure. Conventional optical microscopy (COM) relies on visible light and due to the random scattering of light at the floc surface, COM may not be able to reveal the internal structure of the flocs\textsuperscript{38,85}. Reliability of confocal laser scanning microscopy (CLSM) data is partially dependent upon homogenous diffusion of fluorescent probes, section thickness and specific binding of the fluorescent probes to the targeted epitope\textsuperscript{90,165}. 

18
TEM includes sample preparation steps with harsh chemicals such as glutaraldehyde and osmium tetroxide for chemical fixation followed by treatment with acetone for dehydration. The processing causes protein reconfiguration, lipid extraction and finally nucleic acid condensation. Each of the above mentioned microscopic methods have built in limitations, biases and artifacts. Therefore, to have results with minimal bias, multiple microscopy tools must be used in conjunction with one another. Use of multiple microscopic tools was first defined by Leppard (1979) as correlative microscopy and successfully applied in the field of wastewater treatment to flocs by Liss et al. (1997).

1.2.2.1. Conventional optical microscopy

Conventional optical microscopy (COM) utilizes transmitted visible light, unlike other modern techniques that may employ reflected laser beams or transmitted electrons. This hinders the maximum image magnification and resolution; i.e. it can only reveal objects larger than 1 µm in size. Floc researchers use COM to study features such as the size of a floc, shape, porosity/density, settling characteristic and identification of filamentous microorganisms. Morphological features such as relative amount of floc formers as compared to filamentous microorganisms can be observed using COM. Despite its shortcoming, COM is a fast and inexpensive method to reveal general features of flocs.

1.2.2.2. Transmission electron microscopy

Transmission electron microscopy (TEM) works much like light microscopy, however, instead of visible light, a beam of electrons is used to image the specimen providing resolutions about 10000 times greater than COM. Typically biological materials are relatively opaque to a beam of electrons and require negative staining to
help define the cellular compartments and to provide contrast to the image. Negative stains contain water soluble heavy metals with salts that provide contrast to the specimen. 

Traditionally, methods used for floc preservation for TEM visualization include dehydration. Conventional methods include a dehydration step and harsh chemical treatment, which may lead to artifacts as opposed to high pressure freezing (HPF). High pressure freezing followed by freeze substitution (HPF-FS) excludes dehydration, which is critical for hydrated structures such as flocs. HPF-FS is not a flawless protocol either. Problems with this protocol include low contrast of subcellular structure and the preparation time of the sample. HPF-FS has successfully been implemented to study the internal architecture of *P. aeruginosa* biofilms, the EPS structure of diatoms, and distribution of bacterial protein. Furthermore, when TEM is used along with other microscopic tools (COM, CLSM) and combined with physicochemical data, it may add information to visualize the nano-scale ultra structure of the floc interior.

Understanding the internal architecture and the compactness of the floc interior may reveal important information about the role played by physicochemical stability of flocs and their settleability.

1.2.3. Evaluation at the sub-floc scale

At the sub-floc level the EPS composition becomes important. A floc is composed of EPS, microorganisms, and organic and inorganic material absorbed into the flocs. The composition plays a strong role in settleability of a floc, because it determines hydrophobicity, surface charge, bound water and free water.
1.2.3.1. Sub-floc level examination with extraction based method

Traditionally, many methods have been employed to examine mixed microbial community EPS. This has included various extraction methods like resin-based extraction, harsh acid and alkaline-based extractions, heat, centrifugation and sonication based extractions \(^1\).

Floc EPS can be classified into bound and soluble EPS \(^9\). Bound EPS can be further sub-classified into loosely bound, tightly bound, and the core region known as the pellet \(^1\). Each of these layers may have different bacterial and EPS compositions \(^9\,10\,12\,16\). Variability in the EPS and bacterial composition suggests that each layer has a different relationship with water and potentially impacts settleability differently \(^10\,16\). EPS extraction is non-specific and extracts all the layers and their constituents simultaneously. Therefore extraction of EPS would not answer questions about each layer and how each layer contributes to settleability.

1.2.3.2. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) exploits light beams at various narrow wavelength ranges to excite fluorescent dyes, termed the excitation wavelength. These excitation wavelengths cause photons to be given off from a fluorescent molecule, which is referred to as emitted light. Thus, fluorescent microscopy relies on emitted light and as a result the thickness of the specimen does not distort the image. CLSM can optically section a sample allowing the capture of multiple cross-sections at different focal planes called Z-stacks. These captured images can be stacked to reconstruct a three dimensional model of the sample, in this case a floc \(^1\). The 3D model can then be used for qualitative data or semi-quantitative data output.
Chen et al. (2007) used multiple staining techniques along with confocal microscopy to study the distribution of EPS constituents within aerobic granules. They found differential EPS distribution in phenol-fed granules versus acetate-fed granules. Granules are similar to flocs in that, they are both suspended microbial agglomerates; however granules are highly dense and are very well settling structures as compared to flocs. Blonk et al. (1995) translated fluorescent intensity to gray scale and furthermore translated the gray scale to computable numbers to measure properties of cells. Utilizing CLSM along with multiple staining techniques, such as EPS specific stains, followed by computation of fluorescent intensity should allow the determination of the EPS composition at different regions within a floc. The CLSM data will complement the physicochemical and compositional analysis through the bulk extraction method.

Researchers have used multiple staining techniques with CLSM to study the three-dimensional structure, and other characteristics of biofilms, flocs and granules. Qualitative assessments include microbial identification by fluorescence in situ hybridization (FISH), and the distribution of cells and cellular components, and the adhesion of microorganisms to flocs. Quantitative measurements include: porosity, volume and weight measurements of biomass. Finally, semi-quantitative evaluations include the heterogeneity of floc constituents such as microorganisms and EPS.

CLSM techniques rely on fluorescent dyes that bind to specific constituents of flocs and make them visible when excited at their specific wavelengths, see Table 1.2. The dyes bind to specific epitopes; however, some of these epitopes could be part of other EPS constituents as well, leading to non-specific binding, which is inevitable. EPS
constituents tagged in this thesis are: proteins using FITC, Concanavalin A used as a universal tag for polysaccharides, DAPI as a universal DNA tag, and finally SBA lectin labeling N-acetyl galactosamine. FITC binds to an amine groups that stains proteins and other amine-containing compounds such as amino-sugars. Concanavalin A binds to α-mannopyranosyl & α-glucopyranosyl sugar residues. DAPI binds to AT rich regions of DNA. SBA lectin specifically binds to α- and β-N-acetyl galactosamine and galactopyranosyl residues.

1.3. RESEARCH SUMMARY

Mixed microbial communities form flocs in WWTP. Flocs are responsible for the biodegradation of complex carbon forms and reduction in concentration of environmentally toxic nutrients such as phosphorus and nitrogen. The nutrients are used for biomass production. Biomass production includes cell creation and secretion of EPS.

The flocs then need to be separated from the water column to enable water to be released back into the environment. This separation of flocs from the water column is the most expensive process in the WWTP. The separation could be achieved by chemical settling induction, such as the use of divalent cations, or through physical processes such as centrifugation. The settling of bacteria is dependent upon physicochemical properties such as surface charge, hydrophobicity, amount of bound water and floc size. The population of filamentous microorganism versus floc formers may also play a role, but this is considered an ecological characteristic, and not a physicochemical property. Given that EPS makes up the largest surface area of floc, the chemical composition of EPS may govern the physicochemical properties.
<table>
<thead>
<tr>
<th>Stain</th>
<th>Cellular component</th>
<th>Location</th>
<th>Sample types</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacLight Live-Dead staining kit,</td>
<td>DNA</td>
<td>In cells with and without compromised cell membranes and other DNA outside cells with compromised membrane</td>
<td>floc/biofilm/granule/river snow</td>
<td>157, 162, 163, 164</td>
</tr>
<tr>
<td>FITC</td>
<td>Protein</td>
<td>Outside cell and EPS constituents</td>
<td></td>
<td>154, 155, 104, 163, 164, 166, 167, 170, 171, 172</td>
</tr>
<tr>
<td>Lectins</td>
<td>Carbohydrates</td>
<td>Outside cell and EPS constituents</td>
<td></td>
<td>153, 152, 155, 156, 158, 159-163, 166-168, 170, 171</td>
</tr>
<tr>
<td>DAPI</td>
<td>DNA</td>
<td>Outside and inside cells</td>
<td></td>
<td>155</td>
</tr>
</tbody>
</table>
EPS consists proteins, carbohydrates, humic acid and DNA. The largest fraction is covered by proteins and carbohydrates\textsuperscript{49}. The chemical composition collectively induces local forces and long-range forces best explained by DLVO theory. DLVO theory explains that there is a double layer of counter ions, a short-range strong force, followed by long-range weak forces.

Given the fact that the major EPS fractions are composed of proteins and carbohydrates, various studies have examined the ratios of EPS\textsubscript{p} to EPS\textsubscript{c} within different systems\textsuperscript{7,25,39,50}. These studies included extraction of EPS and the use of colorimetric analysis to quantify overall quantity of these constituents within flocs from each system. Floc EPS, however, can be classified into bound and soluble\textsuperscript{168,169}. The bound could further be sub-classified into loosely bound, tightly bound and the pellet\textsuperscript{88,169}. Yu et al. (2008) reported that each of these layers have different properties and water binding capabilities by using differential centrifugation to extract the different EPS layers and measuring the water binding capability of each. Furthermore, Li and Yang (2007) reported that loosely bound EPS is responsible for attachment of new cells, and therefore concluded that understanding the properties of this layer is important.

It has been well established that the EPS composition, and more specifically the EPS\textsubscript{p} and EPS\textsubscript{c} ratio, is responsible for the overall physicochemical properties of flocs. Studying the overall EPS composition through the bulk extraction method will not reveal the characteristics of each region\textsuperscript{25}. However, studying the EPS composition within each region of a floc along with overall physicochemical properties has been lacking in the literature. This thesis has attempted to combine the two and to study EPS constituents at various depths of a floc along with a study of EPS constituents using the cation exchange
resin (CER) extraction method to determine the physicochemical properties of each zone within the flocs.

1.3.1. Hypothesis

I hypothesize that the EPSp:EPSc ratio will vary within mixed microbial flocs and that a higher relative abundance of EPSp will be located at the core of the flocs, as compared to the outer layers of flocs. To test this hypothesis, I propose to use the following objectives to evaluate and compare flocs from three distinct wastewater treatment sites:

**Objective 1.** Obtain floc samples from three distinctive sources (Guelph wastewater treatment plant from the full-scale conventional system and the pilot-scale membrane bioreactor system and the Rothsay wastewater treatment plant). Evaluate the performance and operational parameters of each of the three selected sampling sites. Evaluation of operational parameters would allow for close examination of the environmental conditions of each sampling site. The examination of performance parameters would relay information about the performance of microorganisms and about utilization of electron donors and electron acceptors by the microorganisms in WWTPs.

**Objective 2.** Determine the eubacterial composition of the flocs from the various systems using standard molecular techniques. This allows for examination of overall variability in the bacterial composition of each sampling site.

**Objective 3.** Determine the macroscopic (bulk) physicochemical characteristics and the chemical compositions of microbial flocs from the three sampling sites. This will reveal the overall physicochemical characteristics of flocs and will allow for verification
of the correlations between the physicochemical characteristics and the chemical composition of EPS.

Objective 4. Visualize the gross morphological features, internal architectures and distributions of EPS content (EPSp:EPSc ratio), using correlative microscopy and small angle x-ray scattering (SAXS). The architecture will reveal important information about the relationship between settleability and the architecture of the flocs. Furthermore, revealing the exact location of EPS constituents may reveal information about zones within flocs that may contribute more to the settleability and physicochemical stability of the flocs.
CHAPTER 2
MATERIALS AND METHODS
CHAPTER 2. MATERIAL AND METHODS

Sample collection

For all of the tests, the required volume of grab sample of mixed liquor from aerated reactors was collected in clean autoclaved glass jars, from each of the sampling sites. The selected sampling dates are stated in the specific method section pertaining to the test. The sampling events are meant to capture a snapshot of microorganisms within an aerated basin of a secondary wastewater treatment unit from each sampling site for a comparison of the three sampling sites. The sampling sites include: the municipal waste treatment unit Guelph wastewater treatment plant (GWWTP) and the membrane bioreactor (MBR) unit were both treating municipal waste from Guelph, Ontario. Rothsay wastewater treatment plant (RWWTP) unit was treating effluent from a rendering plant; RWWTP is located in Dundas, Ontario. The samples were carefully poured in wide neck sample containers and stored at 4 °C in a cooler with ice packs inside and carried to the lab for further analysis.

2.1. ECOLOGICAL COMPOSITION
2.1.1. DNA extraction

The samples collected for DNA extraction were immediately processed. The biomass was concentrated by centrifuging 50 mL from each sample at 20000X g for 5 min at 4 °C. The supernatant was decanted and 200 mg of concentrated biomass was weighed in 2 mL Eppendorf tubes to the nearest 5 mg. The DNA was extracted from the concentrated biomass (200 mg) using the QIAamp DNA Stool Mini Kit, following the pathogen extraction protocol. 1.4 mL ASL buffer was added to the biomass and vortexed for 2 min until completely homogenized and then heated at 95 °C for 5 min to lyse the cells. Samples were then vortexed for 15 sec and centrifuged at 13000X g for 1 min.
mL of the supernatant was transferred to a new sterile microcentrifuge tube, and 1 inhibitEX tablet was added (QIAGEN, Valencia, U.S.A.). The sample was vortexed for 1 min. The resulting matrix was centrifuged at 13000X g. The supernatant was transferred to a new tube and centrifuged at 13000X g again to remove any remaining traces of the inhibitEX tablet. Then 200 µL of supernatant was transferred to a new tube and 15 µL of proteinase K and 200 µL of AL Buffer were added to digest all proteins. The mixture was vortexed for 15 seconds and incubated at 70 °C for 10 min. Following the incubation, 200 µL of ethanol was added to the sample and mixed by vortexing. The mixture was then transferred to a QIAamp spin column and centrifuged to bind the DNA to the column. The spin column was washed with 500 µL of AW1 buffer first and then with 500 µL AW2 buffer. The DNA was eluted with 200 µL of AE buffer. The presence of DNA in the extracts was verified by running the samples on a 0.8 % agarose gel to compare band intensities with 2 µL of DNA ladder. DNA samples were stored at -20 °C until further analyses.

2.1.2. DNA amplification

Platinum PCR SuperMIX High Fidelity kit (Invitrogen, Burlington, ON) was used for amplification of the 16S rDNA gene, the isolated genomic DNA from the three sampling sites. The kit included optimized concentrations of Taq polymerase, magnesium sulphate, deoxyribonucleotide triphosphates (dNTP’s) (dATP, dCTP, dGTP and dTTP) and buffer. The primers 341F + GC clamp and 534R \(^{30}\) 341F (CCTACGGAGGCAGCAG) with GC clamp at the 5’ end and 534R (ATTACCGCGGCTGCTGG) were optimized for concentration 250 nm and a final concentration of 500 nM was used for both forward and reverse amplification. For each primer a concentrated stock solution (100 µM) was prepared and stored at -20 °C.
Working solutions were made from stock solutions such that when 3 µL of working solution was added to the 50 µl PCR reaction, the PCR reaction tube would have a total of 250 nM primer concentrations. To make 50 µL PCR reaction, 40 µL of PCR master mix was added to 3 µL of each of the forward and reverse primers and 4 µL of template DNA which would make for a total primer concentration of 250 nM. The positive control included 4 µL of genetic material extracted from a pure culture of *E. coli* and the negative control included 4 µL of RNA/DNA free water. Amplification was performed in a BioRad C1000 Thermo cycler and the overview of reaction sequence is summarized in Table 2.1.

PCR products were verified by running 2 µL aliquots on 2% agarose gel electrophoresis in TAE buffer with a 100 bp DNA ladder (Promega®, Madison, WI). A single clean band at about 200 bp was expected.

2.1.3. *Denaturing gradient gel electrophoresis*

Denaturing gradient gel electrophoresis (DGGE) solutions were prepared in advance and stored away from UV light at 4 °C. A 30%-70% urea denaturing gradient was selected to examine the bacterial diversity of the 200 bp PCR products prepared as outlined in section 2.1.2. The 30% gradient was composed of the following constituent: 12 mL formamide, 12.6 g of urea, 20 mL of 40% acrylamide : Bis, 2 mL of 50X TAE and NANOpure water to make the final volume 100 mL. The 30% gradient was composed of the following constituents: 28 mL formamide, 29.4 g of urea, 20 mL of 40% acrylamide : bis, 2 mL of 50X TAE and NANOpure water to make the final volume 100 mL. The reagents were prepared and filtered through 0.45 micron pore size Nitrocellulose (Cat #166-2807EDU, BioRad, Mississauga, Canada) filter paper. The gel solutions were
Table 2.1: PCR cycle sequence for mixed microbial community for 3 sampling sites.

<table>
<thead>
<tr>
<th>Step #</th>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Time (min:sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initiation</td>
<td>94</td>
<td>2:00</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>00:30</td>
</tr>
<tr>
<td>3</td>
<td>Anealing</td>
<td>58.2</td>
<td>00:30</td>
</tr>
<tr>
<td>4</td>
<td>Elongation</td>
<td>68</td>
<td>01:00</td>
</tr>
<tr>
<td>5</td>
<td>Final elongation</td>
<td>72</td>
<td>5:00</td>
</tr>
<tr>
<td>6</td>
<td>Storage</td>
<td>12</td>
<td>∞</td>
</tr>
</tbody>
</table>
prepared and casted in between glass plates as per the manufacturer’s instructions (BIO RAD, Mississauga, Ontario).

PCR products were prepared for DGGE using the Wizard® SV Gel and PCR Clean-Up System (Promega®, Madison, WI). The DNA concentration of the PCR products was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware) at 260 nm. Preliminary tests indicated that 1500 µg of PCR product was the optimal concentration for DGGE and therefore 1500 µg was loaded into each DGGE well. The gel was run using a DCode™ Universal Mutation Detection System (BIO RAD, Mississauga, Ontario) at 100 V for 16 hr in 1X TAE buffer at 60 ºC. Next, the gel was stained in a bath of 1% (v/v) SYBR SAFE gel stain (Invitrogen, Burlington, ON) with shaking for 30 min. The gel was imaged on a Gel Doc™ XR+ System (BIO RAD, Mississauga, Ontario) under UV illumination.

The gel image was saved in TIFF format and imported into GeneTools (Syngene, Frederick, MD) to define boundaries of each band in the gel. The boundaries are defined in 2 dimensions: the width of bands and the distance traveled from the bottom of well to the bottom of gel. Furthermore, discrepancies such as contaminated spots were removed from analysis using the software to define contamination spots and exclude them from any further calculations. Contaminating spots were not defined by particular size or intensity threshold but by their position away from the DNA bands. GeneTools translated the bands as peaks with defined height and volume under peaks. The intensity of the band was translated into peak height and the width of band into volume of the curve. This information was then exported to the GeneDirectory software (Syngene, Frederick, MD) to generate a cluster analysis, a similarity matrix and a dendrogram as outputs with
default settings and no further adjustments. Finally, the information provided by GeneTools was submitted to Primer6 (Primer-E®, Fullerton, CA) to determine the correlation coefficient between each sampling site and sampling event, the coefficient of correlation was established based on a 95% confidence level.

2.2 PHYSICOCHEMICAL CHARACTERISTICS

2.2.1. Physical analysis

2.2.1.1. Mixed liquor suspended solid (MLSS)

One nalgene glass prefiter (Cat#0974023, Thermo Fisher Scientific, NY, USA) was placed in each separate aluminum weigh boats, weighed individually and the weights recorded as B in equation 2.1. The filter paper was then mounted on a filtration apparatus and 5 mL of mixed liquor sample was filtered through the filter paper by vacuum suction. The filter paper was placed in its respective aluminum weigh boat and dried in an oven at 103-105 °C for 4 hours. The filter papers and weigh boats were weighed again, the value represented by A in equation 2.1. These values were used to compute the MLSS as in equation 2.1.

\[
MLSS (mg/L) = \frac{(A-B) \times 1000}{sample \ volume} \quad [Eq. 2.1]
\]

2.2.1.2. Sludge volume index (SVI)

1 L of mixed liquor sample was poured into a graduated cylinder. The graduated cylinder was then incubated on a flat surface at 23 °C without any physical disruption for 30 min and the SVI was computed using Equation 2.2. The settled sludge and the
suspended sludge was visually assessed by looking at the solid/liquid interface formed \(^{48}\).

Three biological replicates were collected and each biological replicate included only one
technical replicate and sampling events included samples collected on June 12\(^{th}\), August
20\(^{th}\) and September 8\(^{th}\).

\[
SVI (mL/mg) = \frac{settled sludge volume (mL/L) \times 1000}{suspended solids (mg/L)}
\]  \hspace{1cm} [Eq. 2.2]

2.2.1.3. Bound water measurement in mixed microbial flocs

Bound water was determined using a dilametric technique first developed by
Weisberg and Heukelekian\(^{74}\). 500 mL sludge samples were concentrated to 10000 ±
2000 mg/L by centrifugation at 13000X g for 5 min followed by resuspension of the
pellets in smaller volumes of water. The amount of water used to resuspend the sludge
sample was determined by the original MLSS i.e. if the original MLSS was 5000 mg/L,
the sludge was resuspended in half as much water to achieve 10000 ± 2000 mg/L. Total
water content was measured for the concentrated sample at 105 °C using a 10 mL sample
to ensure 10000 ± 2000 sample concentration was reached for the bound water
measurements. The dilameter (Knutes Chemistry and Life Science Products, Mississauga,
Canada) was filled with 5 mL of concentrated sludge sample and then filled with 20 mL
hydraulic jack oil (AW32, Motomaster, China). The hydraulic jack oil was tested for the
four selection criteria put forward by Robinson and Knocke\(^{132}\), namely: (1) immiscible
with water, i.e. would not form a homogenous mixture with water, (2) specific gravity
less than 1.0, which allows formation of a distinct layer on top of the water column, (3)
linear expansion/contraction over temperature range -20 °C to 20 °C, and (4) to ensure
that the fluid does not freeze at -20 °C. The dilameter was filled with the sample and was immersed in a constant temperature water bath circulator (Model # 1150A, VWR, Mississauga, Canada) and allowed to stabilize at 20 °C for 15 mins. The initial reading was recorded and the circulator was cooled gradually from 20 °C to -20 °C in 10-degree increments while the dilameter was immersed in the water bath circulator. At each 10°C interval the change in expansion/contraction of the sample and hydraulic oil was recorded. Linear expansion/contraction of hydraulic oil over the temperature range -20 °C to 20 °C was previously determined, so that only the change in expansion and contraction of the concentrated sludge sample were accounted for in Equation 2.3. Finally, the bound water was computed using equation 2.3. Three biological replicates were collected and each biological replicate included only one technical replicate and sampling events included samples collected on June 14\textsuperscript{th}, August 25\textsuperscript{th} and September 12\textsuperscript{th}.

\[
\text{Bound water} \left( \frac{g}{g \text{ MLSS}} \right) = \text{Total water} - \text{free water} \quad [\text{Eq. 2.3}]
\]

2.2.1.4. Hydrophobicity of mixed microbial flocs

The hydrophobicity of the flocs was assessed by the microbial adhesion to hydrocarbons (MATH) method\textsuperscript{133}. 100 mL of sludge sample was pelleted by centrifugation at 3000 g for 5 min and then suspended in 50 mL of NANOpure water. The suspension was then brought back to the 100 mL original volume using NANOpure water; this procedure was repeated 3 times. The sample was then diluted, sonicated for two-minute intervals to disperse the particulate matter evenly and adjusted to initial absorbance (I\textsubscript{0}) of 1.5 ± 0.02 using NANOpure water at a wavelength of 400 nm. The samples were sonicated multiple times before the required initial absorbance was
achieved. While sonicating, the sample container was placed in an ice bath. 10 mL of adjusted sample were then mixed with 1 mL of hexadecane using a vortex mixer for 2 min. The mixed sample was then poured in a separatory funnel and allowed to stand for 10 min at 20 °C. The aqueous phase was collected, the absorbance was measured at 400 nm and values were recorded as (I) final absorbance. Percentage hydrophobicity was then calculated using Equation 2.4. Three biological replicates were collected and each biological replicate included only one technical replicate Sampling events included samples collected on June 15th, August 26th and September 13th.

\[
\% \text{Hydrophobicity} = \frac{(I_f - I)}{I_0} \times 100 \quad [\text{Eq. 2.4}]
\]

2.2.1.5. Surface charge of mixed microbial flocs

The surface charge was measured by the zeta potential method based on electrophoretic mobility developed originally by Forster. The activated sludge sample was diluted using NANOpure water and adjusted to total MLSS of 2000 mg/L ± 1000 mg/L. The sample was then mixed well without vortexing and 5 mL of the adjusted sample was carefully injected into specially designed cuvettes (CAT# DTS0012, Malvern Instruments, Worcestershire, United Kingdom). The sample was injected slowly to avoid injection of air bubbles. The cuvette was then inserted into a zeta potential reader (Zeta Sizer NanoZ, Malvern Instruments, Worcestershire, United Kingdom). The Zeta potential instrument was connected to a computer, where previously setup settings for WWTP sample were selected. The instrument examines the surface charge of hundreds of flocs at the same time to compute the surface charge of each sampling site by inducing a charge to the entire chamber and monitoring changes in OD of one region within the chamber.
Three biological replicates were collected and each biological replicate included only one technical replicate and sampling events included samples collected on June 15th, August 26th and September 13th.

2.2.1.6. Particle size distribution of wastewater treatment flocs

The particle size distribution was measured using a Mastersizer 2000 Hydro MU (Malvern Instruments, Worcestershire, United Kingdom). The beam and laser path was washed 6 times with 800 mL distilled water, primed with an additional 800 mL distilled water, primed with an additional 800 mL of distilled water and the pump speed was then adjusted to 500X g. Each run measured particle size for 2000 particles or more within the sample. The instrument was calibrated on a monthly basis therefore no positive control was run. Samples were then added slowly to allow for laser obscuration of 15 % and then particle size was measured for each sample. Three values were determined for each sample: the distribution of the smallest particles in a sample in d(0.1), the mean particle size in d(0.5) and the distribution of the largest particle size in d(0.9) \(^{33}\). Three biological replicates were collected and each biological replicate included only one technical replicate Sampling events included samples collected on June 16th, August 27th and September 14th.

2.2.2. Chemical analyses

2.2.2.1. Extracellular polymeric substance extraction via cation exchange resin method

An effective EPS extraction procedure is one that causes minimal cell lysis and that does not disrupt the exopolymer layer \(^{60}\). However, there is no single standard protocol for optimum EPS extraction \(^{152,168}\); Frolund’s cation exchange resin (CER) method was used in this thesis for EPS extraction \(^{57}\). EPS extraction buffer was prepared
as follows: \( \text{Na}_3\text{PO}_4 \) (0.7603 g/L); \( \text{NaH}_2\text{PO}_4 \) (0.55195 g/L); \( \text{NaCl} \) (0.5260 g/L); KCl (0.07455 g/L); \( \text{ddH}_2\text{O} \) (pH=7). 200 mL of activated sludge was settled by gravity to 50 mL and the total volume was reduced to 66 mL. The sample was then washed with 10 mL of EPS extraction buffer 3 times by centrifugation at 2000 g at 4 °C. The final pellet was then resuspended in 5 mL EPS extraction buffer, vortexed and then brought up to the original volume (200 mL) using the extraction buffer. MLSS was measured with the standard method by using 5 mL of the washed sludge solution. Since the amount of sodium based cation exchange resin-Dowex (CAS # 150604-77-6, Sigma-Aldrich, Inc., Oakville, Canada) to be used for EPS extraction depends upon the sample MLSS, Equation 2.5 was utilized to determine the specific amount of CER to add to each individual sample.

\[
\text{CER(g)} = \text{MLSS (g/L)} \times \text{Volume of Sludge (L)} \times 80 \quad \text{[Eq. 2.5]}
\]

Following the addition of CER, the sample and CER was stirred for 2 hours in a beaker at 500X g on an ice bath to prevent excessive heat. Lastly, the sample was centrifuged at 12000X g at 4 °C to pellet the biomass and the supernatant was aliquoted into 1.5 mL Eppendorf tubes, such that each tube contained 1.5 mL of sample and stored at -20 °C until further analysis. Three biological replicates were collected and each biological replicate included only one technical replicate. Sampling events included samples collected on June 20\textsuperscript{th}, August 29\textsuperscript{th} and September 15\textsuperscript{th}.

All chemical analyses to determine polysaccharide (both total and acidic), protein, humic acid, and DNA contents were performed in microtitre plate assays (Cat#07-200-706, Fisher scientific, Ontario, Canada). Each assay included EPS from each sampling site and included a technical replicate from each of the three biological replicates and
triplicate standard curves were performed. The plates were then read in a FLUOstar OPTIMA microplate reader (BMG LABTECH GmbH, Offenburg, Germany) at an appropriate wavelength as indicated in the sections below.

2.2.2.2. Determining total polysaccharide concentration

Total polysaccharide content was measured using Gaudy’s method\textsuperscript{58}. A solution of 400 mg/L D-glucose was used to prepare a 6 point standard calibration curve in the range of 0-200 mg/L\textsuperscript{57}. Fresh anthrone solution (0.08 g anthrone in 10 mL of 97% H\textsubscript{2}SO\textsubscript{4}) was made before each quantitation experiment, which was stored at -20 °C until use. Appropriate amounts of D-glucose solution were mixed with NANOpure™ water (Cole-Parmer Canada Inc., Quebec city, Canada) to a final volume of 66 µL to produce a 6 point standard curve containing concentrations from 0-200 mg/L. Next, 1 mL of EPS extraction from each sample was thawed and 66 µL from each sample was transferred to individual wells in the microtitre plate for polysaccharide. The rest was used for other tests. To each reaction well, 166 µL of anthrone reagent was added and mixed by pipetting up and down several times. The microtitre plates were then incubated at 100 °C for 15 min and immediately cooled in an ice bath for 20 min before the colorimetric measurement was completed. The results of the colorimetric assay were measured in a microplate reader at a wavelength of 625 nm. Note that the unknown concentration of polysaccharides in the sludge samples was determined by comparing to the glucose standard curve.

2.2.2.3. Determining acid polysaccharide concentration

The acid polysaccharide content was measured using a modified $m$-hydroxydiphenyl sulfuric acid method of Filisetti-Cozzi and Carpita\textsuperscript{47}. A standard
solution of 200 mg/L D-glucuronic acid solution was used to produce a 6 point standard curve of 0-100 mg/L. The following reagents were made a day prior to the experiment and stored as indicated until use: (1) 4 M of sulfamic acid-potassium sulfamate: prepared by mixing 39.16 g of sulfamic acid powder in 100 mL of NANOpure water and dissolved using saturated KOH. The final pH was adjusted to 1.6 using KOH solution and the solution stored at 4 °C. (2) 75 mM of sodium tetraborate (w/v) in 97% H₂SO₄ was prepared and stored at -20°C. (3) 0.15% (w/v) m-hydroxydiphenyl in 0.5% (w/v) NaOH was prepared and stored at 4 °C. The assays were prepared as follows: 40 μL of either solutions with known concentration of D-glucuronic acid or extracted sludge sample were mixed with 4 μL of sulfamic acid-potassium sulfamate by pipetting up and down several times. 240 μL of sodium tetraborate reagent was then added to each reaction well and the microtitre plate incubated at 100 °C for 20 min followed by cooling in an ice bath. Next, 8 μL of m-hydroxydiphenyl was added to each reaction well, mixed by pipetting and incubated at 4 °C for 10 min. Lastly, the absorbance was measured at 525 nm.

2.2.2.4. Determining protein concentration

The protein content of EPS was quantified using the Lowry method. A standard solution of 500 mg/L bovine serum albumin (BSA, concentration of BSA) was used to produce a 6 point standard curve of 0-250 mg/L. The following reagents were made fresh before the experiment and not exposed to light until used: (1) 0.25 g CuSO₄.5H₂O dissolved in 50 mL of 1% (w/v) sodium tartrate; (2) 20 g Na₂CO₃ in 1L of 0.1N NaOH; (3) solution 2 and 1 were mixed in a 25:1 ratio, respectively; (4) Diluted Folin and Ciocalteu’s Phenol (FCP) reagent was prepared by mixing the FCP reagent in a
1:1 ratio with NANOpure water. The microtitre plate protein assays were prepared as follows: in the appropriate wells 100 µL of either protein standard or extracted sludge sample was mixed with 50 µL of reagent 3 by pipetting several times. The assays were incubated at room temperature (22 °C) for 10 min. To each assay, 5 µL of diluted FCP reagent was added and mixed by pipetting. The assays were incubated for 30 min at room temperature (22 °C). Finally the absorbance was measured at a wavelength of 750 nm in a plate reader.

2.2.2.5. Determining humic substance concentration

The humic substance content of EPS was quantified using a modified Lowry et al. method. Standard solutions of 400 mg/L humic acid were used to produce a 6 point standard curve of 0-200 mg/L. The following reagents were made fresh before the experiment and not exposed to light until use: (1) 20 g Na₂CO₃ in 1L of 0.1N NaOH; (2) Diluted FCP reagent was prepared by mixing the FCP reagent in a 1:1 ratio with NANOpure water. The microtitre plate assays were prepared as follows: in the appropriate wells 40 µL of either a humic acid standard or extracted sludge sample was mixed with 200 µL of reagent 1 and mixed by pipetting several times. The assays were incubated at room temperature (20 °C) for 10 min. Next, 2 µL of diluted FCP reagent was added to each assay and mixed by pipetting. The assays were incubated for 30 min at room temperature (20 °C). Finally, the absorbance was measured at a wavelength of 735 nm in a plate reader.

2.2.2.6. Determining double stranded DNA concentration

The double stranded DNA content within the EPS samples was quantified using a fluorescence based DNA Quantitation kit (Product #DNAQF-1KT, Sigma-Aldrich, Inc,
A 6 point DNA standard curve with concentrations between 0-1 mg/L was prepared using calf thymus DNA (1 mg/mL solution, Product #DNAQF-1KT, Sigma-Aldrich, Inc, Oakville, Canada).

Prior to each experiment a 1 µg/mL bisbenzimide H33258 solution was made by mixing 10 µL bisbenzimide H33258 solution with 1 mL of 10X fluorescent assay buffer and 9 mL of NANOpure water to make up a total volume of 10 ml (Reagent 1). Each reaction consisted of 200 µL of Reagent 1 and 5 µL of either DNA standard or extracted EPS sample (as described in section 2.2.2.1) that was mixed by pipetting several times. The assays were incubated at room temperature (20 °C) for 1 min. Black 96 well microtitre plates (Product #3991, Corning Inc, NY, USA) covered with aluminum foil were utilized to avoid photobleaching. Lastly, the fluorescence was measured in a plate reader, at an excitation wavelength of 360 nm and emission was detected at 460 nm.

2.3. MORPHOLOGICAL EXAMINATION OF MIXED MICROBIAL FLOCS

2.3.1. Conventional optical microscopy (COM)

Fresh sludge samples were obtained from the three sampling sites. For COM analyses, 10 µL of a sludge sample was placed on a glass slide and covered with a cover slip. Samples were collected on June 23, 2010, August 7, 2010 and September 20, 2011. Each sampling event included examination of three slides of 10 µL sludge samples and 3-4 flocs were examined per slide. Samples were examined unstained using a Reichert Leica 410 Microstar IV Laboratory microscope by phase contrast at magnifications of 400X and 200X.
2.3.2. Confocal laser scanning microscopy (CLSM)

10 mL of fresh samples were fixed with neutral buffer formalin (NBF) at pH 7.3, then embedded in cryomatrix embedding resin (product# 6769006, Thermo fisher scientific, Wilmington, Delaware) and sectioned in 50 µm thick sections with a cryo-microtome. The sections were produced at -20 °C (Cryostat Leica CM3050 S, Wilmington, Delaware). The sectioned slices were placed on microscope slides and stored at -20 °C until stained; slides left for more than a week were discarded 13.

Some of 20-30 flocs from each sampling site were stained with FITC, ConA, SBA lectin and DAPI for visualization of different components of the EPS (Table 2.2). One negative sample was carried with an absence of flocs and while all the stains were added and no fluorescent was observed. 300 µL of each of FITC, ConA and SBA lectin stains were pipetted onto the floc sections attached to the microscope slides respectively 25. The final concentration and staining times are stated in Table 2.2. Following staining, the sections were washed with (1 mL) phosphate buffered saline (PBS) (0.1 M, pH 7.4). The samples were then stained with DAPI, (300 µL) were pipetted onto the sections for the time and at the final concentration indicated in Table 2.2. Following DAPI staining, the sections were washed with indicated volumes of PBS. One drop of ProLong® Gold antifade reagent (Cat# P36934, Invitrogen, Burlington, Canada) was added to each slide and slides were covered with cover slides and sealed with nail polish. The slides were left overnight in a dark room at 20 °C before they were examined by CLSM.

Separate 8-15 floc sections from each sampling site were stained with the Bacto Live/Dead stains to assess the amount of live cells in flocs of different sampling sites 153. Manufacturer’s instructions were followed without any further modification. The times
and concentration were the same as in Table 2.2. One drop of ProLong® Gold antifade reagent (Cat# P36934, Invitrogen, Burlington, Canada) was added to each slide and slides were covered with cover slides and sealed with nail polish. The slides were left overnight in a dark room at 20 °C before they were examined by CLSM. To assess cell membrane integrity of the bacteria within flocs of various sampling sites a pure culture biofilm of *E. coli* JM109 was used as a positive control of PPI. The pure culture of *E. coli* JM 109 was in the exponential growth phase as determined by measurements of OD<sub>600</sub>.

A Leica DM 6000B confocal laser-scanning microscope equipped with a Leica DFC350FX digital camera was used to image the stained floc sections. The Leica DM 6000B confocal laser-scanning microscope consisted of 8 lasers with the following wavelengths: 458 nm, 476 nm, 488 nm, 496 nm, 514 nm, 543 nm, 594 nm, 633 nm and a 690-1040 nm tunable laser. The LAS AF software was used to capture images. Excitation and emission wavelengths were inputted into the software and each dye was separately excited and two emission images were captured by the software to allow for averaging. 20-30 flocs were examined for each sampling site to compute EPS constituent analysis and 8-15 flocs were examined for live/dead analysis from each system. Flocs were located using the phase contrast component of a Leica DM 6000B confocal laser-scanning microscope. Then using the LAS AF software, a floc was selected and clear contrast was made between the floc and the scattered signal around the floc. This was repeated for each stain to ensure that the exposure and imaging parameters were optimized. The depth of field was defined by manually focusing through the floc depth once and the information was stored in the LAS AF software. The imaging software was setup to automatically obtain images every 0.3 µm and create a series of virtual cross-
sections through the entire floc (Leica TCS SP5 LASAF New User Guide, Leica, Germany). The software averaged the images captured at each depth of floc and the resulting output was stored on the computer for Live/Dead analysis and EPS composition analysis.

The images were then imported to the LAS AF software and the intensity stack profile option was selected to obtain numerical values for the intensity of PPI and Syto9. The software converts the intensities of PPI and Syto9 separately into grey scale and computes the intensity of each as a numerical value. The ratio of the two was then computed as the relative number of live cells using Equation 2.5. The same procedure was followed on floc samples and the ratio for the positive control was normalized to 100%. The fluorescent intensity of Syto9 and PPI values obtained from the test flocs were computed against the positive control \(^{153}\). The percentage of live cells was calculated using Equation 2.5.

\[
\text{%Live cell} = \left( \frac{\text{Fluorescent intensity of Syto9} - \text{Fluorescent intensity of PPI}}{\text{Fluorescent intensity of Syto9}} \right) \times 100\% \quad [\text{Eq. 2.5}]
\]

To assess EPS\(_p\):EPS\(_c\) ratios, the stack images were loaded into the LAS AF software and the intensity stack profile option was selected to obtain numerical values for the intensity of FITC and ConA. The software converts the intensities of FITC and ConA separately into grey scale and computes the intensity of each as a numerical value \(^{16}\). Average values for thickness of floc material within each section was computed and found to be \(\sim 15 \, \mu m\), and therefore a random unit-less value of 15 was selected to rate the thickness of flocs. All flocs were given a standard size score from a minimum of 0 to a maximum of 15. The intensity average for all flocs for FITC and ConA signal was plotted
from standardized values between 0-15 at increments of 1. Then the average ratio of proteins to carbohydrates was plotted for the flocs from three sampling sites.

2.3.3. Transmission electron microscopy (TEM)

1.5 mL of fresh floc samples were obtained and settled by gravity in 1.5 mL Eppendorf tubes. As much supernatant water as possible was removed carefully using a pipette. The thickened sludge was resuspended in 1.5 mL of 10% (w/v) sucrose solution. The samples were then placed on sapphire disks and immobilized by a Leica EMPact high-pressure freezer. The sample was then transferred to cryovials containing 2% (w/v) osmium tetroxide (OsO₄) and freeze substituted using Leica EM AFS2 using the protocol outlined in Table 2.3.

The sample was then washed in acetone for 1 hr and infiltrated with Epon-Acetone mixtures as follows: 1:3 for 2 h, 1:2 for 2 h and 3:1 2 h. Finally, 100% Epon was added and samples were incubated overnight at 60 °C. Thin sections (~5 µm) were prepared with a microtome and samples were viewed under TEM CM-10 (Philips) operating at 80 kV and images were captured using SIS Morada CCD with iTEM Software (Olympus, Canada).

2.4. STATISTICAL ANALYSIS

2.4.1. One-way ANOVA

A one-way ANOVA test with a 95% confidence level was done on each parameter tested from the three-selected sampling sites to test statistical significance and variability in floc properties. The physicochemical parameters such as surface charge, hydrophobicity, floc size distribution and bound water were tested for statistical significance. EPS constituents, mainly EPSp:EPSc ratios, were also tested using two
methods: micro analysis using CLSM and macro analysis via EPS extraction followed by colorimetric analysis.

2.4.2. Correlation and regression analysis

Correlation and regression analysis were done on pairs of parameters tested from the three selected sampling sites to test statistical significance and the relationship in floc properties. Regression and correlation tests were done on pairs of data where literature suggested that there might be correlation. The physicochemical parameters such as surface charge, hydrophobicity, floc size distribution and bound water were tested for statistical significance. EPS constituents, mainly EPSp:EPSc ratios, were also tested using two methods: micro analysis using CLSM and macro analysis via EPS extraction followed by calorimetric analysis. EPS constituents using the microscopy method were also tested for their statistical correlation with physicochemical properties.
Table 2.2: Stains used for tagging EPS constituents of WWTP flocs.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Emission (λ)</th>
<th>Target component</th>
<th>[C]</th>
<th>Time (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC (fluorescein isothiocyanate)</td>
<td>575</td>
<td>Universal protein</td>
<td>2 mg/mL</td>
<td></td>
</tr>
<tr>
<td>Concanavalin A (ConA)</td>
<td>647</td>
<td>α-mannopyranosyl &amp; α-glucopyranosyl</td>
<td>100 µg/mL</td>
<td>45</td>
</tr>
<tr>
<td>Soybean agglutinin (SBA)</td>
<td>617</td>
<td>N-acetylglactosamine</td>
<td>200 µg/mL</td>
<td></td>
</tr>
<tr>
<td>DAPI (4′-6-Diamidino-2-phenylindole)</td>
<td>461</td>
<td>Universal DNA</td>
<td>1 mg/mL</td>
<td>60</td>
</tr>
<tr>
<td>Bacto Live/Dead</td>
<td>500 635</td>
<td>EPSDNA/BactoDNA</td>
<td>4 µg/mL</td>
<td>30</td>
</tr>
</tbody>
</table>

Note: the time allowed for staining were all in dark storage to minimize decay of fluorescent signal.

Table 2.3: Freeze substitution program for WWTP flocs \(^7\).

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Time (hr)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>-90</td>
</tr>
<tr>
<td>2 (Steady increase)</td>
<td>3 h @ 10 °C/h</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>-60</td>
</tr>
<tr>
<td>4 (Steady increase)</td>
<td>3 h @ 10 °C/h</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>-30</td>
</tr>
<tr>
<td>6 (Steady increase)</td>
<td>5 h @ 10 °C/h</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>20</td>
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CHAPTER 3
EXPERIMENTAL RESULTS
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Activated sludge grab samples from three WWTPs, namely Guelph wastewater treatment plant (GWWTP), Rothsay wastewater treatment plant (RWWTP), and Membrane bioreactor (MBR) were collected at three different sampling events. The samples were treated as outlined in the materials and methods chapter, to obtain operational parameters, eubacterial ecological composition, physicochemical characteristics, microscopic and bulk chemical composition and finally microscopic visualization.

3.1. OPERATIONAL PARAMETERS

The GWWTP and RWWTP both use conventional treatment facilities to treat municipal waste and waste from rendering plants, respectively. The MBR treats the same influent waste as GWWTP, however, rather than settling tanks to remove microbial flocs they use a membrane technology to capture microbial flocs. Each respective facility management team provided operational information for their respective treatment facility: 1) the performance of the facility based upon differences between influent and effluent waters; and 2) the parameters of the activated sludge present at each of the sites. The performance parameters are important to examine how well the microorganisms are functioning in organic waste removal. The operational parameters are equally important in understanding the environmental conditions under which the microorganisms function.

The performance parameters for influent and effluent waters are presented in Table 3.1 and were evaluated by the following: biochemical oxygen demand (BOD) as an indirect measure of organic pollution (that can be oxidized biologically), total suspended solid (TSS) is defined as the amount of dried solid trapped by a defined filter size (in this case 45 micron filter), total phosphorus (TP) and total nitrogen (TN). Note
that TN represents the sum of nitrite, nitrates, and ammonia forms and TP represents the sum of suspended, dissolved, organic and inorganic phosphorus. Canadian regulations require effluent water to contain no more than 5-15 mg/L of BOD, 0.5-1.5 mg/L TP, 15-35 mg/L TN and 10-30 mg/L TSS\textsuperscript{125}. Overall, the GWWTP produced an effluent within the outlined limits. However, the RWWTP did not meet the required limits for TP and the MBR exceeded the required limits for TSS and BOD. The inefficiencies in microbial bioconversion determined for the three plants may be related to one or more aspects of the flocs such as the physicochemical properties, including surface charge or hydrophobicity and the microbial ecology, which may have a strong influence on bioconversion inefficiencies\textsuperscript{17,86,167}.

The results of the operational parameters of the activated sludge for each of the sampling sites are presented in Table 3.2. The standard activated sludge parameters examined included: mixed liquor suspended solids (MLSS), sludge retention time (SRT), sludge volume index (SVI), total daily flow, pH and temperature. For each wastewater system the MLSS, pH, and temperature values were within normal operational ranges. The variability in MLSS values between the different reactor types was expected, since it is known that MBR typically have higher MLSS values than conventional systems. The temperature of all the reactor types ranged from 18-25 °C. The SRT, or the time spent by solids in the reactor, and was only available for the GWWTP. Thus, comparisons to the RWWTP and MBR were not available. For total daily flow, into and out of the WWTPs, the GWWTP and RWWTP had typical ranges of daily flow, while the MBR system had approximately 100-fold less flow. Typically, full-scale reactors have up to 800 million liters per day and full scale MBRs have up to 15 million liters per day\textsuperscript{126}. The difference
in flow is likely due to the MBR being a pilot-scale project and the other two reactors being full-scale. Sludge volume index (SVI) is an indirect measure of settleability and flocculation. Generally, good settling sludge is considered to have an SVI value between 80-120 mg/mL \(^{149}\), with lower values considered to indicate better settling flocs. The MBR had the best settleability, which fell within the desired range. The GWWTP had an intermediate value between the MBR and RWWTP, however, its SVI of 122.33 mL/mg fell just outside of the optimal range. The RWWTP had the worst SVI relative to the GWWTP and MBR, with an SVI of 170.29 mL/mg that fell well outside of the optimal range.

### 3.2. BACTERIAL COMPOSITION

The biological component of WWTP is comprised of diverse members from all three major branches of the phylogenetic tree of life, namely eukaryotes, prokaryotes and archa. Prokaryotes are the most dominant microorganisms in the biological reactor and are responsible for most of the bioconversion process \(^{14,107}\). Specific bacteria with defined metabolic needs may only perform one function such as nitrification, while others may have unique shapes that are better suited to enhancing flocculation and settleability in the last stage of the process.

The changes in the relative eubacterial biodiversity between the 3 different sampling events for a particular wastewater system and among different wastewater sites were analyzed using PCR amplification of 16S ribosomal DNA followed by DGGE (Figure 3.1).

**Table 3.1:** Performance of three wastewater treatment process. The data represent average performance of the reactors for August 2010 to August 2011.
<table>
<thead>
<tr>
<th>Influent (mg/L)</th>
<th>Effluent (mg/L)</th>
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<tr>
<td>TSS</td>
<td>TN</td>
</tr>
<tr>
<td>GWWTP</td>
<td>350</td>
</tr>
<tr>
<td>MBR</td>
<td>310</td>
</tr>
<tr>
<td>RWWTP</td>
<td>843</td>
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*N/A – not available

Samples collected for specific test is mentioned along with respective sample collection date. However the performance parameters shown above are averaged over a year, for the year when samples were taken.

**Table 3.2:** Operation parameters for three activated sludges. The data represent average operational parameters of the reactors for August 2010 to August 2011.

<table>
<thead>
<tr>
<th></th>
<th>GWWTP</th>
<th>MBR</th>
<th>RWWTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total daily flow (L/D)</td>
<td>6.4×10^7</td>
<td>3.24×10^5</td>
<td>5.8×10^7</td>
</tr>
<tr>
<td>SVI (mL/mg)</td>
<td>122.33</td>
<td>98.12</td>
<td>170.29</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.19</td>
<td>6-8</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient^f</td>
<td>Ambient^f</td>
<td>Ambient^f</td>
</tr>
<tr>
<td>MLSS</td>
<td>3550</td>
<td>8910</td>
<td>2000-5000</td>
</tr>
<tr>
<td>SRT (hrs)</td>
<td>6.5</td>
<td>N/A*</td>
<td>N/A*</td>
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Notes:
*N/A – not available
^# Ambient – 18-25 °C

Samples collected for specific test is mentioned along with respective sample collection date. However the operational parameter values shown above are averaged over a year, for the year when samples were taken.
The DGGE profiles are presented in Figure 3.1. Visual inspection of the profiles suggested the replicates for a particular sampling site appeared consistent, with similar dominant bands present in all replicates (two replicate DGGE runs of all samples and samples from each WWTP represented three different sampling events) from a particular site (Figure 3.1). Visual inspection of the profiles from different sampling sites, revealed variable dominant bands. Differential band intensities indicate variable concentration of the bacterial community represented by the bands in different samples. Furthermore absence of a band indicates, absence of a bacterial species or total genomic material extracted for the specific bacterial specie is less than 1 % (Figure 3.1) \(^9\).

To better evaluate the PCR-DGGE results presented in Figure 3.1, a cluster analysis was performed and a dendrogram was produced (Figure 3.2). These analyses indicated that the eubacterial profiles in the RWWTP and GWWTP were more closely related than samples from the MBR. There is about 54 % similarity between RWWTP and GWWTP and 49 % similarity between MBR and GWWTP or RWWTP, as the similarity matrix shows. Based upon the DGGE profiles the results were unexpected; it was expected that the same influent water would develop similar groups of bacteria. It was therefore surprising that similar treatment processes, the conventional treatment process used at the GWWTP and RWWTP sites, produced profiles with greater similarity. However, it must be noted these are preliminary results and a more detailed analysis of the microbial communities from each of the sampling sites is required to confirm the results and to establish the statistical significance of these results, which was outside the scope of this work.
To aid in confirming the preliminary eubacterial results outlined above additional studies can be carried out. At this point, it is unclear if the dominant bands represent multiple bacteria with a similar range of GC content or a single dominant microorganism present in the flocs. Previous studies have indicated that some bands may yield no useful sequencing information, making it difficult to conclude whether the band had contained genetic information from multiple organisms. Future studies, outside of the scope of this work, should aim to excise the dominant bands and sequence the genetic material to determine the nature of the dominant microorganisms. Furthermore, the DNA extracted from the flocs included DNA from viable and dead cells and from extracellular sources. To better determine which bands in the profiles originate from viable cells, extraction of mRNA followed by DGGE analysis of RNA profile could be compared to that of DNA profile, to establish the microbial ecology of live cells versus dead cells in mixed microbial floc. Additionally, profiles for all sampling sites appeared to indicate that the full width of the gel (using 30% to 70% denaturant) should suffice to capture diverse microbial communities. These additional steps will aid to confirm the differences in the microbial ecology of the GWWTP, MBR, and RWWTP demonstrated in this thesis.
Figure 3.1: A representative PCR-DGGE result for eubacterial present in Guelph wastewater treatment plant (GWWTP), a membrane bioreactor (MBR) at the GWWTP and the Rothsay wastewater treatment plant (RWWTP). A grab sample for each sampling site on the following dates: October 8th, 2010, January 10th, 2011 and February 21st, 2011 was collected and DNA free water was used as negative control and *E. coli* Jm 109 was used as positive control. (i) denotes a dominant band present in all sampling sites and dates, while (ii) denotes a band that represents a bacterial species with high dominance in GWWTP. (iii) denotes a band that represents a bacterial species with relative increased dominance in MBR. (iv) denotes a band that represents a bacterial species unique to RWWTP.
Figure 3.2: Dendrograms for three sampling sites. GeneDirectory® software was used with 3% well height distance tolerance and jacquard statistical method at 90% confidence level to establish dendrograms, representative result of two DGGE gels.
3.3. PHYSICOCHEMICAL CHARACTERISTICS OF WWTP FLOCS

3.3.1. Chemical content analyses for floc EPS

Bulk extracellular polymeric matrix (EPS) analysis generally involves EPS extraction from a floc followed by colorimetric analysis. While there are various methods for probing for each of the chemical constituents in activated sludge flocs, the most common tests were utilized for this project: Guady’s method for total polysaccharide, the Filisetti-Cozzi and Carpita method for acidic polysaccharide, the Lowry method for protein, the modified Lowry method for humic acid and finally fluorescence assays for DNA quantification. Results here are shown for the bulk chemical composition of EPS and thus do not provide any information regarding localization of the specific constituents within a floc.

Bulk EPS composition analysis revealed that the largest constituents within mixed microbial flocs include protein, polysaccharide and humic content and the smallest constituents are acidic polysaccharides and DNA. All of the chemical constituents were within the expected range for wastewater treatment flocs. The DNA content was observed to be highest in the MBR sample followed by GWWTP and finally RWWTP, and the same trend was observed for acidic polysaccharides. Finally, humic substance content is significantly lower in RWWTP as compared to the other sites and this may be due to differential influent water for the systems.

All individual physicochemical properties were shown to be statistically significant from the different sampling sites (one-way ANOVA, P<0.05). The overall bulk EPS content analysis that included a test of overall DNA, polysaccharide, acidic
polysaccharide, protein and humic content was observed to not be statistically significant
($P = 0.606$).

An interesting feature of this analysis was the EPS protein content (EPSp) to EPS carbohydrate (EPSc) ratio. The bulk EPS content analysis shows an interesting trend, with protein to carbohydrate ratios that differed significantly among the different samples. The ratios for the different plants were: 6.5 for the GWWTP sample, 4.9 for the MBR sample and 3.9 for the RWWTP sample. The hydrophobicity of the floc is known to increase with a decrease of EPSc and hydrophobicity would increase with an increase of EPSp. Therefore the higher the EPSp:EPSc ratio, the more hydrophobic flocs we would expect.

3.3.2. Mixed liquor suspended solids concentration for WWTP samples

Mixed liquor suspended solids (MLSS) was assessed to estimate the biomass concentration in each sample. The results (Figure 3.4 A) obtained for each sampling site were in agreement with values provided by the plant operators management team as outlined in Table 3.2. The MLSS value gives an idea of biomass concentration and it is necessary to standardize other quantitative measures against the MLSS value. The MLSS values in Figure 3.4.A were used as they are the values determined in our lab rather than values presented in Table 3.2 which are the average values for the treatment plants from August 2010 to August 2011. The GWWTP and RWWTP sites employ conventional treatment processes and have similar MLSS values. However MBR demonstrates a much higher MLSS value that is consistent for MBR technologies. MBRs operate at higher biomass concentrations given the fact that microorganisms are suspended within a membrane$^{154}$ (Table 3.2).
3.3.3. Hydrophobicity of WWTP flocs

The microbial adhesion to hydrocarbons (MATH) method is one of the most commonly used and most reliable methods to measure hydrophobicity and was therefore used in this study. According to Urbain et al. (1993), hydrophobicity can be measured for either intact flocs or disintegrated flocs. The hydrophobicity measurements of disintegrated flocs provide crucial information about the hydrophobicity of the floc interior, and thus floc formation. Flocs used in this research were disintegrated by ultrasonication to reveal the interior of the floc prior to hydrophobicity measurements. GWWTP has the highest hydrophobicity (32 %) among all sampling sites followed by MBR (19 %) and then RWWTP (6 %) (Figure 3.4 B). The lower the floc hydrophobicity, the greater the number of flocs are expected to remain suspended in the water, resulting in slower settling. So, the RWWTP flocs are expected to have poor settling characteristics. Hydrophobicity is dictated by the chemical composition of flocs, which is discussed later.

3.3.4. Surface charge of WWTP flocs

Surface charge is another important parameter to be measured as microorganisms within flocs constantly secrete EPS material with anionic functional groups such as carboxylics and phosphates. The negative charge influences the overall floc-water relationship and settleability. In this study, the zeta potentials were determined in an electrophoretic chamber to measure the electrophoretic motion of particles within the chamber. The movements are reported as the distribution of charges in mV of zeta potential. The values reported here are the averages of the charge distribution.
Zeta potential values for mixed microbial flocs have been reported to vary from -7 mV to -38 mV\textsuperscript{52,77,98}, and values obtained in this study fall within this range. The most electro-negative flocs were observed in the RWWTP samples (-37 mV) followed by the MBR (-19 mV) and finally GWWTP (-12 mV). The highest deviation in surface charge was observed for the RWWTP sample (Figure 3.4 C). The MBR sample settled best followed by GWWTP and finally RWWTP. Although the trend of electronegativity does not follow settleability, clearly the most electronegative flocs, i.e. from RWWTP, did not settle well.

3.3.5. Particle size distribution of WWTP flocs

Floc size can be determined either microscopically or by using a particle size analyzer, which utilizes a laser to determine particle size. The advantage of microscopy over a particle size analyzer is the ability to specifically examine the size of flocs, while excluding all other particles in the sample. The amount of the solid material other than flocs and flocculating organisms depends on the source of the water influent and the age of the activated sludge. The presence of solid material other than flocs would obscure the view of a particle size analyzer. The obscuration by material other than flocs would skew the results presented by the particle size analyzer. The primary benefit of using the particle size analyzer is that thousands of particles are measured within a few minutes as opposed to the microscopic method which could take up to a day for a single analysis. For this study floc size was determined via a particle size analyzer.
Figure 3.3: Bulk EPS content in three engineered systems: EPS was extracted from flocs derived from GWWTP, RWWTP and MBR using the CER extraction method and each constituent was measured by colorimetric analysis. These results represent an average EPS content for three sampling sites for three independent sampling events, error bars indicate standard deviation. (n=3)
For the purpose of this study the following particle size distributions were determined as: the distribution of the smallest particles in a sample in d(0.1), the mean particle size in d(0.5) and the distribution of the largest particle size in d(0.9). The d(0.1), d(0.5) and d(0.9) values indicate that 10%, 50% and 90% of the particles measured were less than or equal to the size stated. The d(0.5) value is the diameter at which half of the particles obscure the laser beam, and half pass through; therefore, this would give the average floc size in a system. Understanding the average size allows us to study the correlation between settleability and average floc size. The d(0.5) for RWWTP is 200 µm, for GWWTP it is about 170 µm and finally for MBR it is 80 µm. On average, RWWTP flocs are largest (200 µm) followed by GWWTP (170 µm) and finally MBR (80 µm) which have pinpoint flocs (Figure 3.5 A). Pinpoint flocs are defined by flocs ≤ 80 µm, which may be an indication of fragile flocs that have broken into many smaller flocs. This was not expected as flocs from the MBR exhibit physicochemical properties that are similar to the other two sampling sites. In addition, pinpoint flocs were not observed in the other two sampling sites despite the same handling and storage criteria. Additionally the distribution of the smallest particle size d(0.1) were 72 µm for RWWTP, 53 µm for GWWTP and 26 µm for MBR. The distribution of the largest particle size d(0.9) were 371 µm for RWWTP, 421 µm for GWWTP, and 301 µm for MBR.
Figure 3.4. Physicochemical properties of WWTP flocs: A. MLSS of biomass in three different engineered wastewater treatment systems. (N=3). B. Relative hydrophobicity of biomass from three engineered system using MATH. (N=3). C. Surface Charge of biomass in three engineered systems. Surface charge values were obtained using Zeta-potential. Values were obtained for the three sampling sites at three independent sampling events and the average is reported, error bar indicate standard deviation. (N=3).
Figure 3.5. Physicochemical properties of WWTP flocs: A. Average floc size distribution in three different engineered wastewater treatment systems. (N=3). B. Bound water content for flocs from three engineered system using diametric analysis in g/g MLSS. (N=3).
3.3.6. **Bound water measurements**

EPS is a sponge-like polymer with a high capacity for holding water. Therefore, bound water is yet another significant physical parameter to be taken into consideration. There are various techniques for examining bound water. The dilatometric method used in this study is based on the theory that bound water does not freeze below the freezing point of free water. Therefore, if the total water and free water content are known, the bound water can be calculated. The dilatometric method is the most widely used method for determining the bound water content of WWTP flocs. The bound water content in the samples tested are within the range of (5-12 g/g MLSS) reported for fresh activated sludge. Bound water is strongly correlated to the hydrophobicity and surface charge with $r^2$ values of 0.98 and 0.82 respectively. Therefore, lower bound water content may indicate better settleability. For example, GWWTP had the lowest bound water and also had the best settling property as compared to RWWTP flocs. RWWTP had the largest amount of bound water 7.8 g/g MLSS, followed by MBR at 7.2 g/g MLSS and finally GWWTP at 6.0 g/g MLSS (Figure 3.5 B).

**3.4. MICROSCOPIC VISUALIZATION**

3.4.1. **Determination of floc gross morphology using conventional optical microscopy**

Conventional Optical Microscopy (COM), operating under phase contrast mode, is a common technique used to illustrate the various shapes and morphological characteristic features of flocs. These features include: density of floc formers, filamentous outgrowth, porosity, and incorporation of organisms other than prokaryotic life as part of flocs. The COM images of flocs from the three WWTP are presented in Figure 3.6.
Figure 3.6: Gross morphology of WWTP flocs. Images obtained at 200X total magnification, the white scale bar indicates 100 µm. A & B are representative of RWWTP. C & D are representative of MBR. E & F are representative of GWWTP. i filamentous microorganism, ii floc formers, iii rotifers, iv protozoa, v plant cells. These representative images are based upon the examination of greater than 20 flocs per sample. Samples were collected June 23, 2010, August 7, 2010 and September 20, 2011.
Flocs from GWWTP appear to have a dense structure with a lower proportionality of projectile filaments on their exterior. MBR flocs relative to GWWTP flocs have a lower density of floc formers and a higher density of filamentous microorganisms. Lastly, RWWTP flocs have a relatively higher proportion of filaments projecting outward and much lower density than flocs of the other WWTPs (Figure 3.6). For the purpose of this thesis, the proportionalities of floc formers to filamentous microorganisms are not measured against any scale and are assessed by comparing micrographs of one sampling site to another. The compactness of the flocs and also the reduced relative amount of projectile structures extending outward indicates a relatively lower density of filamentous microorganisms in the GWWTP as compared to the RWWTP. The relative outgrowth of filamentous bacteria in one sampling site as compared to another is likely due to variations in two important microbial populations: floc formers and filamentous microorganisms. Where one would have a higher population of filamentous microorganisms, such as RWWTP flocs, and other would have a relatively higher population of floc formers, such as GWWTP flocs.

3.4.2. Determination of internal architecture of WWTP flocs using transmission electron microscopy

High pressure freezing (HPF) followed by freeze substitution (FS) is known to preserve most of the internal architecture of microbial consortia\textsuperscript{111}. Various methods for TEM preparation of mixed microbial flocs have been explored\textsuperscript{101}. However, it has been shown that HPF-FS provides superior preservation of the ultra-structure of surface-associated biofilms\textsuperscript{71,78}. No study of HPF-FS preservation of mixed microbial flocs has been observed in literature\textsuperscript{72,78}. Thus, for the purpose of this thesis HPF-FS was used to prepare the floc samples for TEM analysis. Due to lack of time and resources, direct
attempts have not been made to test HPF and FS on selected samples and compare them to the traditional preservation methods\textsuperscript{101}. Generally, the flocs examined here exhibited good structural preservation as was observed by the detailed EPS texture and its encapsulation of the microorganisms. Furthermore, retention of bacterial position was also maintained, another hallmark feature of this preservation method. The HPF-FS method was not as successful with the RWWTP samples, as the staining did not provide as much contrast as it did with the GWWTP and MBR floc samples.

The GWWTP sample was observed to have the largest flocs with the greatest concentration of floc formers and the smallest amount of filamentous microorganisms (Figure 3.7 A & B). The well-structured flocs were characterized by higher densities of cells that were closely packed. The RWWTP sample was observed by COM (Figure 3.6) to have a relatively higher concentration of filamentous microorganisms and this paralleled the TEM findings. The RWWTP flocs were observed to have a loose distribution of microorganisms with a dispersed pattern of EPS. The pattern may have been due to a higher relative proportion of filamentous microorganisms (Figure 3.7 E & F). MBR flocs were observed to have a much smaller floc size, however, they had a much greater density of cells and a more defined structure relative to RWWTP flocs. This also corresponded to the COM findings (Figure 3.6) where a greater concentration of floc formers and fewer filamentous microorganisms were observed for the MBR flocs as compared to the RWWTP samples. In addition, the MBR samples were found to have greater concentrations of higher microorganisms such as nematodes and algae, which was previously observed with COM.
3.4.3. Summary of microscopy findings

Better settleability of WWTP as demonstrated by GWWTP’s SVI value could be due to a lower density of filamentous microorganism and higher density of floc formers as can clearly be seen in COM and TEM micrographs (Figure 3.6). Lower density of the filamentous microorganism eases the hydrodynamic drag force around the flocs and thereby enhances settleability. Flocs from MBR have been shown to have a lower density of floc formers. Furthermore, MBR samples seem to contain many smaller flocs known as pin-point flocs, which is in agreement with floc size distribution analysis. MBR samples have also been observed to have relatively higher concentration of higher organisms such as nematodes and other eukaryotes. To conclude, data obtained by microscopy correlate with SVI data, as RWWTP had higher SVI than other sampling sites and RWWTP had a relatively higher amount of filamentous microorganisms and flocs with irregular structures.

3.4.4. Characterization of mixed microbial flocs using confocal laser scanning microscopy with multiple staining technique.

3.4.4.1. EPS constituent localization within mixed microbial community

Microbial consortia including flocs, granules and biofilms are routinely examined by CLSM using multiple fluorescent staining techniques. The EPSp:EPSc ratio and its correlation to floc physicochemical stability and settleability has been well studied. Multiple stains combined with CLSM to localize constituents has also been extensively studied, however, here a novel approach is made to combine the two and map the localized EPSp:EPSc ratio within mixed microbial flocs.

Flocs for CLSM imaging were stained for the following EPS constituents: FITC tagging universal protein (Green), Concanavalin A tagging α-mannopyranosyl & α-
glucopyranosyl (Yellow), DAPI tagging universal DNA (Blue) and SBA lectin tagging N-acetylgalactosamine (Red). Flocs from the various sampling sites were of different sizes as indicated by the size distribution results (Figure 3.5.A) and furthermore microtome cuts were not precise, cuts ranged from 5 µm to 50 µm and this introduced further heterogeneity to the thickness of the samples. The variability in sample thickness did not allow for comparison of flocs from different sampling sites. To compensate for this heterogeneity, it was determined that the average thickness of the floc slices was 15 µm. This value was utilized to normalize all floc sizes. The standardization procedure eliminated the possibility of direct compositional analysis in micrometers because all the values were normalized units, but this allows flocs from one site to be compared to those of another.

CLSM confirmed the findings from COM and TEM; each sampling site had a variable proportion of floc formers and filamentous microorganisms. Note that microbial populations were not identified with fluorescent probes but with the distinctive pattern that each of these two groups of microorganism create, i.e. filamentous microorganisms have outward projectile structures and floc formers are characterized by dense structures. Flocs from the various sampling sites also differed in their porosity. For instance, RWWTP are more porous than samples from GWWTP as determined by TEM and confirmed with CLSM. Finally there is no information available to verify, the location at which the flocs sections were sliced from, however, the pattern of spacing and outward growth is confirmed through multiple micrographs using multiple microscopy technique.

Through visual inspection it was determined that all floc samples appeared to have lower intensities for DAPI and SBA lectin as compared to FITC and ConcA (Figure
Lower intensities in the DAPI and SBA lectin could be due to fewer binding sites caused by lower concentration of these EPS constituents. The lower concentration of DNA and acidic polysaccharides had been previously confirmed with bulk EPS analysis (Figure 3.3). Furthermore, through the bulk EPS extraction method, it was determined that one sampling site may have a relatively higher concentration of one constituent than another sampling site. For instance, protein was found to be more abundant in MBR flocs as compared to RWWTP. Therefore, the fluorescent intensity of specific constituents was variable for one sampling relative to another; for example MBR was observed to be brighter for the FITC signal as compared to GWWTP and bulk extraction revealed that MBR samples contained a higher concentration of protein (Figures 3.8 to 3.10).

The determination of protein to carbohydrate ratios for floc samples from each WWTP was conducted by examining the relative fluorescent intensity of the FITC and ConA signals obtained from 3D stacked images of the CLSM examination of floc slices. In addition these data will allow assessment of the distribution of EPS, specifically the distribution of proteins and carbohydrates, within mixed microbial flocs. The flocs examined from all three sources in this study had higher concentrations of both proteins and carbohydrates in the middle of the floc in comparison to the exterior (see Figure 3.13). The similarity in the central peak was expected, as they are all mixed microbial flocs from different systems and are colloidal particles that should have concentrated core structures and loosely bound exteriors. Furthermore, as was hypothesized, the exterior of the flocs had lower concentrations of EPS constituents that were more loosely packed. The lower signal intensity at the exterior of the flocs as compared to the interior of flocs can be related to loose packing of EPS at the exterior. Observing a dynamic high and low
on the two sides of the central peak was interesting and this happened perhaps due to the porosity and filamentous bacteria on the exterior of the flocs (Figure 3.14). As discussed earlier, filamentous microorganisms grow unidirectionally and act as a backbone on which floc formers can grow. These branches of filamentous microorganisms covered by floc formers may be the reason for observing fluctuations of the separate signals of EPSp and EPSc at various depths of the flocs. The central peak indicates a higher concentration of EPS constituent at the core. The DLVO theory explaining laws that govern colloidal particles could be similar to the laws governing of mixed microbial flocs; such that flocs contain EPS constituents that are charged particles and are closely packed at the center and loosely packed on the outer layer.

Researchers have examined microbial flocs for EPSp:EPSc ratios and reported a range of ratios between 0.5 to 21.4 for bulk analyses \(^{168}\). For the sludge samples analyzed in this thesis, via the bulk extraction method, the calculated EPSp:EPSc ratios were: 6.56 for GWWTP, 4.94 for MBR and 3.96 for RWWTP. For the sludge samples analyzed through microscopic analysis however, the calculated EPSp:EPSc ratios were between 0.5 at the exterior and 0.85 at the core of the flocs.

The bulk analysis has revealed that the bulk EPS constituent of all sampling sites were not significantly different according to a one-way ANOVA, (p= 0.66). EPSp:EPSc ratios examined through the microscopic method for all the sampling sites revealed similar patterns with a one way ANOVA, 95% confidence level, (p= 0.21). Therefore, the chemical composition of the three sampling sites is not significantly different.
**Figure 3.7:** Survey TEM images for the visualization of the internal architecture of flocs. 5 μm thick sections were prepared by high-pressure freezing-freeze substitution, and stained with 2 % (w/v) osmium tetroxide. A & B, GWWTP, A. length of the bar indicates 10 μm and B. length of bar indicates 20 μm C & D. MBR, length of the bar indicates 5 μm. E & F. RWWTP, length of the bar indicates 10 μm. Samples from each site were collected February 24, 2011. These representative images are based upon the examination of greater than 20 flocs per sample. i. bacteria, ii. Nematode. iii. EPS matrix.
3.4.4.2. Live/dead assessment within mixed microbial flocs

The distribution of live cells was determined using a live/dead staining kit. Propidium iodide (PPI) stains dead cells i.e. the cells with comprised cell membranes, and extracellular DNA but is impermeable to intact cells in the floc. Syto9 was used to stain total available DNA (present in live cells, dead cells and extracellular DNA in the flocs) (Figure 3.11). In general for all floc samples, the Syto9 and PPI signals were distributed throughout the flocs sections. However, the intensity of the two signals varied from one region of a floc to another, likely due to the differential number and localization of live versus dead cells within the flocs.

Stacks of images were imported into the Leica software to mathematically compute relative intensities of the Syto9 and PPI signals, and to calculate the relative percentile of live cells per floc. The relative live cell percentile was standardized against a control to determine the PPI signal for a pure culture of *E. coli* in exponential growth, as determined by OD$_{600}$. The relative distribution of dead/live cells is shown in Figure 3.11 and relative percentiles can be observed in Figure 3.12 for three sampling sites.

Ten or more flocs from each sampling site were examined and MBR samples had the most intact cells, followed by GWWTP and then RWWTP. This may indicate the overall health and well being of cells within flocs of different origin. Higher amount of intact cell in the RWWTP may be due to sampling regimen at RWWTP. The samplings took place on Tuesdays; GWWTP and MBR reactors were running seven days a week, whereas RWWTP reactor was shutdown on Saturdays and Sundays. The microorganisms in the RWWTP reactor would go through logarithmic growth phase after the nutrients are...
supplied on Mondays, therefore higher amount of intact cells observed in the RWWTP sample.

Visual inspection in Figure 3.11 reveals that there is little difference in signal intensity between the total DNA (i.e. live, dead, and extracellular DNA) and dead signals. Therefore, we expect the percentage of the live cells relative to dead cells to be low and this is demonstrated in Figure 3.12; where viable cells accounted for about 7%-15% of the different mixed microbial flocs. It was established by researchers in the field that the ratio of live to dead cells is normally between a factor of one to four. The finding here is contradictory to their findings given that the percentage of dead cells is more than live cells, Lawrence et al. (2005) used live dead staining and found contradictory findings for their sampling sites.

3.5. STATISTICAL EXAMINATION OF PHYSIOCOCHEMICAL PROPERTIES OF MIXED MICROBIAL FLOCS

The settleability and physiochemical parameters are attributed to the EPSp:EPSc ratio. The EPS constituents for all sampling sites were examined by extracting EPS from mixed microbial flocs via the CER extraction method, which will be referred to as the macroscopic method. In addition, the EPSp:EPSc ratio was also determined for all sampling sites using CLSM and for the purpose of this thesis will be termed microscopic assessment. The data was supplemented with physicochemical tests. The physicochemical tests included settleability measured via SVI, particle size analysis, MLSS, surface charge, hydrophobicity and bound water.
Figure 3.9: CLSM images of the EPS distribution within GWWTP flocs. Images were obtained at 400X total magnification. A. DAPI staining total DNA B. FITC Staining universal protein. C. SBA lectin staining N-acetylgalactosamine. D. ConcA staining α-mannopyranosyl & α-glucopyranosyl. Images are representative of 20-30 flocs examined.
Figure 3.10: CLSM images of the EPS distribution within MBR flocs. Images were obtained at 400X total magnification. A. DAPI staining total DNA. B. FITC Staining universal protein. C. SBA lectin staining N-acetylgalactosamine. D. ConA staining α-mannopyranosyl & α-glucopyranosyl. Images are representative of 20-30 flocs examined.
Figure 3.11: CLSM images of the EPS distribution within RWWTP flocs. Images were obtained at 400X total magnification. A. DAPI staining total DNA B. FITC Staining universal protein. C. SBA lectin staining N-acetylgalactosamine. D. ConA staining α-mannopyranosyl & α-glucopyranosyl. Images are representative of 20-30 flocs examined.
Figure 3.12: CLSM image of live cell distribution within WWTP flocs. A & B. GWWTP. C & D. MBR. E & F. RWWTP. Flocs were stained with syto9 for total available DNA (green) and PPI staining DNA for dead cells and EPS DNA(red). Images obtained at 400X total magnification. These representative images are based upon the examination of 10-17 flocs per sample.
Figure 3.13: Relative number of live cells in three WWTP flocs. The percentile was determined by measuring fluorescent intensities of Syto9, which labels all DNA in a sample, and PPI, which labels DNA from cells with compromised membranes and extracellular DNA. The calculation is based on measurements of 10-17 flocs from each sampling site. Bars represent average and error bars indicate standard deviation of each sampling site.
Figure 3.14: Average protein and carbohydrate dynamic within WWTP flocs. A. GWWTP flocs (n = 17), B. MBR flocs (n = 25), and C. RWWTP flocs (n = 17). The protein signal (FITC staining) included all amino groups and the carbohydrate signal includes all α-mannopyranosyl and α-glucopyranosyl polysachrides (Concavalin A staining).
Figure 3.15: Average protein and carbohydrate dynamic within all systems’ flocs. A. GWWTP flocs (n = 17), B. MBR flocs (n = 25), RWWTP flocs (n = 17). Protein signal included all amino groups and carbohydrate signal includes all $\alpha$-mannopyranosyl and $\alpha$-glucopyranosyl polysachrides. Y-axis is measured in ESp:ESc ratio and X-axis normalized depth of specimen.
The settleability data, measured through SVI, showed a stronger correlation with the EPSp:EPSc ratio examined through the microscopic method \( (r^2 = -0.998) \) rather than the macroscopic method \( (r^2 = -0.538) \). The negative correlation means improved settleability could be achieved by a higher EPSp:EPSc ratio. The fact that there is stronger correlation between EPSp:EPSc and the microscopic method than the macroscopic method may imply that the former is a better tool for examining the EPSp:EPSc ratio than the latter. Overall, these results are consistent with previously published data \(^{89,168}\).

Table 3.3 summarizes the correlation between the macroscopic and microscopic EPSp:EPSc ratios to the physicochemical parameters tested. Three + or – signs indicate an \( r^2 \) value of 0.8 and above, two indicate 0.4 to 0.8 and one indicates 0 to 0.4 \(^{61}\). The table very clearly shows that there are stronger correlations between the microscopic EPSp:EPSc ratio to settleability than that of the macroscopic method through EPS extraction and colorimetric analysis.
Table 3.3: Statistical correlation between Macroscopic and microscopic EPS_p:EPS_c to physicochemical parameters

<table>
<thead>
<tr>
<th>Physical Parameter</th>
<th>Settleability</th>
<th>EPS_p:EPS_c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Macroscopic</td>
</tr>
<tr>
<td>Particle Size</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>MLSS</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SVI</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>Surface Charge</td>
<td>---</td>
<td>+++</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>-</td>
<td>---</td>
</tr>
<tr>
<td>Bound Water</td>
<td>++</td>
<td>---</td>
</tr>
</tbody>
</table>
CHAPTER 4

Discussion
CHAPTER 4. DISCUSSION

The goal of biological wastewater treatment is to utilize mixed microbial flocs for bioconversion of contaminants into simple carbon forms and microbial cells. An important aspect of this process is the settling of microorganisms or separation of microorganisms from the water column after contaminant removal. The settling of microorganisms is dependent on suitable physiochemical conditions within flocs, which is dictated by chemical composition within the flocs. This thesis has encompassed three sampling sites for collecting activated sludge, namely the Guelph wastewater treatment plant (GWWTP), the Rothsay wastewater treatment plant (RWWTP) and the Membrane bioreactor (MBR). Good settling sludge is considered to have an SVI value between 80-120 mg/mL. By that standard GWWTP has good settling sludge, MBR is slightly above 120 mg/mL and RWWTP has poor settling sludge. Further, we examined their ecological composition, through microscopic examination of all samples followed by an array of physicochemical tests. The physicochemical tests included floc size, hydrophobicity, surface charge, bound water and EPS chemical composition (bulk examination). Many other studies have examined physicochemical characteristic, but, unique to this thesis is the determination of the localization of the EPS constituents, specifically the EPSp:EPSc ratio, at various depths of the flocs (microscopic examination). This information was used to examine the local distribution of constituents and comparison of the microscopic approach with the bulk approach. Thus, the focus of this discussion will be the examination of the bulk EPS determinations relative to the microscopic examination via microscopy.
4.1. PHYSICOCHEMICAL CHARACTERISTICS

Determination of the physicochemical properties of flocs is key to understanding the floc/water relationship and more specifically, settleability. Water molecules attract hydrophilic solutes and due to the partial negative charge of water, the more electro-positive the surface charges of a flocs, the better the settleability. Therefore, floc surface charge and hydrophobicity is an important factor in the settleability of flocs. Finally, one should not underestimate the effect of gravity on settleability. Gravity is influenced by floc size, density and hence bound water content. Assessing floc size and water content may also indirectly provide information about floc volume and density. Given that EPS occupies the largest surface area in the flocs, understanding of the properties of EPS is important to the overall understanding of a floc’s physical characteristics.

The tests of physicochemical properties are well established in the literature and the methodologies are commonly used within the field of wastewater treatment. The hydrophobicity analysis in this study resulted in values between 6%-37% using MATH. Activated sludge flocs have relative hydrophobicities that can range between 0-80%\(^\text{168}\). Zeta potential is a well established method for determining surface potential; the method has been implemented for determining surface charge for a single cells and activated sludge flocs\(^\text{164,168}\). Ranges of values between -7 to -40 mV have been reported for activated sludge flocs\(^\text{168}\). The values obtained for the flocs examined in this thesis ranged between -12 to -37 mV, spanning the range measured for activated sludge. Activated sludge flocs are known to have particle sizes ranging between 1 \(\mu\text{m}\) to 1000 \(\mu\text{m}\). Generally membrane bioreactors samples may have variable particle sizes based on recirculation protocols, where more rigorous recirculation process would result in lower
average floc sizes \(^{15,166}\). On average, floc sizes for the membrane reactor samples range between 20 \(\mu\)m to 500 \(\mu\)m \(^{166}\). Flocs observed in the three sampling sites are within the reported range, however MBR samples with \(d(0.5)\) of about 85 \(\mu\)m seem to be on the lower end of average floc size expected for membrane bioreactors. Liao et al. (2000), have shown that when activated sludge is stored for a long time, bound water content decreases \(^{97}\). Fresh activated sludge is known to have 7-8 g of bound water per gram MLSS \(^{97}\). Bound water examined with dilatometric analysis has revealed that flocs studied here are within the range of fresh flocs. There is a strong correlation between the physicochemical properties and EPS constituents of a floc \(^{21,98}\).

Overall, the three sampling sites exhibited significantly unique physicochemical properties (\(P<0.05\)): MLSS, SVI, size distribution, hydrophobicity, surface charge and bound water. Examining the overall EPS composition analysis, however, did not prove to be significantly different from one sampling site to another for both macroscopic and microscopic analysis as is discussed further in section 4.4. The macroscopic method is considered to be quantitative measure, microscopic method however is regarded as semi-quantitative method. While one may not compare the two methods, both have led to the same conclusion in this thesis i.e. the EPS composition of flocs from wastewater treatment plants are the same, despite differential microscopic features and significantly different physicochemical properties.

**4.2. MICROSCOPIC VISUALIZATION**

The aim for this experiment was: (1) to obtain the gross morphological data of the flocs in the different systems using conventional optical microscopy (COM), (2) to image the distribution of various the EPS constituents using fluorescent dyes able to bind to
specific EPS components and confocal laser scanning microscopy (CLSM), and finally (3) to obtain high resolution survey images of the interior of the flocs architecture using transmission electron microscopy (TEM). The microscopic analysis allowed for the comparison of morphological characteristics and examined the role played by morphological characteristics on settleability. Morphological characteristics such as porosity and filaments may add to the drag force and hinder settleability \(^{28,103,148}\).

The correlative microscopy approach revealed the internal and external features of mixed microbial flocs. The three microscopic tools yielded similar outcomes. The GWWTP flocs have a dense interior structure with tightly packed cells and EPS on the inside and outside. In addition, the GWWTP flocs appeared to have a smaller population of filamentous microorganisms, relative to other sampling sites. Followed by MBR, which had relatively lower floc formers and higher filamentous microorganisms as compared to GWWTP. Finally, the RWWTP flocs were observed to have loosely packed cells and EPS on the inside and outside, relative to other sampling sites with a larger population of filamentous microorganisms.

4.3. MICROSCOPIC AND MACROSCOPIC EPS CHARACTERIZATION

According to DLVO theory, there are double layers of counter ions made up of charged particles stabilizing each other and lead to formation of colloidal particles. Researchers studying mixed microbial flocs have attempted to apply DLVO theory to study the laws governing mixed microbial flocs and therefore defined the EPSp and EPSc as counter ions that stabilize floc formation. Furthermore DLVO theory defines a double layer of counter ions, the inner stable layer is tightly packed and the outer layer is loosely packed. Here it was observed that the core of the floc contained a higher EPSp:EPSc ratio.
and higher concentrations of each of EPSp and EPSc as compared to the exterior of the floc. Therefore implying that the interior of floc having higher ratio reflects floc physicochemical properties that suggest better settleability.

Well settling flocs are known to have higher EPSp:EPSc ratios than poorly settling flocs. This can be dictated by optimal concentrations of EPSp and EPSc within each region of the flocs, which influences the local physicochemical properties. Higher EPSp:EPSc ratio indicate better settleability and mixed microbial flocs from WWTPs are unknown to settle better when compared to river flocs. Higher EPSp:EPSc result in surface charge, hydrophobicity and bound water and therefore impact water/floc relationship and lead to better settleability. Cyr and Morton (2006) studied mixed microbial flocs in rivers and found EPSp:EPSc ratios between 0.1-3.4. For mixed microbial flocs sourced from wastewater treatment plants, however, values are typically between 0.5-21.4. Generally, flocs from wastewater treatment have better settleability than that of river flocs. Traditional methods for examining EPS composition included EPS extraction followed by the colorimetric analysis that yielded an averaged chemical composition value. This thesis attempted to study local compositional analysis within the mixed microbial flocs.

Comparing the EPSp:EPSc ratio in the interior and exterior of mixed microbial flocs from three sampling sites revealed a significant difference between the interior and exterior of mixed microbial flocs (p<0.05). The microscopic method revealed a good central peak at the core of the flocs which is an indication of a higher EPSp:EPSc ratio which is expected as stated in DLVO theory. In addition, at first glance it may seem like microscopic methods yielded results that are not typical for wastewater treatment but
rather for river flocs i.e. the ratios obtained are lower than expected for mixed microbial flocs originating from wastewater treatment plants. Closer examination revealed that the bulk examination is a quantitative measure while microscopic examination is semi-quantitative. Thus, one cannot conclusively compare the two methods and expect EPSp:EPSc ratios for the two methods to yield the same numerical results. Since the studies by include Yan (2003) examined the EPSp:EPSc ratio using bulk examination method, it was not surprising to observe that they yielded different results.

4.4. SUMMARY AND FUTURE STUDIES

The results presented here suggest the use of CLSM and fluorescent staining of specific EPS constituents provide a novel method for assessing fluctuation of EPS content within flocs. To further validate this method a larger study population to evaluate more sampling sites to encapsulate more treatment plants with different influent waters and flocs is needed. Larger population size would allow for better statistical examination. In addition, a larger sample size to assess more flocs from each sampling site for CLSM microscopy is needed. More flocs would minimize the standard deviation and lead to better-averaged curves. The work done here could also be expanded, by doing a complete chemical content analysis using the microscopy method. A comprehensive EPS constituent examination would give more in-depth knowledge of the chemical composition dynamic within flocs. Mapping of EPS constituents of lower concentrations such as N-acetylgalactosamine using SBA lectin or nucleic acids using Syto 63 may yield important information about bioconversion or adsorption of humic acid at the core of floc versus the exterior of the floc.
Microscopic assessment of the EPSp:EPSc ratio correlated to physicochemical properties of the mixed microbial flocs. For future studies it is recommended to study mixed microbial flocs with microprobes to study charge and other properties with accuracy inside the mixed microbial flocs. Santegoeds et al. (1998) examined chemical gradients at specific location within flocs using micro-sensors, the probed for concentrations of oxygen, nitrogen and sulfides to establish the reactions undergoing within floc and found relatively lower concentrations at the core of the floc. This method could also be used to study the correlation of floc properties and EPSp:EPSc ratio within each region of a floc. Currently, bulk parameter measurements are recommended for an overall assessment of flocs and for troubleshooting wastewater reactors, if, for example, sludge bulking occurs. These methods are less time consuming, widely described in the literature, and the correlation between the chemical content and the physicochemical constituents is well established. Alternatively, the fluorescence intensity is semi-quantitative, while the bulk assessments are quantitative. One approach to make the test quantitative could be to devise methods to specifically quantify the fluorescence intensity relative to specific protein or carbohydrate concentrations. Standardization with known amounts of protein and carbohydrate would provide a frame of reference and ultimately establish concentration of each constituent, relative to a fluorescent intensity, within floc.

Small angle x-ray scattering (SAXS) examination may be used to examine the EPS matrix within mixed microbial floc to assess compactness of EPS matrix with each sampling site. This result may complement CLSM data to further complement the data found in this thesis. Unfortunately due to shortness of time the SAXS examination
yielded no useful information. The background signal was higher than that of the buffer plus EPS sample indicating that there is more scattering in background buffer solution than that of the buffer plus EPS sample, which falsifies the experiment. Scattering of photon should occur when photons hits a non-transparent object, therefore more constituents being present in buffer control indicates that the experiment was not successful. The SAXS data can compliment CLSM data and show the differential packing of EPS matrix produced by mixed microbial flocs and work is in progress to achieve better results with the SAXS examination of mixed microbial floc.
CHAPTER 5

Appendices
### CHAPTER 5. APPENDICES

#### 5.1. UNIVERSAL PRIMERS USED TO STUDY MICROBIAL ECOLOGY OF MIXED COMMUNITY

<table>
<thead>
<tr>
<th>Primer(s)</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>338f and 518r with a GC clamp on the forward primer</td>
<td>PCR of bacterial genes 16S rDNA</td>
<td>Dearman et al. 2006</td>
</tr>
<tr>
<td>UNIV519F and UNIV1406R</td>
<td>PCR of bacterial and archaeal 16S rDNA</td>
<td>Cheon et al. 2008a</td>
</tr>
<tr>
<td>63F and 1390R</td>
<td>PCR of bacteria 16S rDNA</td>
<td>Chouari et al. 2005</td>
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<tr>
<td>341F(GC) and 906R</td>
<td>PCR of bacteria 16S rDNA</td>
<td>Diaz et al. 2006</td>
</tr>
<tr>
<td>19-38f and 1581-1541R</td>
<td>PCR of 16S rDNA for Bacteria clone library</td>
<td>Fernandez et al. 1999</td>
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<tr>
<td>UNIV519F and UNIV1406R</td>
<td>Universal</td>
<td>Lee et al. 2009</td>
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<tr>
<td>EC9-26f and 926r</td>
<td>Bacteria</td>
<td>Leven et al. 2007</td>
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<tr>
<td>w18 and w02</td>
<td>bacteria</td>
<td>Moletta et al. 2008</td>
</tr>
<tr>
<td>w18 and w31</td>
<td>nested PCR of bacteria genes</td>
<td>Moletta et al. 2008</td>
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<tr>
<td>27F and 1522R</td>
<td>16S rRNA bacterial</td>
<td>Syutsubo et al. 2008b, Rincon et al. 2008</td>
</tr>
<tr>
<td>20bF and 1429bR</td>
<td>16S rRNA archaeal</td>
<td>Rincon et al. 2008</td>
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<tr>
<td>341F and 534R</td>
<td>V3 Region of bacterial 16S rDNA</td>
<td>Li et al. 2008</td>
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<td>341F with GC clamp and 534R</td>
<td>Eubacterial 16S rDNA</td>
<td>Muyzer et al. 1993</td>
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</table>
CHAPTER 6

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CHAPTER 6. REFERENCE


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