CHARACTERISTICS  

OF  

A DEVELOPING BIOFILM  

IN A  

PETROCHEMICAL  

WASTEWATER TREATMENT PLANT  

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B Sc (Hons), M App Sc  

Thesis submitted for the Degree of Doctor of Philosophy  

University of Western Sydney, July 2003
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
DECLARATION

I hereby declare that this thesis is my own work except where acknowledged. This work has not been submitted for any degree at another institution.

Kalyani Perera
This thesis is dedicated to my father, who always believed that beyond each challenge lied another and another.....
SUMMARY

A study was undertaken to investigate developing biofilms in a petrochemical wastewater treatment plant encompassing the architecture, microflora and the chemical nature of the matrix. Biofilms were developed on glass slides immersed in the activated sludge unit and analysed at known time intervals using a range of techniques. Initially, biofilms were investigated using conventional and emerging microscopic approaches to select a suitable technique. Scanning Confocal Laser Microscopy (SCLM) allowed visualisation of biofilms in situ with minimal background interference and non-destructive optical sectioning which were amenable to quantitative computer-enhanced microscopy. SCLM was superior over Light microscopy and Scanning Electron Microscopy. This study demonstrated biofilm growth, presence of extracellular polymer substances (EPS) in early biofilms associated with cells and the development of porous nature of mature biofilms including channel-like structures.

A viable culturable bacterial count of 2.3 – 24% of the total bacterial count and a succession of bacterial colonisation in developing biofilms were demonstrated in this study. Bacillus cereus and Aeromonas hydrophila were isolated from 1 day old biofilms, while Aeromonas caviae, Pseudomonas testosteroni, and Pseudomonas vesicularis were isolated from 2 day old biofilms. Weekella virosa, Xanthomonas campestris, Flavobacterium yabuuchiae, Rhodococcus equi and a species of Brevibacterium were isolated from the biofilm after 5 days. Sphingobacterium multivorum was detected in the wastewater but could not be detected in the biofilm over the 7 day period.

Colonisation of Bacillus cereus in the biofilm was followed using Fluorescent in situ Hybridisations (FISH) using 16S rRNA targeted probe RDR 502 and SCLM. B.
cereus was not present in early 4h and 8h biofilms, but was present in the 24h biofilms. Other bacterial cells were present in these early biofilms.

Differences in cell surface hydrophobicity (CSH) among biofilm isolates were demonstrated using Bacterial adherence to hydrocarbon assay (BATH), Salt aggregation Test (SAT) and Hydrophobic Interaction Chromatography (HIC). Their appearance in the biofilm at a particular stage appeared unrelated to the CSH. Studies conducted on aging pure culture biofilms demonstrated higher CSH in cells harvested from early biofilms than cells harvested from aging biofilms in all except P. vesicularis and F. yahuuchiae.

Analysis of polysaccharide fractions (PF) of EPS demonstrated glycoconjugation to proteins. Presence of hexoses, pentoses, hexosamines, sialic acids and uronic acids in PF was demonstrated using colourimetric and HPLC analysis. Western Blotting and Fluorophore Assisted Carbohydrate Electrophoresis (FACE) demonstrated that PF isolated from EPS of 1d old biofilm contained a 25kDa polysaccharide attached to N-linked oligosaccharides having at least 7 bands with 4-7 glucose monomers. PF isolated from EPS of 3d old biofilm contained a 60kDa polysaccharide attached to N-linked oligosaccharides with at least 4-10 glucose monomers in varying concentrations. Attempts to sequence N-linked oligosaccharides were unsuccessful.

Overall new information has been obtained on developing biofilms in an Australian petrochemical wastewater treatment plant.
ACKNOWLEDGMENTS

I thank University of Western Sydney, Nepean now known as University of Western Sydney, for accepting me for a Doctor of Philosophy in Biological Sciences and providing valuable resources and a scholarship.

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I also thank Dr Nicolle Packer (MUCAB, Macquarie University) who provided me with valuable information and advice in polysaccharide chemistry, HPAEC analysis, FACE techniques and allowing me to use the facilities at the Macquarie University; Dr Peter Devine for his advice on analytical protocols for polysaccharide analysis.

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Scanning Confocal Laser Microscope. The hints given by both of you regarding sample preparation and using the microscopes were invaluable.

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I have always been challenged by microbes, “the unseen majority”. At this stage I wish to acknowledge my mentors, Professor Uma Coomaraswamy (University of Colombo) and Professor Graham Fleet (University of New South Wales) for guiding me through the world of Microbiology. I learnt to question “Why?” and “Why not?” of what appeared to be the norm.
Last, but not least, the constant love and support of my parents have stepped me up to where I am now. The encouragement, patience and tolerance of my husband Duminda, and my son, Hasitha, during the past years meant the world to me.

Thank you.
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<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>$A_i$</td>
<td>Initial absorbance</td>
</tr>
<tr>
<td>$A_c$</td>
<td>Absorbance of control</td>
</tr>
<tr>
<td>$A_t$</td>
<td>Absorbance after treatment</td>
</tr>
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<td>$A_{480}$</td>
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<td>$A_{490}$</td>
<td>Absorbance at 490nm</td>
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<td>$A_{570}$</td>
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<td>ANTS</td>
<td>8-aminonaphthalene-1,3,6-trisulfonic acid</td>
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<tr>
<td>APHA</td>
<td>American Public Health Association</td>
</tr>
<tr>
<td>AT</td>
<td>Adenine-Thymine</td>
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<td>AS</td>
<td>Australian Standard</td>
</tr>
<tr>
<td>ASB</td>
<td>Activated Sludge Basin</td>
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<tr>
<td>BATH</td>
<td>Bacterial Adhesion To Hydrocarbon assay</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BD</td>
<td>Blue Dextran</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BSP</td>
<td>Biomass Support Particle</td>
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<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CSH</td>
<td>Cell Surface Hydrophobicity</td>
</tr>
<tr>
<td>DAF</td>
<td>Dissolved Air Flotation</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of Polymerisation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetraacetic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------------------------------------------</td>
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<td>EPS</td>
<td>Extracellular polymer substances</td>
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<td>EPS isolated from 1 day old biofilm</td>
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<td>EPS isolated from 2 day old biofilm</td>
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<td>EPS7</td>
<td>EPS isolated from 7 day old biofilm</td>
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<td>FACE</td>
<td>Fluorophore Assisted Carbohydrate Electrophoresis</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent In Situ Hybridisation</td>
</tr>
<tr>
<td>fuc</td>
<td>Fucose</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>G3</td>
<td>Polymer of 3 glucose molecules</td>
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<td>Polymer of 4 glucose molecules</td>
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<td>Polymer of 5 glucose molecules</td>
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<td>Polymer of 9 glucose molecules</td>
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<td>GC</td>
<td>Guanine-Cytosine</td>
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<td>GC-GC</td>
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<tr>
<td>gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>galNH₂</td>
<td>Galactosamine</td>
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<tr>
<td>glc</td>
<td>Glucose</td>
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<tr>
<td>glcNH₂</td>
<td>Glucosamine</td>
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<td>GLC</td>
<td>Gas Liquid Chromatography</td>
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<td>Description</td>
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<td>-------------</td>
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<tr>
<td>HIC</td>
<td>Hydrophobic Interaction Chromatography</td>
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<tr>
<td>HPAEC</td>
<td>High Pressure Anion Exchange Chromatography</td>
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<td>HPAEC-PAD</td>
<td>High Pressure Anion Exchange Chromatography – Pulse Amperometric Detection</td>
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<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
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<td>HR</td>
<td>Hydrophobicity Ranking</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>LM</td>
<td>Light microscopy</td>
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<tr>
<td>man</td>
<td>mannose</td>
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<tr>
<td>MBTH</td>
<td>3-methyl-2-benzothiazolinone hydrazone hydrochloride</td>
</tr>
<tr>
<td>MRD</td>
<td>Modified Robbin’s device</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>NANA</td>
<td>N-acetyl-neuraminic acid</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue Tetrazolium</td>
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<tr>
<td>NGNA</td>
<td>N-glycolyl-neuraminic acid</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>Non PF</td>
<td>Non Polysaccharide Fraction</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PEMBA</td>
<td>Polymixin Egg Yolk Methylene Blue Agar</td>
</tr>
<tr>
<td>PF</td>
<td>Polysaccharide fractions</td>
</tr>
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<td>PF1</td>
<td>Polysaccharide fractions isolated from EPS of 1 day old biofilm</td>
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<td>PF2</td>
<td>Polysaccharide fractions isolated from EPS of 2 day old biofilm</td>
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<td>PF3</td>
<td>Polysaccharide fractions isolated from EPS of 3 day old biofilm</td>
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<td>Description</td>
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<td>PF5</td>
<td>Polysaccharide fractions isolated from EPS of 5 day old biofilm</td>
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<td>PF7</td>
<td>Polysaccharide fractions isolated from EPS of 7 day old biofilm</td>
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<tr>
<td>PNGase F</td>
<td>Peptide N-glycosidase F</td>
</tr>
<tr>
<td>PUM</td>
<td>Phosphate urea magnesium buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose Nucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SAP</td>
<td>Streptavidin Alkaline Phosphatase</td>
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<tr>
<td>SAT</td>
<td>Salt Aggregation Test</td>
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<td>SCLM</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis</td>
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<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<td>Std</td>
<td>Standard</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>TIFF</td>
<td>Tagged Image Format File</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
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<tr>
<td>TSB</td>
<td>Tryptone Soya Broth</td>
</tr>
<tr>
<td>(w/v)</td>
<td>Weight per volume</td>
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LITERATURE REVIEW
1.1 HISTORICAL PERSPECTIVE

As far back as the 14th century, Guy de Chauliac, a French surgeon, recorded the relationship between the presence of foreign bodies and delayed wound healing (cited in Voorhees, 1985). However, the first recorded observation that described biofilms was in a 1933 paper by Henrici, in which he stated "...it is quite evident that for the most part water bacteria are not free floating organisms, but grow upon the submerged surfaces" (cited by O'Toole et al., 2000). Subsequently, the first detailed description of microbial attachment to surfaces appeared in literature in 1943 (Zobell, 1943). However, it was not until late 1970s that the word biofilm made an appearance in the scientific literature. Following these pioneering observations, Costerton et al. (1987) recognised and described the ubiquity of biofilms in nature, identifying the need for interdisciplinary research in this area.

1.2 THE DEFINITION OF A "BIOFILM"

Characklis and Marshall (1990) defined a biofilm as a "surface accumulation consisting of cells immobilized at a substratum which is frequently embedded in an organic polymer matrix of microbial origin".

Subsequently, Costerton et al. (1995) defined biofilms as matrix-enclosed populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and flocules and also adherent populations within the pore spaces of porous media.

Wimpenny (2000) described that "any reasonable definition needs to incorporate the idea of a surface or interface on or at which microorganisms proliferate; it should also invoke the unifying effect of extracellular polymers which can envelop and probably protect the microbial colonies forming. It might also embrace a sense of community with
the implication of emergent properties”. In this context, a vast number of microbial aggregates fall into the definition of “biofilm” as these are found in almost every environment with surfaces, sufficient nutrient and some water.

Microorganisms form natural assemblages at air-water interfaces and in suspensions in which they preferentially aggregate to form “flocs” (MacLeod et al., 1990; Wu et al., 1996). Although the substrata for attachment are difficult to discern in these flocs, these assemblages are also viewed as biofilms. Thus, biofilms are broadly defined as assemblages of microorganisms and their associated extracellular products at an interface and typically attached to an abiotic or biotic surface (Davey and O’Toole, 2000).

The term biofilm is chosen to mean those associations of microorganisms that are largely microbial biomass plus varying amounts of extracellular polymeric material that are produced by the microbes themselves. This definition excludes communities which are associated with significant amounts of inanimate or other materials, for example soil and many sediments (Wimpenny, 2000).

In essence, biofilms represent an interdependent community-based existence. Biofilms can be composed of a population that developed from a single species or a community derived from multiple microbial species, and they can form on a vast array of abiotic and biotic surfaces or at a phase boundary. Although most phase boundaries can be colonised, the commonest type of biofilm appears at a liquid:solid interface (Wimpenny, 2000).
1.3 IMPORTANCE OF BIOFILMS

The significance of biofilms in nature and industry has been long recognised (Denyer et al., 1993). Some biofilms processes are beneficial and have wide industrial applications whilst some biofilm associated colonisations have harmful effects.

1.3.1 Beneficial effects

Bacterial communities play a key role in the production and degradation of organic matter and the cycling of nitrogen, sulfur and many metals. These natural processes require the concerted effort of biofilm bacteria with different metabolic capabilities (Davey and O'Toole, 2000). Biofilms are involved in the processing of sewage, in the treatment of groundwater contaminated with petroleum (Massol-Deya et al., 1995), and in nitrification (de Boer et al., 1991). “Mats” which are biofilms in natural waters determine water quality by influencing dissolved oxygen content, and by serving as a sink for toxic and hazardous material. In addition these “mats” play a significant role in the cycling of chemical elements (Characklis and Marshall 1990).

Biofilms also form in extreme environments, such as in acid mines where they contribute to the cycling of sulfur (Edwards et al., 2000), and in ice cover in Antarctica (Paerl and Pinckney, 1996). Biofilms in these extreme environments have been found to undertake a variety of biological processes such as photosynthesis, nitrogen fixation and fermentation. Similarly, it has been shown that bacterial assemblages associated with suspended particles of organic and inorganic material in the marine environments, often referred to as “marine snow”, are involved in biogeochemical transformation of particulate organic carbon in the pelagic environment (Caron et al., 1986; Paerl and Priscu, 1998).
Biofilms are responsible for removal of dissolved and particulate contaminants from natural and wastewater treatment plants. Such processes use immobilized microorganisms to maintain stability and increase productivity of the process. In activated sludge treatment, trickling filters and fluidized bed reactors, biofilms or biofilm covered particles kept in suspension by strong agitation are employed to catalyse organic substrates (Bryers and Characklis, 1990).

Deliberate immobilization of pure or mixed cultures of microorganisms on surfaces in gels to provide fixed film fermenters for specific transformation is yet another example. Bryers (1990) reported a number of beneficial biofilm applications in biotechnological processes such as the use of biofilm reactors in vinegar fermentation and the use of biofilms to extract minerals from ores and in oil recovery.

The colonization of most sections of the gastrointestinal tract by normal bacterial flora results in naturally occurring biofilms in humans and most animals. Such biofilms are believed to perform a protective function by providing a degree of resistance to invasion by pathogenic bacteria (Patrick and Larkin, 1993).

1.3.2 Deleterious effects

Biofilms cause fouling of industrial equipment, such as heat exchangers, pipelines and ship hulls resulting in reduced heat exchange, increased frictional resistance and deposition of unwanted organic and inorganic chemicals on surfaces resulting in reduction of the life time of equipment (Characklis and Marshall 1990).

Similarly, accumulation of biofilms in drinking water systems leads to increased health risks (Mackerness et al., 1993) while biofilm detachment in cooling towers increase the risk of Legionella infection (Keevil et al., 1993). Formation of biofilms on
food contact surfaces in manufacturing plants result in contamination of product (Stanley, 1983).

In medicine, attachment of potentially pathogenic microorganisms to the urinary tract, intestines and to medical devices (Wilcox, 1993) and biofilm accumulation on teeth and gums (Addy et al., 1993) cause increased health risks. A relatively new development in medical practice resulting from the extensive use of metal and plastic prosthetic devices, is the rapid bacterial colonization and biofilm formation on the surfaces of these devices. In all instances of biofilm formation on prosthetic devices, the apparent antibiotic resistance of the bacteria is increased because of limitations in the diffusion of antibiotics through the biofilm polymer matrix.

Some examples of biofilm processes and their effects are listed in Table 1.1.
<table>
<thead>
<tr>
<th>Biofilm Process</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm accumulation on heat exchanger tubes and cooling tower fill</td>
<td>Increased heat transfer resistance, energy loss, reduced performance</td>
</tr>
<tr>
<td>Biofilm formation in water conduits, porous media, ship hulls</td>
<td>Increased fluid frictional resistance, energy losses, reduced performances</td>
</tr>
<tr>
<td>Accelerated corrosion due to microbial processes at the biofilm-substratum interface</td>
<td>Material deterioration, reduced equipment lifetime</td>
</tr>
<tr>
<td>Biofilm accumulation in drinking water distribution systems</td>
<td>Decreased water quality, increased health risk</td>
</tr>
<tr>
<td>Detachment of microorganisms from biofilms in cooling water towers (e.g. Legionella)</td>
<td>Increased health risk</td>
</tr>
<tr>
<td>Biofilm accumulation on teeth, gums, urinary tract and intestines</td>
<td>Increased health risk</td>
</tr>
<tr>
<td>Extraction of organic and inorganic contaminants from water and wastewater</td>
<td>Reduced pollutant load to the receiving waters</td>
</tr>
<tr>
<td>Benthal biofilm stream activity</td>
<td>Maintenance of instream water quality</td>
</tr>
<tr>
<td>Extraction of minerals from ores mediated by biofilms</td>
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</tr>
<tr>
<td>Immobilised microorganisms in biotechnological processes</td>
<td>Improved productivity and stability of processes</td>
</tr>
</tbody>
</table>

Adapted from Characklis and Marshall (1990)
1.4 BIOFILM FORMATION

Biofilms form on available surfaces in virtually all aquatic ecosystems that can support microbial growth (Lappin-Scott et al., 1993). Biofilm development involves phases of attachment, growth and polysaccharide production, maturation and the import of other components, and the detachment from the surface.

During the complex process of biofilm formation, bacterial cells, after adhering to a surface, find themselves in a suitable juxtaposition with cells of the same species and with those of other species (Fletcher, 1991). These cellular juxtapositions and the production of a matrix containing extracellular polymer substances (EPS) within the biofilm, condition the microenvironment of each biofilm bacterium (Van Loosdrecht et al., 1990). Different biofilm bacteria respond to their specific microenvironmental conditions with different growth patterns resulting in a structurally complex mature biofilm (Hamilton, 1987).

A model illustrating different stages of a cycle of biofilm development is shown in Fig 1.1 (adapted from Stoodley et al., 2002). These stages include attachment of planktonic cells to a surface following initial interactions, production of extracellular polymers, development and maturation of the biofilm and detachment of cells from the biofilm.

Biofilm formation can occur by many mechanisms. One mechanism is via the binary division of attached cells. Heydorn et al. (2000) showed that as cells divide, daughter cells spread outward and upward from the attachment surface to form cell-clusters, in a similar manner to colony formation on agar plates. A second mechanism was suggested by Tolker-Nielsen et al. (2000), where “recruitment” of cells from the bulk fluid to the developing biofilm facilitated the aggregation of cells. Thirdly, results
from O'Toole and Kolter (1998b) on studies of *P. aeruginosa* mutants suggest that type IV pili-mediated twitching motility played a role in surface aggregation for this organism.

There is evidence to suggest that environmental signals triggering biofilm formation vary among organisms. It has been shown that *P. aeruginosa* and *P. fluorescens* will form biofilms under almost any conditions that allow growth (O'Toole and Kolter, 1998b) while some strains of *E. coli* K-12 and *Vibrio cholerae* did not form biofilms in minimal medium unless supplemented with amino acids (Pratt and Kolter, 1998). Other factors that influence biofilm formation include nutrient content, temperature, osmolarity, pH and oxygen availability (Costerton *et al.*, 1978; Fletcher and Pringle, 1986).

In summary, these observations suggest that the formation of biofilms depend on the organisms involved, the nature of the surface being colonised and the physical and chemical conditions of the surrounding environment.

Aspects of biofilm formation are discussed in detail in the following sections.
Fig 1.1  Diagram showing the development of biofilm as a five-stage process

1  Initial attachment of cells to the surface
2  Production of EPS resulting in more firmly adhered “irreversible” attachment
3  Early development of biofilm architecture
4  Maturation of biofilm architecture
5  Dispersion of cells from the biofilm
1.4.1 Adsorption of bacteria to surfaces

Biofilm formation is thought to begin when bacteria sense environmental conditions that trigger the transition to life on a surface (O'Toole and Kolter, 1998a). Therefore a significant phenomenon of biofilm formation is the adsorption of microorganisms to the surface.

Many early studies on the initial attachment of bacteria suggested that simple chemical models could account for the behaviour of bacteria during their initial stages of attachment (Marshall et al., 1971). Although these chemical interactions must contribute to cell-surface interactions, these early events are now known to be much more complex than originally assumed.

The steps for the early stages of biofilm formation were described by Palmer and White (1997) and included cell-surface and cell-cell interactions, followed by the development of the mature biofilm. At the time the molecular determinants required for these steps had not been identified, but many aspects of their models still hold true (reviewed by O'Toole et al., 2000).

Microorganisms are far from being ideal particles, they have neither a simple geometry nor do they have a simple uniform molecular composition. In some cases they are deformable and internal chemical reactions can lead to changes in molecular composition both in the interior and at the surface (Rutter and Vincent, 1980).

A study of the attachment of bacteria to surfaces therefore requires an understanding of the physicochemical characteristics of the two surfaces and the interaction between them. The bacterial cell surface is a highly dynamic surface responding strongly to environmental changes through adsorption of ions and macromolecular components. Pooringa et al. (2002) reviewed and summarised this as
“the bacterial cell surface is structurally and chemically more complex and heterogeneous than the surface of synthetic colloidal particles”.

With a few exceptions, the bacterial cell surface carries a net negative charge under most physiological conditions (Busscher et al., 1990). As most natural surfaces are negatively charged as well, bacteria generally experience electric double layer repulsion when approaching these surfaces. Thus, for attachment to take place, the resulting electrostatic repulsion barrier must be overcome by attractive forces (Pooringa et al., 2002). The shorter the distance between the substratum and the bacterial cell, the greater the adhesive forces begin to predominate. This is enhanced by the presence of cellular appendages such as fimbriae, pili, flagella and extracellular polysaccharides. Henrichsen (1972) suggested that flagella composed of protein subunits and fimbriae which are non-flagellar proteinaceous structures are important in bacterial attachment to surfaces. This attachment is the first step in the colonization of a surface and precedes the process of consolidation, during which time the initially weak adhesive forces are strengthened by extracellular polysaccharide formation and finally growth to form an established biofilm (Davies, 2000).

1.4.1.1 Microbial cell surface and types of interactions

Mozes et al. (1991) described the cell surface as the zone that is in direct contact with the external environment. Thus the microbial surface and surface structures that are in direct contact with the external environment must be able to withstand rapid changes in the environment. It has been hypothesized that some microbial surface structures might be regarded as probes to monitor the ever-changing external environments and to relay the information on such changes to the internal regulatory mechanisms of the organisms (Marshall, 1991).
Due to their small size, microorganisms possess a large surface-to-volume ratio. Combined with this feature, rapid growth, nutrient utilization and excretion of metabolic byproducts, microorganisms can quickly alter their own microenvironments. This feature often results in the maintenance of their competitive status within the community despite drastic alterations to the external environments (Mozes et al., 1991).

The substratum surface to which bacteria adhere can vary across a wide range of inanimate and animate surfaces. Adhesion of microorganisms to surfaces is preceded by transport of the cells by fluid dynamic forces to the point where long-range forces may be important in positioning the cells near a surface (Busscher and Weerkamp, 1987). At separation distances greater than 50 nm, Van der Waals forces are the only forces that are operative. This distance is considered to be too large for the opposing surfaces to recognize specific surface components. At separation distances between 10 and 20 nm, secondary minimum interactions occur as a result of Van der Waals and electrical double layer forces. At this point hydrophobic groups on the cell and substratum surfaces play a major role in removing the water film from between the interacting surfaces enabling short range interactions to occur. At separation distances of less than about 1.5 nm most potential energy barriers to attachment have been overcome and a variety of specific and non-specific short-range forces occur that lead to adhesion. Marshall et al. (1971) termed this phenomenon as “the phase of reversible sorption” since the bacteria could be removed from the surface by the application of a shear force such as flagellar movement.

Fletcher and Marshall (1982) demonstrated two types of adsorption with a marine strain of Pseudomonas NCMB 2021. This strain exhibited a time dependant irreversible adsorption to a hydrophobic substratum, but resulted in almost an instantaneous adhesion to a hydrophilic substratum. This observation suggests that the response of bacteria to a surface is more complicated than indicated above. Characklis et al. (1990) suggested that
this could result from 1) prior production of extracellular polymers capable of bridging to a hydrophilic surface, but requiring surface-induced modifications for bridging to a hydrophobic surface 2) production of two different polymers by the same cell with only one of the polymers stimulated by surface contact 3) the presence of a heterogeneous population of cells, some of which are constitutive for the production of polymer capable of binding to a hydrophilic surface.

Once attachment to a surface has been effected, by reversible attachment, the bacterium must maintain contact with substratum and grow in order to develop a mature biofilm. This change from reversible to irreversible attachment was noted by Zobell (1943), and has been characterised by Characklis (1990) as the transition from a weak interaction of the cell with the substratum to a permanent bonding, frequently mediated by the presence of extracellular polymers. A schematic presentation of the initial steps of adsorption in biofilm formation is presented in Fig 1.2.

Recent investigations suggest that profound physiological changes may accompany the transition to permanent attachment at a surface. One means of transition from reversible to irreversible attachment is mediated by type IV pili. Twitching motility is a mode of locomotion used by *P. aeruginosa* in which type IV polar pili are believed to extend and retract, propelling bacteria across a surface. In *P. putida*, irreversible attachment to a surface was demonstrated using differential gene expression and immunoblot analyses to induce a surface-regulated switch from flagella-based motility to type IV pili-based twitching motility (Sauer and Camper, 2001). Twitching motility is also speculated to be involved in the formation of microcolonies (O'Toole and Kolter, 1998b). These authors suggested that interactions of bacteria with one another at a surface, forming groups of cells, help to strengthen the degree of attachment to the surface. Working with *Staphylococcus epidermidis* Gerke et al. (1998) showed that
adherent cells produce a polysaccharide intercellular adhesin that bonds the cells together and facilitates the formation of microcolonies and the maturation of biofilms.
Fig 1.2 Schematic sequential representation of the initial steps in biofilm formation

Adapted from Bos et al. (1999)

Schematic sequential presentation of the initial steps in biofilm formation.

A: Adsorption of conditioning film components  B: Microbial transport and aggregation
C: (reversible) adhesion of organisms  D: Adhesion between microorganisms
E: Establishment and irreversible adhesion through exopolymer production  F: Growth
1.4.1.2 Bacterial cell surface hydrophobicity

Bacterial cell surface hydrophobicity is a term used to describe the hydrophobic properties conferred on bacterial cells by their outermost cell surfaces (van der Mei et al., 1991). Bacterial cell surface hydrophobicity plays an important role in complex interactions that occur between bacteria and surfaces during initial stages of attachment.

In physical chemistry, the implication of the words “hydrophilic” or “hydrophobic” seldom go beyond the macroscopic wetting behaviour of a surface by water in air. Thus teflon and paraffin wax are called hydrophobic because a water droplet does not spread, and clean glass is called hydrophilic for the opposite reason. Hydrophobicity is not necessarily associated with repulsion between water and a surface (Tanford, 1979). Even between water and hydrocarbons attractive interactions exist, which are however smaller than the attraction of water for itself. This relative lack of attraction between water and a surface forms the basis for the term “hydrophobicity” (Tanford, 1979).

It was initially thought that the hydrophobic surface properties of a given strain could be related to a single cell surface component. Rosenberg and Kjellberg (1986) proposed that the degree of hydrophobicity of the microbial surface depends on the surface components which either promote or reduce cell surface hydrophobicity which are termed respectively, as hydrophobins and hydrophilins. They also proposed that amphipathic surface properties of microorganisms are usually determined by the presence, distribution, configuration and juxtaposition of combinations of hydrophobins and hydrophilins. Rosenberg et al. (1991) showed that non-hydrophobic mutants isolated from relatively hydrophobic wild type cells are often deficient in not one, but several surface components. This suggests that the underlying mechanisms may be much more complex than originally assumed.
1.4.2 Production of Extracellular polymer substances (EPS)

One of the essential features of bacterial biofilms that differentiates them from bacteria simply attached to a substratum is the presence of extracellular polymers surrounding the resident bacteria in biofilms.

The terminology used in literature to describe the extracellular material associated with cell aggregates or biofilms varies, and includes as slime, capsule, sheath, glycocalyx and EPS (Wimpenny et al., 1993).

Microorganisms are capable of producing complex macromolecules, including polyelectrolytes, which become deposited on the surface during initial stages of attachment. This adsorbed layer sometimes called the conditioning film, plays a major role in the attachment of the microorganism to the surface they colonize (Rutter and Vincent, 1980).

Attachment appears to occur when polymers at the cell surface act as adhesives bridging the gap between the bacterial and solid surfaces (Costerton et al., 1985). Busch and Stumm (1968) suggested that the fine polymer fibrils may assist to overcome the repulsive mechanisms. Proteins have also been shown to be involved as various proteases have been found to interfere with adhesion (Stenström, 1989). Thus it is possible that surface adhesives may be mixed polymers consisting of both polysaccharides and proteins (Fletcher et al., 1991).

Bacterial EPS appear to be produced in larger quantities in biofilms when bacteria adhere to surfaces than when they grow in liquid phase (Characklis et al, 1990). It is possible that these polymers are produced constitutively by bacteria as a response to "sensing" a surface. Thus a primary question was, whether an initial adhesive polymer differs chemically from the polymers which are produced later in a mature biofilm.
Fletcher et al., (1991) addressed this problem and their studies with Pseudomonas species indicated that there may be differences in initial adhesive polymers and intercellular matrix polymers that developed later during biofilm formation.

1.4.2.1 Structure of extracellular polymer matrix

The vast majority of the bacterial EPS are biosynthetic polymers that can be highly diverse in chemical composition. These may include substituted and unsubstituted polysaccharides, substituted and unsubstituted proteins, nucleic acids and phospholipids (Wingender et al., 1999). Common sugars such as glucose, galactose, mannose, fructose, rhamnose, N-acetylglucosamine, glucuronic acid, galacturonic acid and mannuronic acid are typical constituents of bacterial polysaccharides (Costerton et al., 1981).

Three basic shapes, namely spherical, stiff rods and random coils have been identified in bacterial extracellular polymers (Characklis and Marshall, 1990). Polysaccharides may also adopt a variety of different structures, including single stranded and multi-stranded helices (Rees et al, 1982).

Most polysaccharides and phospholipid accumulation was demonstrated to occur late in the stationary phase, when the physiological status of the cells showed the maximum stress (Uhlinger and White, 1983). Wrangstadh et al. (1986) induced starvation in exponentially growing cells of a marine Pseudomonas sp and observed that soluble, viscous polysaccharide was released, whereas the same polysaccharide was not produced when cells were growing under non-starvation conditions. These observations suggest that the production of polysaccharide depends on the available nutrients as well as the growth phase of the bacterium.

There are many functions attributed to EPS within biofilms, apart from involvement in adhesion. EPS has been shown to adsorb dissolved organic compounds
such as diclofop methyl (a herbicide) and other xenobiotics from the bulk fluid, thereby providing a mechanism by which the community can concentrate essential growth components (Woolfaardt et al., 1998). EPS can serve as a nutrient trap, especially under oligotrophic conditions (Costerton et al., 1981).

The EPS matrix has the potential to physically prevent access of certain antimicrobial agents into the biofilm by acting as an ion exchanger, thereby restricting diffusion of compounds from the surrounding environment into the biofilm (Gilbert et al., 1997). This effect appears to be pronounced with antibiotics that are hydrophilic and positively charged such as aminoglycosides suggesting that this characteristic is largely dependent on the nature of the EPS matrix and the antimicrobial agent (Nicholas et al., 1989).

In one study, the copper-binding characteristics of capsular polysaccharide of an unidentified bacterium isolated from metal-laden sediments were examined and found that a highly purified EPS preparation was capable of binding copper (Mittleman et al., 1985).

EPS has also been reported to provide protection from a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation (Flemming 1993). The response of P. aeruginosa biofilms to UV radiation was examined utilising a whole-cell bioluminescent biosensor (Elasir and Miller, 1999). It had been shown previously that the recA gene of P. aeruginosa was induced by DNA damaging agents. A P. aeruginosa strain containing a transcriptional fusion of the P. aeruginosa recA gene to the lux operon from Vibrio fischeri was constructed, creating a bioluminescent biosensor for monitoring the response to DNA damage. When they immobilised the biosensor strain in Alginate to mimic a biofilm, they found that the EPS matrix protected the cells from DNA damage, as indicated by the lack of induction of expression of the biosensor.
The role of EPS in protection from desiccation was examined in another study (Ophir and Gutnick, 1994). In these experiments, mucoid strains of *E. coli*, *Acinetobacter calcoaceticus* and *Erwinia stewartii* were compared to non-mucoid variants in their resistance to desiccation. It was demonstrated that EPS producing mucoid strains of all three bacteria showed better survival under conditions of dehydration.

1.5 ARCHITECTURE OF BIOFILMS

Biofilms are not simply organism-containing slime layers on surfaces; instead biofilms represent biological systems with a high level of organisation where bacteria form structured, coordinated and functional communities (O’Toole *et al.*, 2000). Mature biofilms display spatial heterogeneity with respect to both organisms and physicochemical micro-environments (Zhang and Miller, 1994). As a result, the structure, activities and composition of biofilms vary with biofilm depth.

The movement of latex particles in *P. aeruginosa* biofilms has suggested that the conceptual model that treats biofilms as a uniformly thick aggregate was inadequate (Drury *et al.*, 1993). They also suggested that a hypothesis regarding thickness variations and heterogeneity of biofilms and additional theoretical and experimental work was required to address this.

In pure cultures of *P. putida* and *E. coli* it has been shown that the activity of cells in the centres of the cell clusters diminished as the clusters grew larger, but their activity could be restored by the addition of a more readily utilizable carbon source (Sternberg *et al.*, 1999). This indicates that cell activity in the interior of the clusters may be controlled by the availability of nutrients.

A model biofilm consisting of *P. aeruginosa*, *P. fluorescens* and *K. pneumoniae* was used for studying the relationships between the structural heterogeneity and
hydrodynamics (Stoodley et al., 1994). They used fluorescent latex particles and scanning confocal laser microscopic techniques to describe the presence of cell clusters and channels in these biofilms. The presence of open channel like structures was demonstrated in mature biofilms of many commercial reactors including four ground water treatment facilities contaminated with petroleum waste (Massol-Deya et al., 1995). It was also shown that the channels surrounding the cell clusters of biofilms could increase the supply of oxygen and other nutrients to bacteria within the biofilm, thus relating structure to function (de Beer et al., 1994).

It has been demonstrated that mucoid strains of *P. aeruginosa* produced more structurally differentiated biofilms than nonmucoid strains and more specifically, that O-acetylation of alginate (a principal component of the EPS of mucoid *P. aeruginosa* strains) was required for structural development (Nivens et al., 2001). It was found that structural complexity in a normally flat undifferentiated wild-type nonmucoid strain of *P. aeruginosa* could be induced by causing the overexpression of alginate (Hentzer et al., 2001). These alginate over-producing biofilms formed mound and mushroom shaped cell clusters separated by water channels similar to those formed by the wild type mucoid strains (Nivens et al., 2001). These observations suggest that the biofilm structure is largely determined by the EPS, and EPS also provides the structural support for the biofilm.

Most laboratory biofilms are grown under low laminar flow conditions and no evidence of directionality on the surface of the biofilms have been observed so far (Stoodley et al., 2003). However, under higher unidirectional flows the influence of increased shear was apparent and the biofilm cell clusters became elongated in the downstream direction forming filamentous streamers. Biofilms growing in hot springs (Reysenbach and Cady, 2001) and acid mine drainage run-off (Edwards et al., 2000) are
examples of such biofilms containing elongated filamentous cell clusters. These observations suggest that the physical growth environment may also play a significant role in the determination of the structure of the biofilms.

1.6 BACTERIAL COMMUNITIES OF BIOFILMS

"In nature, colonisation of habitats by mixtures of bacterial populations is the rule rather than the exception" (Marsh and Bowden, 2000). Evidence is accumulating that such mixtures of organisms are not merely passive neighbours but that they are involved in a wide range of dynamic physical and metabolic interactions. These interactions appear to be essential for the attachment, growth and survival of species at a site, and also enable organisms to persist in what often appears to be overtly hostile environments. Such interacting mixtures of microorganisms are termed microbial communities.

Early studies of microbial communities tended to focus primarily on isolating species from samples on culture media and then characterising the properties of these organisms in pure culture under artificial conditions in the laboratory. The characteristics of the organisms determined in this way were then extrapolated back to their activities in the natural habitat. Examples of citations indicating the inadequacy of selective enrichment and pure cultures for describing microbial communities have been summarised in Table 1.2.

One current model is that the development of biofilms on surfaces proceeds as a succession of adhesion and multiplication events (Rickard et al., 2003). The first organisms to attach are the primary colonisers. Primary colonisation is mediated through specific and non-specific physico-chemical interactions with components of an adsorbed, organic conditioning film (Dang and Lovell, 2000). These early events of colonisation are represented in Fig 1.3. If conditions are suitable the primary colonisers can multiply on
the substratum to form microcolonies. As environmental conditions change within the early biofilm and the substratum becomes covered by bacteria, secondary late colonisers are able to attach to the primary colonisers, and the biofilm becomes a multi-species community. Coaggregation interactions are believed to contribute to the development of biofilms by two routes. The first route is by single cells in suspension specifically recognising and adhering to genetically distinct cells in the developing biofilm. The second route is by the prior coaggregation in suspension of secondary colonisers followed by the subsequent adhesion of this coaggregate to the developing biofilm. In both cases bacterial cells in suspension (planktonic cells) specifically adhere to cells in the biofilm in a process known as coadhesion. Coadhered cells can then become a part of the biofilm. After the initial and late phases of colonisation, several bacterial species will coexist within the habitat and interactions can occur.

Davey and O'Toole (2000) stated that the identification and quantification of members of biofilm microbial communities, as well as a clear understanding of the functional relationship between members are required to appreciate the complex processes that these communities perform. Amann et al. (1995) reported the percentage of culturable bacteria in any sample in comparison with the total cell counts was in the range of 0.001% - 15% and only a minor fraction of a microbial community could be isolated by conventional methods. A number of molecular approaches have been developed and used to identify these non-culturable organisms (Wagner et al., 1993; Amann et al., 1995; Embley and Stackebrandt, 1996).

Studies combining fluorescent in situ hybridisation (FISH) with microelectrode analysis for determining pH, oxygen or sulfide profiles have been conducted in order to evaluate the distribution of different populations in relationship to chemical profiles. FISH techniques have been used to locate propionate oxidising bacterial species and
various types of methanogens in sludge granules (Harmsen et al., 1996b). They showed that the outer layers of the granules were populated with a variety of bacterial colonies involved in hydrolysing complex organic matter, while the interior of the granule contained methanogenic microcolonies. These experiments provided convincing evidence of a layered microbial architecture whereby the bacteria on the surface of the granule hydrolysed complex organic material, thereby providing the anaerobic bacteria in the interior of the biofilm with an energy source.
<table>
<thead>
<tr>
<th>Citation</th>
<th>Source</th>
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<tbody>
<tr>
<td>&quot;....it is thus impossible to isolate a truly representative fraction of the bacteria and fungi living in natural environments for taxonomic purposes. All of these factors, together with taxonomic difficulties, make a conventional description of the number and species composition of microorganisms from natural environments difficult, if not impossible&quot;</td>
<td>Roswell and Kvillner (1978)</td>
</tr>
<tr>
<td>&quot;....It should be readily apparent from the foregoing discussions that it is currently impossible to determine accurately the species diversity of the heterotrophic bacteria in typical aquatic habitats because of identification limitations and enumeration problems&quot;</td>
<td>Staley (1980)</td>
</tr>
<tr>
<td>&quot;.....bacteriologists, who rely on cultural methods to identify species, face the problem of selectivity and thus the inevitable underestimation of community diversity. Just as viable plate counts underestimate the number of bacteria in a sample, diversity measurements of bacterial communities are also a biased parameter&quot;</td>
<td>Atlas (1984)</td>
</tr>
<tr>
<td>&quot;....It is clearly difficult to devise objective procedures for the isolation of unknown microbes&quot;</td>
<td>Williams et al. (1984)</td>
</tr>
<tr>
<td>&quot;....Enrichment and isolation of community members depends on preconception of an organisms's niche and on the ability of the investigator to devise culture conditions which duplicate the niche successfully. Thus the organisms we know from a community are those which we can grow&quot;</td>
<td>Ward et al. (1987)</td>
</tr>
<tr>
<td>&quot;....one isolates pure cultures from nature and then categorises them...then....one determines a diversity index. Diversity of what? Not of the natural population, since we have already shown that culture procedures do not provide representative samples of the natural populations&quot;</td>
<td>Brock (1987)</td>
</tr>
</tbody>
</table>

*Adapted from Ward (1989)*

26
Fig 1.3  Diagram illustrating the possible roles of coaggregation in the development of multi-species biofilms

(a) Primary colonizers

(b) Microcolonies

(c) Secondary colonizers

(d) Mature multi-species biofilm

Adapted from Rickard et al. (2003)

(a) Primary colonisation of a substratum covered in a "conditioning film" composed of polysaccharides and proteins
(b) Cell growth, division and production of EPS leading to the development of microcolonies
(c) Coadhesion of single cells, coaggregated cells and groups of identical cells into the early biofilm
(d) Maturation and the formation of clonal mosaics within the multi-species biofilm
1.7 BIOLOGICAL WASTEWATER TREATMENT IN THE PETROCHEMICAL INDUSTRY

Biological wastewater treatment is an important industrial application of biofilm science. In general, biofilms and biomass support particle (BSP) systems are used on all scales of water purification, from fundamental laboratory systems to technical scale municipal and industrial treatment facilities (Bryers and Characklis, 1990). There are three main types of biological processes used in the petrochemical industry to purify oil-contaminated water. These include activated sludge process, trickling filter process and granular bed biological filter process (Berne and Cordonnier, 1995).

Activated sludge process is widely used for treatment of both domestic and industrial wastewater including petrochemical wastewater. The bioflocculated microbial aggregates are the essential components of the activated sludge system (Li and Ganczarczyk, 1989). These microbial aggregates known as “flocs” are composed of microorganisms aggregated and embedded in EPS. As such sludge flocs are biofilms, and activated sludge process relies on these floc microbial community for the degradation and uptake of organic matter under aerobic conditions. The biomass is finally separated from the purified water by gravitational settling prior to the recirculation of part of this sludge back into the aeration basin (Schramn et al., 1999). Activated sludge processes in modern plants are often supplemented with anaerobic reactor stages to enhance nitrogen and phosphorous removal, however, this is not common in petrochemical wastewater treatment where most of the effluent is of hydrocarbon origin.

The biofilms of aerobic activated sludge process have received less attention compared with the biofilms formed in anaerobic sludge digestors (Tay et al., 2001). In activated sludge flocs, EPS have been implicated in determining floc structure. Floc formation is considered the result of interaction of EPS with microbial cells and other
particles forming the structure of the floc (Flemming et al., 2000). The concept of EPS mediating microbial aggregation has been applied in field trials by using a slime producing *Bacillus* species in aerobic wastewater treatment systems of the paper industry which resulted in improved flocculation and effluent quality (Volpe et al., 1998).

A comprehensive study conducted on the community structure of aerobic and anaerobic zones of a wastewater treatment plant indicated presence of *Aeromonas* spp., *Acinetobacter* spp, *Pseudomonas* spp, *Shewanella putrefaciens*, *Acidovorax* spp, *Comamonas* spp, and *Alcaligenes* spp (Kampfer et al., 1996). Species of *Nocardia* and *Rhodococcus* have been isolated from activated sludge aeration basins (Blackall, 1994), whilst methanogens and propionate oxidising bacteria have been characterised using 16S rRNA probes (Harmsen et al., 1996a). The presence and spatial distribution of several filamentous Gram negative bacteria within activated sludge has been demonstrated using SCLM techniques (Wagner et al., 1994).

In a recent comprehensive study by Schramn et al. (1999), multiple methods were used to investigate the occurrence of anaerobic processes, such as denitrification and sulfate reduction, in well aerated sludge samples. It was discovered that anoxic microniches and denitrification can occur in well-aerated activated sludge, but this situation appeared to be the exception rather than the rule. In addition, sulfate reduction was not detected in any of the flocs, but sulfate reducing bacteria were present in small numbers.

The trickling filter process depends on attached microorganisms for the purification of industrial wastewater. In this process water is allowed to trickle through masses of material contained in towers where an air countercurrent is circulated by natural draft. The material requires a large specific area and supports a thick film of
microorganisms much more complex in community structure than the sludge flocs. This process is not commonly used in the petrochemical industry for wastewater treatment.

Granular bed biological filters contain microorganisms attached to grains or particles of several millimeters in size. These particles may be sand, anthracite or light material. Particles with attached microorganisms are placed in tanks on support floors that are equipped with a water and air countercurrent washing system. There are different types of biofilters in this category but these will not be discussed. These biofilters are not commonly used in the petrochemical industry for wastewater treatment.

1.8 Technologies for Studying the Biota and Chemical Composition of Biofilms

Research on biofilms has depended on a range of techniques available to investigate their structure and function. Selected techniques are discussed in this section.

1.8.1 Developing biofilms: Methods and devices

Robin's device was one of the earliest apparatus used in the laboratory to develop biofilms (McCoy et al., 1981). This consisted of a long hollow steel pipe with sampling ports. The device was used to study both changes in biofilm formation with time, and methods of controlling biofilm growth with antimicrobial agents. This prototype was also developed as an in situ sampling device for monitoring biofilms in industrial pipelines, however, it was cumbersome and could not be efficiently used to study environmental systems. A Modified Robbin's Device (MRD) was used to determine the effective concentration of antimicrobial agents, such as β lactam and tobramycin, in studies of the
control of biofilms (Nickel et al., 1985). This device was smaller, contained easily removable sampling studs and was autoclavable.

The RotoTorque or the Rotating Annular Reactor has been used to study the engineering aspects of biofouling (Trulear and Characklis, 1982; Bakke et al., 1984). This apparatus consisted of two cylinders, a stationary outer cylinder with removable glass slides attached to the wall (biofilm surface) and a rotating inner cylinder. This system allowed the study of fluid frictional resistance of known shear forces on biofilms. An adapted version of the RotoTorque with a rotating drum has been used to study growth of biofilms but this device was not designed specifically to apply known shear fields (Arcangeli and Arvin, 1995; 1997). The Gilbert Rotator, a modification of RotoTorque with four chambers was used to study the growth of biofilms at four different shear rates (Allison et al., 1999). Although these devices were useful in studying biofilms, they were too complex for routine microbiological use.

Membrane reactors containing a permeable membrane immersed in the growth medium have been used by some investigators to study biofilm growth receiving nutrients from each side of the membrane (Rothemund et al., 1994; Wilderer, 1995; Watanabe et al., 1997).

The simplest in vitro method of producing a biofilm is to inoculate the surface of an agar plate containing an appropriate medium to produce a lawn culture. Shapiro (1987) demonstrated the high degree of morphological and biochemical organization of agar grown colony of E. coli in a series of scanning electron micrographs. As the colonies aged, morphologically distinguishable zones involving cells of different shapes, sizes and orientation were identified. The simplest in situ approach, adopted and described by Keevil et al. (1987) was to suspend small flat test pieces (tile, glass) in continuous culture vessels. Submerged test pieces were fastened by thread and lowered into the fermenter
and secured by the stopper of the vessel. The method was simple, convenient and permitted the study of attachment of microorganisms onto various surfaces.

These systems, their values and limitations are summarised in Table 1.3.
<table>
<thead>
<tr>
<th>System</th>
<th>Key features</th>
<th>Value and Limitations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robbin's device</td>
<td>Channel is an open or closed tube. Sample ports at intervals along the channel.</td>
<td>Cumbersome and cannot be used in many environmental systems</td>
<td>McCoy <em>et al.</em> (1981); Nickel <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>Rototorque</td>
<td>Contained two concentric cylinders, outer stationary and the inner rotating</td>
<td>Has been used to study effect of fluid frictional resistance of known shear forces on biofilms. Too complex for routine use</td>
<td>Truear and Characklis (1982); Bakke <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>Rotating drums</td>
<td>Similar to Rototorque, but growth on inside of the outer cylinder and the outside of the inner cylinder</td>
<td>As above, but shear force was not known. Too complex for routine use</td>
<td>Arcangeli and Arvin (1995, 1997)</td>
</tr>
<tr>
<td>Gilbert Rotator</td>
<td>Contained four chambers formed by four sets of intercalating cylinders</td>
<td>Has been used in laboratory biofilm studies. Too complex for routine use</td>
<td>Allison <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>Membrane reactors</td>
<td>Biofilms grow on the membrane receiving essential nutrients from each side</td>
<td>Used in nutrient limitation studies. Limited application in <em>in situ</em> studies in environment</td>
<td>Rothemund <em>et al.</em> (1994); Wilderer (1995); Watanabe <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Transparent surface allows optical microscopy</td>
<td>Convenient and can be used for <em>in situ</em> studies in most environmental systems. Simple system</td>
<td>Keevil <em>et al.</em> (1987); Caldwell and Lawrence (1988); Bos <em>et al.</em> (1994)</td>
</tr>
</tbody>
</table>
1.8.2 Biofilm architecture: Methods of study

An important step in biofilm development is the formation of the characteristic biofilm architecture. It has been emphasised that there is a need for analytical methods to visualise biofilms in situ as well as measure physical properties such as thickness of biofilms in a non-destructive way (Hamilton and Characklis, 1989). Following is a summary of different approaches used and their limitations and values in the field of biofilm study.

1.8.2.1 Light microscopic techniques

Structural studies of microbial biofilms and their formation have been performed by using light microscopy to examine wet mounts and stained preparations (Pescod and Nair, 1972, Korber et al., 1989). Considerable heterogeneity of Pseudomonas aeruginosa biofilms has been demonstrated using light microscopy, however, the interior of the biofilm could not be examined using this method (Stewart et al., 1993). The major disadvantage of use of this conventional microscopic technique is the inability to view internal composition of a thick biofilm causing out-of-focus blur.

Biofilm thickness has been determined using the stage micrometer of a light microscope (Truear, 1983). The main disadvantage of this method is the inability to measure the thickness at different points of the biofilm as biofilms vary in thickness at various points. Similarly, locating the point of attachment of the biofilm to the substratum through a light microscope is not a trivial endeavour, thus adding cumulative errors to the data collected.

An approach to automating biofilm thickness measurements by light microscopy based on variance analysis of images was described by Lauvvik and Bakke (1994). The
method was based on the hypothesis that the variance of a series of images in a biofilm is a function of the vertical position of the image within the biofilm at a fixed horizontal position. They found that the results obtained by this method was consistent with manually determined thickness measurements.

The area of cells occupying a biofilm has been estimated using positive prints of photographs obtained through a light microscope (Lester et al, 1978). This involved measuring the cell images of a positive print by projecting the outlines of the object using a micro projection apparatus or a camera lucida onto millimeter ruled graph paper and then counting the number of squares enclosed within each profile. A number of variations of this application has been reported in literature (Hader, 1992). Instead of calculating the area on graph paper, some methods included measuring the areas of traced profiles with a planimeter or tracing the projected outlines of the object onto a uniform thickness paper and cutting each profile and weighing on a microbalance. Apart from the human errors inherent in these manual tracing and interpretations, these approaches were very slow, tedious and impractical.

1.8.2.2 Scanning electron microscopic (SEM) techniques

Scanning Electron Microscopy (SEM) techniques have been used extensively to determine the structure of biofilms (Eighmy et al., 1983; Richards and Turner, 1984; Chang and Rittman, 1986; Kellogg, 1989). These techniques were found to be excellent for studying surface structures as they offered high magnifications, but of limited value for studying thick hydrated biofilms, as internal structures could not be examined in situ and sample preparation caused artifacts and dehydration. Preparation of biofilm samples by air drying was attempted to avoid dehydration associated with critical control point
drying, but this method did not preserve the original biofilm structure (Keevil et al., 1987). Subsequently, application of a wet SEM method to observe unstained, untreated wet samples using a Electroscan® SEM was reported and compared with conventional SEM, this method generated images relatively free of shrinkage and artifacts and showed cells embedded in the matrix of the biofilm (Sutton et al., 1994).

1.8.2.3 Scanning Confocal Laser Microscopy (SCLM) and associated computer aided techniques

Scanning Confocal Laser Microscopy (SCLM) has been widely applied to analyse structural heterogeneity of biofilms (Lawrence et al., 1991). Thickness has been measured by optically sectioning the biofilm at desired points on a xy plane using a laser beam. The advantages of this method are many, permitting visualisation of biofilms in situ with no apparent sample destruction and allowing any part of the biofilm to be optically sectioned.

SCLM, in conjunction with fluorescent stains, appears by far to be the best method to study natural biofilms in their hydrated state in a non-destructive manner (Davey and O'Toole, 2000). In the old layered biofilm theory it was thought that liquid flow occurred only above the biofilm and that nutrients, waste products, and other chemicals moved into, out of and through the biofilm by diffusion alone. The presence of interstitial voids or channels within biofilms has been documented using particle tracking techniques by following the movement of a single latex particle through a biofilm water channel using SCLM (Stoodley et al., 1994). This clearly demonstrated the open structure of biofilms.
1.8.2.4 Image analysis and associated techniques

Image analysis techniques have been employed to analyse image regions and area calculations in biomedical applications (Bovik et al., 1992). The first step in most image-processing systems is the object isolation process. In this process the object of interest is isolated from the background with varying optical complexity. This is achieved by marking the edges, partitioning the image according to homogeneity criteria or by gray-level thresholding. Once the object of interest has been isolated, the image is digitised and processed through image processing software in several stages. The digitised image is reduced to 256 x 256 spatial resolution and a gray level thresholding operation is applied. A correction factor is then applied to both object and background pixels. Once this has been achieved and boundaries of objects are marked, area calculations can be conducted based on the number of pixels. The information then can be exported to spreadsheets for further analysis.

1.8.3 The composition of EPS: Analytical techniques

There are two major analytical steps involved in studying EPS. The first step is to isolate EPS from the biofilms containing cells and unbound material. The second step is to conduct detailed analysis to characterise the structure and components of isolated EPS. Methods that have been employed in achieving these have been summarised below.

1.8.3.1 Isolation of EPS from biofilms

A number of methods have been employed to isolate EPS from biofilms. Some of these techniques include:
1) cold ethanol precipitation (Evans and Linker, 1973; Read and Costerton, 1987)

2) ammonium sulphate precipitation (Sar and Rosenberg, 1989)

3) ion exchange resin (DOWEX in Na form) (Frølund et al., 1994)

4) dialysis (Goldman et al., 1982)

5) selective precipitation with cetyl trimethyl ammonium bromide (MacCormick et al., 1993)

6) propanol precipitation (Mian et al., 1978)

7) EDTA precipitation (Omar et al., 1983)

All of the above methods have been successfully employed to isolate EPS and the choice very much depended on the nature of the biofilm and the growth environment.

1.8.3.2 Analysis of EPS

Similarly, there are a number of widely applied techniques to characterise composition and structure of EPS. These include polyacrylamide gel electrophoresis (PAGE) (Corpe, 1970), Thin Layer Chromatography (TLC) (Corpe, 1970), GC-MS and GLC analysis (Wrangstadh et al., 1986; MacCormick et al., 1993; Beech et al., 1991). Using these techniques the presence of acid polysaccharides and neutral sugars such as N-acetylglucosamine and N-acetylglactosamine in EPS has been demonstrated.

The use of lectins, a group of nonenzymatic proteins with high affinity for mono and oligosaccharides, has been used for separation and analysis of glycoconjugates and oligosaccharides present in EPS (Brush 1999). Lectin affinity chromatography using lectins immobilised on agarose beads also have been used to characterise EPS (Beeley, 1984; Peacock et al., 1990). These methods yielded good results if the glycoconjugate
was stable at high salt concentrations and room temperature. The chemical nature and spatial arrangements of exopolymers in a biofilm community has been studied in situ using fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate conjugated probes coupled to lectins (Wolfaardt et al., 1998, Johnsen et al., 2000). Lectin binding to a variety of sites within the EPS matrix in these biofilms has been detected and quantified using image analysis and dual channel imaging, in conjunction with SCLM.

Attempts to identify sugar chains in polysaccharides using metabolic radiolabelling were widely described in literature in the 1980s and valuable information on polysaccharide structure has been obtained using these methods (Diaz and Varki, 1985; Esko et al., 1986; Cummings et al., 1989; Roux et al., 1988).

Analysis of EPS on membranes subsequent to gel electrophoresis and western blotting has been employed to detect glycoconjugation in polysaccharides (Bayer and Wilchek, 1990). This detection was based on a specific carbohydrate oxidation reaction that involved labeling the terminal monosaccharide with biotin and subsequent detection using a system such as streptavidin-alkaline phosphatase and colour development. The reactions involved in this method are shown in Fig 1.4. A number of commercial kits and gel imaging systems (Bio-Rad and Glyko) are also available for detection, quantitation and characterisation of glycoconjugates.
Fig 1.4 Detection of glycoconjugation of EPS

This process involves oxidation of terminal non reducing monosaccharide with periodate, labelling with biotin and detection using a colour development system such as NBT/BCIP (Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)).

SAP – Streptavidin Alkaline Phosphatase

Source: Instruction Manual BioRad catalog 170-6490
The monosaccharide composition of EPS has been characterised and quantified using chemical colorimetric methods (Dubois et al., 1956), High performance liquid chromatography (Hardy et al., 1988), and fluorophore assisted carbohydrate electrophoresis (FACE) (Jackson and Williams, 1991; Jackson, 1994; Hu and Valee, 1994; Basu et al., 1994).

The use of colorimetric methods for the detection and quantification of hexoses, pentoses, sialic acids, hexosamines, and uronic acids in EPS has been demonstrated (Christensen et al., 1985; Read and Costerton, 1987). These methods were valuable in elucidating the monosaccharide composition of EPS, but were time consuming.

High performance anion exchange chromatography with pulse amperometric detection, (HPAEC-PAD), has been used to determine the monosaccharide composition of glycoconjugated EPS (Hardy et al., 1988). Although similar results can be obtained with Gas Chromatography (GC), such methods require derivatisation of monosaccharides to alditol acetates or methyl glycosides prior to detection, which cause destruction of more sensitive sialic acids. HPAEC-PAD methods do not require derivatisation prior to analysis. A HPAEC column from Dionex Corporation has been used to detect monosaccharides at the same level of sensitivity as GC for separation of closely related monosaccharides (Hardy et al., 1988).

Most FACE analysis to date has been conducted on plant and animal secretory polysaccharides. FACE is a sensitive method for determining the presence and relative abundance of individual oligosaccharides (Goins and Cutler, 2000). This technique involves tagging the released terminal aldehyde groups of a polysaccharide with the charged fluorophore, 8-aminonaphthalene-1.3.6-trisulfonate (ANTS) followed by separating with high resolution on the basis of size by PAGE. FACE gels allow
separation, visualisation and quantification of monosaccharides present in macromolecules in one experiment, and are therefore convenient. The structure of Glycosaminoglycan disaccharides present in urine has been characterised using FACE techniques (Masada et al, 2002).

The analysis of polysaccharides can be approached using a number of different techniques and the choice depends on the questions being addressed and the nature of the biofilm.

1.8.4 The biota of the biofilm: Methods of analysis

Viable plate count or most-probable-number techniques are still used extensively for quantification of viable cells in environmental samples. However, these methods are inadequate in facilitating the understanding of the microbial ecology of an environment. The restrictions and potential biases of these methods have been reviewed by Brock (1987). Conventional isolation techniques described in American Public Health Association (1989) consist of cultivation using nutrient agar, plate count agar or trypticase soy agar. The use of such culture media fails to appropriately reflect the conditions that such microorganisms experience in their natural habitat. Use of such media favours the growth of organisms best adapted to grow under these artificial conditions (Embley and Stackebrandt, 1996) leaving the others that do not grow underestimated.

Physiological and biochemical tests are often ambiguous and require the isolation of pure cultures, a task not easily achieved for many microorganisms (Brock, 1987). Attempts to establish a systematic classification on the basis of phenotypic characters have therefore been of limited success (Woese, 1994).
A range of molecular techniques has been used for the analysis of the structure and species composition of microbial communities. The use of nucleic acid probes including a PCR step targeting specific DNA regions to amplify the region of interest has been widely used to determine the composition of microbial communities (Saiki et al., 1988).

The use of rRNA genes as biomarkers in microbial biosystematics has been employed in the study and identification of members of microbial communities. rRNA genes are universally present in all known microorganisms and contain conserved as well as variable regions. Thus rRNA genes contain general as well as specific target sites for probes and contain sufficient sequence information to be used as phylogenetic markers which are well described in literature (Giovannoni et al., 1988; Ward et al., 1990;穆泽尔 and Ramsing, 1995; Amann et al., 1995; Embley and Stackebrandt, 1996). The sequence variation in the 16S rRNA gene has been exploited for inferring phylogenetic relationships among microorganisms and for designing specific nucleotide probes for the detection of individual microbial taxa in natural habitats (Giovannoni et al., 1990; Amann et al., 1992). These techniques have been used to examine the distribution of sulfate reducing bacteria in microbial mats (Risatti et al., 1994), as well as to analyse microbial composition of photosynthetic biofilms isolated from a wastewater trickling filter (Ramsing et al., 1993). A genus specific 16S rRNA probe to target all known sulfate reducing species of Desulfotomaculum and five specific probes to target subclusters within the genus have been used to perform comparative analysis of this genus (Hristova et al., 2000).

In situ hybridisation of fluorescence labelled 16S or 23S rRNA probes in combination with SCLM is a powerful tool for visualisation of the spatial distribution of
important groups of organisms in bacterial communities (Christensen et al., 1998). The presence of many thousands of copies of rRNA per cell facilitates the incorporation of sufficient dye to be visualised by fluorescence microscopy. The application of rRNA techniques in community analysis can be approached in many ways (Blackall, 2002). These approaches are presented in Fig 1.5. FISH has been applied to the study of a range of microbial ecosystems such as marine environments, limnology, wastewater treatment, rhizosphere, bulk soil and numerous symbioses as well as in clinical applications (Blackall, 2002). Figure 1.6 shows the principle involved in detecting 16S rRNA in whole cells using fluorescence microscopy.
*In situ* hybridisation probing with published domain and phylum level probes

"Global" community structure

Stable isotope probing

Nucleic acid extraction

Pure cultures

Probes and Primers from Clone Library

Partial and Full Sequences from Clones

DNA sequencing

Phylogenetic Trees

Mixture of Nucleic Acids Genomic DNA and all RNAs

Density Gradient Centrifugation To collect Isotope Labelled DNA

Specific Amplified Nucleic Acid from 16S rDNA

Clone Library of Community 16S rDNA

RFLP for screening library

Fig 1.5  Use of rRNA techniques in community analysis
Full cycle rRNA analysis shown in grey arrows with two modifications; Pure culture approach in green and the use of stable isotope probing in black

*Adapted from Blackall (2002)*
Synthetic oligonucleotide probe labelled with fluorochrome
(≈ 20 bases)

Ribosomes containing 16S rRNA

DNA

Ribosomal RNA operon

Cell wall and membrane

TAGCTGGCAGT
CGUACGACCGUCAUA

fluorescein

Adapted from Blackall (2002)

Fig 1.6 Diagrammatic representation of Fluorescence *in situ* Hybridisation (FISH) probing of fixed whole microbial cells
1.8.5 Bacterial Cell Surface Hydrophobicity: Methods of study

A number of methods have been developed to study microbial cell surface hydrophobicity. Some of these methods include electrophoretic mobility, colloid titration, electrostatic interaction chromatography, bacterial adherence to hydrocarbons, partitioning in an aqueous two-phase system, hydrophobic interaction chromatography, contact angle measurement and X-ray photoelectron spectroscopy (van der Mei et al., 2002). However, not all these methods have been applied in microbiology. The most popular method in microbiology has been bacterial adhesion to hydrocarbon (BATH) assay (Rosenberg and Kjellberg 1986), while hydrophobic interaction chromatography (HIC) (Smyth et al., 1978) and salt aggregation test (SAT) (Lindahl et al., 1981) also have been used extensively.

BATH and HIC assays measure actual binding of a cell to a hydrophobic ligand while SAT assay gives an estimate of the overall surface properties of the bacterial cell surface. The availability of various techniques for measuring bacterial hydrophobicity has provided flexibility of approaches for investigation of bacterial adhesion.

Contact angle and surface tension data converted to surface free energies, eventually leading to the calculation of free energy of adhesion have been used to obtain an indication of the hydrophobicity of bacterial surfaces (Absolom et al., 1983; Busscher et al., 1984 and van Loosdrecht et al., 1987). Contact angle measurement to determine the cell surface hydrophobicity of mixed bacterial strains of an anaerobic sludge has demonstrated different hydrophobicities within the layers of the biofilm (Daffenchio et al., 1995).

The binding of fluorescent hydrophobic microspheres to the cell surfaces of wastewater microflora \textit{in situ} has been used to examine the cell surface hydrophobicity of
this community members (Zita and Hermansson, 1997). This method involved enumerating the number of microspheres attached to cells directly using epifluorescence microscopy.

1.9 THIS STUDY

Wastewater treatment is an important process in petrochemical industry. Hydrocarbon contaminated water generated during production must be treated to ensure compliance to legislative requirements before discharging to public waterways. This is achieved by purification of contaminated wastewater using an activated sludge process that depends on microorganisms immobilised on flocs within aeration tanks.

This study is a holistic approach towards elucidating the structure of developing biofilms in the activated sludge units of a petrochemical wastewater treatment plant from initial stages to maturation over a selected time period. The aim of this study was to encompass the architecture, the nature of extracellular polymer substances and the biofilm bacterial community of these developing biofilms with a view of investigating the heterogeneity of this important biological niche with respect to time and space. Further, physico-chemical properties of isolated bacterial cells from developing biofilms at various maturation stages were also included in this study to understand their cell surface properties important in attachment.

This approach can be visualised as shown in figure 1.7.
Fig 1.7 Approach of this study
The approach used to provide this understanding and knowledge can be summarised as follows:

**Biofilm architecture**
- Compare, evaluate and select suitable microscopic techniques to study biofilms *in situ*
- Use the selected technique to examine the architecture of developing biofilms of various stages in the petrochemical wastewater treatment plant

**Bacterial community of the biofilm**
- Isolate bacteria from developing biofilms
- Understand the community structure and primary colonisers of the biofilm using molecular techniques

**Cell surface properties of bacterial isolates**
- Investigate cell surface properties of isolated culturable bacteria from developing biofilms

**Nature of the EPS**
- Compositional and structural analysis of EPS isolated from developing biofilms using various biochemical techniques
The body of the work presented in this thesis aims to provide a comprehensive study of the main features of a developing biofilms in an important industrial setting and the reader is directed to the sections as given in the flowchart below.

Biofilms developing in the wastewater treatment plant (Chapter 2)

Structure and architecture of these biofilms (Chapter 3)

Bacterial inhabitants of the biofilm (Chapter 4)

Physico-chemical properties of the cell surfaces of biofilm bacteria (Chapter 5)

Nature of the extracellular polymer matrix (Chapter 6)

Concluding remarks (Chapter 7)
CHAPTER 2

SITE DESCRIPTION AND BIOFILM DEVELOPMENT
2.1 **Crude oil processing**

Crude oil or “unprocessed oil” is a mixture of various hydrocarbons containing paraffins, aromatics, naphthalenes, alkenes and alkynes. These hydrocarbons are separated using distillation processes to obtain various marketable products. At the time of the study, the product range of Shell refinery included petroleum gas, naphtha, gasoline, kerosene, diesel, lubricating oil, fuel oils (heavy gas for marine vessels) and residual by-products such as coke, asphalt, tar and waxes. Hydrocarbon contaminated water generated during production had to undergo treatment before reusing or discharging out of the refinery and wastewater management therefore had to be effective to ensure sufficient quality to meet legislative requirements.

2.2 **Site Description**

Shell commenced operating in Australia in 1901 as Shell Refining (Australia) Pty Ltd. In 1928 the Clyde oil refinery was acquired and in 1930s the exploration of oil and gas began. As shown in Fig 2.1, the Shell refinery is located within the industrial area east of Rosehill Gardens Racecourse. Access to the Refinery is via private roads exiting Grand Avenue. The Duck River is located at the east-west border of the site. At the time of the study, 4000 tonnes of treated water was allowed to be discharged to Duck River. Designated collecting areas within the plant were operational in order to collect plant wash down water and rainwater to avoid overflow of untreated water into the Duck River.
Fig 2.1 Location of Shell Petrochemical Refinery

The refinery occupies the area within red lines
Treated water is discharged into Duck River
2.3 On site wastewater treatment process

In petroleum refining and subsequent chemical processing, water is used for many purposes. Apart from using as a cooling medium, water is used in process units for scrubbing, steam stripping and steam ejectors. During these operations the water becomes contaminated with hydrocarbons. Hydrocarbon contaminated water also comes from water runoff from plant surfaces during rainfall or washing. Most hydrocarbons can be removed by plant interceptors because hydrocarbons are generally insoluble in and lighter than water. Some hydrocarbons pass through these gravity separators because either they are well dispersed or are dissolved in water.

This contaminated water must be treated before it can be reused or discharged from the refinery. The treatment of wastewater streams is carried out in the biotreater to ensure that the water is of sufficient quality to be compatible with the public water ways adjacent to the refinery. Biotreatment of water in this process is aerobically conducted in the Activated Sludge Basin (ASB) units by microorganisms.

The Refinery plant layout is shown in Fig. 2.2 with emphasis on the wastewater treatment section.

The system consists of 2 buffer basins, a dissolved air flotation (DAF) unit, an activated sludge basin (ASB) and a clarifier. Hydrocarbon contaminated water is pumped into one of the buffer basins. The second basin is held in reserve to store highly contaminated water that may cause problems to the plant. The contaminated water is pretreated for pH correction, toxic chemical removal and flocculation in the buffer basin.

The effluent from the basin flows by gravity to the DAF where it is combined with aerated water. The fine bubbles created in the DAF lifts the floc and oil to the
surface where it is skimmed and clear water is lifted to the ASB for treatment. The ASB contains 1370 m$^3$ of liquid. Oxygen is dissolved into the liquid to sustain the microorganisms by using three mechanical surface aerators that are capable of transferring 1,400 kg of Oxygen per day. The flow to the ASB has a temperature alarm set at 35°C.

Many compounds are removed from the wastewater during the treatment process. These include sulphides, sulphates, iron, ammonia, zinc, nitrates, phosphates, phenols, oil and grease.

After digestion in the ASB the microorganisms and treated water are separated in the clarifier and allowed to settle. The clear water is removed from the top of the clarifier and pumped into sand filters. From sand filters the effluent water filters into the cooling tower basin or to the Duck River.
Fig 2.2 Clyde Refinery Plant layout
(Diagram kindly provided by Shell (Australia) Pty Ltd. Photographs were not taken for security reasons)
Fig 2.3  Schematic diagram of the Activated Sludge Basin
2.4 Biofilm development

Biofilms were developed in the secondary treatment tank of activated sludge basin (ASB) at a depth of 2m. A schematic diagram of this section is shown in Fig. 2.3 (This drawing was kindly provided by Shell Refining (Australia) Pty Ltd).

The method used for biofilm development was selected for its practicality and simplicity and was based on the method described by Keevil et al. (1987). Glass microscope slides (76.2 x 25.4mm) and glass microscope coverslips (76.2 x 50mm) cleaned in 20% HCl acid were placed vertically inside a small stainless steel basket with grooves to hold slides. The basket was immersed in the ASB tank suspended by strong nylon thread at a depth of 2m. Biofilms were allowed to develop on the glass surface under natural conditions and samples were removed carefully after 1, 2, 3, 5, and 7 days (unless otherwise specified) to observe gradual development of the biofilm. Freshly collected samples were immediately placed in a sterile jar containing wastewater and transported to the laboratory in an esky. Samples were kept in the dark and at 4°C until further processed as described in each chapter. Unattached cells and debris were removed from the biofilm by gently immersing the slide in a beaker containing phosphate buffered saline (PBS, pH 7.2).
CHAPTER 3

STRUCTURAL CHARACTERISTICS OF DEVELOPING BIOFILMS
CHAPTER 3

STRUCTURAL CHARACTERISTICS OF DEVELOPING BIOFILMS
3.1 INTRODUCTION

There appear to be some disagreement as to what constituted the "typical" architecture of a biofilm. In water distribution systems where the substrate concentration was very low biofilms were described as simple stalked or irregularly branched structures, well separated from their neighbours (Kevel, 1989; Walker and Keenvil, 1994). In the laboratory situation using media containing significant nutrient concentrations biofilms were shown to be mushroom or tulip shaped structures penetrated by large and small pores (reviewed by Costerton et al., 1994, 1995). In contrast dense relatively uniform and homogeneous biofilms have been described in dental plaques where nutrient levels were generally high (Nyvad and Fejerskov, 1989). Wimpenny and Colasanti (1997) formulated a simple all encompassing model based on the premise that the final structure of a biofilm was largely dependent on resource concentration. Thus, the biofilm structure is not a chance occurrence but represents an optimal arrangement for the influx of nutrients and various other intrinsic and extrinsic parameters.

In this study a range of microscopic techniques were used to investigate structural properties and architecture of biofilms developing in the petrochemical wastewater treatment plant with a view of selecting the most appropriate technique to apply throughout the study.
3.2 MATERIALS AND METHODS

3.2.1 Biofilm development

Biofilms were allowed to develop in the wastewater treatment plant as described in section 2.4.

3.2.2 Microscopic examination of biofilm structure

Biofilm samples were prepared and observed using various microscopic techniques to investigate the biofilm structure as described in following sections. In order to observe thick biofilms in their natural hydrated condition under the Scanning Confocal Laser Microscope, a chamber was prepared as described in section 3.2.2.3.

3.2.2.1 Sample preparation and examination under light microscopy

Biofilms were stained using Alcian Blue method described by Gurr (1963). Glass slides removed from the wastewater treatment plants were immersed in 0.1% Alcian Blue solution (w/v) for 1 minute followed by a rinse in tap water and air drying. The slide was then counter stained with 10% Carbol Fuschin (w/v) for 3 minutes and gently rinsed under running tap water to prevent overstaining.

Prepared slides were examined using an Olympus CX40RF200 microscope at x150 and x600 magnification. Colour photomicrographs were taken using an Olympus SC36 type 12 camera using Kodacolor 100 films attached to the microscope. These films were then converted to digital images using Kodak Pro Photo CD software and an IBM compatible computer (Digital Masters, Australia).
3.2.2.2 Sample preparation and examination under Scanning Electron Microscope

Glass slides removed from the wastewater treatment plant were immediately fixed with 0.5% gluteraldehyde for one hour followed by overnight fixation in 0.1M cacodylate buffer (pH 6.2). The samples were dehydrated in a series of ethanol solution, 30%, 50%, 70%, 95%, 30 minutes each and in 100% twice for 20 minutes. Dehydration was completed by transferring the samples to 100% acetone followed by critical control point drying in liquid carbon dioxide. Samples were sputter coated with gold using a Polaron sputter coater with a Magnetron head and examined in a Scanning Electron Microscope model Stereoscan 360 at 20 kV.

3.2.2.3 Sample preparation and examination under Scanning confocal laser microscope (SCLM)

Glass coverslips removed from the wastewater treatment plant were mounted on glass slides leaving a gap between the slide and the coverslip. This was achieved by fixing 1mm thick glass pieces along the edge of the glass slide using glue (Sellys Supaglue 3ml). A sufficient amount of phosphate buffered saline (PBS, pH 6.8) was placed in the “chamber” in order to keep the biofilm wet during observations. Rhodamine X was injected into the chamber at a concentration of 0.1% without disturbing the biofilm, to allow visualization of the biofilm microorganisms.

Images were obtained using an Olympus BH2 RFCA Scanning Confocal Laser Microscope in conjunction with a x100, 1.3 numerical aperture (NA) oil immersion lens. Phase contrast microscopy was conducted with a 100 W tungsten lamp (Zeiss illuminator 100) and a green interference filter (Zeiss VG-9). An Argon/Krypton laser with optimum emission line at 586nm was used as the excitation source for the fluorophore Rhodamine X.
The images were acquired using the compatible computer software LSM – GB 200 provided by the Olympus microscope system. Beam scanning through the x and y directions was facilitated through the use of galvinometrically controlled mirrors. Intervals between optical thin sections (xy sections) and the positions of sagittal sections (xz positions) were user definable by using the software in conjunction with the computer controlled, motor-driven focusing system connected to the microscope. The images were photographed using a Kodak camera equipped with a 49mm lens with Kodacolor AS 100 film. Images were also saved in the computer for later analysis.

3.2.3 Measurement of biofilm thickness

Only two techniques using light microscope and scanning confocal laser microscope were used for measurement of biofilm thickness. Scanning electron microscopic techniques were not used to obtain biofilm thickness.

3.2.3.1 Using the light microscope

Biofilm thickness was measured using a modification of the method described by Truelar (1983).

This involved the measurement of the thickness of microbial film using a microscopic calibration as follows. A slide containing the biofilm was removed from the wastewater treatment plant and placed on the stage of an Olympus BH 200 microscope. The x10 objective (x150 total magnification) was lowered until the biofilm surface was in focus and the adjustment dial setting was recorded. The objective was then lowered until a mark on the glass slide was in focus. Biofilm thickness was determined by comparing the difference in the adjustments. The difference in fine adjustment settings was
compared with a calibration curve and the thickness was determined. The reported biofilm thickness was the mean of 25 measurements along the slide.

3.2.3.2 Using the Scanning Confocal Laser Microscope

Saggital sections along xy positions of all biofilms were performed using the Olympus BH2 RFCA Scanning Confocal Laser Microscope as described in section 3.2.2.3. The scanning laser beam was directed through the upper surface of the chamber to section the biofilm. The top of the biofilm was located, focused and the stage elevation position was noted. Without changing the position of the biofilm, the bottom of the biofilm was located and the stage elevation was noted. The difference between the top and bottom stage elevation parameters of the Piezo stage was used to calculate the biofilm thickness. The process was repeated for 25 randomly selected locations on the biofilm.

3.2.4 Examination of surface topography of biofilms by construction of contour maps using Scanning confocal laser microscopic techniques

Contour maps were constructed by selecting the contour maps option from the Tool bar of LSM GB 200™ software provided with the confocal system. Selection of this option allowed one to calculate the surface topography of the whole surface compared with the thinnest point of the surface projected onto a plane.
3.2.5 Analysis of void and occupied space of biofilms using Scanning Confocal Laser Microscopy

Images obtained via Scanning Confocal Laser Microscope were analysed using Image ProPlus® application by plotting gray-level histograms. The number of pixels for each intensity level in the range of 0 (black) to the maximum gray-level (white) was recorded and boundaries were marked for each object. Digitised images were then discriminated to capture cellular and noncellular material in the biofilm along the whole slide. Captured data was transferred to an Excel data file. This data file contained information on the area of each cellular and non-cellular object form. The data was used to construct scatter diagrams representing the object number versus the area of the object. These diagrams were then used to estimate the total area occupied by cellular and non-cellular object forms of the image. Void fraction included the difference between the total area and occupied area of a particular image. Percentage occupied area and void area of each biofilm image were calculated by dividing the occupied and void areas by the total area of the image.

3.2.6 Construction of three dimensional stereoscopic image of a biofilm using Scanning Confocal Laser Microscopy

Optically thin sections along $xy$ axis were obtained by collecting images at 1.2μ intervals for the construction of three-dimensional image of the biofilm. These sections were then serially arranged and stereo pairs were created with LSM GB 200 software provided with the Olympus BH2 RFCA Scanning Confocal Laser Microscopic system.
3.3 RESULTS

3.3.1 Biofilm structure

The structure of developing biofilms was examined using light microscopy (LM), scanning electron microscopy (SEM) and scanning confocal laser microscopy (SCLM). Biofilm samples were collected from the wastewater treatment plant on day 1, day 2, day 3, day 5 and day 7.

LM photomicrographs of 1, 2 and 3 day old biofilms showed the distribution of two major components of the biofilm: microbial cells stained in red and extracellular polymer substances (EPS) stained in blue (Figs 3.1a, 3.1b, 3.1c and 3.1d). Colonisation of the surface by wastewater microflora appeared to be patchy and more EPS accumulation was observed on the biofilm when more cells were present. It was not possible to view the internal structure of the biofilm using light microscopic techniques when the biofilm was mature and thick. Heavy stain accumulation and dark areas with no apparent structure were observed in 5 day and 7 day old biofilm samples (Figs 3.1e and 3.1f). Different cell types could not be identified at lower magnifications (x150) under the light microscope. A 2 day old biofilm examined under the light microscope at higher magnifications (x600), showed different cell types, however, the background was unclear due to out-of-focus haziness (Fig 3.1c). Large rods, spherical cocci and short rods were observed in this biofilm. Majority of the rod shaped cells at this stage had formed chains and cell aggregates. The cells were stained in red while the surrounding EPS was clearly stained in blue.

The SEM photomicrograph of a 1 day old biofilm showed cells attached to the surface, but EPS was not visible (Fig 3.2a). In this biofilm the cells attached to the surface were primarily rod shaped, and some cocci and short rods were observed. Mature
biofilms observed under SEM showed cell aggregations and shrunken EPS (Figs 3.2b, 3.2c, 3.2d and 3.2e). The cell aggregation appeared to be less in these biofilms viewed under SEM compared to biofilms examined under the light microscope. A 1 day old biofilm viewed under SEM at a higher magnification is shown in Fig. 3.3a. This initial biofilm showed the presence of flagella in some cells, but EPS was not visible. Fig 3.3b is a 3 day old biofilm at a higher magnification showing shrunken EPS and collapsed structure.

SCLM images obtained through the confocal system as described in section 3.2.2.3 demonstrated the presence of cells and EPS without background blur (Figs 3.4a, 3.4b and 3.4c). Cells embedded in the EPS were distinctly visible. The morphology of cells was not clear at the magnifications observed (x150). In 5 and 7 day old mature biofilms some areas were densely covered with cells embedded in EPS while some areas were empty and devoid of cells and EPS (Figs 3.4d and 3.4e) or any other structures.

3.3.2 Biofilm internal structure and architecture

The internal structure of biofilms was examined using non-destructive SCLM techniques. An optical section taken across the xz plane, parallel to the attached surface, of a 3 day old biofilm showed colonised areas as well as porous areas within the biofilm (Fig 3.5). A series of optical sections taken across xz plane of a mature 7 day old biofilm showed hollow areas and densely covered areas with cells and EPS (Fig 3.6). This biofilm was 26.2μm thick and sections were taken at 1.2μm intervals. The internal structure of the biofilm appeared to be porous in the cross sections examined. It was not possible to observe the internal structure of thick biofilms using LM and SEM techniques.
Fig. 3.1  Light Microscopic photomicrographs of biofilm samples collected from the wastewater treatment plant stained with Alcian Blue and Carbol Fuschin

(a)  1 day old biofilm (x150). EPS was stained in blue and cells were counterstained in red. Cell aggregation was seen in some areas (arrow). Cells and EPS of different planes caused out-of-focus haziness

(b)  2 day old biofilm (x150) showing an increase in cell numbers and EPS clearly visible. More EPS (arrow) was seen where cell density was higher

(c)  2 day old biofilm (x600) showing cell morphology of biofilm colonisers. Large and small rods and cocci were observed as pointed by arrows

(d)  3 day old biofilm (x150) showing increasing cell aggregation

(e)  5 day old biofilm (x150). Thick biofilm heavily colonised with cells embedded in EPS. The structure was not clear due to the thickness of the biofilm. Heavy stain accumulation was observed as indicated by arrow

(f)  7 day old biofilm (x150). Biofilm appeared dark and structure was not clear due to increased thickness. Heavy stain accumulation was observed as pointed by arrow
Fig. 3.2 Scanning Electron Micrographs of biofilm samples collected from the wastewater treatment plant prepared by Critical Control Point Drying

(a) 1 day old biofilm. Rods and cocci were present in this biofilm as a monolayer. Cell aggregation was not observed. No EPS was visible

(b) 2 day old biofilm showing an increase in cell aggregation. EPS was visible but the structure appeared to be shrunken (arrow)

(c) 3 day old biofilm showing increased cell aggregation and shrunken EPS

(d) 5 day old biofilm. Some areas of the biofilm showed cells embedded in EPS (arrow)

(e) 7 day old biofilm. The biofilm structure appeared to have collapsed, but cells were clearly visible (white arrow). In some areas cells embedded in EPS was observed (black arrow).
Fig. 3.3  Scanning Electron Micrographs of biofilm samples collected from the wastewater treatment plant prepared by Critical Control Point Drying

(a)  1 day old biofilm showing morphology of initial colonisers of the biofilm. Large rods, short rods and some cocci were present in the biofilm. Flagella were evident in some cells (arrows) suggesting involvement of such appendages in attachment. EPS was not visible

(b)  3 day old biofilm showing cell aggregation. EPS was visible but the structure was collapsed and shrunken (arrow).
Fig. 3.4 Scanning confocal laser microscopic (SCLM) images of biofilm samples collected from the wastewater treatment plant stained with Rhodamine X

(a) 1 day old biofilm. Cells were very clear and mostly adhered to the surface as a monolayer. Cells embedded in EPS were visible and the structure of EPS appeared to be “hydrated”. No background blur was observed.

(b) 2 day old biofilm showing an increase in cell aggregation (arrow). EPS was visible and distinct.

(c) 3 day old biofilm showing increased cell aggregation and cells embedded in EPS

(d) 5 day old biofilm. Cells embedded in EPS (arrow) and hollow areas forming channels (arrow) were evident in this biofilm.

(e) 7 day old biofilm. The biofilm was heavily colonised and structure appeared to be thick and hydrated with cells embedded in EPS. Hollow channels were also visible.
Fig 3.5  

An optical section of a 3 day old biofilm taken across $xz$ plane, parallel to the attached surface, using SCLM.

The biofilm was stained with Rhodamine X to visualise cells and EPS. Arrows point towards porous areas. The internal structure of the biofilm appears to contain open channels and dense areas.
Fig 3.6  SCLM micrographs of horizontal optical thin sections (along xz plane), taken at 1.2μm intervals of a 7 day old biofilm developed in the wastewater treatment plant stained with Rhodamine X

Section marked “0” is the biofilm attached to the surface whilst the last section shown in this photomicrograph was at the opposite end 26.2μm away from the attached surface. Hollow areas representing channels were present within the dense biofilm.
3.3.3 Biofilm thickness

Biofilm thickness measurements were obtained by light microscope according to the method of Trulear (1983) and using SCLM sections. These results were obtained by averaging twenty-five random measurements taken across five replicate biofilm samples. Results obtained using light microscope showed an increase of biofilm thickness over time, however, this method did not detect differences in thickness when the biofilms were thin (Table 3.1). According to this method a 1 day old biofilm showed a thickness of 4.0 (± 1.2)μm while the thickness of a 2 day old biofilm was (3.2 ± 1.1)μm. The thickness of 3, 5 and 7 day old biofilms was 8.2 (± 2.5)μm, 16.8 (± 1.8)μm and 19.4 (± 2.9) μm respectively.

Optical sections of biofilms obtained along xy plane perpendicular to the surface using SCLM interactive software are presented in Fig 3.7. The system recorded the top and bottom elevation positions of the microscope stage and the difference between these two readings was used to calculate the thickness of biofilm at that position. The thickness of biofilms gradually increased as the biofilms matured with time. The thickness of 1, 2, 3, 5 and 7 day old biofilms when measured using SCLM methods were 5.6 (± 0.8)μm, 7.2 (± 1.5)μm, 13.2 (± 3.7)μm, 22.4 (± 5.1)μm and 24.8 (± 6.2)μm respectively. The vertical sagittal section of a 1 day old biofilm taken along the xz plane was thin and non-confluent (Fig 3.7a). The yellow areas in the image indicate stain accumulation in cell free areas. Optical sections of 3 day and 5 day old biofilms clearly showed the confluent nature of the biofilm due to cells and EPS with varying thickness along the length of the slide.

It was not possible to obtain any measurements for biofilm thickness using the SEM methods.
Table 3.1  Biofilm thickness measured by light microscopy according to the method described by Trulear (1983) and using Scanning Confocal Laser Microscopy.
(Five samples were analysed for each day and 25 measurements were measured across each biofilm)

<table>
<thead>
<tr>
<th>Age of the Biofilm (days)</th>
<th>Biofilm thickness (μ m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light Microscopy</td>
</tr>
<tr>
<td>1</td>
<td>4.0 (± 1.2)</td>
</tr>
<tr>
<td>2</td>
<td>3.2 (± 1.1)</td>
</tr>
<tr>
<td>3</td>
<td>8.2 (± 2.5)</td>
</tr>
<tr>
<td>5</td>
<td>16.8 (± 1.8)</td>
</tr>
<tr>
<td>7</td>
<td>19.4 (± 2.9)</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviations
Fig 3.7  SCLM images of optical sections of biofilms taken perpendicular to the attached surface.

Arrows indicate the attached surface

(a) 1 day old biofilm showing almost a monolayer of cells
(b) 3 day old biofilm showing cell aggregations
(c) 5 day old biofilm showing a confluent biofilm with varying thickness along the length of the slide.
3.3.4 Surface topography of biofilms and contour maps

The surface topography of biofilms was examined using SCLM-simulated contour maps as described in section 3.2.4 (Fig 3.8). Any accumulation on the surface is represented as a peak in contour maps. The 1 day old biofilm showed little variation in surface topography with a few peaks (Fig 3.8a) while the 2 day old biofilm showed many peaks originating from the surface (Fig 3.8b), yet planar areas were also present on this contour map. A large number of peaks were observed in the 3 day biofilm, which covered almost the whole surface (Fig 3.8c). The 5 day and 7 day biofilms on the other hand showed broad peaks due to increase in dense areas and variations in surface topography was observed along the image (Figs 3.8d and 3.8e).
Fig. 3.8  Contour map representation of biofilm surfaces showing topography, obtained by SCLM. Peaks (ridges) are indicated by yellow arrows, while red arrows indicate planar areas

(a)  Topography of a 1 day old biofilm surface appeared to be mostly flat indicating few small peaks where cells were attached. Two broad peaks were present in this biofilm where cell aggregates were present

(b)  Topography of a 2 day old biofilm surface showing increased number of peaks due to increased colonisation

(c)  Topography of a 3 day old biofilm surface showing a large number of peaks due to increased colonisation and cell aggregation

(d)  Topography of a 5 day old biofilm surface showing large broad peaks due to cells embedded in EPS and cell aggregation

(e)  Topography of a 7 day old biofilm surface showing heavy peaks due to extensive coverage of the surface by cells and EPS

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3.3.5 Occupied and void space area in the biofilms

The % occupied area and void area of biofilm images were calculated by image analysis techniques described in section 3.2.5. The resultant scatter diagrams are presented in Figs 3.9 and 3.10. Occupied and void area calculations derived from these scatter diagrams are presented in Table 3.2.

Image analysis resulted in counting cells, cell aggregates and EPS as "objects". A 1 day old biofilm contained 532 objects on the image and majority of these objects occupied an area below 400\(\mu\text{m}^2\) each. The largest object occupied an area of 488\(\mu\text{m}^2\) (Fig 3.9a). The total occupied area of this biofilm was 2.69 (± 0.54)\% of the total image. A 2 day old biofilm contained 969 objects. Majority of these objects occupied an area below 600\(\mu\text{m}^2\) each while few objects covered larger areas ranging from 1039\(\mu\text{m}^2\) to 4016\(\mu\text{m}^2\) (Fig 3.9b). The total coverage of this biofilm was 8.37 (± 1.22)\% of the total image.

Scatter diagrams of 5 day and 7 day old biofilms are shown in Fig 3.10. The 5 day old biofilm contained 2197 objects where majority of them covered an area of less than 1500\(\mu\text{m}^2\) each. Two large objects were shown on the scatter diagram, which occupied an area of 8444\(\mu\text{m}^2\) and 24435\(\mu\text{m}^2\) each (Fig 3.10a). The coverage of this biofilm was 33.54 (± 3.85)\% of the total area. The 7 day old biofilm had 4909 objects on the scatter diagram (Fig 3.10b). The two largest objects occupied an area of 28951\(\mu\text{m}^2\) and 28903\(\mu\text{m}^2\) each. The coverage of this biofilm by objects was 46.19 (± 7.42)\% of the total area suggesting presence of void unoccupied area amounting to 53.84\% of the total biofilm.
**Fig. 3.9**  
**Biofilm coverage presented as scatter diagrams.**

Cells, cell aggregates and EPS were defined as "objects" for the purpose of analysis. The area occupied by each object was estimated using Image Proplus™ software after defining boundary limits. Captured data was exported to excel files and scatter diagrams were constructed to estimate the total area of biofilm covered by "objects".

(a) Scatter diagram of a 1 day old biofilm – captured data showed that there were 532 objects on the selected image area. The largest object covered an area of $488 \mu m^2$, while the other objects were below $400 \mu m^2$.

(b) Scatter diagram of a 2 day old biofilm – captured data showed that there were 969 objects. Majority of these objects occupied an area below $600 \mu m^2$ each. Largest object covered an area of $4016 \mu m^2$.  

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Fig. 3.10  Biofilm coverage presented as scatter diagrams

Cells, cell aggregates and EPS were defined as “objects” for the purpose of analysis. The area occupied by each object was estimated using Image Proplus® software after defining boundary limits. Captured data was exported to excel files and scatter diagrams were constructed to estimate the total area of biofilm covered by “objects”.

(c) Scatter diagram of a 5 day old biofilm – captured data showed that there were 2197 objects on the selected image area. The largest object covered an area of 24435μm², while most of the other objects were below 1500μm².

(d) Scatter diagram of a 7 day old biofilm – captured data showed that there were 4909 objects. Majority of these objects occupied extensive areas of the biofilm suggesting aggregation. Largest object covered an area of 28951μm².
Table 3.2  Percentage of void and occupied areas of developing biofilms calculated by Image Analysis. Five biofilms were analysed and averaged to obtain these values

<table>
<thead>
<tr>
<th>Biofilm Age (days)</th>
<th>% Occupied Area</th>
<th>% Void Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.69 (± 0.54)</td>
<td>97.31 (± 0.54)</td>
</tr>
<tr>
<td>2</td>
<td>8.37 (± 1.22)</td>
<td>91.63 (± 1.22)</td>
</tr>
<tr>
<td>3</td>
<td>21.56 (± 4.59)</td>
<td>78.54 (± 4.59)</td>
</tr>
<tr>
<td>5</td>
<td>33.54 (± 3.85)</td>
<td>66.46 (± 3.85)</td>
</tr>
<tr>
<td>7</td>
<td>46.19 (± 7.42)</td>
<td>53.84 (± 7.42)</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviations
3.3.6 Visualisation of the biofilm as a three-dimensional image

The reconstructed three-dimensional image of a 7 day old biofilm is shown in Fig 3.11. This image was created by arranging the series of 1.2 μm optically-thin sections shown in Fig 3.6 in order, to form a stereoscopic image. Reconstructed image allows visualization of the entire data set together on a 3D plane.
Fig 3.11 3D Image compiled from the 25 sections shown in Fig 3.6

This was constructed by serially arranging the 25 sections shown in Figure 3.6 to create stereo pairs using the LSM GB 200 software provided by the confocal system.

Red and green glasses must be used to view the depth of this image which shows void areas within the biofilm
3.4 DISCUSSION

This study adopted a simple approach to develop biofilms in the wastewater treatment plant. Precleaned microscopic slides or glass coverslips suspended in the Activated Sludge Basin (ASB) for the required period as described in section 3.2.2.3 was a simple convenient way of collecting biofilms from the wastewater treatment plant at known time intervals without disruption. This study also utilised a "chamber" prepared using glass slides to observe biofilms in situ. The chamber contained a sufficient quantity of buffer to keep samples hydrated, thus eliminating problems of shrinkage and artifact formation. Unlike previous studies that utilised Robin's Device (McCoy et al., 1981) or reactors (Bakke et al., 1984; Marshall, 1980) as surfaces to develop biofilms this simple method was convenient and allowed visualisation of biofilms without disruption, still attached in a hydrated state with no apparent collapse in the structure.

Further, this study has utilised light microscopy (LM), Scanning Electron Microscopy (SEM) and Scanning Confocal Laser Microscopy (SCLM) to examine the structure and architecture of developing biofilms.

All three microscopic methods used in this study allowed visualisation of cells and extracellular polysaccharides (EPS) of the biofilms. Examination of biofilms using the light microscope after staining with Alcian Blue and Carbol Fuschin proved to be a valuable method in identifying and locating the distribution of EPS during early stages of biofilm development (Fig 3.1). This staining technique allowed visualisation of EPS which was stained in blue against cells that were counter-stained in red within biofilms. Using this technique distribution of EPS in early biofilm samples were observed to be closely associated with cells (Fig 3.1a-d). More EPS was observed where greater cell aggregation was observed consistent with early observations that EPS in biofilms was produced by microorganisms (Costerton et al., 1985). Further, observation of EPS in very
early biofilms associated with single cells attached to the surface of the biofilm confirmed that EPS production was one of the early events of attachment (Fig 3.1a) in agreement with Stenström (1989) and Fletcher et al. (1991). Preparation and staining of biofilm samples for light microscopy by Alcian Blue method was simple and less time consuming and was valuable in locating the distribution of EPS within biofilms less than 10μm in thickness. Main disadvantage of light microscopy was the inability to reduce the out-of-focus blur with samples having a thickness of greater than 10μm. Although this could be partially corrected by flattening the biofilm on the microscopic slide, this caused disruption of the biofilm structure. Mature biofilm samples examined under the light microscope appeared black with heavy stain accumulation (Fig 3.1e and f). The internal structure of mature biofilms exceeding 10μm thickness could not be examined in situ using light microscopy.

Scanning Electron Microscopic (SEM) techniques allowed visualisation of biofilms at higher magnifications, thus cell morphology of biofilm colonisers was visible when samples were examined under SEM (Fig 3.2). Presence of flagella in some bacterial cells was observed in a 1 day old biofilm sample using SEM suggesting the possible involvement of flagella during the initial stages of attachment and biofilm formation (Fig 3.3a). The presence of EPS in early biofilms could not be demonstrated when samples were examined under SEM. Although EPS was visible in mature biofilms, the structure had collapsed due to shrinkage (Fig 3.3b) occurred during the dehydration steps. Sample preparation for examination of biofilms under SEM was laborious and produced artifacts and shrinkage of structure during dehydration and fixation of samples (Figs 3.2 and 3.3). These observations are in agreement with Kinner et al., 1983; Robinson et al., 1984; Costerton et al., 1987 and Stewart et al., 1993. SEM techniques can scan the surface of a sample but not the interior (unless TEM techniques are used) limiting the application of
such techniques to a thick biofilm. SEM techniques may act as an invaluable tool to study the surface characteristics of isolated microorganisms, especially in pure/mixed culture form.

Photomicrographs obtained from Scanning Confocal Laser Microscope (SCLM), clearly showed the cellular components of biofilms and surrounding EPS without the focal problems and shrinkage, providing excellent contrast (Fig 3.4). SCLM microscopy reduces the thickness of the focal region by using lenses with high numerical apertures to achieve this (Lawrence et al., 1989). The simple staining procedure employed in this study did not permit visualisation and location of EPS attached to single cells in early biofilms, however, cells embedded in EPS were evident in areas where cell aggregation was observed. The magnifications available with the SCLM system were not adequate to examine morphological features associated with early stages of attachment such as involvement of microbial appendages during early biofilm formation.

Biofilm thickness was measured by SCLM techniques using a series of vertical optical sections made perpendicular to the attached surface. Unlike the method adopted by Trulēar (1983), which relied on manual measurement of biofilm thickness using a light microscope, SCLM techniques provided a simple, effective method of measuring the thickness of developing biofilms. Although both methods showed the development of a biofilm as indicated by increasing thickness with time, the light microscopic technique was not as sensitive as the SCLM method in discriminating the differences between relatively thin biofilms due to errors involved in manual techniques and visual judgement. SCLM techniques proved to be a convenient and reliable method for measuring biofilm thickness compared with the light microscopic techniques. These results are consistent with observations made by Lawrence et al. (1991) and Caldwell et al. (1992). It was assumed in SCLM techniques that the attached surface was reached
when there was no fluorescence observed. Whether the drop-off of fluorescence was due
to reaching the limit of the biofilm, or due to the depth of penetration of excitation light,
is questionable.

The application of SCLM-simulated fluorescence processing or shadow imaging
showed topographical variations in the biofilm containing ridges and grooves (Fig.3.8).
Observations made by Gorman et al. (1991) suggested considerable variations in surface
roughness of biofilms formed on catheter surfaces. Our results demonstrated
heterogeneous nature of the biofilm at different points varying with time. These images
confirm that biofilms are not planar structures, but are structurally heterogeneous, with
organised niches developed by microorganisms over time. Shadow imaging and contour
map construction was only possible using SCLM techniques and hence this information
could not be gathered from light microscopy or SEM.

Application of image analysis techniques to SCLM images and condensing
extracted data to plot scatter diagrams allowed the calculation of the percentage of the
occupied and unoccupied areas of the biofilms. The results suggest that biofilms growing
in the wastewater treatment plant were not tightly packed but actually contained ‘porous’
unoccupied areas (Table 3.2). Examination of a series of optical sections taken parallel to
the surface of a thick biofilm developed in the wastewater treatment plant using SCLM
techniques also showed that the internal structure of this biofilm was not densely packed,
but was porous and contained channel like structures (Fig 3.6). Davey and O’Toole
(2000) suggested that biofilms are pierced with channels that allow nutrients to reach the
interior and waste to be carried away. Optical sectioning and non destructive examination
of biofilms in situ was only possible with SCLM techniques and therefore this
information was valuable and could not be gathered from light microscopy and SEM
techniques.
3.5 CONCLUSION

The nature of the information about biofilm structure that each microscopic method yielded and limitations inherent with the three approaches differed. SCLM techniques offered a method for detailed visualization of thick biofilms eliminating the focal problems associated with light microscopic and SEM techniques. SCLM techniques also permitted visualization of the internal structure of the biofilm in situ non-destructively. This method involved less sample processing and probably provided the most accurate picture of the biofilm structure. The biofilm remained hydrated and attached to the substratum eliminating dehydration and artefact formation with this technique. SCLM techniques also provided optical sectioning allowing examination of the internal structure and architecture of biofilms. SCLM techniques provided tools for analysing topography of surfaces and to extract quantitative data readily for analysis. Perhaps the unique feature of CSLM is its ability to image biofilms in situ and in real time. The disadvantage of using SCLM was the inability for the system to provide very high magnifications to the cellular level.

Conventional SEM preparation techniques were laborious and lead to shrinkage, loss of EPS and artefact formation. Apart from these limitations the interior of the biofilm was not accessible and quantitative analysis of SEM images was difficult because they condensed three-dimensional information into two dimensions. The advantage of this technique was the availability of very high magnifications to the cellular level.

Light microscopy, on the other hand was found to be a simple technique, involved least sample preparation requirements and convenient for quick observations. The Alcian Blue staining method used was convenient and showed the distribution of EPS in early biofilms. The disadvantages were the inability to visualise thick biofilms and problems of out-of-focus blur as discussed previously.
In summary, using microscopic techniques the presence of EPS and growth of biofilm by cell aggregation over time was well demonstrated. Using non-destructive optical sectioning the internal structure of the biofilm was demonstrated to be porous and containing channel-like structures. In summary, SCLM proved to be a convenient non-destructive method to examine biofilms \textit{in situ} as demonstrated by this study.
PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.
CHAPTER 4

MICROFLORA OF THE BIOFILM
4.1 INTRODUCTION

Bacterial communities play a key role in the production and degradation of organic matter and many environmental pollutants. Most of these processes require the concerted effort of bacteria with different metabolic capabilities, and it is therefore likely that bacteria residing within biofilm communities carry out many of these complex processes (Davey and O'Toole, 2000). The activated sludge process, in terms of total metabolized matter, is an important biotechnological process that uses bacterial biofilms for the purification of wastewater.

A current model is that the development of biofilms on surfaces proceeds as a succession of adhesion and multiplication events (Rickard et al., 2003). Under suitable conditions the primary colonisers multiply to form microcolonies, secondary colonisers then attach to the primary colonisers or to the conditioned surface forming a multi-species biofilm community.

The objectives of this section of the study were:

1) to compare the culturable versus the total number of microorganisms over time in biofilms growing in the wastewater treatment plant

2) to isolate and identify predominant culturable bacteria using conventional techniques

3) to follow the colonisation pattern of a key bacterial member using cultural and molecular techniques.

The flowchart below summarises the approach used in this section to study biofilm microorganisms (Fig 4.1).
Biofilms developed in the wastewater treatment plant (Section 2.4)

Culturable viable bacterial cell count and total cell count of biofilm and planktonic populations (Sections 4.2.1 and 4.2.3)

Is it possible to identify a succession of bacteria during the development of biofilms?

Isolation and identification of culturable bacteria from biofilms (Section 4.2.2)

If so, is it possible to follow the colonisation pattern of a key bacterial member?

Using Culture methods (Section 4.2.5.1) Using 16S rRNA probes (Section 4.2.5.2)

Probe design and synthesis (Section 4.2.5.2.1)

Optimising probe hybridisation conditions (Section 4.2.5.2.2)

Evaluation of probe specificity (Section 4.2.5.2.3)

Fluorescent In situ Hybridisation (FISH) of biofilms to follow colonisation patterns (Section 4.2.5.2.4)

Fig. 4.1 Overview of conceptual approach taken in biofilm community analysis; relevant chapter sections in methods are given in brackets
4.2 MATERIALS AND METHODS

4.2.1 The culturable viable bacterial cell count

The viable bacterial count of sessile biofilm population and planktonic population was determined using culture methods as described below.

4.2.1.1 Sessile biofilm population

Biofilms were allowed to develop on glass microscope slides placed in the wastewater treatment plant as described in section 2.4.

Five replicate glass slides were removed from the wastewater treatment plant after 1, 2, 3, 5, and 7 days and placed in an insulated container maintained at 4°C while transported to the laboratory. The samples were analysed for culturable viable count upon arrival in the laboratory as follows. Each glass slide was placed inside a sterile blender bag containing 50ml of sterile phosphate buffered saline (PBS pH 7.2) and the biofilm was removed from the glass slide by rubbing the bag from outside whilst holding the slide with fingers. The washed glass slide was removed aseptically and placed inside another sterile bag containing 50ml of PBS and the above procedure was repeated. The washed glass slide was observed under the light microscope to ensure that no more bacterial cells were attached to the slide. All washes from one glass slide were combined and the dilution factor was noted. 0.1ml of each wash was plated on nutrient agar (Oxoid, Australia) using spread plate technique. One ml of the first dilution was transferred into 9ml of PBS and serial dilutions (1/10 – 1/100,000) were prepared and plated on nutrient agar as before. All plates were incubated at 30°C for 48h under aerobic conditions. The
number of separate colonies formed on each plate after incubation was counted and recorded.

4.2.1.2 Planktonic population

At the same time that the slides were removed a wastewater sample was collected from the same depth at the test site. The sample was placed in an insulated container maintained at 4°C and transported to the laboratory. Each sample was analysed in five replicates. The spread plating technique as described in section 4.2.1.1 was used for enumeration of total viable culturable bacteria in each wastewater sample.

4.2.2 Isolation and identification of culturable bacteria

Isolates with different colonial characteristics on nutrient agar were isolated and purified by restreaking onto sterile fresh nutrient agar plates. The culture purity was checked macroscopically and by the gram stain procedure. Isolated pure cultures were identified using a series of preliminary screening tests consisting of Gram stain, catalase and / or oxidase reaction, motility and fermentation of glucose. The standard procedures used in these analyses are described in Cowan and Steel (1974) and McFaddin (1980). On the basis of the results of the preliminary screening tests, an appropriate array of biochemical tests was subsequently undertaken using the API system (BioMerieux Vitek, Australia) and incubated according to the manufacturer’s guidelines. API profiles generated by biochemical reactions were checked with reference numbers provided by the manufacturer. Some organisms could not be identified to species level on the basis of these biochemical tests and therefore in the text are only designated to the genus level.
Identified cultures were routinely subcultured and maintained at 4°C for further use and also maintained as stock cultures stored in 10% glycerol at -70°C for reference purposes.

4.2.3 Enumeration of total bacteria in biofilms developed in the wastewater treatment plant using direct microscopy

4.2.3.1 Collection of samples

Biofilms were allowed to develop in the wastewater treatment plant and transported to the laboratory as described in Section 2.4.

4.2.3.2 Direct microscopic count

Glass slides containing biofilm samples were fixed in a fresh 1% solution of paraformaldehyde in phosphate buffered saline (10mM NaPO₄, 120mM NaCl pH 7.2) followed by two rinses with MilliQ water. Slides were then stained for 5min in a 0.1% solution of Rhodamine X (Sigma Aldrich, Australia) in PBS and washed twice with 10ml of MilliQ water to remove excess stain. Samples were examined and the total cell number was determined using an Olympus BH2 RFCA Scanning Confocal Laser Microscope (SCLM), fitted with an Argon/Krypton laser with optimum emission at 586nm.

4.2.4 Measurement of biofilm thickness

Thickness of biofilms collected for population enumeration was measured as described in section 3.2.3.2 using an Olympus BH2 RFCA Scanning Confocal Laser Microscope (SCLM). This measurement was required to determine the total microscopic count per cm³ of biofilm.
4.2.5 Detection of *Bacillus cereus* in biofilms

As *Bacillus cereus* was identified as a key bacterial member that appeared in the 1 day old biofilm samples examined by culture techniques (Section 4.2.2), it was decided to study the colonisation pattern of this microorganism in detail.

4.2.5.1 Detection of *Bacillus cereus* using culture methods

Biofilms were collected and prepared as described in section 4.2.1.1. In addition to 1, 2, 3, 5 and 7 day old biofilms additional samples were collected after 4h and 8h from the wastewater treatment plant. This was undertaken to identify early colonisers of the biofilm formed in the wastewater treatment plant.

The cultural method described in Australian Standard, AS 1766.2, was used to estimate the viable count of *B. cereus*. The biofilm was removed from the surface of the slide as described in section 4.2.1.1 and serial dilutions in PBS were cultured on sterile PEMBA (Polymixin Egg yolk Methylene Blue Agar – Oxoid, Australia) agar plates using spread plate technique. Plates were incubated at 37°C for 48h followed by incubation at room temperature for a further 48h as described in the media manufacturer’s manual. *B. cereus* colonies form large peacock blue colour colonies surrounded by a white precipitate on the PEMBA medium. After incubation the peacock blue colour colonies with a white precipitate were counted as presumptive *B. cereus* as described in AS 1766.2. These colonies were isolated and purified on nutrient agar (Oxoid) and confirmed as *B. cereus* using API 50 CHB (Biomerieux, Australia) bacterial identification kit.
4.2.5.2 Detection of *Bacillus* species using 16S rRNA targeted oligonucleotide probes

4.2.5.2.1 Oligonucleotide probe design and synthesis

The oligonucleotide probe RDR 502 (Greisen *et al.*, 1994), complementary for the 16S rRNA region of *Bacillus* species (at the domain level), and corresponding to nucleotide position 1354 to 1378 of *Escherichia coli* sequence was selected for this purpose. The probe sequence was 5’GTA TTC ACC GCG GCA TGC TGA TCC G-3’ as described in Greisen *et al.*, 1994. The synthesised probe was purchased from Genset K.K © (1-3-18 Akasaka, Minato Ku, Tokyo 107, Japan).

The *Bacillus* probe RDR 502 was synthesized with a C6-TFA aminolinker [6-(trifluoroacetyl-amino) -hexyl- (2-cyanoethyl) - (N, N-diisopropyl) phosphoramidite] at the 5’ end and labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS). The base sequence and labeling requirements for probe RDR 502 were provided to Genset and purchased as purified labeled oligonucleotide probes.

The tube containing lyophilised probe was centrifuged lightly for 5 seconds using a bench top Beckmann centrifuge and resuspended in sterile MilliQ water at a concentration of 50ng μl⁻¹. The tube was gently mixed by hand for 1 minute followed by vortexing for 15 seconds on a Beckman vortex. The resuspended probe was stored in clean microcentrifuge tubes in aliquots of 25μl at -70°C until further use.

4.2.5.2.2 Optimising probe hybridisation conditions

The probe hybridisation conditions were optimised using a pure culture of isolated target *B. cereus* cells as described below. This culture was isolated from a 1 day old biofilm developing in the waste water treatment plant.
The culture was prepared according to the method of Amann et al. (1990). A culture of the *B. cereus* isolated from biofilm was grown in TSB (Tryptone Soya Broth – Oxoid, Australia) for 18 h at 37°C. The cells were harvested by centrifugation at 5000xg for 10 min. The supernatant was discarded and the pellet containing cells was washed twice in MilliQ water. Culture purity was checked by microscopic examination.

One of the pre-requisites for successful hybridisation between the probe and 16S rRNA sites is the permeabilisation of bacterial cell to the probe. Paraformaldehyde and ethanol were evaluated for this purpose using 4% paraformaldehyde at 4°C for 2 h and 16 h, and 50% ethanol at 4°C for 2 h and 16 h. It was found that 50% ethanol for 16 h at 4°C yielded best hybridisation signals.

The washed cells were treated by adding 1 volume of 98% ethanol to 1 volume of bacterial cell suspension followed by incubation at 4°C for 16 hours. The treated cells were centrifuged at 5000xg for 15 min at 4°C. The supernatant was discarded and the pellet of cells was resuspended in PBS (pH 7.2) to give a fixed concentration of approximately $10^8 - 10^9$ cells per ml. One volume of ice-cold ethanol was added to one volume of cell suspension in PBS to fix the cells. Fixed cells were stored at −20°C until hybridisation experiments were conducted.

Glass slides with a Teflon coating with eight separate hybridisation wells (Cellline product, Biofusion Pty Ltd, Australia) were used to prepare bacterial smears as described by Amann et al., (1990). The slides were soaked in ethanolic KOH (10% potassium hydroxide in ethanol) for 1 h and rinsed in MilliQ water. Cleaned air-dried slides were placed inside a container to avoid dust and stored inside a desiccator.

3 µl of fixed cell suspension was transferred into each well of pretreated glass slides and allowed to air dry. The cells were dehydrated in a series of ethanol by
immersing the slide in 50%, 80% and 98% ethanol jars leaving for 3min in each. Fixed samples were stored in the dark at \(-20^\circ\text{C}\), if not used immediately.

Hybridisation conditions were optimised for the probe as follows:

The melting temperature of duplex nucleic acids are affected by the solvent, salt concentration, pH and the G and C composition in the nucleic acid (Stackebrandt and Goodfellow, 1991). Melting temperature of the probe was provided by the supplier and was also calculated using the Wallace rule:

\[
T_m = 2 \times (A+T) + 4 \times (G+C) = 80^\circ\text{C}
\]

Where;

\[
T_m = \text{Melting Temperature} \quad A = \text{Adenine} \quad T = \text{Thymine}
\]

\[
G = \text{Guanine} \quad C = \text{Cytosine}
\]

It was necessary to determine stringent conditions required for complementary nucleic acid strands of the probe and target cells to hybridise. Formamide content of the hybridisation buffer and the hybridisation temperature were varied to determine the optimum hybridisation conditions for probe RDR 502 to hybridise with the *Bacillus cereus* cells. Preliminary experiments were carried out using cells grown in culture to optimise hybridisation conditions as described below.

A series of hybridisation buffer in 2ml aliquots, containing 360\(\mu\)l of 5M NaCl, 40\(\mu\)l of 1M Tris-HCl, 2\(\mu\)l of 10% SDS, \(x\)\(\mu\)l of formamide and \(y\)\(\mu\)l of sterile MilliQ water was prepared according to the following table (Table 4.1).
Table 4.1  Volumes of formamide and MilliQ water required for preparation of formamide solutions varying from 0 – 25%

<table>
<thead>
<tr>
<th>Volume of Formamide</th>
<th>% Formamide in the well</th>
<th>Volume of MilliQ water</th>
</tr>
</thead>
<tbody>
<tr>
<td>x µl</td>
<td>y µl</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1598</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>1498</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td>1398</td>
</tr>
<tr>
<td>300</td>
<td>15</td>
<td>1298</td>
</tr>
<tr>
<td>400</td>
<td>20</td>
<td>1198</td>
</tr>
<tr>
<td>500</td>
<td>25</td>
<td>1098</td>
</tr>
</tbody>
</table>

At each formamide concentration, the temperature was varied from 45°C to 60°C to establish optimum stringent conditions for the probe-cell hybridisation. The temperature of the wash buffer was maintained 5°C greater than the selected hybridisation temperature.

The hybridisation chamber was prepared for the whole cell hybridisation as follows. A piece of cotton wool was moistened and soaked with hybridisation buffer and inserted into a clean 50ml polypropylene screw cap tube. The cap was tightened and left to equilibrate for 2 hours at the selected temperature. This experimental apparatus is shown in Fig 4.2.

1µl of RDR 502 probe at a concentration of 50ng µl⁻¹ was mixed in 8µl hybridisation buffer and added to each well containing immobilised cells to allow hybridisation between cells and the probe. The slide was gently rotated to mix cells with
the probe. The slide was then placed inside the pre-warmed hybridisation chamber, the cap was tightened and transferred into the incubator set at the selected temperature.
Fig 4.2 Hybridisation technique

a  Teflon coated 8 well slides used in hybridisation experiments
b  Hybridisation chamber with moistened cotton wool inside a polypropylene tube
    Prior to hybridisation the tube was allowed to equilibrate to the hybridisation temperature
After hybridisation the slides were carefully removed from the chamber and immediately rinsed in warm washing buffer (50µl 10% SDS, 1ml Tris-HCl, 2.15ml 5M NaCl in 46.8ml MilliQ water), followed by pipetting a small amount of wash buffer gently over the slide. The slide was then transferred into a tube containing wash buffer warmed to the selected temperature and then placed in a waterbath at the same temperature for 30min. All transfers were done rapidly to prevent cooling to avoid non-specific binding of the probe.

The slides were quickly immersed in cold tap water three times, air dried and mounted in Vectashield (Lab Supply, Australia) and viewed immediately using an Olympus BH2 RFCA Scanning Confocal Laser Microscope fitted with an argon/ krypton laser with emission lines. The slides were viewed for fluorescein bound cells at 528nm. If samples were not viewed immediately, the slides were stored at −20°C after drying without mounting.

4.2.5.2.3 Evaluation of probe specificity using target and non-target cells

The probe RDR 502 was checked against cultures of B. cereus isolated from biofilms, reference B. cereus (ATCC 10876), Staphylococcus aureus (ATCC 6571), and other cultures isolated from the biofilm to investigate the probe specificity. Optimised hybridisation conditions described in section 4.2.3.4.2 were used for these experiments.

4.2.5.2.4 In situ hybridisation of biofilms

Biofilms growing on glass slides were collected as described in section 4.2.5.1. The samples were fixed and prepared for in situ hybridisation as detailed above under conditions optimised for B. cereus using probe RDR 502.
A secondary staining process was carried out with Rhodamine X as described below to visualise the unhybridised background microorganisms present in the biofilms.

After hybridisation the slides were air dried and quickly immersed in a solution of 0.1% Rhodamine X for 1 minute. The slides were drained to remove excessive stain and immersed quickly in a container with MilliQ water to remove any residual stain. Care was taken to avoid excessive washing. If samples were not viewed immediately, the slides were stored at −20°C after drying.

The samples were viewed and scanned using an Olympus BH2 RFCA Scanning Confocal Laser Microscope fitted with an argon/krypton laser with emission lines. The Rhodamine stained cells were viewed at 586nm while Fluorescein stained cells were viewed at 528nm.
4.3 RESULTS

4.3.1 Total and culturable bacterial number

Biofilm samples collected at 1, 2, 3, 5 and 7 days old were analysed. Five replicates of each sample were analysed by culture techniques for determining the culturable bacterial count. Five replicates of each were analysed microscopically to determine the total bacterial count. Twenty five microscopic fields were counted from each sample. These results are summarised in Table 4.2. The images of biofilms acquired using SCLM are shown in Figs 4.3 – 4.6.

After 1 day, the culturable bacterial count of biofilms was $1.2 \times 10^6$ per cm$^3$ while the total bacterial count was $5.0 \times 10^6$ per cm$^3$. After 2 days the total count increased to $1.2 \times 10^7$ per cm$^3$ of biofilm while the culturable bacterial count was $2.8 \times 10^6$ per cm$^3$ of biofilm. After 3 days the culturable bacterial count was $1.8 \times 10^6$ per cm$^3$, while the total count was $9.1 \times 10^7$ per cm$^3$. The % of culturable bacterial counts of 1, 2 and 3 day old biofilms were 24%, 23.3% and 19.7% of the total bacterial counts respectively. As shown in Table 4.2, the difference between the two counts increased with the maturation of the biofilm. It was difficult to obtain a total direct microscopic count when the biofilms were older than 3 days due to the large number of cells that were present in the biofilm. This is shown in Fig 4.6. At this stage the culturable bacterial count had increased up to $5.2 \times 10^7$ per cm$^3$ of the biofilm.
4.3.2 Biofilm thickness

Biofilm thickness was estimated in all biofilm samples using the method described in section 3.2.3.2. Fig 4.7 shows vertical optical sections of biofilms taken along the xy plane using SCLM. Mean biofilm thickness calculated using this method is presented in Table 4.3. The results presented are mean values of 25 microscopic fields of one biofilm sample. Five replicates for each sample were analysed. These results show that an initial biofilm sample after 1 day had a thickness of 7.1(±0.9) μm while the thickness gradually increased over the 7 day period up to a value of 29.2 (±9.8) μm. These values were used in calculating the total microscopic bacterial count in the biofilm samples.

Fig 4.8 is a series of optically thin sections taken at intervals of 1.3μm along the xz plane of a 3 day old biofilm. This was constructed using SCLM software. Fig 4.9 shows the area of a 3 day old biofilm of 8208μm² calculated by the interactive CLSM software. This value was used to calculate the volume of the biofilm, which was necessary for area and thickness calculations of the biofilms to compare the total and culturable bacterial counts directly.

4.3.3 The total and culturable number of planktonic microorganisms

The number of total and culturable planktonic bacteria isolated from the wastewater at the same time as biofilms were collected are shown in Table 4.4. The culturable bacterial count over the 7 day period remained between 1.1 (±0.4) x 10² per ml – 3.5 (±0.01) x 10² per ml, while the total bacterial count was in the range of 1.3 (±0.15) x 10³ per ml – 5.5 (±0.14) x 10³ per ml of wastewater sample. Consistently throughout the seven day period the total number of bacteria was higher by one order of
magnitude than the culturable bacterial numbers isolated from the wastewater samples and the percentage of culturable bacterial counts varied from 5.2% - 19.2% of the total bacterial count.

Fig 4.10 shows the microflora present in the wastewater at the depth biofilms were growing. Bacterial cells were stained in red and other debris and oil particles were unstained in the background. Morphological differences were also noted between the cells growing in the biofilm and wastewater. The cells growing in the biofilm were relatively larger than the planktonic cells.
Table 4.2  Total and culturable number of bacteria in biofilms developed in the wastewater treatment plant – attached biofilm population

<table>
<thead>
<tr>
<th>Age of Biofilm (days)</th>
<th>Culturable Bacterial Count a ((1 \times 10^7 \text{ per cm}^3))</th>
<th>Total Bacterial Count b ((1 \times 10^7 \text{ per cm}^3))</th>
<th>% Culturable Bacterial Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0012 (\pm) 0.00019</td>
<td>0.005 (\pm) 0.00019</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>0.0028 (\pm) 0.00023</td>
<td>0.012 (\pm) 0.0011</td>
<td>23.3</td>
</tr>
<tr>
<td>3</td>
<td>1.8 (\pm) 0.14</td>
<td>9.1 (\pm) 0.26</td>
<td>19.7</td>
</tr>
<tr>
<td>5</td>
<td>5.2 (\pm) 0.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
<td>61.1 (\pm) 3.2</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

a  determined by culture techniques;  \(n = 5\) samples  
b  determined by SCLM;  \(n = 5\) samples (x 25 microscopic fields)  
--  could not determine due to heavy confluent nature of the biofilm

Values in parentheses are standard deviations
Fig 4.3  Rhodamine X stained 1 day old biofilm growing in the wastewater treatment plant

The cells are stained in red and were observed using a SCLM at 586nm to visualise cells stained with Rhodamine X. Stain accumulation at the background was observed in this image.
Fig 4.4 Rhodamine X stained 2 day old biofilm growing in the wastewater treatment plant

The cells are stained in red and were observed using a SCLM at 586nm to visualise cells stained with Rhodamine X. Cell aggregates are shown by arrow.

Bar = 10μ
Fig 4.5  Rhodamine X stained 3 day old biofilm growing in the wastewater treatment plant

The cells are stained in red and were observed using a SCLM at 586nm to visualise cells stained with Rhodamine X  

Bar = 10μ
Fig 4.6 Rhodamine X stained 5day old biofilm growing in the waste water treatment plant

The cells are stained in red and were observed using a SCLM at 586nm to visualise cells stained with Rhodamine X. Note arrow (a) pointing towards cells embedded in EPS (extracellular polymer matrix) and arrow (b) showing void non-cellular spaces within the biofilm.

Bar = 10μm
Fig 4.7  Vertical optical sections of biofilms taken along xy plane using SCLM. Arrows indicate the attached surface.

a  1 day old biofilm: the mean thickness of 25 points of this biofilm was 7.1µ

b  2 day old biofilm: the mean thickness of 25 points of this biofilm was 10.8µ
Fig 4.8  Rhodamine X stained 3 day old biofilm growing in the wastewater treatment plant

Optically thin sections were taken using SCLM at 1.3u intervals along xz plane and arranged serially.

Cells are stained in red and were observed using SCLM at 586nm to visualise Rhodamine stained cells
Fig 4.9  3 day old biofilm growing in the wastewater treatment plant

The area within yellow lines of this photomicrograph include an area of 8208μm² (calculated by the interactive SCLM software) which was used to calculate the volume of the biofilm.
<table>
<thead>
<tr>
<th>Age of Biofilm (days)</th>
<th>Biofilm thickness $^a$ (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.1 (± 0.9)</td>
</tr>
<tr>
<td>2</td>
<td>10.8 (± 0.5)</td>
</tr>
<tr>
<td>3</td>
<td>17.9 (± 1.9)</td>
</tr>
<tr>
<td>5</td>
<td>25.6 (± 6.1)</td>
</tr>
<tr>
<td>7</td>
<td>29.2 (± 9.8)</td>
</tr>
</tbody>
</table>

$^a$ determined by SCLM; $n = 5$ samples (x 25 microscopic fields)
Values in parentheses are standard deviations
<table>
<thead>
<tr>
<th>Age of Biofilm when the water sample collected a (days)</th>
<th>Total Bacterial Count ( b ) (1 x 10^3 per ml)</th>
<th>Culturable Bacterial Count ( c ) (1 x 10^3 per ml)</th>
<th>% Culturable Bacterial Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.1 (± 0.18)</td>
<td>0.11 (± 0.04)</td>
<td>5.2</td>
</tr>
<tr>
<td>1</td>
<td>2.4 (± 0.29)</td>
<td>0.35 (± 0.01)</td>
<td>14.5</td>
</tr>
<tr>
<td>2</td>
<td>1.4 (± 0.73)</td>
<td>0.27 (± 0.08)</td>
<td>19.2</td>
</tr>
<tr>
<td>3</td>
<td>1.7 (± 0.12)</td>
<td>0.15 (± 0.04)</td>
<td>8.8</td>
</tr>
<tr>
<td>5</td>
<td>5.5 (± 0.14)</td>
<td>0.21 (± 0.06)</td>
<td>3.8</td>
</tr>
<tr>
<td>7</td>
<td>1.3 (± 0.15)</td>
<td>0.16 (± 0.03)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

a water samples were collected when the experiment was set up (day 0) as well as each time biofilm samples were collected for bacterial counts

b determined by SCLM; \( n = 5 \) samples (x 25 microscopic fields)

c determined by conventional plating; \( n = 5 \) samples

Values in parentheses are standard deviations
Fig 4.10  Rhodamine X stained image of wastewater microflora

Note bacteria stained in red. Other particles are debris and oil globules in the water in the background shown by arrows

Bar = 20µ
4.3.4 Identification of bacteria using culture techniques

During the study period 11 culturable bacterial species were isolated on nutrient agar medium and identified using procedures described in section 4.2.2. These results are presented in Table 4.5. In 1 day old biofilms the most abundant growth on nutrient agar plates was due to Bacillus cereus and Aeromonas hydrophila. In 2 day old biofilms, A. caviae, P. testesteroni and P. vesicularis were detected. By 5 days, Weeksella virosa, Xanthomonas campestris, Flavobacterium yabuuchiae, Rhodococcus equi and a species of Brevibacterium were present in the biofilm.

All these species were also isolated from the planktonic population in wastewater except the species identified as Weeksella virosa. On the other hand, Sphingobacterium multivorum was present in the planktonic population, but was not detected in the biofilms during the 7 day period.

The relative abundance of isolated culturable bacteria from the biofilm samples and the wastewater samples is presented in Table 4.5. Based on culture methods B. cereus was found to be the most abundant bacterial species, while A. caviae, P. testesteroni and R. equi were the second most abundant species present in the biofilm. A. hydrophila, P. vesicularis, W. virosa and F. yabuuchiae were less abundant than those previously mentioned, while X. campestris and Brevibacterium sp were the least abundant in the biofilm.

In contrast, A. hydrophila and A. caviae were the most abundant species detected in the planktonic population, while F. yabuuchiae, P. testesteroni, B. cereus, X. campestris, R. equi, S. multivorum were isolated in relatively low numbers. P. vesicularis and Brevibacterium species were the least abundant bacteria in the planktonic population.
*Weeksella virosa* was not detected in the planktonic population at all during the study period, although this bacterium was present in the biofilm.
### Table 4.5  Identities and abundance of culturable bacteria isolated from planktonic and biofilm populations of the wastewater treatment plant at various stages of biofilm development

<table>
<thead>
<tr>
<th>Identified microorganism</th>
<th>Isolated stage from biofilm (days)</th>
<th>Relative abundance in the biofilm a</th>
<th>Relative abundance in the wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>1, 2, 3, 5, 7</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>1, 2, 3, 5, 7</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td><em>Aeromonas caviae</em></td>
<td>2, 3, 5, 7</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td><em>Pseudomonas testesteroni</em></td>
<td>2, 3, 5, 7</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>Pseudomonas vesicularis</em></td>
<td>2, 3, 5, 7</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>Weeksellia virosa</em></td>
<td>5, 7</td>
<td>++</td>
<td>Not detected</td>
</tr>
<tr>
<td><em>Xanthomonas campestris</em></td>
<td>5, 7</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>Flavobacterium yabuuchiae</em></td>
<td>5, 7</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Rhodococcus equi</em></td>
<td>5, 7</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>Brevibacterium species</em></td>
<td>5, 7</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>Sphingobacterium multivorum</em></td>
<td>Not detected</td>
<td>NA</td>
<td>++</td>
</tr>
</tbody>
</table>

---

a  n = 5 samples  
+++ Most abundant isolate  
+ Least abundant isolate  
NA Not applicable as not detected
4.3.5 Detection of *Bacillus cereus* in biofilms

4.3.5.1 Optimisation of probe hybridisation conditions

It was necessary to optimise stringent hybridisation conditions for Probe RDR 502 (complementary to genus *Bacillus*) to whole cells of isolated *B. cereus* before applying *in situ* hybridisation techniques to detect *B. cereus* in naturally developed biofilms.

The formamide content of the hybridisation buffer was varied from 0% to 30% whilst keeping the hybridisation temperature constant. This experiment was repeated at temperatures 45°C, 50°C, 55°C and 60°C in order to determine the most stringent conditions for the probe to hybridise to *B. cereus* cells. These results are tabulated in Table 4.6. When the hybridisation temperature was kept at 45°C and the formamide content was varied from 0 – 30%, the probe RDR 502 did not appear to have bound to *B. cereus* cells. When viewed under fluorescein specific filter sets no green colour was observed suggesting that no hybridisation took place between the probe and cells. A similar result was obtained when the hybridisation temperature was elevated to 50°C. At 55°C, no hybridisation occurred when the formamide content was kept at 0 – 20%, but hybridisation occurred when the formamide concentrations were 25% and 30%. At 60°C, hybridisation was observed at lower formamide content of 20%. Based on results obtained from these experiments 20% formamide at 60°C was used in subsequent hybridisation experiments.
4.3.5.2 Probe specificity

Probe specificity to *B. cereus* was verified against target and non-target cells using optimised probe hybridisation conditions. These test cultures included all isolated and identified biofilm bacterial cultures and a reference culture of *B. cereus* (ATCC 10876).

Results of this experiment are shown in Table 4.7. Only *B. cereus* cells hybridised with probe RDR 502 under the stringent hybridisation conditions employed. *Staphylococcus aureus* and *Rhodococcus equi* cells displayed autofluorescence, but this could be easily differentiated from the signal of hybridised probe. The hybridisation experiments with *S. aureus* and *R. equi* were repeated to ensure that no binding occurred to probe RDR 502 under the optimised conditions.
Table 4.6  Optimisation of conditions for whole cell hybridisation of *Bacillus cereus* isolated from waste water treatment plant with 5'-flourescein-labelled probe RDR – 502

<table>
<thead>
<tr>
<th>Hybridisation Temp °C</th>
<th>Wash Temp °C</th>
<th>% of Formamide used</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>50</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>50</td>
<td>55</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>55</td>
<td>60</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>65</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

_: hybridisation was not observed

+_+: hybridisation was observed

133
Table 4.7  Hybridisation results (whole cell) of biofilm isolates and reference bacterial cultures to probe RDR 502

<table>
<thead>
<tr>
<th>Target microorganism</th>
<th>Hybridisation Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference cultures:</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (ATCC 6571)</td>
<td>Negative</td>
</tr>
<tr>
<td><em>E. coli</em> (ATCC 11775)</td>
<td>Negative</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> (ATCC 10876)</td>
<td>Positive</td>
</tr>
<tr>
<td>Biofilm isolates:</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Aeromonas caviae</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Pseudomonas testosteroni</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Pseudomonas vesicularis</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Sphingobacterium multivorum</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Weeksellia virosa</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Xanthomonas campetris</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Brevibacterium sp.</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Flavobacterium yabuuchiae</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Rhodococcus equi</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> (biofilm isolate)</td>
<td>Positive (autofluorescence was detected)</td>
</tr>
</tbody>
</table>
4.3.5.3 Detection of *Bacillus cereus* in biofilms

Two approaches were used to detect *B. cereus* in biofilms. The culture method involved the use of Polymixin Egg yolk Methylene Blue agar (PEMBA), which was a selective growth medium for detecting *B. cereus*. The other approach was a molecular based method involving the use 16S rRNA targeted fluorescent oligonucleotide DNA probes to detect *B. cereus* in biofilms. Although both these methods were applied to naturally growing biofilms in the wastewater treatment plant, the culture method showed presence or absence of this microorganism in the biofilm while the molecular approach not only detected but also allowed visualisation of the location of this microorganism within the biofilm.

Since *B. cereus* had been already isolated and identified as the most abundant bacterium in 1 day old biofilm samples by culture methods, the question asked was “is this bacterium one of the initial colonisers of these biofilms?” In order to test this, it was decided to collect biofilm samples after 4h and 8h to investigate the types of microorganisms that were present in these biofilms. As in previous cases, both culture and 16S rRNA approaches were applied to detect *B. cereus* in these early biofilms.

Fig 4.11a and b show a 4h biofilm growing in the wastewater treatment plant hybridised with RDR 502 probe and then stained with Rhodamine. All microorganisms present in this biofilm stained with Rhodamine are visible in Fig 4.11a. Fig. 4.11b is the same field viewed under fluorescein specific filter sets. Any cells binding to probe RDR 502 should appear green in colour. No green cells were visible, suggesting that there were no *B. cereus* cells present in this biofilm at this stage. Fig 4.11a and b represent the same microscopic field viewed under rhodamine and fluorescein specific filter sets respectively. Fig 4.11c shows a 4h biofilm plated on PEMBA plates. No growth was
present on PEMBA plates suggesting that there were no *B. cereus* in this biofilm. All five replicates analysed gave similar results.

Fig 4.12a and b show the same microscopic field of an 8h biofilm using rhodamine and fluorescein specific filter sets respectively. No cells appeared to have hybridised with RDR 502 probe suggesting absence of *B. cereus* cells in this 8h old biofilm.

Fig. 4.13a and b represent a 1 day old biofilm removed from the wastewater treatment plant. Fig 4.13a was viewed under rhodamine specific filter sets while Fig 4.13b is a composite image of the same microscopic field viewed under fluorescein specific filters, and then superimposed on the previous red image. Thus 4.13a represents all microorganisms inhabiting the biofilm, while 4.13b shows *B. cereus* cells in green as a result of binding to probe RDR 502. A colony of *B. cereus* as well as single cells were observed in this image. Fig 4.13c shows blue *B. cereus* colonies on PEMBA plates with a peacock blue colour surrounding. Non-*Bacillus cereus* colonies on this agar could be easily identified due to the absence of peacock blue colour halo.

Fig 4.14a and b represent a 2 day old biofilm, of the same microscopic field viewed under different filter sets specific for rhodamine and fluorescein. A large number of *B. cereus* cells were present in this biofilm. The corresponding PEMBA plate represented in Fig 4.14c, showed confluent *B. cereus* colonies.

Fig 4.15a and b represent a 3 day old biofilm, of the same microscopic field viewed under different filter sets specific for rhodamine and fluorescein. A large number of *B. cereus* cells were present in this biofilm based on the growth on PEMBA plates shown in Fig 4.15c. However, direct microscopic observation showed that the number of other background cells in this biofilm was far more greater in number than the number of *B. cereus* cells.
A 5 day old biofilm hybridised with probe RDR 502 and stained with Rhodamine is shown in Fig 4.16. The superimposed red and green image shows that the number of other bacterial types increased with time relative to \textit{B. cereus} cells. Both 3 day old biofilm and 5 day old biofilm (Fig 4.15 and 4.16) show colonisation of other types of bacteria to a large extent when compared to the number of \textit{B. cereus} colonies. The relative abundance of \textit{B. cereus} cells (green) was low when compared to the total number of other inhabitants of the biofilm (red).

The objective of these experiments was to investigate whether \textit{B. cereus} was a primary coloniser in early biofilms. Enumeration of \textit{B. cereus} for direct comparison of molecular and culture methods was not undertaken in this study.
Fig. 4.11 a  Bacterial cells of a 4h biofilm growing in the waste water treatment plant stained with Rhodamine

Arrows on the image show red colour cells adhered onto the surface, representing the total microflora present in the biofilm at this stage

Fig. 4.11 b  Bacterial cells of the above biofilm, hybridised with fluorescein tagged probe RDR 502 to detect Bacillus cereus.

No Bacillus cereus cells were detected as indicated by the absence of any green cells

Fig. 4.11 c  PEMBA plate of a 4h biofilm growing in the wastewater treatment plant

No growth was observed suggesting absence of Bacillus cereus in the biofilm at this stage
Fig. 4.12a Bacterial cells of a 8h biofilm growing in the waste water treatment plant stained with Rhodamine

Red colour cells adhered onto the surface, representing the total microflora present in the biofilm at this stage

Fig. 4.12b Bacterial cells of the above biofilm, hybridised with fluorescein tagged probe RDR 502 to detect *Bacillus cereus*.

No cells were detected as indicated by the absence of any green cells
Fig. 4.13 a  Bacterial cells of a 1 day biofilm growing in the wastewater treatment plant stained with Rhodamine

Red colour cells adhered onto the surface represent the total microflora present in the biofilm at this stage

Fig. 4.13 b  Bacterial cells of the above biofilm, hybridised with fluorescein tagged probe RDR 502 to detect *Bacillus cereus*.

The same microscopic field was observed under specific filter sets and the green image was superimposed onto the red image to visualise presence of *Bacillus cereus* and the background microorganisms.

Arrows indicate a colony and single cells of *Bacillus cereus* stained in green

Fig. 4.13 c  PEMBA plate of a 1 day biofilm growing in the wastewater treatment plant.

Arrows on the image shows peacock blue *Bacillus cereus* colonies.

Note dull green non *Bacillus cereus* colonies
Fig. 4.14 a  Bacterial cells of a 2 day biofilm growing in the waste water treatment plant stained with Rhodamine

Note the large number of total microflora present in the biofilm at this stage. Autofluorescence was noted in some areas of this biofilm image (green).

Fig. 4.14 b  Bacterial cells of the above biofilm, hybridised with fluorescein tagged probe RDR 502 to detect *Bacillus cereus*

The same microscopic field was observed under specific filter sets and the green image was superimposed onto the red image to visualise presence of *Bacillus cereus*.

Fig. 4.14 c  PEMBA plate of a 2 day biofilm growing in the wastewater treatment plant.

Note the large number of blue colour *Bacillus cereus* colonies present on the plate.
Mature biofilms also showed the important architectural features of biofilms such as channels and cells embedded in the extracellular polymer matrix (EPS). These features are evident in Fig 4.15 and 4.16.

Interference and background autofluorescence was noted in some areas of biofilms (Fig 4.14a), however such false signals could be easily differentiated from the signal emitted by true fluorescein labeled hybridised B. cereus cells.

Optical sections of biofilms taken along xy plane using a SCLM are shown in Fig 4.17. These biofilms were subjected to hybridisation with fluorescein tagged probe RDR 502 to detect B. cereus and then stained with Rhodamine X to detect the background microorganisms. The arrows indicate attached surface of the biofilm. Although not very clear, B. cereus cells hybridised to the probe RDR 502 were visible as green cells, while the background microorganisms were stained in red. Colonisation of Bacillus cells from the attached surface to the exterior of the biofilm was observed in these optical sections as demonstrated by the green colour.
Fig. 4.15 a  Bacterial cells of a 3 day old biofilm growing in the waste water treatment plant stained with Rhodamine
Note the mixed cell population stained with Rhodamine representing the total microflora in the biofilm at this stage

Fig. 4.15 b  Bacterial cells of the above biofilm, hybridised with fluorescein tagged probe RDR 502 to detect *Bacillus cereus*
The same microscopic field was observed under specific filter sets and the green image was superimposed onto the red image to visualise presence of *Bacillus cereus*.
Bacillus cereus cells are stained green

Fig. 4.15 c  PEMBA plate of a 3 day old biofilm growing in the wastewater treatment plant
Heavy growth of *Bacillus cereus* was observed on the plate
Fig. 4.16 a  Bacterial cells of a 5 day old biofilm growing in the waste water treatment plant stained with Rhodamine

Note the heavy growth of mixed cell population stained with Rhodamine representing the total microflora in the biofilm at this stage.

Arrows point towards channel like structures within the heavily colonised biofilm

Fig. 4.16 b  Bacterial cells of the above biofilm, hybridised with fluorescein tagged probe RDR 502 to detect Bacillus cereus

The same microscopic field was observed under specific filter sets and the green image was superimposed onto the red image to visualise presence of Bacillus cereus.

Bacillus cereus cells are stained green, but the number of Bacillus cereus is low relative to the background microorganisms
Fig. 4.17 a  Optical section of a 3 day old biofilm along xy plane.

Arrow indicates the attached surface. *Bacillus cereus* cells are stained in green, and are present on the attached surface progressing to the exterior of the biofilm

Fig. 4.17 b  Optical section of a 5 day old biofilm along xy plane.

Arrow indicates the attached surface. *Bacillus cereus* cells are stained in green, and are present on the attached surface progressing to the exterior of the biofilm
4.4 DISCUSSION

The characterization of a biofilm community is an essential part of any study aimed at understanding the structure and function of this complex survival strategy of microorganisms. Jones (1996) argued that a good understanding of members in a community as well as physical and chemical properties surrounding the growth and colonisation of these microorganisms was a key component of any community study. The protocol described in this section of the study is an attempt to understand the community structure and to follow the colonisation pattern of a key bacterial member of the community using cultural and molecular approaches.

The results of bacterial enumeration studies presented in this section has shown that the culturable bacterial count varied from 2.3 - 24% of the total bacterial count, in agreement with previous studies by Wagner et al. (1994), Amann et al. (1995) and Alfreider et al. (1996). Similarly, samples of wastewater had a ten-fold lower count when enumerated using culture techniques compared with direct microscopic counts. These results are in agreement with previous studies that have demonstrated the inadequacy of culture dependent detection protocols, which often underestimate total numbers of bacteria and the diversity of the bacterial community. However, there is no evidence to suggest that all cells counted by direct microscopic methods are viable, and therefore an alternative possibility is that a percentage or all of these organisms were non-viable, dead cells.

Scanning Confocal Laser Microscopy (SCLM), in conjunction with fluorescent staining, proved to be a valuable technique to study total number of microorganisms in these complex community structures. The ability to perform optical sectioning without disrupting the sample allowed the same sample to be analysed using a number of techniques thus contributing to a more holistic view of the biofilm community structure.
Similarly, optical sectioning along different axes allowed the thickness of biofilm samples to be determined efficiently. The biofilm thickness measurements given in Table 4.3 are similar to those obtained previously (Table 3.1), although not identical. Comparison of these results suggests the reproducibility as well as the variability inherent to the technique.

Eleven bacterial species were identified from the biofilm samples during the study period using culture methods. Different bacterial species were identified from biofilm samples collected at different times suggesting a possible succession of bacterial colonization within the biofilm. As shown in Table 4.5, *B. cereus* and *A. hydrophila* were identified from all biofilm samples during the 7 day period suggesting that these are possibly the initial colonisers of the biofilm community. On the other hand, *A. caviae*, *P. testesteroni* and *P. vesicularis* were isolated from the biofilms only after two days, while *Weeksella virosa*, *Xanthomonas campestris*, *Flavobacterium yabuuchiae*, *Rhodococcus equi* and *Brevibacterium* sp were isolated from biofilms after 5 days. These results may suggest a possible "pattern of colonization", however, as these observations are based on culture techniques, it could be argued that culture conditions, nutrients present in the culture medium and incubation temperatures may have affected the growth and isolation of these species at different times and numbers.

Interestingly, the species *Weeksella virosa* was isolated from the biofilm samples after 5 days; however, it was not possible to isolate this microorganism from the planktonic population (Table 4.5). It is possible that this species was present in the wastewater in very low numbers below the sensitivity and the detection limit of the methods applied, preventing detection, although there is no data to support this argument. Similarly, *Sphingobacterium multivorum* was isolated from the wastewater, but was not isolated from the sessile adherent biofilm samples. It is possible that this species did not
colonise the biofilm, or was present in the biofilm in very low numbers and was not detected by the methods employed.

Although nutrient agar is widely accepted as a general culture medium to examine total bacteria in industrial and wastewater, it fails to reflect natural conditions required for the proliferation of all bacteria present. As a consequence, it may not be possible to determine accurately the species diversity of a biofilm by completely depending on such techniques. Van Neil (1955) found that there were many microbes whose natural functions were unknown, since they had not been encountered in culture media. Atlas (1984) reiterated this point saying "bacteriologists who rely on culturable methods to identify species face the problem of selectivity and thus inevitable underestimation of community diversity”.

In order to follow the colonization pattern of a key biofilm community member, a molecular approach using a 16S rRNA specific probe and a culture method based on plating on a selective agar medium were applied in this section of the study. This involved selection of a "predominant" bacterium from the microflora isolated from biofilm samples. B. cereus (identified as B. cereus using API and biochemical methods) was selected for this purpose as this bacterium was isolated from 1 day old biofilms during initial studies conducted. A suitable probe sequence was sought from published data, and trialled against the target and reference cultures of B. cereus to optimise stringent probe hybridisation conditions. Evaluation and optimization of the fixation procedure was crucial for the success of fluorescence in situ hybridisation (FISH). Paraformaldehyde is the most commonly used fixative for FISH studies in microbial ecology and has been demonstrated to be effective for most Gram negative bacteria (Amann et al., 1992). It was not possible to permeabilise Gram positive B. cereus cells by the use of paraformaldehyde. The use of paraformaldehyde and ethanol as fixatives were
evaluated for fixing biofilm samples and it was found that 50% ethanol was required as a fixative to permeabilise Gram positive cells to obtain a maximum signal.

Experiments conducted to optimise hybridisation conditions revealed that the optimum conditions required for hybridisation were 20% formamide content in the hybridisation buffer at 60°C. These conditions were employed throughout in all in situ hybridisation experiments to visualise B. cereus cells. Guanine-Cytosine (GC) pairs containing 3 hydrogen bonds are more stable than Adenine-Thymine (AT) pairs containing 2 hydrogen bonds. Therefore GC rich double stranded nucleic acids have a higher melting temperature than AT rich nucleic acids.

Interestingly, B. cereus was not detected from biofilms collected after 4h and 8h by culture methods or by 16S rRNA targeted probe application. Direct microscopic observations showed that there were other bacterial cells present in these biofilms at very initial stages of development. These may or may not belong to any of the other bacterial species that had been isolated by culture methods, and the possibility for these microorganisms to be unculturable was not investigated and was beyond the scope of this study.

It was noted that progression of B. cereus cells occurred starting from a small colony or individuals, towards chains of cells. This may relate to cells lining a distinct interface within the biofilm to their advantage. It is possible that such spatial arrangement positioned B. cereus cells towards areas where nutrients are abundant, away from toxic chemicals in the multi-species biofilm.

The introduction of rRNA targeted oligonucleotide probes by Stahl et al. (1988) facilitated the detection, identification and quantification of microorganisms without the limitations of culture dependant methods (Lipski et al., 2001). However, it must be noted that there are limitations in the 16S rRNA approach. The conserved nature of rRNA
sequences limits the resolving power of rRNA based hybridization probes (Ward et al., 1992). Comparison of 16S rRNA sequences and DNA-DNA hybridisation results of three psychrophilic Bacillus strains, B. globisporus W25T, B. psychrophilus W16A\textsuperscript{T} and B. psychrophilus W5T has led to the generalisation that highly specific rRNA probes are not necessarily always possible (Fox et al., 1992). This suggests that effective identity of 16S rRNA sequences is not necessarily a sufficient criterion to guarantee species identity. In this study, the selected probe was checked for hybridisation signals against the other species isolated to minimise the possibility of having false signals from species other than B. cereus. But it must be assumed that unknown microorganisms may cross react with a probe thought to be specific, or that undiscovered relatives may fail to react with a probe designed to hybridise with rRNA from all members of a group. Thus it must be considered that probes should be regarded as tools subject to refinement.

Slow growing members of natural communities with the possibility of having a low rRNA content may also not be detected by 16S rRNA targeted probes as the target molecules are not in abundance. Helper probes attached to the target probe have proved to be useful in such circumstances (Fuchs et al., 2000). Another problem that may affect the RNA-probe hybridisation is accessibility of target which requires the penetration of probe through environmental and cell barriers. In spite of such problems, 16S rRNA probes still offer a convenient rapid means of detecting predominant populations, culturable or unculturable.

4.5 CONCLUSIONS

The work presented herein provides an approach of detection and enumeration of bacterial populations in biofilms with respect to initial colonisation patterns. Results of
this study have indicated that not all microorganisms that are present in the wastewater environments necessarily become community members of a biofilm growing in that environment.

A possible succession of bacterial colonisation within the biofilm was demonstrated in this study using culture techniques, but culture conditions and nutrients present in the growth medium may have affected the isolation of species from biofilms.

It was demonstrated that selective cultural techniques as well as 16S rRNA methods can be employed to obtain a more comprehensive picture of the colonisation of *B. cereus* in biofilms growing in the wastewater treatment plant. The two methods were in agreement throughout, however, the use of 16S rRNA *in situ* techniques also showed the location of *B. cereus* within the biofilm. Although it was suspected that *B. cereus* was one of the initial colonisers of these biofilms, it was not possible to detect this bacterial species before 24h. It was demonstrated that other bacterial species were present in the biofilm before the colonisation of *B. cereus* occurred using 16S rRNA hybridisation methods.

Limitations associated with culture techniques, in enumeration of populations were also highlighted in this study, whilst the use of SCLM proved to be an extremely valuable non destructive technique in obtaining information on this complex community structure growing in the wastewater treatment plant. Other molecular techniques involving PCR steps have allowed information to be gathered on the community structure of biofilms, but FISH techniques allowed the visualisation of cells *in situ*, without disrupting the biofilm.
CHAPTER 5

CELL SURFACE HYDROPHOBICITY OF BIOFILM

MICROORGANISMS
5.1 INTRODUCTION

It has been observed that bacteria grow mainly as adherent microcolonies embedded within extensive polysaccharide matrices in nature (Allison and Sutherland 1987). From an ecological perspective, this survival strategy, known as biofilm production offers advantages for bacterial colonization, growth and survival. One of the most significant steps in biofilm formation is initial microbial adhesion to surfaces and bacterial cell surface hydrophobicity is thought to play an important role in this process (Gilbert et al., 1991).

The term bacterial hydrophobicity describes the hydrophobic properties conferred on bacterial cells by their outermost cell surfaces. A positive correlation between bacterial hydrophobicity and their adhesion to substrata have been reported in dental plaque formation (Pratt-Terpestra et al., 1988) and in microbial adhesion to minerals (Stenström 1989). Despite the apparent significance of hydrophobic interactions in adhesion phenomena there is no general consensus concerning scales of hydrophobicity (Rosenberg and Kjellberg 1986).

The surface of microbial cell is of paramount importance for survival as this controls association between cells and other surfaces. Some microbial surface structures have been regarded as probes to monitor the ever-changing external environments and to relay the information on such changes to the internal regulatory mechanisms of the organisms (Marshall, 1991). Characterization of these surfaces is important to understand the macromolecular constitution and physicochemical properties such as hydrophobicity and surface charge (Pembury et al., 1999).

The objective of this section of the project was to study the cell surface hydrophobicity of bacteria isolated at different stages from developing biofilms in the
wastewater treatment plant.Measurement of the cell surface hydrophobicity of the isolates in aging pure culture biofilms was also included in this study to assess any changes that may occur in the cell surface of isolates during aging and maturation of the culture.

The flowchart below summarises the approach used in this section to understand the cell surface hydrophobicity of bacterial species isolated from developing biofilms in the wastewater treatment plant.
Bacterial isolates of biofilms growing in the wastewater treatment plant

(Section 4.2.2)

Development of pure culture biofilms of isolates in the laboratory (Section 5.2.2)

Measurement of bacterial cell surface hydrophobicity in laboratory biofilms

Comparison of cell surface hydrophobicity of pure culture biofilms after 1 day

1. Salt Aggregation Test (Section 5.2.3.1)

2. Bacterial Adhesion to Hydrocarbons (Section 5.2.3.2)

3. Hydrophobic Interaction Chromatography (Section 5.2.3.3)

Change in cell surface hydrophobicity over 3 days in aging pure cultures of isolates forming biofilms (Section 5.2.4)

Figure 5.1 Approach taken in cell surface hydrophobicity analysis of biofilm isolates
5.2 MATERIALS AND METHODS

5.2.1 Isolation and identification of bacterial flora

Bacteria isolated from biofilms as described in section 4.2.2 were used for these experiments.

5.2.2 Biofilm production and preparation of cell suspensions

Pure cultures of bacterial isolates were inoculated into 250 ml of tryptone soya broth (TSB, Oxoid) containing suspended sterile glass microscope slides in Erlenmeyer flasks and incubated for 1 day at 25°C. The slides were removed from the culture vessel after incubation and biofilms were washed with sterile phosphate buffered saline (PBS, pH 7.2) collecting the wash into sterile flasks. The collected PBS wash was centrifuged at 5000xg for 20 min at 25°C. The supernatant was discarded and the resultant pellet of cells was washed with MilliQ water three times prior to suspending in the appropriate buffers for hydrophobicity assays as described in sections below.

5.2.3 Measurement of bacterial cell surface hydrophobicity

5.2.3.1 Salt aggregation test (SAT)

A bacterial suspension obtained as described in section 5.2.2, containing $10^9$ cells ml$^{-1}$ was prepared in 2 mM sodium phosphate buffer (pH 6.8) after the method of Lindahl et al. (1981). Twenty five µl of $(NH_4)_2SO_4$ (Sigma Aldrich) at concentrations varying from 0.02 M to 4.0 M in 2 mM sodium phosphate buffer (pH 6.8) was added to an equal volume of cell suspension in a cavity slide placed on a black tile. The slide was gently rocked for 2 min, watching carefully for bacterial cell aggregation and thickening. The
lowest concentration causing bacterial aggregation was recorded as the SAT value. Bacterial suspensions mixed with 2 mM sodium phosphate buffer (pH 6.8) minus (NH₄)₂SO₄ were used as negative controls.

5.2.3.2 Bacterial adherence to hydrocarbon test (BATH)

Bacterial cells obtained as described in section 5.2.2, were suspended in phosphate urea magnesium buffer (PUM pH 7.1). The PUM buffer contained 1 g l⁻¹ each of K₂HPO₄ 16.87, KH₂PO₄ 7.26, MgSO₄ 7H₂O 0.2 and urea 1.8. The culture suspension was prepared to an initial absorbance (A₀) of approx. 1.0 at 550 nm.

1.2 ml of above bacterial suspension in PUM buffer was dispensed into a series of round bottom test tubes after the method of Rosenberg et al. (1980). The test hydrocarbon n-hexadecane (Sigma Aldrich) was then added at five different volumes of 50, 100, 150, 200 and 250 µl in five replicates. These were incubated at 25°C for 10 min, and vortexed for 2 min, followed by standing at room temperature for 10 min to allow for phase separation. The absorbance (A₅₅₀) of aqueous phase was measured before (A₀) and after treatment (A₁) using a Hitachi U2000 UV-VIS spectrophotometer. The results were recorded as the absorbance of the aqueous phase after treatment relative to the initial absorbance of the bacterial suspension. A representative sample from each treatment was examined under the microscope to ensure that there was no cell lysis.

% Hydrophobicity was calculated according to the formula given below.

\[
\% \text{ Hydrophobicity} = \left(\frac{A₀ - A₁}{A₀}\right) \times 100
\]

5.2.3.3 Hydrophobic interaction chromatography (HIC)

Washed cells, obtained as described in section 5.2.2, were suspended in 4.0 M NaCl in sodium phosphate buffer pH 6.8 at a concentration of approximately 10⁹ cells per
ml. The initial absorbance (Ai) of this suspension was adjusted to approx. 0.600 at 550
nm using a Hitachi U 2000 UV-VIS spectrophotometer. These cell suspensions were
applied to a column of octyl-sepharose CL-4B (Pharmacia) as described in the method of
Smyth et al. (1978). The absorbance of eluates (Ar) were compared with the original
bacterial suspensions (Ai) by measuring the absorbance (A550,1.0cm) using a Hitachi U
2000 UV-VIS spectrophotometer. Sepharose CL-4B was used as a control for non-
specific adsorption (Ac). The results were recorded as the percentage of retained bacteria
on the hydrophobic gel relative to the initial cell suspension applied.

% Hydrophobicity was calculated according to the formula given below:

\[
\% \text{ Hydrophobicity} = \left[ \frac{(Ai - Ac - Ar)/(Ai - Ac)} \right] \times 100
\]

5.2.4 Change in cell surface hydrophobicity of bacterial isolates in
aging biofilms

Based on results obtained from the three assays, BATH assay was selected to
measure cell surface hydrophobicity of isolates. Bacterial cells obtained as described in
section 5.2.2, were inoculated into 250 ml TSB in Erlenmeyer flasks containing
suspended sterile glass slides. Slides were removed after 1 day, 2 days and 3 days from
the flask, and biofilms were washed in PBS and cells were harvested as described
previously in section 5.2.2. Surface hydrophobicity of harvested cells was measured by
BATH assay using 250 \( \mu \)l of n-hexadecane measuring the decrease in absorbance
(A550,1.0cm) of the aqueous phase. Only one concentration of n-hexadecane was used for
this experiment, and this concentration was selected based on the results obtained from
the previous trial described in section 5.2.3.2.
5.2.5 Data analysis

Data from each experiment were analyzed by one way Anova and significant differences (P < 0.05) in cell surface hydrophobicity between isolates were determined by Fisher PLSD test using Statview® software (Abacus Concepts, USA).
5.3 RESULTS

5.3.1 Bacterial flora isolated from biofilms

Bacteria isolated from naturally developing biofilms at various stages of maturity, varying from 1-7 days and the stages at which they were originally isolated from biofilms are listed in Table 4.5. Cells were harvested from pure culture biofilms of those isolates grown in the laboratory and cell surface hydrophobicity was measured to investigate whether any differences existed among the isolates.

5.3.2 Bacterial Adhesion to Hydrocarbon Assay

Estimates of cell surface hydrophobicity were obtained using a range of concentrations of n-hexadecane and results are shown in Figs 5.2 and 5.3. This method identified W. virosa, B. cereus and R. equi to be significantly more hydrophobic while F. yabuuchiae, X. campestris and A. caviae were found to be relatively less hydrophobic. Anova analysis of these results indicated that significant differences in cell surface hydrophobicity existed among these isolates, however, two general groups of isolates having low and high cell surface hydrophobicity were identified. Increasing the volume of n-hexadecane resulted in increasing the number of cells attached to n-hexadecane up to about 200µl, with no further increase when larger volumes of n-hexadecane were added (Fig. 5.2).
Fig 5.2  Adherence of 10 bacterial biofilm isolates to varying degrees of n-hexadecane in the BATH assay. Results are expressed as the % hydrophobicity (% of cells excluded from aqueous phase) at various volumes of n-hexadecane. At each data point n = 5; bar = SE
Fig. 5.3 Adherence of 10 biofilm isolates to 250 μl of n-hexadecane in the BATH assay. Results are expressed as the % hydrophobicity (% of cells excluded from the aqueous phase) at various volumes of n-hexadecane. Bars represent standard errors.

Different letters (a-f) above the error bars indicate significant differences (P < 0.05) between cell surface hydrophobicity of isolates as determined by Fisher PLSD. Based on this analysis cell surface hydrophobicity of 

e > b > d > a > c > f
During the course of the experiment, one representative sample from each treatment was examined under the microscope to check for cell lysis that could potentially interfere with the absorbance readings. No evidence of such damage to cells was observed for any of the isolates.

5.3.3 Salt aggregation test

The lowest concentration of (NH₄)₂SO₄, which caused visual aggregation of cells is shown in Fig. 5.4. The SAT assay identified isolates having significant differences in their cell surface hydrophobicity. Using this technique W. virosa was identified to be the most hydrophobic while A. hydrophila and A. caviae were found to be the least hydrophobic among the isolates. Anova analysis indicated that significant differences in cell surface hydrophobicity existed among these isolates and two general groups having low and high cell surface hydrophobicity were identified based on these results. The other isolates demonstrated cell surface hydrophobicity to varying degrees. R. equi and F. yabuuchiae demonstrated the second highest cell surface hydrophobicity, while B. cereus, P. testesteroni, P. vesicularis, X. campesris and Brevibacterium species were third in the order of decreasing hydrophobicity.
Fig. 5.4  Lowest molarity of ammonium sulphate causing visual cell aggregation of isolates in SAT assay. Bars represent standard errors. Different letters (a-d) above the error bars indicate significant differences (P < 0.05) between cell surface hydrophobicity of isolates as determined by Fisher PLSD. Based on this assay cell surface hydrophobicity of a < c < b < d.
Fig. 5  Percentage adsorption of cells (% retention of cells) of biofilm isolates on hydrophobic gel, Octyl-sepharose. Bars represent standard errors. Different letters (a-e) above the error bars indicate significant differences (P < 0.05) in cell surface hydrophobicity between isolates as determined by Fisher PLSD. Based on this assay, cell surface hydrophobicity of b > d > e > a ≥ c.
5.3.4 Hydrophobic interaction chromatography

The percentage of cells adsorbed to octyl-sepharose as a measure of cell surface hydrophobicity is presented in Fig. 5.5. In this assay *W. virosa*, *B. cereus* and *R. equi* were significantly more hydrophobic, while *P. testosteroni*, *P. vesicularis*, and *Brevibacterium* species were observed to be relatively less hydrophobic. *X. campestris* and *A. hydrophila* demonstrated a lower cell surface hydrophobicity than the second group while *A. caviae*, and *F. yabuuchiae* demonstrated the least cell surface hydrophobicity.

5.3.5 Comparison of hydrophobicity values obtained by the three methods

To allow comparison between the three methods a hydrophobic ranking (HR) was given to each isolate and the summarised results are tabulated in Table 5.1. The most hydrophobic isolate was given a ranking of HR 1, while the least hydrophobic isolate was given a ranking of HR 10. Based on this arbitrary ranking, *Weeksella virosa* had the highest cell surface hydrophobicity while *B. cereus*, showed the second highest cell surface hydrophobicity by all three methods. The other isolates showed different degrees of cell surface hydrophobicity with the three methods employed. *R. equi* showed a higher cell surface hydrophobicity by BATH and HIC methods but SAT assay gave a lower value. *P. vesicularis*, *P. testosteroni* and *Brevibacterium* species were ranked at a median level in the scale of cell surface hydrophobicity. *A. hydrophila*, *A. caviae* and *F. yabuuchiae* demonstrated low cell surface hydrophobicities by all three methods. *X. campestris* on the other hand showed a relatively low cell surface hydrophobicity by BATH and HIC methods, but SAT assay gave a higher cell surface hydrophobicity value.
5.3.6 Change in cell surface hydrophobicity of bacterial cells in aging biofilms

The BATH assay was selected to study the cell surface hydrophobicity of aging isolates in pure culture biofilms. This method was chosen due to its reliability (Vanhaecke and Pijck, 1988). Only one volume, 250μl of n-hexadecane, was used based on results of previous experiments, where a saturation point was observed (Fig 5.2).

Cells isolated from pure culture biofilms growing in the laboratory for 3 days were used for these experiments. Percentage cell surface hydrophobicity of cells harvested from aging biofilms of 1 - 3 d old was determined using the BATH assay and results are presented in Fig 5.6.

In all isolates the cell surface hydrophobicity gradually declined over the three day period except for *F. yabuuchiae* and *P. vesicularis*. In *F. yabuuchiae* and *P. vesicularis* an increase in cell surface hydrophobicity was observed after 3 days, following the decrease observed in cells harvested from 2 day old biofilms.
Table 5.1 Comparative cell surface hydrophobicity of biofilm isolates obtained by different methods (indicated by hydrophobic ranking HR) and their isolation stages from developing biofilms in the wastewater treatment plant

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>**Age of biofilm (days)</th>
<th>Hydrophobic ranking (HR)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>1</td>
<td>7 8 10</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>1</td>
<td>2 2 2</td>
</tr>
<tr>
<td><em>Aeromonas caviae</em></td>
<td>2</td>
<td>9 9 9</td>
</tr>
<tr>
<td><em>Pseudomonas testesteroni</em></td>
<td>2</td>
<td>5 5 2</td>
</tr>
<tr>
<td><em>Pseudomonas vesicularis</em></td>
<td>2</td>
<td>4 4 5</td>
</tr>
<tr>
<td><em>Xanthomonas campestris</em></td>
<td>5</td>
<td>8 7 4</td>
</tr>
<tr>
<td><em>Weekella virosa</em></td>
<td>5</td>
<td>1 1 1</td>
</tr>
<tr>
<td><em>Rhodococcus equi</em></td>
<td>5</td>
<td>3 3 8</td>
</tr>
<tr>
<td><em>Flavobacterium yabuuchiae</em></td>
<td>5</td>
<td>10 10 7</td>
</tr>
<tr>
<td><em>Brevibacterium sp</em></td>
<td>5</td>
<td>6 6 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> HR - Hydrophobicity ranking of isolates. 1-10 decreasing hydrophobicity

<sup>b</sup> Bacterial adherence to hydrocarbon assay. n = 5

<sup>c</sup> Hydrophobic interaction chromatography n = 5

<sup>d</sup> Salt aggregation test n = 5

** The age of the biofilm when bacteria were first isolated from the wastewater treatment plant
Fig. 5.6  Percentage cell surface hydrophobicity of isolates as a function of age of biofilm. BATH assay measurements were performed on populations of 1, 2 and 3 day old cultures.

Bars represent standard errors, n = 5.
5.4 DISCUSSION

The results suggest differences in the cell surface hydrophobicity of the bacterial species isolated from the wastewater treatment plant. While the BATH and HIC assays gave similar overall ranking of species (Table 5.1), the results of the SAT assay gave a different order. However, using all three methods W. virosa and B. cereus were found to possess cell surfaces with a relatively high hydrophobicity. The order of the hydrophobic ranking obtained with the SAT assay was different for intermediate and less hydrophobic isolates such as P. testesteroni, Brevibacterium sp., X. campestris and F. yabuuchiae, from the hydrophobic ranking obtained by BATH and HIC assays (Table 5.2).

The BATH assay is widely used and regarded as a reliable method to evaluate cell surface hydrophobicity. Studies have indicated the reliability of n-hexadecane as an organic test fluid, compared to xylene and toluene which caused cell lysis and absorbance readings as a measure of cells retained by the organic phase compared to viable counts in adhesion assays (Rosenberg and Kjellberg 1986, Vanhaecke and Pijck, 1988). During the present study the possibility of n-hexadecane being toxic to isolates was eliminated by microscopic examination which indicated no cellular lysis.

Only one method, the BATH assay, was used to study the variation of cell surface hydrophobicity of isolates in aging pure culture biofilms. This method was selected due to its reliability (Vanhaecke and Pijck, 1988) and also due to the fact that the bacteria were isolated from a petrochemical wastewater treatment plant. Only one volume of n-hexadecane was used in the BATH assay to study the variation of cell surface hydrophobicity of isolates in aging pure culture biofilms. This volume was selected on the basis of results seen in Fig.5.2, where the volume of n-hexadecane had no impact upon effective measurement of hydrophobicity. This observation is consistent with the
results reported previously (Lichtenberg et al., 1985) involving kinetics of BATH assay. In the BATH assay when cell suspensions are vortexed in the presence of the hydrocarbon and phase separation is allowed, cell coated oil droplets rise to the top. When low hexadecane volumes are employed, a larger number of cells may adhere to oil droplets than when larger volumes of hydrocarbon are used. It was shown that flotation of oil droplets was not hampered by the adherence of bacteria, even at high bacteria : hydrocarbon ratios.

Although the results of SAT assay was consistent with the results of the HIC and BATH assays with respect to species with a high hydrophobic ranking, the trend was somewhat different in species with low hydrophobic ranking (Table 5.2). It has been suggested previously that the SAT assay can only be used to detect strongly hydrophobic microorganisms and that it lacks sensitivity when the hydrophobicity decreases (Lichtenberg et al., 1985). Our results are in agreement with Dillon et al. (1986) and Mozes and Rouxhet (1987) but in contrast with those of Lindahl et al. (1981) who found a good correlation between the HIC and SAT assays. The SAT assay measures the (NH₄)₂SO₄ concentration required to neutralize the overall surface charge to produce cell aggregation and therefore is strongly related to the properties of the molecular groups responsible for the surface charge. In contrast, the BATH and HIC assays estimate the hydrophobic properties based on actual binding to a hydrophobic ligand. A better correspondence between the assays is obtained when they are applied to a homogeneous set of bacterial strains (Van der Mei et al., 1991).

Although A. hydrophila, B. cereus and A. caviae were isolated after 1 day from developing biofilms in the wastewater treatment plant, only B. cereus was found to possess a cell surface with relatively high hydrophobicity. Table 5.4 shows the age of the biofilm from which these bacteria were isolated. Similarly W. virosa was suggested to
possess the highest cell surface hydrophobicity by all three methods but was isolated only after 5 days from the biofilms growing in the wastewater treatment plant. Thus it appears that there is no obvious relationship between the relative cell surface hydrophobicity of isolates and the age of the biofilm when the bacterium was isolated from the wastewater treatment plant. However, two groups, a group of bacterial isolates possessing significantly higher cell surface hydrophobicity as well as a group possessing significantly lower cell surface hydrophobicity from the wastewater treatment plant were isolated and identified in this study.

The variation of cell surface hydrophobicity of isolates in aging pure culture biofilms was studied using the BATH assay to assess any changes that may occur on the cell surface with time. Cells isolated from all pure culture biofilms, except *F. yabuuchiae* and *P. vesicularis* biofilms, became less hydrophobic as the biofilms matured from 24 h to 3 d. The observation that cells harvested from most pure culture biofilms as they matured had less cell surface hydrophobicity indicates that the hydrophobicity changes occur during the life cycle of bacteria. This could be a result of many factors. The production of EPS during biofilm formation may have contributed to changes in cell surface hydrophobicity of bacterial isolates during aging. Microbial surface-active compounds bound to the cell surfaces as they age may also contribute to changes of hydrophobicity of the cell surface. Similarly changes in cell surface hydrophobicity may occur due to surface appendages or proteins associated with adhesion. The observation that *F. yabuuchiae* and *P. vesicularis* showed a higher cell surface hydrophobicity when the biofilm matured from 1 day to 3 days, suggest that differences occur among species.

It has been reported that newly formed cells, shed from *P. aeruginosa* biofilms were found to be more hydrophilic than their biofilm counterparts suggesting that changes in the cell surface hydrophobicity occur during the life cycle of a bacterium
(Allison et al., 1990a). Further, the observation that daughter cells dislodged from *E. coli* biofilms were observed to be more hydrophobic than those remaining in the biofilm suggests that differences in cell surface hydrophobicity may occur depending on whether the bacterial cells reside within a sessile or planktonic population (Allison et al., 1990b). These data as well as the data of the present study suggest that the changes in cell surface hydrophobicity is not only species-dependent but also depends on many other environmental and physical parameters.

It must be noted that hydrophobicity of bacteria *in situ* may differ from results obtained with laboratory designed experiments. Bacterial cells in the natural environments may adsorb proteins and other polymers from the surroundings, which affect their surface properties.

5.5 CONCLUSIONS

In summary, the results of the present study indicate that a biofilm may contain organisms having varying degrees of cell surface hydrophobicity and that their appearance in the biofilm at a particular stage appears unrelated to their cell surface hydrophobicity. Furthermore, whilst bacteria harvested from single species biofilms at early stages of development may demonstrate relatively high cell surface hydrophobicity, this character may be species-dependent and may decline or increase as the biofilm matures. This variation observed may be a result of changing the immediate physico-chemical environment of the cells due to production of EPS, surface-active components, adsorption of molecules and many other environmental factors.
CHAPTER 6

CHARACTERISATION OF POLYSACCHARIDES IN EPS ISOLATED FROM BIOFILM MATRIX
6.1 INTRODUCTION

There are many advantages for microorganisms in forming a biofilm versus living as individual cells. Bacteria experience a degree of homeostasis when residing within a biofilm, and one of the key components of this microniche is the surrounding extracellular matrix. This matrix is composed of a mixture of components, including extracellular polymer substances (EPS), proteins, nucleic acids, and other substances (Davey and O'Toole, 2000). The presence of EPS helps to maintain the integrity of the biofilm, allowing large numbers of bacteria to coexist under flowing conditions. EPS also helps to cement bacteria to one another and to the substratum, and is therefore important in creating the biofilm architecture. In addition EPS provides a matrix which can trap nutrients, protect the resident microorganisms from the activity of grazing organisms, concentrate intercellular communication molecules and allow the development of high cell densities required for degradation of complex substances.

Microbial EPS are biosynthetic polymers that can be highly diverse in chemical composition. The vast majority of bacterial EPS are polysaccharides (Wimpenny et al., 1993). Typically, EPS includes substituted and unsubstituted polysaccharides, substituted and unsubstituted proteins and nucleic acids (Wingender et al., 1999). The structure of EPS is known to differ from species to species and is largely uncharacterised, with the exception of a small number of EPS with commercial significance (Davies, 2000).

Understanding the physical and chemical characteristics of EPS and its relationship to resident organisms in biofilms is crucial to the understanding of the structure and function of the biofilm under investigation. The characterisation of EPS associated with activated sludge process has received very little attention and the aim of
this section therefore was focused on characterising the chemical components of EPS isolated from biofilms growing in the wastewater treatment plant.

The structural characterisation of EPS can be approached in a number of different ways. As polysaccharides and glycoconjugates have been shown to be major components of EPS, a sequential analysis of these macromolecules was undertaken in this study. Emphasis was on the analysis of polysaccharide fraction. A summary of the approach taken is shown in Figure 6.1.
Biofilms developed in the wastewater treatment plant

\[\downarrow\]
Isolate EPS from biofilm (Section 6.2.1)

\[\downarrow\]
Isolate and purify polysaccharide fractions (Sections 6.2.2; 6.2.3)

\[\downarrow\]
Polysaccharide fractions (PF) \hspace{1cm} Non-polysaccharide fractions (Non PF)

\[\downarrow\]
Are they proteins? (Section 6.2.6.2)

Are there any differences in polysaccharides isolated from EPS of biofilms of different ages?
(Sections 6.2.4; 6.2.5; 6.2.6)

\[\downarrow\]
Are they glycoconjugates? Are they conjugated to proteins? (Sections 6.2.6.1; 6.2.6.2; 6.2.6.3)

\[\downarrow\]
Approximate molecular weight of polysaccharides in EPS? (Section 6.2.6)

\[\downarrow\]
What is the monosaccharide composition of polysaccharides in EPS?
(Sections 6.2.5; 6.2.7; 6.2.8; 6.2.9)

\[\downarrow\]
Is it possible to profile oligosaccharides? (Section 6.2.10)

Fig. 6.1 Overview of conceptual approach taken in biofilm polysaccharide analysis; relevant chapter sections in methods are noted in brackets
6.2 MATERIALS AND METHODS

6.2.1 Isolation of biofilm EPS

Biofilms were allowed to develop on glass microscope slides placed in the wastewater treatment plant as described in section 2.4. Collected glass slides containing biofilms were placed inside a sterile bag containing phosphate buffer saline (PBS pH 7.1). Biofilms were removed from glass slides into PBS by rubbing the bag from outside whilst holding the slides with fingers. The washed glass slides were removed from the bag and the PBS containing biofilm was centrifuged at 4800xg at room temperature for 20min to remove cells and other debris.

To undertake the analysis of EPS, it was necessary to separate cells and other debris from the matrix containing EPS. A number of techniques previously described in the literature were assessed to allow selection of a suitable method for isolation of EPS from the biofilms. Presence of carbohydrates in isolated fractions was used as a measure to select a suitable method, as the emphasis was on characterising the polysaccharide fraction of EPS. These methods are as follows:

6.2.1.1 Dialysis (Christensen et al., 1985)

The supernatant containing EPS was filtered through a Whatmann GF/F filter and dialysed exhaustively against distilled water. The nondialysable fraction was collected and tested for the presence of carbohydrates using phenol sulfuric assay described in section 6.2.5.1. This method resulted in low recovery of EPS due to dilution and was not selected for use in further studies.
6.2.1.2 Extraction with NaCl (Read and Costerton, 1987)

This method involved addition of 1M NaCl to the EPS containing supernatant to precipitate proteins. After adding NaCl, the contents were centrifuged at 12000xg for 30min at room temperature, and the pellet containing proteins was discarded. The resultant supernatant was concentrated by evaporating in a SpeedVac. The supernatant was tested for presence of carbohydrates using phenol sulfuric assay described in section 6.2.5.1. This method also yielded very low amounts of EPS and therefore was not selected for subsequent studies.

6.2.1.3 Cold ethanol precipitation (Evans and Linker, 1973)

As will be discussed further in section 6.3.1 this was the method of choice for isolation of EPS from biofilms as polysaccharides were detected in the precipitate obtained after cold ethanol addition. This method involved the following procedure. The collected supernatant was centrifuged at 20,000x g for 1h at room temperature. The pellet was discarded and the supernatant containing the polysaccharide fraction was decanted into a fresh clean tube. The supernatant was recentrifuged for 2 h at 20,000x g at room temperature. The resultant pellet was discarded and the supernatant was decanted into a clean tube. Three volumes of cold 95% ethanol were added slowly to this supernatant fraction while stirring to precipitate the polysaccharides. The precipitate thus formed was collected by cold centrifugation (4°C) at 3000x g for 30 min. The precipitate was washed twice with 95% ethanol, followed by one wash with absolute ethanol. The precipitate was transferred onto preweighed filter paper placed in a dish and allowed to dry at room temperature. Once the ethanol had evaporated the filter paper containing the EPS was weighed using an analytical balance. The yield of crude EPS per cm² of biofilm was calculated using the difference in weight.
6.2.2 Isolation of polysaccharide fraction (PF) from EPS

By Gel Filtration on Sepharose 4B

Solutions containing 10mg of crude EPS prepared as above were dissolved in 10ml of PBS. Blue dextran and glucose were added to this solution at a concentration of 1% to set criteria for fraction collection. It was assumed that the EPS fractions containing the polysaccharides would be eluted from the column after the high molecular weight Blue dextran, but before glucose.

Solutions of EPS were separated on a column of Sepharose 4B (Pharmacia) as described below.

Sepharose 4B was available as a pre-swollen gel. A sufficient quantity of Sepharose 4B was washed in 50mM ammonium acetate buffer (pH 7.0) containing 0.02% NaN₃ (Sigma Aldrich). The gel was degassed before packing into the column. The swollen gel was packed carefully avoiding the generation of air bubbles into a 100 x 2.5cm chromatography column, which was mounted vertically on the bench. When the gel was packed to the desired bed height a small amount of the ammonium acetate buffer was poured on top of the gel and the bed was allowed to settle.

Solutions of EPS were layered carefully onto the surface of the column and eluted using buffer at a rate of 20ml per hour. 5 ml fractions were collected and analysed for carbohydrates by the method of Dubois et al. (1956) as described in section 6.2.5.1.

Fractions that were positive for carbohydrates, collected after blue dextran and before glucose were eluted from the column, were kept separate as these were polysaccharide fractions (PF) of biofilm EPS. PF fractions collected from one EPS sample were pooled and stored at -18°C until further purification and analysis.
6.2.3 Purification of Polysaccharide Fractions (PF)

Desalting to remove salts introduced during extraction

Desalting was conducted according to the method of Fischer (1980). Pooled PF fractions were desalted using 50mM acetic acid on a column of Sephadex – G 25 (Pharmacia), to remove low molecular weight salts introduced during the extraction process. The fraction eluting in the void volume containing polysaccharides was collected and lyophilised for further analysis.

6.2.4 Analysis of Polysaccharide Fractions (PF) by Infrared Spectrometry

PF samples were prepared for Infrared analysis by dissolving 500 μg of purified EPS sample in 10ml of a 1.1% (w/v) solution of potassium bromide (Sigma Aldrich). This mixture was lyophilised and pressed into tablets. Infrared spectra of PF samples in potassium bromide pellets were measured using a Perkin-Elmer Model 21 spectrophotometer and the resultant spectra were saved for subsequent analysis.

6.2.5 Quantitative analysis of PF fractions for carbohydrates and proteins

Isolated polysaccharide fractions (PF) were analysed for monosaccharides using a number of colourimetric methods. Although PF samples primarily contained polysaccharides the possibility of protein conjugation to these fractions was also investigated at this stage by analysing the PF fractions for total protein content.
6.2.5.1 Hexose and pentose content of PF samples

A stock solution of PF fractions was prepared by dissolving 10μg of PF in 1ml of MilliQ water. The hexose and pentose content of samples were estimated using the phenol sulfuric assay method described by Dubois et al. (1956).

150μl aliquots of PF stock solution were transferred into acid washed pyrex test tubes in five replicates. Three tubes containing MilliQ water were used as controls. 150 μl of 5% (w/v) phenol solution was added to each tube and vortexed briefly. 750 μl of concentrated sulfuric acid was rapidly added to each sample tube, directly onto the liquid surface to produce rapid mixing and even heat distribution. Tubes were allowed to stand at room temperature for 10 min. The tubes were then vortexed and allowed to stand for 30 min for colour development. The solutions were transferred to 1 cm quartz cuvettes and absorbance (A_{480} for pentoses and A_{490} for hexoses) was measured using a Hitachi-2000 UV/VIS spectrophotometer. Absorbance values obtained were compared with standard curves to estimate the hexose and pentose content of samples. The standard curve for hexoses was obtained using a series of D-glucose (Sigma Aldrich) solutions containing 10 – 80 ng of glucose per μl of MilliQ water. The standard curve for pentoses was obtained using a series of xylose solutions containing 10 – 80 ng of glucose per μl of MilliQ water.

6.2.5.2 Sialic acid content of PF samples

The sialic acid content of PF samples was estimated using the Ferric Orcinol assay described by Schauer (1978) as detailed below. Bial reagent required for this assay was prepared by adding 0.2g of orcinol (Sigma Aldrich), 81.4ml of conc. HCl acid, 2ml of 1% ferric chloride made up to 100ml with MilliQ water.
150 μl aliquots of PF stock solution were transferred into clean pyrex test tubes in five replicates and 50 μl of deionised water was added to each tube. 200 μl of Bial reagent was added to each tube and vortexed. Test tubes were covered with glass marbles and heated in a boiling waterbath at 100°C for 15 min followed by cooling in tap water for 15 min. After cooling 1ml of isoamyl alcohol was added into the mixture, tubes were vortexed and cooled in ice for 5 min. The contents were then centrifuged for 3 min to separate phases. The upper phase was transferred into 1cm quartz cuvettes with a pasteur pipette and absorbance was measured at 570nm. Sialic acid content of samples was determined by comparing to the absorbance values obtained to a standard curve of sialic acid. The standard curve was obtained using a series of N-acetylneuraminic acid (Sigma Aldrich) containing 5 – 40 nmol of N-acetylneuraminic acid per ml of MilliQ water read against the absorbance of blank at 570nm.

6.2.5.3 Hexosamine (aminosugars) content of PF samples

The hexosamine content of PF samples was estimated using the MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) assay described by Smith and Gilkerson (1979).

One hundred μl aliquots of PF stock solution were placed in small pyrex test tubes in replicates of five. 100 μl of 1M HCl was added, and the tubes were vortexed to mix contents. The tubes were capped and heated in a heating block for 2h at 110°C. After heating, the tubes were cooled to room temperature, and 400 μl of 2.5% sodium nitrite was added to each tube followed by vortexing. The tubes were allowed to stand for 15 min at room temperature. 200 μl of 12.5% ammonium sulfamate was added to the tubes inside a fume hood, the samples were vortexed and then allowed to stand for 5 min at room temperature. Following this, 200 μl of 0.25% MBTH (Sigma Aldrich) was added to
each tube and the tubes were vortexed, capped and then incubated for 30 min at 37°C. After incubation, 200 µl of 0.5% ferric chloride was added to the mixture, the tubes were capped and incubated for 5 min at 37°C. The tubes were allowed to cool to room temperature and the contents were transferred into 1cm quartz cuvettes. The absorbance of samples was measured at 650nm using the reagent blank to zero the spectrophotometer (Hitachi-2000 UV/VIS). The hexosamine contents of samples were determined by comparing the absorbance values with the standard curve obtained using N-acetyl-D-glucosamine. The standard curve was obtained using the absorbance of a series of N-acetyl-D-glucosamine containing 5 – 30 nmol of N-acetyl-D-glucosamine per ml of MilliQ water read against the absorbance of blank at 650nm.

6.2.5.4 Uronic acid content of PF samples

The uronic acid content of PF samples was determined using the method described by Bitter and Muir (1962).

One hundred µl aliquots of PF stock solution were placed in pyrex culture tubes with caps in replicates of five. 100 µl of deionised water was added to all samples followed by 20 µl of 4M ammonium sulfamate (Sigma Aldrich) and the mixture was vortexed. 1ml of 25mM sodium tetraborate in H2SO4 was added slowly into the mixture, the tubes were covered and heated at 100°C for 5 min. Samples were cooled to room temperature, 40 µl of 0.1% carbazole (Sigma Aldrich) was added and the mixture was heated for 15 min at 100°C. Samples were allowed to cool and transferred to 1cm quartz cuvettes. The absorbance was measured at 520nm. Uronic acid contents of samples were determined by comparing absorbance values to a standard curve. The standard curve was obtained using the absorbance of a series of glucuronolactone containing 10 – 100
glucuronolactone (Sigma Aldrich) nmol per ml of MilliQ water read against a blank of water at 520nm.

6.2.5.5 Quantitative analysis of proteins in PF samples

The method described by Bradford (1976) was used to determine the protein content of EPS samples.

Coomassie Brilliant Blue solution was prepared as following. 100mg of Coomassie Brilliant Blue G-250 (Sigma Aldrich) was dissolved in 50ml of 95% ethanol and 100ml of 85% phosphoric acid. The total volume of the solution was made up to 1 litre by adding MilliQ water and the contents were filtered through Whatman No.1 filter paper.

50µl of PF stock solution was transferred into clean glass pyrex tubes in five replicates. 50µl of 0.15M NaCl was added to each tube. 1ml of Coomassie Brilliant Blue solution was added to the mixture and tubes were vortexed to mix the contents. The tubes were allowed to stand for 2 min at room temperature and the samples were transferred to 1cm quartz cuvettes. The absorbance was measured at 595nm. The protein content of samples was determined by comparing to a standard curve of bovine serum albumin. The standard curve was prepared by using the absorbance of a series of bovine serum albumin (BSA purchased from Sigma Aldrich) containing 2.5 – 12.5µg of bovine serum albumin per ml of MilliQ water read against a blank at 595nm.

6.2.6 Qualitative analysis of PF fractions

A number of analytical techniques were used to characterise PF fractions. The two major questions addressed were, whether the polysaccharide fractions (PF) isolated from
biofilm EPS were conjugated to other macromolecules and whether any differences existed in PF fractions isolated from biofilms of different age. Analytical techniques used to achieve this included gel electrophoresis, Western Blotting, HPLC and FACE analysis.

6.2.6.1 SDS PAGE Gel Electrophoresis of PF samples

The PF samples were analysed using 4 – 20% gradient polyacrylamide gels according to the method described by Laemmli (1970). Pre-prepared 4-20% gradient polyacrylamide gels containing 10 wells were purchased from BioRad, Australia.

The gel was aligned into the buffer dam consisting of short and long glass plates by sliding into the clamp assembly. The clamp screws were tightened and snapped onto the inner cooling core of a Mini Protean II cell (BioRad, Australia). The inner cooling core was lowered into the lower buffer chamber and a sufficient quantity of running buffer (pH 8.3) containing Tris Base 15g/l, Glycine 72g/l, SDS 5g/l, was added to the upper buffer chamber until the buffer reached halfway between the short and long plates. A sufficient volume of running buffer was added to the lower buffer chamber to cover about one third of the gel.

10μg of PF sample was dissolved in 1ml of SDS sample buffer containing 1.0 ml of 0.5M Tris – HCl (pH 6.8), 0.8ml glycerol, 1.6ml 10% SDS, 0.4ml 2β mercaptoethanol, 0.2ml of 0.5% bromophenol blue, 4.0ml MilliQ water and heated at 95°C for 4min. 20μl of dissolved sample was loaded into a well with a Hamilton syringe and electrophoresis was conducted with a 60mA at 200V.

Subsequent to electrophoresis the gels were removed from the cell and either stained for detection of proteins or membrane transfers were conducted using Western blotting techniques to detect glycoconjugates as described below (Sections 6.2.6.2 and 6.2.6.3)
6.2.6.2 Detection of proteins in PF samples by Rapid Silver Staining method

The gel was placed in a plastic container and agitated slowly for 10min in 50ml of formaldehyde fixing solution containing 1 litre of 40% methanol and 0.5ml of 37% formaldehyde solution. The fixing solution was removed and the gel was washed twice with water leaving for 5 min in each wash. The water was removed and the gel was soaked in 50 ml of Na₂S₂O₃ solution containing 0.2g of Na₂S₂O₃ per 1 litre of MilliQ water, agitating slowly. The Na₂S₂O₃ solution was removed and the gel was washed twice in water for 20 seconds in each wash. The water was decanted and the gel was soaked in 50ml of 0.1% AgNO₃ solution for 10min, agitating slowly. The AgNO₃ solution was decanted and the gel was washed twice with water and was finally rinsed with a small amount of thiosulfate developing solution containing 3% sodium carbonate, 0.0004% sodium thiosulfate and 0.5ml 37% formaldehyde. The gel was then soaked in 50ml of thiosulfate solution agitating slowly to develop the intensity of bands (for about 1min) and 2.3M citric acid containing 5ml per 100ml of thiosulfate was added and agitated slowly for 10min. The gel was removed from the solution and washed twice in water with gentle agitation. The water was decanted and the gel was soaked in 50ml of drying solution, which contained 10% ethanol and 4% glycerol. The gel was drained and sandwiched between two pieces of wet blotting paper and allowed to dry at room temperature.

6.2.6.3 Detection of total carbohydrates in PF samples using Western blotting

To examine the presence of carbohydrates conjugated to proteins, the macromolecules of PF samples separated using SDS PAGE were transferred onto a nitrocellulose membrane using Western blotting techniques. The presence of
glycoconjugates on the membrane was detected by oxidising the non-reducing terminal monosaccharide using periodate followed by labelling with Biotin. Biotin was detected using Streptavidin Alkaline Phosphatase (SAP) and Nitroblue tetrazolium (NBT)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) colour development system. This reaction is shown in Figure 1.4.

6.2.6.3.1 Electrolytic transfer of separated macromolecules on to membranes

The gel was removed carefully and placed in a dish containing transfer buffer containing 25mM Tris, 192mM glycine, 20% methanol (pH8.3). The gel was fixed in this buffer held at 4°C for 15 min. A 0.45μ nitrocellulose membrane strip cut to fit the gel was gently placed in another dish containing transfer buffer avoiding air bubble formation. The wet membrane was placed against the gel and secured with two cassettes on either side following the instructions provided by the manufacturer of the Mini Trans Blot cell (BioRad Australia). The gel-membrane system was aligned in the Mini Trans Blot cell filled with cold transfer buffer (4°C) with a magnetic stirrer and the whole unit was placed inside an esky filled with ice to reduce the heat generated during the process. The membrane blotting process was carried out at 105V at 250-350mA for 1h.

6.2.6.3.2 Oxidation of terminal non reducing monosaccharide

Following transfer, the membrane was washed in 10ml of PBS (pH 7.2) at room temperature for 10 min with gentle agitation. In order to oxidise the terminal non reducing monosaccharide, the membrane was immersed in 10ml of 10mM sodium periodate in sodium acetate/EDTA (ethylenediamine-tetraacetic acid) buffer (pH 5.5), covered with foil and incubated in the dark at room temperature with gentle agitation.
The membrane was washed 3 times with 10ml of PBS with gentle agitation using fresh buffer each time.

6.2.6.3.3. Labeling the oxidised terminal Carbon with biotin

The membrane was immersed in freshly prepared biotinylation solution containing 2µl hydrazide in 10ml sodium acetate/EDTA buffer, and incubated for 60 min at room temperature with gentle agitation. Following this treatment the membrane was washed 3 times in 10ml of Tris Buffered Saline (TBS pH 7.2) containing 50mM Tris and 27mM NaCl at room temperature to remove excess hydrazide. Fresh buffer was used each time for washing the membrane.

6.2.6.3.4 Detection and colour development

The above labelling reaction was stopped by immersing the membrane in 10ml of blocking reagent. The blocking reagent contained 0.5% non fat dry milk with no biotin in TBS. The membrane was incubated in this solution for 30 min at room temperature. It was then washed 3 times with 10ml of TBS, at room temperature, using fresh buffer each time, to remove excess blocking reagent.

The membrane was then placed in 10ml of TBS (pH 7.2) containing 5µl of Streptavidin-Alkaline Phosphatase for 60 min with gentle agitation at room temperature. The membrane was washed 3 times with TBS buffer to remove excess reagents. Drained membrane was immersed in 10ml of colour development buffer (pH 9.5) at room temperature until a purple colour was developed for about 30min. The colour development buffer contained 1.21g Tris, 1.01g MgCl$_2$$\cdot$6H$_2$O, 0.58g NaCl, made up to 100ml with MilliQ water, with 50µl NBT and 37.5µl BCIP.

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6.2.7 Analysis of PF samples for sialic acids using HPLC techniques

One hundred μl aliquots of PF stock solution were transferred into screw capped microcentrifuge tubes for hydrolysis. 0.1ml of 0.1M trifluoroacetic acid (TFA) was added and tubes were heated in a heating block at 80°C for 40 min to achieve gentle hydrolysis. Samples were allowed to cool and TFA was evaporated in a SpeedVac at room temperature. Five nmol of lactobionic acid was then added as the internal standard. 10μl of this sample was analysed on a HPLC system (Waters) using a Dionex Carbopac PA1 column using a gradient of 50 to 200mM sodium acetate in 100mM sodium hydroxide. The resultant chromatogram was compared to a standard containing N-acetyl neuraminic acid and N-glycolylneuraminic acid.

6.2.8 Analysis of PF samples for neutral sugars using HPLC techniques

One hundred μl aliquots of PF stock solution were pipetted into screw capped microcentrifuge tubes for hydrolysis. 0.1ml of 2M TFA was added into the tubes and heated in a heating block at 121°C for 1 hour. Samples were allowed to cool and the TFA was evaporated in a SpeedVac at room temperature. The dried pellet was washed once with 50μl of water, redried and resuspended in 100μl of water. 20μl of this was centrifuged at 10,000 rpm with 2 nmol of deoxyglucose internal standard. 5μl of sample was analysed using a Dionex HPLC system on a Dionex Carbopac PA1 column eluting with 15mM NaOH and using pulsed amperometric detection. The resultant chromatogram was compared to standards containing glucose, galactose, mannose, glucosamine, galactosamine and fucose (5μl of 1mM solution in MilliQ water).
6.2.9 Analysis of PF samples for amino sugars using HPLC techniques

One hundred μl aliquots of purified EPS stock solution were pipetted into screw capped microcentrifuge tubes. 0.1ml of 4M HCl was added to the tubes and heated in a heating block at 121°C for 1 hour. Samples were allowed to cool and HCl was evaporated in a SpeedVac at room temperature. The pellet was washed once with 50μl of water, redried and resuspended in 100μl of water. 20μl of this was centrifuged at 10,000 rpm with 2 nmol of deoxyglucose as the internal standard. 5μl of sample was analysed using a Dionex HPLC system on a Dionex Carbopac PA1 column eluting with 15mM NaOH and using pulsed amperometric detection. Chromatogram was compared to standards containing glucose, galactose, mannose, glucosamine, galactosamine and fucose (5μl of 1mM solution in MilliQ water).

6.2.10 Analysis of N-linked oligosaccharide profile of PF samples using Fluorophore Assisted Carbohydrate Electrophoresis (FACE)

A BioRad N-linked Oligosaccharide profiling kit (Catalog Number 170-6501) was used to analyse and characterise oligosaccharides of PF samples. This analysis involved enzymatic release of N-linked oligosaccharides from the glycoconjugate sample, fluorescent labelling of the released oligosaccharides, electrophoresis of the labelled oligosaccharides and detection of the separated oligosaccharides under UV illumination.

The first step for oligosaccharide analysis using this kit was to release asparagine linked (N-linked) oligosaccharides from the protein using the enzyme Peptide N-glycosidase F (PNGase F) releasing both intact oligosaccharides and polypeptide chains.
Released oligosaccharides were then labelled with the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) at their reducing termini by reductive amination. Labelled oligosaccharides were separated using gel electrophoresis based on size and bands were detected using UV illumination.

Kit components were reconstituted and stored appropriately according to the manufacturer’s instructions. The numbers within parenthesis in following sections are reference numbers of the kit components.

6.2.10.1 Enzymatic release of N-linked oligosaccharides

50μg of PF sample was dissolved in 45μl of 1x releasing buffer (C3) in a microcentrifuge tube. 1.0μl of 5% SDS and 1.5μl of β-mercaptoethanol (1:10 solution) was added into each sample tube and tubes were heated at 95°C for 5min on a heating block to denature the sample. 4μl of 10% NP-40 was added to the tubes and contents were mixed by gently tapping the sides of the tubes. 2μl of Peptide-N-glycosidase F (PNGase F) was added to tubes containing samples. Contents in the tube were mixed by gently tapping the sides of the tube followed by vortexing for 5 seconds. Tubes were incubated overnight at 37°C to allow digestion of the samples. Following the incubation, 3 volumes of cold 100% ethanol (at 4°C) were added and the tubes placed in ice for 10min. The tubes were centrifuged in a microcentrifuge for 5min and the pellet of proteins was discarded. The supernatant containing the oligosaccharides was transferred into clean microcentrifuge tubes and evaporated in a SpeedVac at room temperature. All samples were stored at –20°C until further treatment.
6.2.10.2 Labeling oligosaccharides with fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)

Five µl of reconstituted Oligo Labelling Diluent (L2) was added to each dried oligosaccharide sample from section 4.2.16.1 and mixed well by vortexing the tubes. Following this, 5µl of reconstituted Labelling Reducing agent was added to each tube, tubes were vortexed and then centrifuged for 5 seconds in a microcentrifuge. Samples were incubated at 37°C overnight. Samples were dried in a SpeedVac at room temperature for approximately 15min.

6.2.10.3 Electrophoresis of labelled oligosaccharides

An N-linked Oligosaccharide gel was removed from the package and the comb was gently removed avoiding damage to the wells. The gel was rinsed with reconstituted Electrophoresis buffer using a disposable 10ml bulb pipette. The gel was placed between the cassettes in the clamp assembly of a Mini Protean II Electrophoresis Cell (BioRad, Australia) and assembled as described in Section 6.2.6.1. The upper buffer reservoir was filled with approximately 250ml of cold (4°C) Oligosaccharide buffer and the clamp assembly was placed carefully ensuring no leakage occurred. Following this 600ml of cold buffer was poured into the lower buffer tank.

Labelled oligosaccharide from section 6.2.10.2 was resuspended in 8µl of MilliQ water and an equal amount of 2x N-Linked sample Buffer (E1) was added to the contents. The wells were loaded with 4µl of samples or controls using a Hamilton syringe. Lane 1 and 2 of the gel was loaded with the tracking dye and glucose ladder (standard) respectively. Electrophoresis was conducted under the constant current mode at 15mA per gel for about 90min, monitoring the movement of the tracking dye. When the electrophoresis was complete, the power supply was disconnected, and the gel was
removed. The gel was placed on the UV lightbox (peak output 360nm) for band detection and photographs were taken using a Polaroid (Spectra) camera on Polaroid 553 film and a Kodak number 8 filter.

6.2.10.4 Quantification of oligosaccharides using image analysis

The photographs were scanned using a Hewlett-Packard ScanJet 6200C at a resolution of 300 dpi and the tagged image format file (TIFF) based images were analysed using Image ProPlus® software. The relative oligosaccharide concentration in the individual bands, defined as regions exhibiting >10% of background, was calculated based on band fluorescence intensity (pixel number). The approximate size of sample oligosaccharides was estimated using the Degree of Polymerisation (DP) by comparing the band position of the oligosaccharide with the standard glucose ladder (E3) provided with the kit, as described below.

The glucose ladder (E3) consists of a mixture of glucose oligomers ranging from glucose$_1$ to greater than glucose$_{20}$. The migration values for oligosaccharides are defined as Degree of Polymerisation, or DP value, which depends on the number of oligomers present in the oligosaccharide. Each oligomer in the glucose ladder corresponds to a specific DP value (eg: glucose$_1$ = 1DP; glucose$_2$ = 2DP; glucose$_3$ = 3DP). The DP value for each oligosaccharide band was estimated by comparing the migration of each band with the adjacent glucose polymers in the glucose ladder.
6.3 RESULTS

6.3.1 Isolation of EPS from biofilms

The amounts of EPS recovered from biofilms at different stages of development are presented in Table 6.1. This analysis was repeated three times. As described in the section 6.2.1, three methods were tried for isolation of EPS. The method described by Christensen et al. (1985) used exhaustive dialysis against distilled water to separate macromolecules from relatively smaller molecules. When the nondialysable fraction was tested for total carbohydrates by the method of Dubois et al. (1956) (section 6.2.5.1), only a weak reaction was observed suggesting loss of macromolecules. The dialysed fraction in distilled water did not give a positive reaction for total carbohydrates, perhaps due to the dilution factor. For these reasons this method was not selected for subsequent use.

The method of Read and Costerton (1987) used 1M NaCl to precipitate and remove proteins and the “supernatant containing polysaccharides” was collected for further analysis. The supernatant when tested for total carbohydrates did not yield a positive reaction and this method was also not selected for subsequent use. The method of choice was the cold ethanol precipitation technique described by Evans and Linker (1973) which resulted in detection of carbohydrates in the supernatant and this method was used to isolate EPS from biofilms for all experiments described in this section. EPS isolated with cold ethanol precipitation, were off white, fluffy, hygroscopic and appeared like flakes.

For convenience, EPS isolated from 1, 2, 3, 5 and 7 days old biofilms will be referenced as EPS1, EPS2, EPS3, EPS5 and EPS7 respectively in this chapter. As shown in the data presented in Table 6.1, the amount of EPS collected from biofilms increased
with the maturation of biofilm. The amount of EPS recovered from a 1 day old biofilm was approximately 2.51 (± 0.7) μg per cm² of biofilm. The amount of crude EPS recovered from biofilms of age 2, 3, 5 and 7 days were 3.07 (± 0.9), 3.12 (± 0.6), 4.09 (± 1.4) and 7.15 (± 2.1) μg per cm² of biofilm respectively.

6.3.2 Isolation of polysaccharide fraction (PF) from EPS

Polysaccharide fractions were isolated from EPS samples using gel filtration on a Sepharose 4B column as described in section 6.2.2 using ammonium acetate buffer. The elution profiles of EPS1, EPS2, EPS3, EPS5 and EPS7 are shown in Fig.6.2. Polysaccharide fractions collected from EPS1, EPS2, EPS3, EPS5 and EPS7 are referred to as PF1, PF2, PF3, PF5 and PF7 in this section. The collected polysaccharide containing fractions were tested for presence of carbohydrates by the method of Dubois et al. (1956) using phenol sulfuric assay described in section 6.2.5.1. Large blue dextran (BD) was eluted in fractions collected from 40 – 90ml as indicated by the positive absorbance at 490nm indicating the first detection of carbohydrate elution. In all cases PF fractions were eluted in the void volume of the column as the PF fractions containing carbohydrates were eluted after the elution of blue dextran and prior to elution of glucose. Glucose molecules were eluted from the column in fractions collected after 230 – 235ml, thus it was assumed that any molecules larger than glucose were eluted prior to this. PF1 was eluted in fractions collected between 90 – 180ml (Fig 6.2a), while PF2 was eluted in fractions collected between 85 – 175ml (Fig 6.2b). PF3, PF5 and PF7 were eluted in fractions collected between 95 – 160ml, 95-165ml and 95 – 125ml respectively (Figs 6.2c-e).
Desalting was conducted to remove salts from collected PF samples. After desalting and drying PF samples containing polysaccharides were off white in colour, hygroscopic and powdery. The samples were stored in desiccators containing silica gel to prevent absorption of moisture.

6.3.3 Infrared spectroscopy

Infrared spectra of alkali treated PF1 differed from PF2, PF3, PF5 and PF7 samples suggesting differences in bonding and molecular vibrations. Comparison of IR spectra of PF1 and PF2 showed significant differences in vibrations indicating differences in bonding (Fig. 6.3a). At frequency levels of 1600 and 1400, absorbance values of PF2 was much higher while PF1 showed no change in absorbance or energy transfers in this region of the IR spectrum. Fig. 6.3b shows overlaid IR spectra of PF3, PF5 and PF7 showing very similar bonding vibration patterns to PF2 suggesting some similarities in structure.
Table 6.1  Yield of EPS isolated from various biofilm samples developed in the wastewater treatment plant

<table>
<thead>
<tr>
<th>Age of Biofilm sample (days)</th>
<th>Yield of EPS** (µg per cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.51 (± 0.7)</td>
</tr>
<tr>
<td>2</td>
<td>3.07 (± 0.9)</td>
</tr>
<tr>
<td>3</td>
<td>3.12 (± 0.6)</td>
</tr>
<tr>
<td>5</td>
<td>4.09 (± 1.4)</td>
</tr>
<tr>
<td>7</td>
<td>7.15 (± 2.1)</td>
</tr>
</tbody>
</table>

** Analysis repeated three times  n=3

Values in parentheses are standard deviations
Fig 6.2  Elution profiles of EPS isolated from biofilms of different ages on a column of Sepharose 4B using ammonium acetate buffer.

The flow rate was 20ml per h. Fractions of 5ml were collected and assayed for presence of carbohydrate by the method of Dubois et al. (1956). Positive absorbance at 490nm indicates presence of carbohydrates. The peaks for elution of blue dextran (BD) and glucose (glc) are indicated. The fractions showing positive absorbance at 490nm collected after blue dextran (the largest molecule) and before glucose (the smallest molecule) was eluted, contained polysaccharide fractions (PF).

(a) PF1 (from EPS of 1 day old biofilm) eluted in fractions of 80 – 180ml
(b) PF2 (from EPS of 2 day biofilm) eluted in fractions of 75 – 170ml
(c) PF3 (from EPS of 3 day biofilm) eluted in fractions of 90 – 160ml
(d) PF5 (EPS of 5 day biofilm) eluted in fractions of 90 – 165ml
(e) PF7 (EPS of 7 day biofilm) eluted in fractions of 90 – 125ml
Fig 6.3 Infrared spectra of Polysaccharide fractions (PF) isolated from biofilm EPS

(a) PF1: from EPS of 1 day old biofilm is shown in red
    PF2: from EPS of 2 day old biofilm is shown in blue
    Note differences in bond vibrations between the two EPS samples

(b) PF3: from EPS of 3 day old biofilm is shown in red
    PF5: from EPS of 5 day old biofilm is shown in green
    PF7: from EPS of 7 day old biofilm is shown in blue

Note similarities in bond vibrations of PF3, PF5 and PF7 samples
similar to PF2 and different from PF1
6.3.4 Quantitative analysis of PF fractions for carbohydrates by colourimetry

Figures 6.4 – 6.8 represent standard curves used for determination of various sugar components of PF fractions presented in Table 6.2. For determination of hexose sugars, D-glucose was chosen as the standard, and as shown in Figure 6.4 the standard curve was constructed by plotting a line of best fit. The hexose content of samples was determined using five replicate samples. Similarly, Figure 6.5 is the standard curve used for determination of pentose sugars in PF samples, using xylose as the standard. In both, the standard curves were linear.

The standard curve for sialic acids was obtained using N-acetyl-neuraminic acid as the standard. It was linear up to concentrations of 30 nmol/ml but values above this were not linear (Figure 6.6). However PF samples contained concentrations of sialic acid between 15 to 20nmol/ml and thus fell within the linear part of the curve.

Figure 6.7 represents the standard curve used for determination of hexosamine content using N-acetyl-D-glucosamine as the standard. The curve was linear and hexosamine contents of samples were determined using the absorbance values obtained. Similarly the standard curve for uronic acid constructed using D-glucuronic acid as the standard (Figure 6.8) was linear and uronic acid contents of samples were determined using the absorbance values obtained.

Results of quantitative analysis of purified PF are presented in Table 6.2. All PF samples tested contained varying levels of hexose sugars, pentose sugars, uronic acids, sialic acids and hexosamines. The hexose sugar content of PF samples were 14.6 (± 2.1), 6.6 (± 1.4), 12.1 (± 2.2), 5.3 (± 1.1) and 14.8 (± 3.2) μg per mg of PF1, PF2, PF3, PF5 and PF7 respectively. The pentose sugar content of PF samples were 21.3 (± 4.6), 10.6 (±
2.2), 20.3 (± 2.7), 19.8 (± 1.9) and 18.4 (± 2.1)μg per mg of PF1, PF2, PF3, PF5 and PF7 respectively. Similarly the uronic acid content of PF samples were 2.64 (± 0.2), 0.704 (± 0.11), 3.52 (± 0.41), 4.71 (± 0.37) and 1.76 (± 0.5)μg per mg of PF1, PF2, PF3, PF5 and PF7 respectively. These results suggest some variation in hexose, pentose and uronic acid content of PF samples isolated from biofilms of different ages. However, the sialic acid content of PF1 was 72.69 (± 6.2)μg per mg, while all the other PF samples contained significantly less sialic acid. The sialic acid content of PF2, PF3, PF5 and PF7 were 2.47 (± 0.4), 1.08 (± 0.2), 1.08 (± 0.4) and 3.71 (± 1.3)μg/mg respectively. A large variation was seen in the hexosamine content in EPS isolated from different biofilms. The hexosamine content of PF1 was 2.65 (± 0.6)μg per mg which was significantly lower than what was detected in other PF samples. The hexosamine content of PF2, PF3, PF5 and PF7 were 221.2 (± 16.9), 331.8 (± 24.9), 584.5 (± 52.2) and 672.8 (± 31.1)μg per mg.

6.3.5 Quantitative analysis of PF fractions for proteins by colourimetry

For determination of protein content in PF samples, bovine serum albumin was chosen as the standard, and as shown in Fig 6.9 the standard curve was constructed by plotting a line of best fit. The standard curve was linear and the protein content of samples was determined using values of five replicate samples. Total protein contents as measured by this assay were 3.0 ± 0.3μg per mg in PF1, 5.8 (± 0.3)μg per mg in PF2, 7.1 (± 0.7)μg per mg in PF3, 10.2 (± 0.5)μg per mg in PF5 and 11.1 (± 0.8)μg per mg in PF7. These values are shown in Table 6.2.
Table 6.2  Summary of component analysis of polysaccharide fractions (PF) of EPS isolated from biofilms of different ages growing in the wastewater treatment plant.

n = 5

<table>
<thead>
<tr>
<th>Sugar/Protein content (µg per mg of EPS)</th>
<th>PF1</th>
<th>PF2</th>
<th>PF3</th>
<th>PF5</th>
<th>PF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexoses a</td>
<td>14.6 (± 2.1)</td>
<td>6.6 (± 1.4)</td>
<td>12.1 (± 2.2)</td>
<td>5.3 (± 1.1)</td>
<td>14.8 (± 3.2)</td>
</tr>
<tr>
<td>Pentoses b</td>
<td>21.3 (± 4.6)</td>
<td>10.6 (± 2.2)</td>
<td>20.3 (± 2.7)</td>
<td>19.8 (± 1.9)</td>
<td>18.4 (± 2.1)</td>
</tr>
<tr>
<td>Sialic acids c</td>
<td>72.69 (± 6.2)</td>
<td>2.47 (± 0.4)</td>
<td>1.08 (± 0.2)</td>
<td>1.08 (± 0.4)</td>
<td>3.71 (± 1.3)</td>
</tr>
<tr>
<td>Hexosamines d</td>
<td>2.65 (± 0.6)</td>
<td>221.2 (± 16.9)</td>
<td>331.8 (± 24.9)</td>
<td>584.5 (± 52.2)</td>
<td>672.8 (± 31.1)</td>
</tr>
<tr>
<td>Uronic acids e</td>
<td>2.64 (± 0.2)</td>
<td>0.704 (± 0.11)</td>
<td>3.52 (± 0.41)</td>
<td>4.71 (± 0.37)</td>
<td>1.76 (± 0.5)</td>
</tr>
<tr>
<td>Protein f</td>
<td>3.0 (± 0.3)</td>
<td>5.8 (± 0.3)</td>
<td>7.1 (± 0.7)</td>
<td>10.2 (± 0.5)</td>
<td>11.1 (± 0.8)</td>
</tr>
</tbody>
</table>

a measured according to phenol sulfuric acid assay (section 6.2.5.1)
b measured according to phenol sulfuric acid assay (section 6.2.5.1)
c measured according to ferric orcinol assay (section 6.2.5.2)
d measured according to MBTH assay (section 6.2.5.3)
e measured according to carbazole assay (section 6.2.5.4)
f measured according to coomassie blue assay (section 6.2.5.5)

Values within parentheses are standard deviations
Fig 6.4 Standard curve for determination of hexose sugars using phenol sulfuric acid assay (Each data point represents the mean of 5 values)

Fig 6.5 Standard curve for determination of pentose sugars using phenol sulfuric acid assay.
(Each data point represents the mean of 5 values)
Fig 6.6  Standard curve for determination of sialic acid content using ferric orcinol assay.
(Each data point represents the mean of 5 values)

Fig 6.7  Standard curve for determination of hexosamine content using MBTH assay.
(Each data point represents the mean of 5 values)
Fig 6.8 Standard curve for determination of uronic acid content using Carbazole assay.
(Each data point represents the mean of 5 values)

Fig 6.9 Standard curve for determination of protein content using method of Bradford
(Each data point represents the mean of 5 values)
6.3.6 Qualitative analysis of PF fractions

Results of experiments conducted to characterise the PF fractions are detailed in this section. The aim was to detect presence of any macromolecules attached to polysaccharides using SDS-PAGE and Western Blotting.

6.3.6.1 Detection of macromolecules attached to carbohydrate moiety

Before any detailed analysis was conducted, it was necessary to determine the optimum quantity of PF sample required for detection of carbohydrates on membranes to avoid any misinterpretation of results. To achieve this and to optimise the technique, trial SDS-PAGE experiments and Western Blotting were conducted as detailed in sections 6.2.6.1 and 6.2.6.3. 100ng of each PF sample (PF1, PF2, PF3, PF5 and PF7) were mixed together in the loading buffer. Dilutions of this solution were prepared to achieve pooled samples that contained 75ng and 50ng of each PF sample. These solutions were loaded into separate wells of the gel. To determine the optimum quantity of the standard biotinylated markers required, quantities of 1, 2, 3, 4 and 5μl of the standard was loaded into separate wells of the gel. Results of this trial are shown in Fig 6.10. The optimum quantity that developed a visible colour reaction with SAP appeared to be 3μl of the reconstituted standard of biotinylated markers (lane 1). Heavily stained bands were observed in lanes 2 and 3, which contained 4 and 5μl of the reconstituted standard respectively. In contrast, faint bands were observed in lanes 4 and 5, which contained 1 and 2μl of the reconstituted standard. Lanes 6, 7 and 8 contained 100ng, 75ng and 50ng of pooled PF sample respectively. Bands appeared in all lanes suggesting presence of carbohydrate moiety conjugated to other macromolecules such as proteins or lipids.
Bands developed in lane 6 were quite intense and streaked suggesting that 100ng of sample was excessive. Based on results of this experiment it was decided that 50 to 75ng of PF sample was sufficient to provide separation and detection on SDS PAGE gels.
Results of trial experiment conducted to determine the optimum quantity of PF sample and the standard marker required for detection of carbohydrates on nitrocellulose membranes.

Lanes 1-8 contained following:

Lanes 1-5:  Biotinylated markers were added to each lane in following quantities.
           Lane 1: 3μl – bands well separated
           Lane 2: 4 μl – bands were separated, but staining was heavy
           Lane 3: 5 μl -bands were separated, but staining was heavy
           Lane 4: 1μl – low level of detection
           Lane 5: 2μl – low level of detection

Lanes 6-8:  PF samples were added to each lane in following quantities
           Lane 6: 100ng - Carbohydrate in PF was detected, but separation was low
           Lane 7: 75ng – Carbohydrates in PF was detected and well separated
           Lane 8: 50ng – Carbohydrates PF was detected and well separated
To further characterise and identify differences in PF samples, PF recovered from biofilms of various stages of development were analysed separately using SDS-PAGE and membrane blotting. To eliminate the possibility of “non polysaccharide” fractions (as determined by colorimetry according to section 6.2.5.1) collected from gel filtration experiment having any glycoconjugates, concentrated “non polysaccharide” (Non-PF) fractions were loaded into the gel along with the PF samples as controls. Results showed presence of glycoconjugates in PF samples of all biofilms (Figs 6.11 – 6.14). Interestingly, the results also clearly indicated presence of at least two different types of polysaccharides. None of the “non polysaccharide” fractions were positive for glycoconjugates.

PF1 contained a polysaccharide with an approximate molecular weight of 25kDa (Fig 6.11, Lane 4) and no other bands were visible on the membrane. None of the “non polysaccharide” fractions showed any bands suggesting absence of carbohydrate in these fractions (Figure 6.11, Lanes 1, 2, 3 and 5) confirming results obtained by colourimetric methods.

PF2 showed a single band on the membrane of approximately a molecular weight of 60kDa (Fig 6.12). A faint band (?) was observed below that, however, whether this was actually due to presence of carbohydrates or due to stain accumulation was unsure. PF3 also showed a single band on the membrane of approximately a molecular weight of 60kDa (Figure 6.13) but no other bands were visible. Similarly, PF5 and PF7 showed bands approximately about 60kDa in molecular weight, suggesting similarities in these PF samples to one another (Figure 6.14).
Fig 6.11  Biotinylated nitrocellulose membrane blot showing presence of glycoconjugation in polysaccharides (PF1) recovered from EPS of 1 day old biofilms

Lanes 1,2,3,5: Fractions from gel filtration which were negative for carbohydrates based on the method of Dubois et al. (1956)
These are “Non PF” fractions collected from gel filtration as detailed in section 6.2.2
No bands were visible in lanes 1,2,3 and 5

Lane 4: PF1 (isolated from EPS1)
A single band of approximately 25kDa was present as indicated by the arrow

Lane 6: Biotinylated markers (range 14.1 - 94.1kDa)
Lane 7: Native Markers (range as above, not biotinylated)
**Fig 6.12** Biotinylated nitrocellulose membrane blot showing presence of glycoconjugates in PF2 recovered from EPS isolated from 2 day old biofilms

Lanes 1, 2, 3, 5, 6 and 7: Fractions from gel filtration which were negative for carbohydrates based on the method of Dubois (1956) ("Non PF" fraction)

These "Non PF" fractions were collected from gel filtration as detailed in section 6.2.2

No bands were visible

Lane 4: PF2 (collected from 2 day old biofilm)

Note band of approximate molecular weight 60kDa, indicated by arrow

(?) stain accumulation or faint band?

Lane 8: Biotinylated markers (range 14.1 - 94.1kDa)

Lane 9: Native Markers (range 14.1 - 94.1kDa not biotinylated)
Fig 6.13  Biotinylated nitrocellulose membrane blot showing presence of glycoconjugation in PF3 isolated from EPS of 3 day old biofilms

Lane 1: PF3 (collected from 3 day old biofilm)  
A single band of approximately 60 kDa was observed as shown by the arrow

Lane 2: Biotinylated markers (range 14.1 - 94.1 kDa)
Fig 6.14  Biotinylated nitrocellulose membrane blot showing presence of glycoconjugation in PF5 and PF7 recovered from EPS of 5 and 7 day old biofilm

Lane 1: PF5 (PF collected from 5 day old biofilm)
A single band was observed having an approximate molecular weight of 60 kDa indicated by arrow

Lane 2: PF7 (PF collected from 7 day old biofilm)
A single band was observed having an approximate molecular weight of 60 kDa indicated by arrow

Lane 3: Biotinylated Markers (range 14.1 - 94.1 kDa)
6.3.6.2 Detection of proteins in PF and “non PF” samples

Previous results from IR spectroscopy (Fig 6.3) and Western Blotting (Fig 6.11-6.14) have suggested that the structure of PF1 (PF isolated from 1 day old biofilm) was different from the other PF samples, i.e. PF2, PF3, PF5 and PF7 (PF isolated from 2,3,5 and 7 day old biofilms respectively). On the basis of these results, PF1 and PF3 were selected for further analysis and characterisation.

The PF and “non PF” containing fractions (fractionated using gel filtration as described in section 6.2.2) were separated by SDS PAGE and stained using rapid silver staining method to visualise any proteins present in those samples. The stained SDS PAGE gel of “non EPS” containing fractions showed presence of large protein molecules (Fig 6.15 - lanes 1, 2, 3 and 4). Similarly, the EPS containing fraction also showed distinct bands suggesting that the carbohydrate moiety of EPS was conjugated to proteins (Figure 6.15 – lane 5). Lane 7 and 8 contained kaleidoscope protein marker and native protein marker respectively, however, due to the time required to detect proteins in test samples the bands of the markers were deeply over-stained and smudged, therefore molecular weight estimations were not possible.

Silver stained SDS PAGE gels of PF1 and PF3 are shown in Fig 6.16. Many bands were observed in PF1 and PF3 suggesting presence of proteins in both. Lane 8 contained the kaleidoscope protein molecular weight markers but molecular weights of proteins were not estimated using these gels. Fig. 6.17 shows an enlarged section of the gel shown in Fig. 6.16 (containing PF3) with the molecular weight markers.
Fig 6.15  SDS PAGE of isolated PF fractions stained with Rapid Silver stain

Lanes 1, 2, 3 and 4:  "Non PF" fractions
Proteins were present, although separation was not clear

Lane 5:  PF containing fraction
Proteins were present in this fraction suggesting conjugation of carbohydrate moiety to proteins

Lanes 6 and 7:  No samples (empty wells)

Lanes 8 and 9:  Molecular weight markers (overstained)
Fig 6.16  SDS PAGE gel of isolated PF1 and PF3 stained with Rapid Silver stain

Lane 1: PF1 (of EPS isolated from 1 day old biofilm)  
Many bands are present

Lane 4: PF3 (of EPS isolated from 3 day old biofilm)  
Many bands are present

Lanes 2, 3, 5, 6, 7: Empty (no samples)

Lane 8: Kaleidoscope protein molecular weight marker
**Fig 6.17**

SDS PAGE of isolated PF3 fraction
(Enlarged from Fig 6.16)

Lane 4: PF3 (from EPS of 3 day old biofilm)

Note: Protein band stained with approximate molecular weight 60kDa shown by arrow

Lane 8: Molecular weight markers (18.4 - 216 kDa)
6.3.7 Detection of sialic acids using HPLC analysis

The presence of sialic acids in PF1 and PF3 was well demonstrated by HPLC analysis using HPAEC-PAD chromatography (Fig 6.18). The chromatogram of PF1 showed a strong peak after 5.95 min, corresponding with the position of N-acetyl neuraminic acid (Fig. 6.18a). The chromatogram of PF3 on the other hand had two relatively smaller peaks (Figure 6.18b). The first peak was observed after 6.0 min while a smaller second peak appeared after 7.3 min suggesting presence of two different types of sialic acid. The first peak at 6.0 min corresponds with the position of N-acetyl neuraminic acid, while the second peak at 7.3 min could belong to a sialic acid related to N-acetyl group, with more than one acetyl group, however the data is inconclusive. The peak for the internal standard lactobionic acid appeared after 8.43 and 8.56 min respectively with PF1 and PF3. Peaks were observed after 6.5 and 14.97 min respectively for the two standard sialic acids N-acetyl neuraminic acid and N-glycolyl-neuraminic acid separated on Carbopac PA 1 column (Fig 6.14c).
Fig. 6.18 HPAEC-PAD chromatograms for detection of sialic acids in biofilm PF samples. Separation was achieved on a Dionex Carbopac PA1 column using a gradient of 50 to 200 mM NaOH with post column addition of 0.4M NaOH.

(a) PF1 (PF of 1 day old biofilm showing peak after 5.95min)
(b) PF3 (PF of 3 days old biofilm showing two peaks after 6.0min and 7.3min)
(c) Standards: NANA (6.5 min) NGNA (14.97 min)

NANA: N- acetyl-neuraminic acid  NGNA: N-glycolyl-neuraminic acid
6.3.8 Detection of neutral and amino sugars in PF using HPLC analysis

Acid hydrolysed PF1 and PF3 samples were separated on a Carbopac PA1 (Dionex Corporation) column for detection of neutral and amino sugars using deoxy glucose as an internal standard. PF samples were hydrolysed under moderate hydrolytic conditions for neutral sugars to avoid destruction of linkages (Section 6.2.8). Both protocols contained the same internal standard and sugar series as described in the methods (sections 6.2.8 and 6.2.9). The internal standard deoxy glucose was detected in all chromatograms as a peak around 8.5min.

Both PF samples contained the two aminosugars galactosamine and glucosamine (Fig 6.19). The presence of other peaks are due to the internal standards. Similarly both PF samples also contained neutral sugars galactose, glucose and mannose (Figures 6.20) and presence of aminosugars in this chromatograms was due to the two aminosugars galactosamine and glucosamine added as internal standards. A few unidentifiable peaks at lower levels were observed in the chromatogram of PF3 suggesting presence of other molecules. The amount of amino and neutral sugars present in PF1 and PF3 were calculated from the peak area compared with the known amounts of standard. The galactosamine and glucosamine content of PF1 and PF3 was 0.319, 0.412nmol/µg and 0.623, 0.783nmol/µg respectively (Figure 6.19). Similarly, the galactose, glucose and mannose contents of PF1 and PF3 were 0.971, 0.989nmol/µg; 0.837, 0.858nmol/µg, 1.168, 1.129nmol/µg respectively.
Fig. 6.19  HPAEC-PAD of amino sugars in PF samples. Separation was carried out on a Dionex Carbopac PA1 column with 15mM NaOH using pulsed amperometric detection.

(a)  PF1 (PF isolated from 1 day old biofilm containing galactosamine – galNH2 and glucosamine – gleNH2 )

(b)  PF3 (PF isolated from 3 days old biofilm containing galactosamine – galNH2 and glucosamine- gleNH2)

(c)  Summarised peak report

<table>
<thead>
<tr>
<th>Amino sugar</th>
<th>PF1</th>
<th>PF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosamine (galNH2)</td>
<td>0.319 nmol/µg</td>
<td>0.412 nmol/µg</td>
</tr>
<tr>
<td>Glucosamine (gleNH2)</td>
<td>0.623 nmol/µg</td>
<td>0.783 nmol/µg</td>
</tr>
</tbody>
</table>

Note: Peaks of neutral sugars due to standards containing fucose (Fuc), galactose (gal), glucose (glc), mannose (man)

Std - Standard
Fig. 6.20  
HPAEC-PAD of neutral sugars in PF samples. Separation was carried out on a Dionex CarboPac PA1 column with 15mM NaOH using pulsed amperometric detection.

(a) PF1 (PF isolated from 1 day old biofilm containing galactose, glucose and mannose)
(b) PF3 (PF isolated from 3 days old biofilm containing galactose, glucose and mannose)
(c) Summarised peak report
Note: Peaks of amino sugars (galNH2 and glcNH2) due to standards containing glucosamine and galactosamine
Std - Standard

<table>
<thead>
<tr>
<th>Neutral sugar</th>
<th>PF1</th>
<th>PF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose (gal)</td>
<td>0.971 nmol/µg</td>
<td>0.989 nmol/µg</td>
</tr>
<tr>
<td>Glucose (glc)</td>
<td>0.837 nmol/µg</td>
<td>0.858 nmol/µg</td>
</tr>
<tr>
<td>Mannose (man)</td>
<td>1.168 nmol/µg</td>
<td>1.129 nmol/µg</td>
</tr>
</tbody>
</table>
6.3.9 Analysis of N-linked Oligosaccharide profile

FACE analytical techniques were used (section 6.2.10) to profile N-linked oligosaccharides released from the PF samples. This involved releasing N-linked oligosaccharides from the protein using the enzyme Peptide N-glycosidase F followed by labelling the oligosaccharides with ANTS. Labelled oligosaccharides were separated using gel electrophoresis based on size along with a standard glucose ladder containing polymerised glucose monomers.

FACE profiles of separated oligosaccharides are presented in Fig 6.21. Lane 1 shows the glucose ladder (E3) consisting of a mixture of glucose oligomers ranging from glucose$_1$ to greater than glucose$_{20}$. Migration values for oligosaccharides are defined by Degrees of Polymerisation (DP value). Each oligomer in the glucose ladder corresponds to a specific DP value (e.g. glucose$_1$ = 1DP, glucose$_2$ = 2DP, glucose$_3$ = 3DP). The DP value for each oligosaccharide band was estimated by comparing the migration of each band with the adjacent glucose polymers in the glucose ladder.

The oligosaccharide band pattern of PF1 showed at least seven oligosaccharides (Fig. 6.21, lane 2). Direct comparison of these bands with the adjacent glucose ladder identified at least two oligosaccharides with 4 DP, two oligosaccharides with 6 DP and one oligosaccharide between 7 and 8 DP.

The oligosaccharide profile for PF3 showed at least eleven bands (Fig. 6.21, lane 3). Comparison of these bands with the glucose ladder identified bands in the lower range as well as in the higher range. These consisted of at least one band with 4 DP, two bands in between 4 and 5 DP, 2 bands in between 5 and 6 DP, 2 bands between 6 and 7 DP, one band similar to 7 DP, one band similar to 8 DP and one band similar to 9 DP.
Although direct quantification of oligosaccharide content in each band from the gel was not possible, image analysis was used to compare the relative concentration of oligosaccharides in individual bands (section 6.2.10.4).

The results of image analysis suggested that PF1 contained oligosaccharides equivalent to 4 and 7-8 glucose monomers at a higher concentration relative to oligosaccharides equivalent to 5 glucose monomers. PF3 on the other hand contained oligosaccharides equivalent to 4 and 5 glucose monomers at a lower concentration relative to oligosaccharides equivalent to 6 and 7 glucose monomers. The results from this analysis are shown in Fig. 6.22 and summarised in Table 6.3.
Fig 6.21 FACE profile of N-linked oligosaccharides released from polysaccharide fractions isolated from biofilm EPS samples

PF1 and PF3 samples were hydrolysed to release N-linked oligosaccharides using PNGaseF, tagged with fluorophore ANTS and separated using gel electrophoresis and visualised under UV illumination.

Lane 1: Glucose Ladder  
G3 = 3 Glucose units; G4 = 4 Glucose units etc...

Lane 2: Separated oligosaccharides of PF1 consisting of 7 distinct bands. DP values were measured using the distance of migration compared to Glucose ladder.

Lane 3: Separated oligosaccharides of PF3 consisting of 11 distinct bands. DP values were measured using the distance of migration compared to Glucose ladder.
**Fig 6.22**  FACE gel analysis of fluorescent bands of oligosaccharides shown in Fig 6.21 to determine relative concentration of oligosaccharide present in each band

The relative oligosaccharide concentration in each band was calculated using image analysis, by defining the bands as regions exhibiting >10% fluorescent intensity of the background.

Lane 2  PF1  
Lane 3  PF3  

+  Least concentration of oligosaccharides in band  
++++++  Highest concentration of oligosaccharides in band
Table 6.3  Relative concentration of various oligosaccharides found in EPS samples
isolated from the waste water treatment plant

<table>
<thead>
<tr>
<th>Length of Oligosaccharide</th>
<th>Relative concentration ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PF1</td>
</tr>
<tr>
<td>Glucose ⁴</td>
<td>++++</td>
</tr>
<tr>
<td>Glucose ⁴-5</td>
<td>+</td>
</tr>
<tr>
<td>Glucose ⁵</td>
<td>+</td>
</tr>
<tr>
<td>Glucose ⁵-6</td>
<td>+++</td>
</tr>
<tr>
<td>Glucose ⁶</td>
<td>++++</td>
</tr>
<tr>
<td>Glucose ⁷</td>
<td>NA</td>
</tr>
<tr>
<td>Glucose ⁷-8</td>
<td>++++</td>
</tr>
<tr>
<td>Glucose ⁸</td>
<td>NA</td>
</tr>
<tr>
<td>Glucose ⁹</td>
<td>NA</td>
</tr>
<tr>
<td>Glucose ¹⁰</td>
<td>NA</td>
</tr>
</tbody>
</table>

1 Length of oligosaccharide with reference to the standard ANTS labelled glucose ladder
The subscript refers to number of equivalent glucose monomer units
eg: Glucose ⁴ = 4 monomers of glucose

2 Relative concentration with reference to the band fluorescence intensity (pixel number)
+ Least concentration of oligosaccharides in band (based on fluorescence intensity)
++ ++ ++ Highest concentration of oligosaccharides in band (based on fluorescence intensity)

NA Not Applicable
6.4 DISCUSSION

The chemical characterization of the extracellular polymeric substances (EPS) of a biofilm community is an essential part of any study aimed at understanding the structure and function of this complex survival strategy of microorganisms. As microorganisms are embedded in EPS, Davey and O'Toole (2000) argued that a good understanding of the chemical nature of EPS was a key component in any biofilm study. EPS is thought to be mainly composed of polysaccharides. The approach described in this section of the study attempts to provide some information on the chemical nature of polysaccharide fractions of EPS.

Most analytical studies of biofilms had been conducted on biofilms developed under laboratory conditions. Although such studies provide valuable information, there are disadvantages in this approach as microorganisms do not behave the same way under natural conditions. The analytical results presented herein were from EPS isolated from biofilms developed in the wastewater treatment plant. Developing biofilms were collected at various stages to investigate any changes in chemical nature, primarily in the polysaccharide fraction of EPS, that occurred with time.

The amount of EPS recovered from biofilms collected over 7 days increased with time indicating growth and development of the biofilm. Many methods have been reported in literature for isolating EPS from biofilms but this study was conducted using method of Evans and Linker (1973). This method was found to be an effective way of recovering EPS from our biofilms compared with the other two methods trialled (Christensen et al., 1985; Read and Costerton, 1987) and was adequate for the purpose of the study. The yield of crude EPS varied from 2.51μg/cm² – 7.15μg/cm² of biofilm
increasing with age. Characklis and Cooksey (1983) reported that most biofilms contained an EPS fraction as high as 50-90% of the biofilm.

Preliminary experiments conducted to screen and detect any differences in the polysaccharide samples isolated from biofilms of various ages suggested possible differences in EPS1 from the other EPS samples. Infrared spectra of alkali treated PF samples showed that PF1 was significantly different in bonding and molecular vibrations from all the other PF samples. This suggested possible chemical and structural differences of PF1 compared to the other PF samples. There are reports in literature which distinguished between the polymers involved in initial adhesion and those involved in the subsequent colonisation (Fletcher and Floodgate, 1973; Leiriche et al., 2000) and this warrants further investigation.

Colorimetric screening assays demonstrated that the polysaccharide fractions of EPS samples contained hexoses, pentoses, uronic acids, sialic acids and hexosamines. These results are consistent with findings of Christensen and Characklis (1990), and Christensen (1989), who reported the presence of common sugars such as glucose, galactose, mannose, rhamnose, N-acetylg glucosamine, glucuronic acid in bacterial extracellular polysaccharides.

The detection of hexosamines confirmed that PF samples contained N-linked oligosaccharide chains attached to other macromolecules such as proteins. Similarly all EPS samples contained sialic acids suggesting that they also contained O-linked oligosaccharide chains. The sialic acid content of PF1 was significantly higher than all other PF samples (PF2, PF3, PF5 and PF7) suggesting possible structural differences in carbohydrate moiety, confirming preliminary results obtained from IR spectra. Hexosamine content varied largely among the PF samples suggesting possible differences in N linked chains.
It is possible that some of the free neutral sugars, amino sugars and sialic acids, if present in the EPS samples, could have been lost during extraction and gel filtration.

Quantification of proteins (using colorimetry) in PF samples resulted in detecting 3.0 - 11.1 µg/mg of proteins in PF samples, increasing with age of the biofilm. These results suggested that the isolated PF samples probably consisted of carbohydrate molecules attached to proteins.

The presence of other macromolecules attached to the carbohydrate moiety was well demonstrated using SDS PAGE and membrane blotting. All PF samples contained proteins attached to the carbohydrate moiety. PF1 isolated from biofilms collected after 1 day showed the presence of a polysaccharide attached to proteins with an approximate molecular weight of 25kDa. All the other PF samples contained proteins conjugated to polysaccharides with an approximate molecular weight of 60kDa. Silver staining of PF samples after SDS PAGE showed presence of proteins in a similar range of molecular weight to the isolated polysaccharide bands, thus providing strong evidence that the PF samples are glycoconjugated to protein moieties. Higgins and Novak (1997) reported the isolation of a 15,000Da polysaccharide, glycoconjugated to proteins from municipal, industrial and laboratory activated sludge. Further analysis of this polysaccharide revealed the presence of a single protein that demonstrated lectin-like activity.

Elucidation or even partial characterisation of chemical structure of macromolecules is not an easy task, and it is even more complicated with branched macromolecules. Considering the above and based on preliminary results which suggested that PF of 1 day old biofilms (PF1) was different from the other PF samples, it was decided to select PF1 and PF3 (PF isolated from 3 days old biofilm) for further structural analysis.
Analysis of PF1 and PF3 using HPAEC – PAD after mild hydrolysis for sialic acids showed that PF1 contained a larger quantity of N-acetyl neuraminic acid compared to PF3. However PF3 contained two different types of sialic acids one of which was identified as N-acetyl neuraminic acid, but the other was unidentifiable. These results suggest that PF1 had at least one type of O-linked oligosaccharide chains while PF3 sample contained more than one type of O-linked oligosaccharide chains.

Both PF1 and PF3 samples contained two aminosugars, galactosamine and glucosamine suggesting the presence of at least two different types of N-linked oligosaccharide chains. PF1 and PF3 also contained neutral sugars galactose, glucose and mannose possibly as component molecules attached to O and N-linked oligosaccharide chains or as free monosaccharides. Two (?) unidentified peaks were observed in the chromatogram for aminosugars of PF3 sample.

The FACE analysis of oligosaccharides released by hydrolysis resulted in at least 7 oligosaccharide bands in PF1 with length varying from G4 to G7 (equivalent to 4-7 glucose monomers). These oligosaccharides were present at varying concentrations based on intensity of fluorescence. On the other hand PF3 contained at least 11 bands, length varying from G4 to G10 (equivalent to 4-10 glucose monomers) in varying concentrations.

Subsequent to this, an attempt was made to further analyse the oligosaccharides by excising the fluorescent bands from the FACE gels and recovering the oligosaccharide bands separately in solution. The oligosaccharides were partially digested with a series of specific enzymes to release the monosaccharides sequentially and the resulting reaction mixture was analysed by HPAEC – PAD technique which had been successfully utilised previously in this work. However, it was not possible to obtain any information on monosaccharide composition of any oligosaccharide band by this method despite many
attempts, and this approach was therefore abandoned. The negative result could have been due to very low concentrations or loss of sugars during fluorescent tagging that made it difficult to recover monosaccharides from discrete bands.

6.5 CONCLUSIONS

Many analytical techniques were successfully used to gain an understanding of the chemical structure of polysaccharide fractions of EPS isolated from biofilms of different ages growing in a petrochemical wastewater treatment plant. High Pressure Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) and Fluorophore Assisted Carbohydrate Electrophoresis (FACE) were proven to be useful techniques in characterising the polysaccharide fraction of EPS, especially with limited amounts of sample.

The work presented here provides an insight into the chemical nature of the polysaccharide fractions of EPS isolated from biofilms of various stages growing in a petrochemical wastewater treatment plant.

Analysis of PF samples isolated from biofilms of various ages indicated that all PF samples analysed were conjugated to a protein moiety. PF samples isolated from 1 day old biofilms was different from the PF samples isolated from biofilms collected after 2, 3, 5 and 7 days. Although all PF samples contained O and N-linked oligosaccharide chains containing hexoses, pentoses, uronic acids, sialic acids and hexosamines, the PF1 sample differed significantly from the other PF samples. The infra red spectrum of PF1 was significantly different from all the other PF samples suggesting structural differences. The sialic acid content of PF1 was much higher than all the other PF samples collected from biofilms that were growing in the wastewater treatment plant for longer periods. A
conjugated polysaccharide of approximately 25kDa was identified in PF1 while the molecular weight of conjugated polysaccharides identified in other PF samples was about 60kDa.

FACE profiles of hydrolysed PF samples showed that PF1 contained at least 7 oligosaccharides while the PF3 contained a minimum of 11 oligosaccharides. Although direct comparison was not possible, the concentration of these oligosaccharides in PF samples varied and was reflected by the intensity of the discrete oligosaccharide bands.

In conclusion, the results of this section of the study has clearly demonstrated that the polysaccharide fraction of initial EPS of a biofilm is structurally different from polysaccharides of the EPS produced later during maturation of the biofilm. A summary of structural components of PF1 and PF3 gathered from our results are presented in Figure 6.23.

It is possible that the structural differences in the initial EPS are related to attachment. Studies conducted on biofilm polymers of a marine Pseudomonas species have suggested possible structural differences between initial adhesive polymers and biofilm matrix polymers (Fletcher et al., 1991). Moreover, it is also possible that different types of polymer may be active with different substrata and different environments.

It is worthwhile to note the possibility of having conjugated or nonconjugated polysaccharides in the PF samples that were not isolated by the methods employed in this study. Loss of polysaccharides could have occurred during extraction processes and during chemical reactions. It is also possible that the polysaccharides were too large to be separated by electrophoresis, and did not enter the gel at all and as such may not have been detected.
O-Linked Oligosaccharides

- Contained relatively high quantity of N-acetyl neuraminic Acid compared to PF3

Aminosugars
- Contained glucosamine and galactosamine

N-Linked Oligosaccharides

- Contained at least 7 oligosaccharides with varying lengths of 4-7 glucose monomers

- Contained a carbohydrate moiety conjugated to proteins of approximately 25kDa

Neutral sugars
- Contained free and or attached galactose, glucose and mannose

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O-Linked Oligosaccharides

- Contained relatively low quantity of N-acetyl neuraminic Acid compared to PF1
  - Two types of sialic acid were present

Aminosugars
- Contained glucosamine and galactosamine

N-Linked Oligosaccharides

- Contained at least 11 oligosaccharides with varying lengths of 4-10 glucose monomers

- Contained a carbohydrate moiety conjugated to proteins of approximately 60kDa

Neutral sugars
- Contained free and or attached galactose, glucose and mannose

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Figure 6.23 A summary of differences between PF1 and PF3 which can be concluded from this study
CHAPTER 7

CONCLUDING REMARKS AND FUTURE DIRECTIONS
7.1 SIGNIFICANCE OF THIS STUDY

As disciples of Koch and Pasteur, we have been taught to extrapolate from single-species laboratory cultures to predict bacterial behaviour in actual environments. Since bacteria can, in a strict sense, be diluted to a single cell and studied in liquid culture, this mode of operation has been frequently exploited and used to study bacterial activities. It has become increasingly clear that biofilms constitute a distinct growth phase of bacteria that is profoundly different from the planktonic growth phase studied so assiduously during the 15 decades following the discoveries of Louis Pasteur.

The ultimate goal and aim of this study was to take a holistic approach into developing biofilms in a petrochemical wastewater treatment plant to understand the extent of heterogeneous nature of this important biological niche. Data presented in this study is an analysis of biofilms developed in the activated sludge units of the Shell refinery over four years which demonstrated the changing nature of biofilms with respect to architecture, community structure, physicochemical properties of isolated bacterial cell surfaces and the structure of the matrix that changed over time. This was achieved by allowing biofilms to develop on immersed glass slides in the activated sludge units and removing the biofilms at known time intervals. This study was the first to describe the nature of biofilms growing in a petrochemical wastewater treatment plant in Australia and therefore the information is new.

The initial approach to this work involved selection of appropriate techniques to study developing biofilms in situ. Many conventional and emerging techniques were evaluated and Scanning Confocal Laser Microscopy (SCLM) was selected as a primary tool to visualise biofilms. Although the value of other techniques such as scanning electron microscopy (SEM) cannot be disregarded, SCLM method was superior, allowed visualisation of the internal structure of biofilms through non-destructive optical
sectioning. This study also utilised a "chamber" prepared using glass slides to observe biofilms under the microscope. This simple method allowed observation of biofilms still attached to the surface without disturbing their structure whilst minimising dehydration. This proved to be a convenient method to study hydrated biofilms in situ and was used for observation of samples under SCLM. Using SCLM techniques in conjunction with fluorescent staining, the presence of channels within the biofilm was demonstrated which suggested a porous type of architecture that developed over time in an organised manner and not by chance. Image analysis of SCLM images demonstrated that a mature biofilm was not densely packed but contained 53.84% void areas confirming the porous nature of the biofilm.

Different bacterial species were identified from biofilm samples collected at different times suggesting a possible succession of bacterial colonization within the biofilm. Further, it was not possible to isolate the planktonic wastewater bacterial species Sphingobacterium multivorum from the biofilm suggesting that this organism did not colonise the biofilm. Both these observations require further investigation using molecular approaches.

Limitations associated with cultural techniques in enumeration of populations were also highlighted in this study. The culturable bacterial count was shown to be 2.3 – 24% of the total bacterial count of the biofilm.

It was also proved that selective culture techniques as well as 16S rRNA methods can be employed to detect B. cereus in biofilms growing in the wastewater treatment plant. It was suspected that B. cereus was one of the initial colonisers of these biofilms, however, investigations conducted on early biofilms using Bacillus probe RDR 502 proved otherwise. Although the biofilm was colonised with bacteria, B. cereus was not detected in early 4h and 8h old biofilms suggesting that other bacterial species colonised
the biofilm before the colonisation by *B. cereus* occurred. The use of FISH with 16S rRNA probes in conjunction with SCLM proved to be a valuable technique in obtaining information about this complex community structure.

Analysis of cell surface hydrophobicity of isolates showed that the biofilm contained microorganisms having varying degrees of cell surface hydrophobicity and that their appearance in the biofilm at a particular stage of development appeared unrelated to their cell surface hydrophobicity. Furthermore, pure culture studies of the isolates showed that most bacteria harvested from single species biofilms at early stages of development demonstrated relatively high cell surface hydrophobicity, but this characteristic was species-dependent and declined as the biofilm matured with the exception of a few.

Presence of free or conjugated hexoses, pentoses, hexosamines, sialic acids and uronic acids was demonstrated in all polysaccharide fractions isolated from EPS samples. Further, it was demonstrated that all polysaccharide fractions isolated from EPS samples were conjugated to proteins. However, detailed analysis of polysaccharide fractions suggested significant differences between initial EPS and EPS produced later during maturation of the biofilm. A polysaccharide fraction with a molecular weight of 25 kDa was present in EPS of early 1 day old biofilms while polysaccharide fractions isolated from late biofilms had a molecular weight of 60 kDa. Structural differences in N-linked and O-linked oligosaccharides of PF1 and PF3 were also demonstrated. Experiments were conducted to sequence N-linked oligosaccharides of PF1 and PF3 using FACE techniques, however was unsuccessful, and this needs further investigation.

In summary this study has demonstrated the heterogeneous nature of naturally developing biofilms in a petrochemical wastewater treatment plant with respect to architecture, community structure and the matrix changing with time. A summary of
findings is presented in Figure 7.1. Overall this study has contributed towards increasing the understanding and knowledge of changing nature of developing biofilms with time.

7.2 FUTURE RESEARCH

Many possible areas of future research arise from this study.

Community analysis using molecular approaches demonstrating the presence as well as location of biofilm microbial flora will further increase the knowledge of this important biological niche. Investigating the distribution of microcolonies within the biofilm using FISH approaches may provide information on juxtaposition of these microcolonies to their advantage.

The absence of some planktonic wastewater bacterial species in the biofilm may have occurred due to cellular level or community level interactions and needs further investigation. Detailed analysis of initial attachment studies and cell surface characteristics of these species may yield information at attachment level whilst studying community interactions such as antagonistic effects may yield information at a community level. These studies may be extended to investigate bacterial communication via quorum sensing which is essential for controlling dynamic biofilm structures.

Further studies on the nature and properties of isolated genera and their association with the removal of waste compounds from the wastewater will add value to understand the involvement of the isolates in the treatment process.

Analysis of the biofilm matrix to determine variations in hydrophobicity within the biofilm may also yield valuable information regarding colonisation patterns that may be species-dependent. Investigations using microelectrodes and in situ approaches in this area may provide invaluable information.
Further work is required in EPS characterisation and can be expanded to include analysis of O-linked oligosaccharides using fluorescent markers and in situ techniques. Sequencing N-linked and O-linked oligosaccharide profiles will yield information on detailed structure of EPS. It has been suggested that production of initial EPS is related to attachment, and based on the results of this study, further characterisation of initial EPS and attachment studies may provide valuable information on EPS mediated initial colonisation events.
**Architecture**
- Thickness increased with time
- 53.84% of the mature biofilm was uncovered, and free of cells, EPS and matrix

**Biofilm community**
Number and Colonisation pattern

**Cell surface hydrophobicity** (CSH)

**Structure of polysaccharide fraction of EPS**
- Initial PF was found to be different to late PF
- All PF fractions were conjugated to protein
- PF1 differed from PF3 in molecular weight, silicic acid content and hexosamine content
- N-linked oligosaccharides were different in PF1 and PF3 in number of glucose monomers and concentrations

- EPS was involved during initial attachment and cell aggregation forming the structure
- A porous internal structure with channels developed over time
- Biofilm bacterial number increased with time
- Culturable bacterial count varied from 2.3-24% of total count
- A succession of bacterial colonisation was identified over time
- *B. cereus*, suspected to be an initial coloniser, did not colonise the biofilm before 24h
- Some resident wastewater microflora were not detected in the biofilm
- CSH of isolates was variable and no correlation of CSH and the age of biofilm when isolated was seen
- In pure culture biofilms CSH of isolates increased with biofilm age with few exceptions

**Figure 7.1** Heterogeneous nature of developing biofilms as demonstrated in this study
“So she went on, wondering more and more each step, as everything turned into a tree the moment she came up to it, and she quite expected the egg to do the same”

Lewis Carroll: *Through the Looking Glass and What Alice Found There*


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