<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Materials Analysis of Bacterial Adhesion and Early-Stage Biofilm Development</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Authors(s)</strong></td>
<td>Allen, Ashley</td>
</tr>
<tr>
<td><strong>Publication date</strong></td>
<td>2017</td>
</tr>
<tr>
<td><strong>Publisher</strong></td>
<td>University College Dublin. School of Chemical and Bioprocess Engineering</td>
</tr>
<tr>
<td><strong>Link to online version</strong></td>
<td><a href="http://dissertations.umi.com/ucd:10174">http://dissertations.umi.com/ucd:10174</a></td>
</tr>
<tr>
<td><strong>Item record/more information</strong></td>
<td><a href="http://hdl.handle.net/10197/9536">http://hdl.handle.net/10197/9536</a></td>
</tr>
</tbody>
</table>
Materials Analysis of Bacterial Adhesion and Early-Stage Biofilm Development

Influence of Surface Topography, Nutrient Concentration and Shear Stress

Ashley Allen
09137025

Principle Supervisor: Professor Eoin Casey
School of Chemical & Bioprocess Engineering
University College Dublin
Ireland

This thesis is submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy.

Doctoral Studies Panel:
Professor Brian Glennon
Professor Susan McDonnell

March 2017
Table of Contents

Chapter 1

*General introduction to the observation and analysis of bacterial adhesion and biofilm development to surfaces*

1.1 Introduction......................................................................................................................1

1.1.1. Water Filtration........................................................................................................1

1.1.2. Biofouling ..................................................................................................................2

1.1.3. Processes of Biofouling ...........................................................................................3

1.2. Bacterial Adhesion onto Surfaces ................................................................................5

1.2.1. Surface Topography ...............................................................................................6

1.2.2. Membrane and Bacterial Interaction ......................................................................9

1.3. Biofilm Development and Structure ............................................................................9

1.3.1. Nutrient Concentration .........................................................................................10

1.3.2. Hydrodynamic Conditions ......................................................................................11

1.3.2. Biofilm Stress Response .......................................................................................12

1.4. Scope of Thesis ............................................................................................................13

Chapter 2

*Quantification of initial bacterial adhesion into Nanofiltration and reverse osmosis surface topographical heterogeneities and surface analysis*

Abstract ...............................................................................................................................15

2.1. Introduction ................................................................................................................16

2.2. Method .......................................................................................................................17

2.2.1. Bacterial Strains, Culture Conditions and Preparation .................................................17

2.2.2. Microbial Adhesion to Solvents ............................................................................19

2.2.3. Zeta Potential .........................................................................................................20

2.2.4. Dynamic Initial Adhesion Assays in the Absence of Pressure ..................................20

2.2.5. Dynamic Adhesion Assays under Permeate Flux Conditions ...............................22
Analysis of vivo and in-vitro biofilm structure with varying nutrient and shear conditions

Abstract .......................................................................................................................................40

3.1 Introduction ...............................................................................................................................41

3.2 Methods ......................................................................................................................................42
  3.2.1 Bacterial Strains, Cultural Conditions and Preparation .........................................................42
  3.2.2 Air-Liquid Interface Biofilm Growth .........................................................................................42
  3.2.3 Air-Liquid Interface Tube Biofilm Staining ..............................................................................44
  3.2.4 Dynamic Biofilm Growth .........................................................................................................44
  3.2.5 Confocal Laser Scanning Microscopy .......................................................................................46
  3.2.6 Statistical Analysis ....................................................................................................................47

3.3 Results and Discussion ................................................................................................................47
  3.3.1 Extracellular Polymeric Substance and Bacterial Staining of Air-Liquid Interface Biofilm Growth .........................................................................................................................47
  3.3.2 Dynamic Biofilm Growth .........................................................................................................53

3.4 Conclusion ..................................................................................................................................59
Chapter 4

Quantitative Atomic Force Microscopy analysis of biofilm formation with differing nutrient factors

Abstract .................................................................................................................. 60

4.1 Introduction ..................................................................................................... 61

4.2 Methods ........................................................................................................... 62
  4.2.1 Bacterial Preparation ................................................................................. 62
  4.2.2 Air-Liquid Interface Biofilm ....................................................................... 63
  4.2.3 Cantilever Materials and Preparation .......................................................... 64
  4.2.4 Atomic Force Microscopy and Nanoindentation .......................................... 65
  4.2.5 Confocal Laser Scanning Microscopy .......................................................... 69
  4.2.6 Scanning Electron Microscopy .................................................................... 70

4.3 Results and Discussion ................................................................................... 71
  4.3.1 Colloid Cantilever Evaluation and Maintenance ......................................... 71
  4.3.2 Biofilm Nanoindentation ............................................................................ 73
  4.3.3 Scanning Electron Microscopy .................................................................... 79

4.4 Conclusion ...................................................................................................... 80

Chapter 5

Conclusion and Future Work

5.1 Surface Topographical Heterogeneities and Initial Bacterial Adhesion .......... 82

5.2 Nutrient Load and Shear Stress ..................................................................... 84

5.3 Biofilm Nanoindentation ............................................................................... 85

5.4 Final Thoughts ................................................................................................. 86

A. Nomenclature .................................................................................................. 88

A.1 List of Abbreviations ...................................................................................... 88

A.2 List of Symbols ............................................................................................... 88

References ............................................................................................................. 90
Acknowledgements

I would like to thank my parents: for their constant support and patience throughout my college years that have culminated with this thesis, for always listening to my problems and helping me to figure them out. I also would like to thank my brother Stephen for the long conversations about anything and everything.

I am grateful to my supervisor Professor. Eoin Casey for his support and guidance throughout this whole endeavour, to the members of the BioFilm group, to Dr Olivier Habimana who mentored me during the project and taught me valuable lab techniques, to Dr Rory Heffernan for his assistance with the MFS system, to Dr. Ashkan Safari for showing me the AFM, to Huayu Cao and his wife Daisy for being my guide on a conference to China, to Giulio Gazzola and Michela Zanoni for the coffee and constant lab music and to Fatima Kavousi for the conversations. Moreover, I would like to thank Mr. Pat O’Halloran for his technical assistance and Ms Aoife Carney for always providing assistance when I needed it.

Thank you to Ines Rauch, the infamous Queen of Mischief, and Daniela Müller for their invaluable lunch breaks and early morning Archery sessions. It was never a dull moment. Finally, I would like to thank my future husband Eoghan who has always been there for me through the best and the worst: I would be completely lost without you.
Materials Analysis of Bacterial Adhesion and Early-Stage Biofilm Development

Influence of Surface Topography, Nutrient Concentration and Shear Stress

Abstract

Bacterial adhesion and the subsequent biofilm formation is a complex phenomenon which has many consequences in water filtration. This aggregation of microorganisms can be difficult to remove from nanofiltration and reverse osmosis membrane surfaces, causing damage and eventual replacement of the membrane. In order to elucidate the cause of this biofilm formation, three influential factors were studied: surface topography, nutrient concentration and shear stress. Analysis was performed on the surface topographical heterogeneities in order to examine the influence of surface topography. Image analysis of the adhesion of *Pseudomonas fluorescens* (*Ps. fluorescens*) and *Staphylococcus epidermidis* (*S. epidermidis*) to the surface topographical heterogeneities was determined for two commercial membranes, NF270 and BW30, using a flow-cell system. Membrane area analysis, using AFM and SEM, showed up to 13% of topographical heterogeneities on the membrane surface with up to 30% of total adhered cells that were discovered within these topographical heterogeneities. For the analysis of the nutrient availability and shear stress on the structural formation of *Ps. fluorescens* biofilm under two different dynamic conditions, an air-liquid interface biofilm and a flow cell grown biofilm were assessed by confocal scanning laser microscopy (CLSM). The analysis showed a three-fold increase in the EPS biovolume of the high nutrient air-liquid interface grown biofilm. However, the flow cell biofilm increased the biovolume for low nutrient and higher shear stress conditions, suggesting harsher growth conditions of the biofilm results in greater biofilm development. Finally, the adhesive and viscoelastic properties of the *Ps. fluorescens* air-interface grown biofilm for two different nutrient dilution factors was determined by nanoindentation. The low nutrient availability showed higher adhesion force and work of adhesion with distributed colonies across the surface, while the high nutrient grown biofilm led to a reduction in the adhesive and elastic nature of the biofilm.
List of Publications

Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Signature:
List of Tables

Table 1: Affinity of Ps. fluorescens and S. epidermidis suspended in RW-C for the four solvents used in the MATS analysis.................................................................25
Table 2: Mean Roughness, Contact Angle and Zeta Potential measurements for BW30 and NF270. .................................................................................................28
Table 3: Surface topographical heterogeneities size characterisation for BW30 and NF270 membranes. .................................................................30
Table 4: Estimated maximum cell loading and deposition rate of Ps. fluorescens and S. epidermidis on NF270 and BW30 under 22.2 mL min\(^{-1}\) and 66.6 mL min\(^{-1}\) volumetric flow rate conditions. .................................................................31
Table 5: Biovolume, substratum coverage, mean thickness and biofilm roughness of 24-hour Ps. fluorescens biofilm grown at the air-liquid interface with low nutrients and high nutrients .................................................................................48
Table 6: Adhesion Force and Work of Adhesion 24-hour Ps. fluorescens biofilm with low nutrients and high nutrients. Error is represented using Standard Error of the Mean............................................................................................................78
List of Figures

Figure 1: SEM image of biofilm forming on nanofiltration membrane........................................3
Figure 2: Biofilm life cycle .............................................................................................................4
Figure 3: Surface topography showing a sample arithmetic average height ..................7
Figure 4: MFS cross flow system..................................................................................................23
Figure 5: Zeta potential of *Ps. fluorescens* and *S. epidermidis* at pH values of 3, 7 and 9 .................................................................26
Figure 6: AFM images of NF20 and BW30 membranes ..........................................................29
Figure 7: Observed adhered *Ps. fluorescens* and *S. epidermidis* cells on NF270 and BW30 membranes following 30 minutes’ adhesion experiments.................................32
Figure 8: Surface area coverage (%) of *Ps. fluorescens* and *S. epidermidis* cells per cm² BW30 and NF270 membrane defect area and the fraction of bacterial adhesion in defect areas. .................................................................34
Figure 9: Representative SEM micrographs depicting the presence of bacterial cells within membrane surface topographical heterogeneities........................................36
Figure 10: Population distribution of the mean number of adhered *Ps. fluorescens* cells, based on the angle at which they adhere on the membrane in relation to the direction of the flow. ..................................................................................38
Figure 11: *Ps. fluorescens* biofilm grown at an air-liquid interface. Biofilms were grown on the coverslip at the interface between the air and King B. ............43
Figure 12: Flow Cell System......................................................................................................45
Figure 13: Flow cell in custom made holder...............................................................................46
Figure 14: CLSM imaging of high nutrient *Ps. fluorescens* biofilm with bacteria stained with Syto 9 and EPS stained with Con A. .........................................................46
Figure 15: Distribution profile of biofilm surface coverage at 1µm biofilm depth increments for four 24-hour *Ps. fluorescens* biofilms with high and low nutrient factors ..............................................................51
Figure 16: Biovolume, substratum coverage, mean thickness and biofilm roughness of 48-hour *Ps. fluorescens* biofilm grown in flow cell with low nutrients (green) and high nutrients (red), two different flow rates of 0.4mLmin⁻¹ (low flow rate) and 0.7mLmin⁻¹ (high flow rate)...............................................................53
Figure 17: Representative CLSM images of high nutrient and low nutrient *Ps. fluorescens* biofilms at high and low flow rate. .................................................................56
Figure 18: Distribution profile of biofilm surface coverage at 1µm biofilm depth increments for four 24-hour *Ps. fluorescens* biofilms with high and low nutrient factors ..........................................................57

Figure 19: Air-liquid interface 24-hour grown biofilm. The fluorescent image was taken using a fluorescent microscope ..............................................................63

Figure 20: Glass slide set-up for colloid and glue application.................................64

Figure 21: Sample force curve measured for 24-hour air-liquid interface biofilm....67

Figure 22: Force vs Distance curve for 10µm silica colloid cantilever on glass in PBS.................................................................................................................71

Figure 23: SEM images of glue contaminated colloid cantilever, no colloid and damaged colloid ..................................................................................................73

Figure 24: Elasticity profiles of selected force-indentation profiles of high nutrient, low nutrient and glass calibration curve.........................................................74

Figure 25: Histogram of the Young’s Modulus (kPa) distribution of 24-hour grown *Ps. fluorescens* biofilm at low and high nutrients ...........................................76

Figure 26: SEM images of 24-hour *Ps. fluorescens* biofilm grown by air-liquid interface.............................................................................................................80
CHAPTER 1

INTRODUCTION

_General introduction to the observation and analysis of bacterial adhesion and biofilm development to surfaces._

1.1 Introduction

1.1.1. Water filtration

Water is an important resource that is required for drinking and sanitation. While a large portion of the Earth’s surface is covered in water, the shortcoming in the availability of clean potable water is the cause of millions of deaths worldwide annually. Many of these deaths are mainly caused by waterborne diseases and contaminants. Billions of people in the developing world have inadequate access to clean drinking water and little or no sanitation (Montgomery _et al._, 2007; Shannon _et al._, 2008). Due to the advancement of developing and industrialized nations, there is an increasing stress on traditional water sources. This growth also indirectly introduces many man-made pollutants into the environment, such as pharmaceuticals, veterinary drugs and cosmetics. The major challenge to remove this large number of contaminants (Schwarzenbach _et al._, 2006) is cost since different appropriate water treatments are required to remove contaminants of various sizes. This introduces costs to developing areas that are unable to afford it.

Another aspect to the strain on water systems is the continuing growth of the world’s population; as the population grows, an increasing demand leads to an expanding requirement for the safe re-use of wastewater. The requirements of filtering water can be influenced largely by location; for instance, arid areas close to the sea may depend upon desalination to provide a fresh water supply (El Aleem _et al._, 1998) Other water supplies, such as ground-water and surface water, are also highly depended upon and, therefore, different filtration processes have developed
over time to remove contaminants such as organic and inorganic particulates and improve water safety.

Two increasingly prominent methods of water filtration are the Nanofiltration (NF) and Reverse Osmosis (RO) processes, which are used in the removal of small particulates including man-made particles previously mentioned (Radjenović et al., 2008). Additionally, RO membranes are used in the removal of salt from seawater to produce drinkable water which can provide an alternative water source to countries with little or no fresh water supplies (Cyna et al., 2002; Lee et al., 2011; Tortajada, 2006). Unlike certain filtration techniques that require changes in temperature and subsequently use large amounts of energy, NF and RO are less energy dependent. Continuous improvement of these filtration techniques due to material or process changes have led to the reduction or recovery of energy. This, in turn, reduces production costs and presents a cheaper alternative source of clean potable water from recycled wastewater (Lee et al., 2011). Nonetheless, a major issue with water filtration is water sources containing bacteria and other organic matter. The accumulation of bacteria on a filtration membrane is a common occurrence in water filtration, and can result in bacterial propagation and formation into a biofilm. Biofilm on the filter’s surface reduces the filter’s efficiency and introduces costly cleaning procedures.

1.1.2. Biofouling

Biofouling is the aggregation of microorganisms which form communities on biotic and abiotic interfaces. These bacterial cells attach to the surface, proliferate and the aggregation of living and dead cells are embedded in a protective matrix. This protective matrix consists of extracellular polymeric substance (EPS) surrounding the bacteria and comprises of polysaccharides, proteins, DNA, nucleic acids, enzymes, lipopolysaccharides and phospholipids among other substances (Flemming et al., 2010). Due to its EPS matrix, the biofilm is sticky in nature and causes a reduction in permeate flux, increased pressure loss and a loss of retention of water filtration membranes (Herzberg et al., 2007; Semiao et al., 2014; Vrouwenvelder et al., 2009). The biofilm matrix can be difficult to remove from membrane surfaces. An example of biofilm formation on a nanofiltration membrane surface is shown
below in Figure 1. Harsh chemical treatments of membrane surfaces that are required to eliminate the biofilm often result in damage to the membrane surface and prevent a full recovery of membrane flux and retention. This, in turn, can lead to an eventual replacement of the membrane which then increases the use of chemicals to treat this problem and leads, as a result, to increased operational costs.

![SEM image of biofilm forming on nanofiltration membrane](image)

Figure 1: SEM image of biofilm forming on nanofiltration membrane

The formation of bacterial micro colonies onto these membrane surfaces during water filtration is a common occurrence and has a significant effect on the performance of membranes during water treatment. In order to combat this, intensive research into possible modifications or methods to protect membranes from biofouling have focused on changing membrane properties. Despite this, no successful techniques that fully prevent biofouling have been discovered.

1.1.3. Processes of Biofouling

Before the adhesion of the bacteria to a submerged surface, a conditioning film forms by organic materials from the water and coats the surface. This coating may alter the physicochemical surface properties and provide favourable adhesive sites for bacterial adhesion (Hwang et al., 2012; Lorite et al., 2011). Research into the initial bacterial adhesion to prevent or diminish bacterial biofilm formation are an
important and continuing subject. The developmental life cycle of a biofilm is described as a five-stage cycle, as depicted in Figure 2.

Figure 2: Biofilm life cycle (Cunningham et al., 2008)

The first stage of the biofilm life cycle is the initial attachment of bacteria to the substrate, where fresh nutrients are readily available and the ideal surface roughness and surface hydrophilicity conditions are met. Wang et al. determined that initial bacterial adhesion was influenced by shear stress, and that increases of adhesion occurred during prolonged times (Wang et al., 2013). Another environmental factor, (i.e.) surface topography, was analysed by Whitehead et al. (Whitehead et al., 2006b), and they postulated that certain feature dimensions of a surface may trap the bacterium. They continued that this occurs only when the features are larger than the bacteria dimension. As biofilm communities can form from a single species or multiple species of cells, various different bacterial dimensions may attach to surface features large enough to retain them. Other factors in initial biofilm development are often a result of environmental factors, such as the temperature, pH and surface properties. For the biofilm to continue its development, these environmental factors as well as bacterial properties must be considered.

The continued attachment and aggregation of these bacterial communities form areas of high density cells. At this second stage, the bacteria become more firmly adhered to the substrate, using the production of a sticky EPS, cover the surface and result in a biofilm (Flemming & Wingender, 2010; Xavier et al., 2007). As discussed in a review by Flemming (Flemming, 2002), bacteria transform
dissolved organic matter within water systems into biomass. The nutrient rich water assists in the growth and proliferation of the attached bacteria (Vanysacker et al., 2014). While bacteria are continuously supplied by nutrients from the raw water supply, biofilm control becomes virtually impossible. As the biofilm continues to grow, it enters the third stage of the biofilm life cycle.

The third stage is the initial development of an EPS matrix with channels that allow for fluid and nutrient transport throughout the matrix. As the biofilm grows thicker, the bacteria deposit onto each other, and potentially become starved due to a lack of availability of nutrients. In order to inhibit this, the biofilm forms the EPS matrix. This protects the biofilm from any external influences, such as biocides, which may be introduced to potentially reduce bacterial growth within the system (Ridgway et al., 1996). This has resulted in a tolerance of biofilms to biocides.

Once the initial development is complete, the biofilm then proceeds to the fourth stage of its life cycle. During this stage, the biofilm’s structural architecture continues to develop and the biofilm forms channels allowing for liquid transport throughout the biofilm structure. Wilking et al. observed channels in *Bacillus subtilis* biofilm, and they noted that, as the biofilm matures, the channels evolve due to bacterial growth on the channel floor (Wilking et al., 2013). These channels provide nutrients, while protecting the biofilm. However, limited nutrients and stress induced by inhospitable conditions may influence the development of these channels as well as the further biofilm architectural development.

Upon increasing in size, the biofilm reaches the final stage in its life cycle, which is the dispersion of single cells from the fully formed biofilm. This can occur due to depletion of nutrients in the area, causing further biofilm development in other areas with fresh nutrients (Costerton et al., 1995; O’Toole et al., 2000; Sauer et al., 2002).

1.2. Bacterial Adhesion onto Surfaces

Due to biofilm growth on surfaces and its subsequent difficulty in removal, greater research is required into biofilm development. Bacteria attach to surfaces in diverse environmental conditions; therefore, studies in biofilm formation further assist in the development of strategies for sustaining submerged surfaces. This can be applied in
using novel cleaning techniques and creating antifouling surfaces. Many studies have developed NF and RO membranes with silver nanoparticles, which inhibit the attachment and growth of bacteria attached onto surfaces but issues due to leaching persist (Ben-Sasson et al., 2014; Liu et al., 2013). From the analysis of various surfaces, properties such as roughness, surface charge and hydrophobicity have been identified as parameters that affect the attachment of bacteria (An et al., 1998; Boussu et al., 2005; Myint et al., 2010), in addition to the response of bacterial cell wall physico-chemical properties and structure (Bos et al., 1999). Studies regarding adhesion of bacteria due to cell size on various surfaces have shown that the dimensions of cells could influence the preference of the adhesion site of cells (Friedlander et al., 2013; Myint et al., 2010; Whitehead et al., 2005; Whitehead et al., 2006a; Whitehead & Verran, 2006b). Medilanski et al. (Medilanski et al., 2002) investigated cells of various sizes on stainless steel by creating defects on the surfaces, and they noted that the cell size influences the site of adhesion. The preference of the cells’ adhesion site due to specific sites was also studied by Díaz et al. (Diaz et al., 2010), who created nanostructures on various substrates (gold, PBCA, Cu) to examine the areas of adhesion of Ps. fluorescens, which adhered in the surface patterns when the sizing was similar to the cells’ dimensions. These interactions between surface and bacteria influence an important process in the formation of biofilms; however, other environmental factors, such as pH, temperature, chemicals, nutrient availability and shear force, must also be considered. Two such properties, which are the focus of this study, are surface topography and nutrient concentration.

1.2.1. Surface Topography

Surface properties is an important parameter in biofilm formation and is investigated as a contributing factor in the adhesion of cells onto surfaces. Surface topography is a property of a surface which is considered an important factor in cell adhesion; it is a parameter used to describe shapes and features of a surface. One of the main parameters used to describe this is surface roughness. Surface roughness, as calculated by the Gwyddion software used in measurements, is determined as the
mean line over one sampling length as shown in equation 1.1 and Figure 3 (Klapetek et al., 2004):

\[ R_a = \frac{1}{N} \sum_{j=1}^{N} |r_j| \]  \hspace{1cm} [1.1]

where N is the number of samples and r is the roughness value.

![Image of surface topography showing a sample arithmetic average height (Ra)](Gadelmawla et al., 2002)

However, the arithmetic average height or the average roughness value are not capable of detecting small changes in the surface topography: the larger the topographical peaks and troughs, the less accurate the measurement. In order to provide a more accurate roughness profile, the roughness root mean squared is used; this parameter allows for a profile that is more sensitive to the large peaks and troughs that deviate from the mean line (Klapetek et al., 2004):

\[ R_{ms} = \sqrt{\frac{1}{N} \sum_{j=1}^{N} r_j^2} \]  \hspace{1cm} [1.2]

This is the measure of the real surface geometry of an area and is used to measure the nanoscale peaks and depressions on the surface of NF and RO membranes (Boussu et al., 2005; Boussu et al., 2006; Johnson et al., 2012). Nanoscale heterogeneities on substrate surfaces present supportive sites for the deposition and accumulation of bacteria (Myint et al., 2010; Subramani et al., 2008).
and other various particulates, such as colloids, organic matter and inorganic matter (Hobbs *et al.*, 2006; Kang *et al.*, 2011; Ramon *et al.*, 2013; Ramon *et al.*, 2012). This colloidal and matter accumulation on the membrane surface can cause further changes in adhesion properties within fouling layers, changing surface roughness and other properties. Studies by Li *et al.*, reviewing cell adhesion to glass surfaces, have shown that various bacterial species tend towards the rougher surfaces (Li *et al.*, 2004a); however, other factors, such as hydrophobicity, must also be considered.

The presence of surface irregularities may result in the misrepresentation of surface roughness measurements of the membrane surface; these surface irregularities can be in the magnitude of several micrometres in width and depth (Boyd *et al.*, 2002). A study conducted by Ghayeni *et al.* noted that “small scale irregularities of 0.3µm” were observed on NF membrane NF45 (Ghayeni *et al.*, 1998); however, further studies on these irregularities or defects on membrane surfaces are required. As the surface roughness is measured on the scale of nanometres, the irregularities are found to be larger (Boussu *et al.*, 2005; Semiao *et al.*, 2013).

Studies in bacterial adhesion and irregularity or defect size on different substrates have shown that bacteria shape is an important factor when considering adhesion within irregularities (Boyd *et al.*, 2002; Hou *et al.*, 2011; Taylor *et al.*, 1998; Whitehead *et al.*, 2005). Different shapes and sizes of modified substrates, replicating the depth and width of membrane surface irregularities, show a preference for bacteria to adhere within valleys of micro structured topographies. This was seen by Hou *et al.* (Hou *et al.*, 2011) for *E. coli* on structured PDMS that adhered within structures of 5µm spacing. Another factor to consider is the areas of low shear stress provided by the irregularities found within the NF and RO (Busscher *et al.*, 2006; Wang *et al.*, 2013). These have been shown to reduce bacterial adhesion in certain conditions in areas of high shear stress (Won *et al.*, 2014). This suggests a promotion of bacterial attachment in the surface irregularities.

Additionally, surface irregularities may cause a change in the localised shear stress along the surface. Scheuerman *et al.* noted that the surface topographies, similar to those seen in Chapter 2, are shielding the cells from harsh hydrodynamic shear forces (Scheuerman *et al.*, 1998). Hydrodynamic flow profiles can be calculated using Computational Flow Dynamics (CFD) and are utilised to predict the bacterial attachment in these areas due to the change in topography. However, these
changes in topographies were mainly calculated for the introduction of feed spacers and used to separate membrane sheets. These studies noted that the spacers changed the flow pattern in the feed channel (Radu et al. 2014; Ngene et al. 2010). As the surface irregularities on the membrane surface are larger than the surface topography roughness, they may have a similar impact on the flow pattern and introduce a sheltered area from shear stress.

1.2.2 Membrane Surface and Bacterial Interactions

The interaction of the membrane and bacteria due to chemical interactions is a factor to consider when determining the adhesion of bacteria. In order to elucidate the adhesion of particles onto a surface, the DLVO theory was created by Derjaguin, Landau, Verwey and Overbeek. This is a theoretical model utilised in the calculation of the adhesive energy involved in bacterial or colloidal adhesion, depending on the distance of the bacteria or colloid from the interacting surface. However, this model assumes that the surface and bacteria are chemically inert and a more accurate model was introduced by Van Oss et al. This extended DLVO theory or XDLVO theory incorporates the short-range Lewis acid-base interactions, this included the electron/donor interactions, the hydrophobic/hydrophilic properties of both the bacteria or colloid and the surface (Van Oss et al., 1986). As the hydrophobicity of bacteria and the surface may have an impact on the bacterial adhesion, Verwey et al. determined that under shear conditions a maximum shear stress can be obtained that overcomes the XDLVO theory and this shear is the maximum required to detach cells (Derjaguin, 1941; Verwey et al., 1999). Therefore, while the adhesion energy calculated in the XDLVO is an important factor to consider, it may have less of an impact on cell adhesion at high shear.

1.3. Biofilm Development and Structure

The morphology of the biofilm under different stressors, such as nutrient concentration, hydrodynamic conditions were observed to influence the biofilm
structure. The biofilm architecture can be assessed by its biovolume, substratum coverage, thickness and roughness, to name a few. This can determine the type of biofilm morphology, such as smooth or flat, rough and filamentous or fluffy, and can depend on EPS production (Flemming & Wingender, 2010). As the EPS consists of a matrix of pores and channels to provide nutrients and protection to the enclosed bacteria, this structure is determined by the environment in which the biofilm is grown. EPS is a viscoelastic, sticky framework to maintain the biofilm’s adhesion to the substrate surface and its presence can be determined by quantification of the adhesive force and elasticity of the biofilm (Beaussart et al., 2014; Touhami et al., 2003).

Different analysis methods have been developed to qualitatively and quantitatively examine biofilm formation, each of which can be limited by the method of biofilm growth. Confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM) allow for in-vitro analysis. Whereas scanning electron microscopy (SEM) requires sample dehydration, which may damage the biofilm structure, it can nevertheless give detailed imaging of biofilm formation on substrates. These techniques are used depending on the method in which the biofilm is grown.

1.3.1. Nutrient Concentration

Nutrients are an important factor in the growth of bacteria. During the biofilm formation, the transfer of nutrients to the biofilm under dynamic conditions has shown an increase in the rate of biofilm’s growth (Moreira et al., 2015; Peyton, 1996). This can be due to the flow of the medium which can provide the biofilm with nutrients and oxygen. With a decrease in the flow rate, this can cause a reduction in the nutrient load, and, subsequently, the biofilm since the bacteria are starved and form tightly packed micro colonies. However, it was observed that, under high shear stress, two different nutrient loads may produce the same biofilm (Teodósio et al., 2011). Nutrient concentration has indicated a change in the biofilm architecture, which may be influenced by the hydrodynamic conditions since slow regimes may not provide the biofilm with the same quantity of nutrients over time when compared to faster flow regimes. The combination of both environmental factors, i.e.
hydrodynamic conditions and nutrient concentration, may result in a different biofilm development (Stoodley et al., 1997). Stoodley et al. observed an increase in biofilm thickness and surface coverage when there is an increase in the nutrients’ carbon and nitrogen for four gram negative bacteria: Pseudomonas aeruginosa, Pseudomonas fluorescens, Klebsiella pneumoniae and Stenotrophomonas maltophilia (Stoodley et al., 1998).

The biofilm may have different spatial and growth rates, resulting in cells obtaining differing quantities of nutrients. The orientation of cells within the biofilm may influence the concentration of any nutrient consumed, with the nutrient concentration decreasing with biofilm depth. The likelihood of cell starvation occurring in the biofilm increases, the further away the cell is from the nutrient source (Stewart et al., 2008). With the reduced nutrient availability, the growth rates of bacteria within the lower section of the biofilm will be suppressed (Gilbert et al., 1997). This may have an effect on the EPS that is produced by individual cells within the biofilm and change the biofilm architecture. EPS has been shown to form pores and channels to absorb nutrients from the bulk liquid and provide a mechanism which can provide a continuous nutrient feed to the cell within the biofilm. If a restriction in nutrient availability were to occur, the biofilm matrix may adapt to its environment by creating a different biofilm structure.

1.3.2. Hydrodynamic Conditions

Biofilms form in a variety of hydrodynamic conditions; wall shear stress is one of the fundamental hydrodynamic conditions (Busscher & van der Mei, 2006; Hu et al., 2011). Initial bacterial adhesion to the surface substrate may be influenced by shear stress. Studies into the shear effects on the attachment of bacteria have shown that bacterial attachment decreases with an increase in the shear stress. The shear stress reaches a balance of attachment and detachment of cells to a substrate surface; this is referred to as the critical shear stress (Nejadnik et al., 2008). Wang et al. (Wang et al., 2013) observed the adhesion of four different strains (P. aeruginos, P. putida, E. coli and S. epidermidis) on two different substrates.

The most noticeable influence of shear conditions on biofilm formation is the surface roughness of the biofilm surface. The evaluation of biofilm surface

11
roughness due to erosion caused by a high flow velocity can result in an observed increase in the biofilm surface roughness (Duddu et al., 2009; Mathieu et al., 2014; Picioreanu et al., 2001). Turbulent and laminar flow regimes during biofilm growth have shown different influences on the biofilm architecture. Under laminar flow, roughly circular micro-colonies were separated by water channels, whereas filamentous streamers were seen with ripple-like structures after prolonged growth in turbulent flow (Sutherland, 2001). Turbulent grown biofilms display stable and rigid biofilms, whereas laminar flow produce thicker and less dense biofilms (Busscher & van der Mei, 2006; Pereira et al., 2002). Horn et al. noted the detachment of the biofilm, once a certain base thickness was obtained, during a simulation of shear stress; this detachment resulted in heterogeneous biofilm growth (Horn et al., 2003). When it is exposed to shear stress, the biofilm is shown to respond by increasing the strength of the matrix, such as the increased production of EPS. This is formed to reduce the bacterial exposure to the external shear stress and, as shown by Shaw et al., can change the viscoelastic properties of the biofilm (Flemming et al., 2010; Shaw et al., 2004).

1.3.3 Biofilm Stress Response

Changes in the various environmental factors were shown to have an influence on the formation and development of the biofilm. When one of these environmental factors in which the biofilm is formed changes or a condition that promotes the biofilm development is at a minimum, such as nutrients, the biofilm becomes stressed. This stressed response was observed by Steinberger et al. as an elongation of the bacteria within the biofilm. *Pseudomonas aeruginosa* cells elongated with the reduction of nutrient availability; this, Steinberger et al. postulated, is to increase the surface area and the nutrient collection of the bacteria (Steinberger et al., 2002). Orruño et al. noted that the stress factor, a protein produced by *E. coli*, induced elongation of the bacteria and reduced the culturability when a significant reduction of carbon occurs (Orruño et al., 2017). By reducing the carbon intake of the biofilm, the bacteria begin to starve and a stress response occurs. However, nutrient intake is only one factor of the environment which influences this stress response; shear stress and antibiotic introduction are two other examples of environmental factors which
may cause a change in the biofilm structure as a stress response (Viveiros et al., 2007; Gefen et al., 2009). Unlike nutrient reduction, the stress response caused by shear stress results in the decrease in the biofilm diversity and a decrease in the growth and propagation of the biofilm; Rochex et al. determined that this would maintain a young biofilm preventing maturation (Rochex et al., 2008). As the stress response of the biofilm differs depending on the stressor applied, this may have implications in the architecture of the biofilms created in Chapters 3 and 4.

1.4. Scope of Thesis

In order to determine bacterial adhesion onto the surfaces of the substrate and the subsequent complex formation of biofilms, there is a need to understand their interactions in different environments. This thesis focuses on the influence of surface topography on bacterial adhesion and further investigates the effect of hydrodynamic shear stress and nutrient availability on the biofilm structure. These analyses were performed in individual sections.

Chapter 2 of this thesis investigates the surface topography of NF270 and BW30 membranes. A technique to detect and measure surface topographical heterogeneities will be demonstrated. The influence of these surface heterogeneities on the adhesion of bacteria will be shown for both systems with and without permeate flux. The experiments were performed using a combination of AFM, SEM as well as fluorescent microscopy and they highlight the influence of surface properties in bacterial adhesion. Moreover, surface heterogeneities were found to be an inherent factor of this and to provide areas of protection from hydrodynamic shear stress. Permeate flux conditions influenced the adhesion patterns of the cells, while membrane properties and bacterial properties had greater influence on bacterial adhesion without the presence of permeate flux.

Chapter 3 analyses the impact of nutrient concentration and shear stress on Ps. fluorescens biofilm on glass. Glass was chosen as a smooth surface to reduce the influence of surface topography investigated in Chapter 2. Chapter 3 focuses on CLSM analysis of the biofilm’s structural morphology with two different techniques: air-liquid interface and flow cell. The CLSM was used to examine the biofilm structure of the biofilm samples. The biofilm architecture was observed to determine
whether nutrient availability influences the structure of the biofilm matrix, with larger clusters of the high nutrient biofilm and the low nutrient biofilm displaying smaller and distributed colonies. The staining of the biofilm highlights the reduced EPS production with the lower nutrient availability. Different biofilm development occurred with changes in both nutrient availability and shear stress.

Finally, Chapter 4 explores the adhesive and viscoelastic properties of biofilms with different nutrient availability. AFM was utilised to determine the mechanical properties of two air-liquid interface *Ps. fluorescens* biofilms grown with high and low nutrients on glass. Nanoindentation of the biofilms in liquid determined the Young’s modulus, adhesion force and work of adhesion. The availability of nutrients was found to influence the Young’s modulus and adhesion force. Biofilms grown in low nutrient conditions displayed high Young’s modulus and adhesion force. This indicates a stiffer and stickier biofilm in comparison to that of the high nutrient grown biofilm.
CHAPTER 2

Quantification of initial bacterial adhesion into nanofiltration and reverse osmosis surface topographical heterogeneities and surface analysis.

Abstract

The role of NF/RO membranes in the adhesion of bacteria has been extensively studied in terms of its physicochemical and surface properties. Despite this, very little is understood of the possible influence membrane topographical heterogeneities can have on bacterial adhesion. Studies into alternative materials have shown the influence of topographical heterogeneities on bacterial adhesion and biofilm development depending on heterogeneity shape and bacteria. In order to elucidate the growth of biofilm onto NF/RO membranes, this study investigates whether bacterial adhesion is influenced by the presence of membrane topographical heterogeneities. Two commercial membranes, NF270 and BW30, were studied for their topographical heterogeneities and physicochemical properties. These were subsequently assessed for their involvement in the attachment of two representative organisms, *Ps. fluorescens* and *S. epidermidis*. These organisms were chosen for their different geometrical shapes. Further testing of the adhesion of bacteria to commercial membranes included the influence of cross-flow velocity and permeate flux, as well as the angle to which bacteria adhered compared to the flow direction. The adhesion of bacteria to the surface topographical heterogeneities was determined using SEM, AFM, fluorescence microscopy and image analysis. Analysis of the membrane area showed that up to 13% of the membrane surface area was covered in topographical heterogeneities with up to 30% of total adhered cells that were found in these membrane defect areas. However, when comparing the angle of attachment of cells within surface heterogeneities to the membrane surface, no difference was observed. The adhesion of cells to surface heterogeneities leads to the supposition that topographical heterogeneities provide environmental recesses that are ideal for attachment during the initial phase of the biofilm development.
2.1. Introduction

The quantification of the membrane topography and surface topographical heterogeneities is difficult since these features can be nanometres to several micrometres in dimensions. This can be challenging (Crawford et al., 2012), as the use of AFM is typically limited to small random areas of membrane surface at a time (Boussu et al., 2005; Boussu et al., 2006). The standard parameter used in the quantification of the membrane surface roughness is the average roughness and the root mean squared roughness. These parameters are obtained by raster scanning the surface and measuring the nanoscale peaks and troughs. This overlooks the large surface heterogenetic areas which provide large areas of membrane with sections of low shear rate. These large surface heterogeneities are areas of membrane surface, which have a height and depth greater than the nanoscale peaks and troughs of the membrane surface roughness. Previous studies on various surfaces analysing the bacterial adhesion to microscale surface heterogeneities have shown to affect cell adhesion (Boyd et al., 2002; Medilanski et al., 2002; Taylor et al., 1998). Additionally, studies have shown that surfaces comprising of surface topographical heterogeneities exhibit preferential bacterial adhesion to those with uniform surfaces (Perni et al., 2013). The adhesion and enhanced biofilm growth of *Escherichia coli* bacteria were observed by Hou et al. (Hou et al., 2011) on features larger than 20µm × 20µm and 40µm × 40µm on PDMS structures. Hou et al. concluded that the cells adhered to surface heterogeneities occurring in flat surfaces require a threshold dimension i.e. the length, width and depth of the surface heterogeneity which does not confine the cells.

The analysis of microscale surface heterogeneities on different materials has enhanced understanding of bacterial adhesion into preferential sites. The origins of these surface heterogeneities may be due to the interfacial polymerisation during the membrane creation. Many factors during interfacial polymerisation, such as time, temperature, curing and monomer concentration, influence the membranes topography (Sorough et al., 2012; Ghosh et al., 2008). While many models have been created to determine the process of the membrane interfacial polymerisation (Freger et al., 2003; Ji et al., 2000) and surface roughness of the membrane was extensively reviewed (Kwak et al., 1999; Ghosh et al., 2009), little or no mention of surface heterogeneities was made. These heterogeneities may be formed during the
interfacial polymerisation process; however, transport and handling of both the Nanofiltration and Reverse Osmosis membranes may result in further damage to the membrane surface. Therefore, surface heterogeneities analysed in this Chapter.

It is unclear the influence micrometre surface heterogeneities on NF and RO membranes have on the initial bacterial adhesion during full scale filtration with flux conditions. Further the contribution the surface heterogeneities have on the rate of bacterial adhesion and the possible attributes the resulting biofilm may develop on the membrane surface with the introduction of permeate flux. This study contributes to the framework in which early stage biofilm on novel membranes can be assessed and can include deliberate micro-topographical features (Maruf et al., 2014; Perni & Prokopovich, 2013; Whitehead et al., 2005; Whitehead & Verran, 2006b). The analysis of bacterial adhesion to membrane surfaces by including surface heterogeneities can also aid in providing further knowledge into the growth and development of the subsequent biofilm.

The objective of this study is to provide further insight into how the surface heterogeneities, located on NF and RO surfaces, may influence the initial adhesion of bacteria. In order to provide a more comprehensive study, two bacteria species of different morphologies were tested: *Ps. Fluorescens* and *S. epidermidis* which frequently foul NF and RO membranes during water treatment (Baker et al., 1998; Flemming & Wingender, 2010; Ghayeni et al., 1998; Wang et al., 2013). These bacteria species were used to determine their capability to adhere to surface heterogeneities, micrometres in size, in areas of two commercial NF and RO membranes, NF270 and BW30 respectively. The adhesion studies were assessed in conditions with and without flux. Additionally, in order to determine if the bacterial adhesion was influenced by flow hydrodynamics, the adhered cells were assessed with respect to their orientation in relation to the flow direction.

2.2. Method

2.2.1. Bacterial strains, culture conditions and preparation

*Pseudomonas fluorescens* (*Ps. fluorescens*) PLC1701 (Institute of Biology Leiden, Netherlands) and *Staphylococcus epidermidis* (*S. epidermidis*) ATCC 12228, were
selected as model strains in this study for bacterial adhesion assays. Gram negative *Ps. fluorescens* is a rod-shaped bacterium measuring approximately 1 µm in width and 2 µm of length, while Gram positive *S. epidermidis* is a cocci bacterium with a diameter of approximately 1 µm. The *Ps. fluorescens* containing the mCherry expressing protein marker (Flemming, 2002) was stored at a temperature of -80°C in a King B broth (King *et al.*, 1954) and supplemented with 20% glycerol. Independent *Ps. fluorescens* cultures were obtained by inoculating 100 mL King B broth supplemented with gentamicin (Sigma Aldrich, Ireland) at a final concentration of 10 µg.mL⁻¹. This was inoculated using a single colony of a previously grown culture on King B agar (Sigma Aldrich, Ireland) at a temperature of 28°C. Independent *S. epidermidis* cultures were obtained by inoculating 100 mL tryptic soy broth (TSB), using a single colony of a previously grown culture on King B agar (Sigma Aldrich, Ireland) at a temperature of 28°C. Both inoculated *Ps. fluorescens* and *S. epidermidis* media were then incubated at a temperature of 30°C for 16 hours, while shaking at 75 rpm until the cell culture reached an optical density (OD) between 0.8-1.2 at OD₆₀₀.

The *Ps. fluorescens* and *S. epidermidis* cultures were centrifuged (Eppendorf Centrifuge 5415C) for 10 mins at 7000rpm. Following this, the supernatant was discarded and the bacterial pallet was re-suspended in Raw Water Medium without carbon (RW⁻C) (Semiao *et al.*, 2013). MilliQ water, Grade 1 pure water (Biopure 15 and Purelab flex 2, Veolia, Ireland), was used in the formulation of the RW⁻C and throughout the study. The *S. epidermidis* cells were stained prior to adhesion assays using 2 µL of 3.34 mM SYTO 9 and incubated in the dark for 15 mins at room temperature. Due to the mCherry fluorescent protein marker, staining of *Ps. fluorescens* was not required. Following staining, the bacterial suspensions were then diluted to an OD of 0.2 in RW⁻C. These were then used in dynamic adhesion assays with and without flux, constituting a feed concentration of approximately 10⁷ CFU.mL⁻¹. CFU was measured by a serial dilution of bacterial culture and plated. The plates were incubated at 30°C for 24 hours and the number of colonies formed on each plate were counted. CFU.mL⁻¹ was calculated using the following equation:

\[
\text{CFU.mL}^{-1} = \frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{Total dilution used}} \tag{2.1}
\]
2.2.2. Microbial Adhesion to Solvents

The Microbial Adhesion to Solvents (MATS) assays were utilised with minor modifications in order to determine the hydrophobic nature and Lewis acid-base characteristics of *Ps. fluorescens* and *S. epidermidis* in this study (Bellon-Fontaine *et al.*, 1996). MATS is based on the correlation between the microbial cell surface affinity to a monopolar solvent and a nonpolar solvent, which both display comparable Lifshitz-van der Waals surface tension components. Two pairs of solvents of the highest purity grade (Sigma-Aldrich, Ireland), Chloroform (electron acceptor solvent) and hexadecane (nonpolar solvent) and ethyl acetate (electron donor solvent) and decane (nonpolar solvent), were chosen as the MATS solvents for this study, as described by Bellon-Fontaine *et al.* (Bellon-Fontaine *et al.*, 1996). These pairs display the electron donor and the electron accepter characteristics of the bacterial surface; additionally, the percentage of bacterial cells adhered to hexadecane indicates the hydrophilicity or the hydrophobicity of the cell surface (Hamadi *et al.*, 2008).

The bacterial cells were incubated to an OD$_{600}$ of 1.0 and centrifuged at 5000rpm for 10 mins and the bacterial platelet re-suspended in RW$^C$; this was repeated. Following this, the bacterial cells were suspended and diluted in RW$^C$ to an OD$_{600}$ of 0.8. 2.4ml of the individual bacterial suspensions and these were mixed using a Vortex Mixer (Stuart, UK) for 60s with 0.4ml of the respective solvent. In order to ensure the complete separation of phases, the mixture was allowed to stand for 15 min, after which 1 ml from the aqueous phase was carefully removed and its final optical density measured at OD$_{400}$. The following equation was used to determine the percentage of adhesion of bacterial cells in the solvent phase:

$$\text{%adherence} = \left(\frac{A_0 - A}{A_0}\right) \times 100$$ \[2.2\]

where $A_0$ is the optical density of the bacterial suspension before mixing at OD$_{400}$ and $A$ is the final optical density after mixing.
2.2.3. Bacterial Zeta Potential

Solutions of 0.001 M NaCl were prepared and pH was adjusted by adding nitric acid or potassium hydroxide to pH 3, 7 and 9. The inoculated bacterial cultures were harvested by centrifuge (5000 RPM, 10 mins) and bacterial pellets were washed twice with 0.001 M NaCl before diluting to an OD600 of 0.2. The bacterial suspension was diluted to a hundredth in the pre-prepared pH solution (2 mL final volume), which was then placed in a capillary cuvette and subsequently placed in a Zetasizer instrument (Malvern Instruments, UK) for Zeta potential measurements. The experiments were performed in triplicate, using three independent cultures.

2.2.4. Dynamic Initial Adhesion Assays in the Absence of Pressure

The initial adhesion assays were performed using both BW30 and NF270 membranes. The selected membranes of dimension 2 by 3 cm were freshly cut and immobilized onto glass slides (VWR, Dublin, Ireland), using double sided tape (3M, ScotchTM, Ireland). These were inserted into individual flow cells (Model BST 81, Biosurface Technologies Corporation, Bozeman, MT, USA) with channel dimensions of 2.35 mm depth, 13 mm width and 50 mm length. The dynamic adhesion system was composed of the flow cell device, a peristaltic pump (Watson-Marlow UK 323E) and a feed container (Falcon Tube VWR 40 mL) with the cells in suspension all connected with silicone tubing (VWR, Ireland) in a closed loop system. The flow cells contained a glass viewing port that allowed for in situ observations by microscopy. Prior to the experimentation, bubbles were removed from the system and “zero point” images at the membrane’s focal plane were observed using an epi-fluorescence microscope (Olympus BX 51) and a 20x objective with a field of view of 1450 µm².

In order to initiate the adhesion experiments, bacterial cells were recirculated at a volumetric flow rate of either 22.2 or 66.6 mL.min⁻¹. A flow rate of 22.2 mL.min⁻¹ corresponds to a Re_{dh} of 26.7, while a flow rate of 66.6 mL.min⁻¹ corresponds to a Re_{dh} of 80.3. Shear stress was calculated using the following equation for wall shear rate (Busscher & van der Mei, 2006):
\[ \sigma = \frac{3Q}{2\left(\frac{h_o}{2}\right)^2 w_o} \]  \[\text{[2.3]}\]

where \(Q\) is the volumetric flow rate (\(m^3\cdot s^{-1}\)), \(h_o\) is the height of the rectangular channel (\(m\)) and \(w_o\) is the width of the rectangular channel (\(m\)). Shear stress is calculated by applying the following equation:

\[ \tau_w = \eta \sigma \]  \[\text{[2.4]}\]

where \(\eta\) is the absolute viscosity (\(kg.m^{-1}.s^{-1}\)) and \(\sigma\) is the wall shear rate (\(s^{-1}\)). Based on the experimental conditions used during the adhesion assays, shear stress was calculated at 0.031 N.m\(^{-1}\) and 0.093 N.m\(^{-1}\) for a volumetric flow rate of 22.2 mL.min\(^{-1}\) and 66.6 mL.min\(^{-1}\) respectively.

Following the inoculation, images were obtained 1 min after initiating the bacterial assay and every 5 min for a total period of 30 min. Fluorescence emissions of adhered \(Ps.\ fluorescens\) and \(S.\ epidermidis\) cells were acquired using the microscope’s U-MNG or U-MWIB excitation/emission filter cube systems. The membrane area observed was randomly selected in the centre of the flow cell area due to varying hydrodynamic conditions at the start and end of the flow cell, which may affect the cell count.

At the end of each adhesion experiment, approximately 40mL of RW\(^{-C}\) was introduced to the system in a non-recirculating mode at the volumetric flow rate of the system. This was to ensure non-adhering cells were removed. Membranes containing adhered cells were retained for further qualitative assessment. Images acquired during the 30 minutes of adhesion were processed using Image J ® to determine bacterial surface coverage over time. \(Ps.\ fluorescens\) and \(S.\ epidermidis\) initial adhesion kinetics on NF270 and BW30 membranes were calculated using the following equation:

\[ q(t) = q_{max}^2 \left(1 - e^{-\beta t}\right) \]  \[\text{[2.5]}\]
where $q(t)$ is the bacterial loading as a function of time ($t$), $q_{\text{max}}$ the maximum cell loading and the accumulation factor $\beta$ obtained by the exponential fit of the adhesion experimental data. By using the following expression, the linear region of the obtained curve was used to calculate the rate of adhesion (Semião et al., 2013):

$$k_d = \frac{\theta(t) \cdot \frac{1}{\Delta t} \cdot C_0}{2.6}$$

where $k_d$ is the deposition rate of *Ps. fluorescens* or *S. epidermidis* on membranes, $\theta(t)$ the number of adhered cells over a time period, $\Delta t$ difference between two time points and $C_0$ the initial bacterial suspension feed concentration.

### 2.2.5. Dynamic Adhesion Assays under Permeate Flux Conditions

A cross-flow system was employed for dynamic adhesion assays under permeate flux conditions, using a Membrane Fouling Simulator (MFS) (Allen et al., 2015; Semiao et al., 2013). The membranes were soaked in MilliQ water for 24 hours at 4°C prior to use in order to remove any preservatives from the surface. As depicted in Figure 4, two MFS devices holding individual membranes of BW30 (FilmTec Corp., USA) and NF270 (FilmTec Corp., USA) were connected parallel to each other. Both MFS cells held a membrane of 102cm² with a slit type channel height of 0.8 mm, width of 40 mm and length of 255 mm. The two individual MFS devices were connected to a 10L autoclavable feed tank (Carboy, Nalgene, VWR Ireland) and a high-pressure pump (P400 from Hydra-Cell, UK). A temperature indicator (Pt 100, Radionics, Ireland) was used to monitor and maintain the temperature in the feed tank at 20°C ± 1°C by a coil inside the tank and connected to a temperature controlled water bath (MultiTemp III, Pharmacia Biotech, Ireland). Pressurisation of the system utilised a back-pressure regulator (KPB1L0A415P20000, Swagelok, UK) and was monitored in both feed and retentate side of the membrane cells with two pressure transducers (PTX 7500, Druck, Radionics, Ireland). A flow meter (OG2, Nixon Flowmeters, UK) measured the flow rate. Data pertaining to the membrane cells’ inlet and outlet pressure, temperature and feed flow was logged, allowing for data collection (PicoLog 1000, PicoTechnology, Radionics, Ireland). The permeate volume was collected using 50ml tubes with a stopper and discarded after
measurements. A conductivity meter measured the feed permeate of each MFS module, using a TetraCon 325 conductivity probe (WTW, Germany). The mass of permeate to determine water flux was measured after 2 min with a balance HCB123 (Adams, Astech Ireland); this was repeated every 30 min until a steady water flux was obtained.

![Diagram of MFS cross flow system](image)

Figure 4: MFS cross flow system

Initially, the membranes were compacted in the MFS at 12 bar pressure and a feed flow rate of 0.66 L.min⁻¹ for 18 hours. The flow rate was calculated using equations 2.3 and 2.4; this corresponds to a velocity of 0.35 m.s⁻¹, a shear rate of 2588 s⁻¹ and a $Re_{th}$ of 579. Following compaction, RW⁻C was used at 8 bar for the adhesion experiments. The system was allowed to equilibrate for 15 min; the bacterial suspension was then added and allowed to recirculate for 30 min. MFS modules were subsequently removed from the system and opened. The membranes were removed and prepared for analysis (Heffernan et al., 2013)
2.2.6. Surface Roughness and Surface Heterogeneities Analysis

The surface roughness and surface heterogeneity analyses were performed using a Nanowizard JPK Instrument (Berlin, Germany). Coupons of BW30 and NF270 were immersed in MilliQ water overnight and stored in the fridge to remove any protective layer and chemical residues. For AFM analysis, membrane coupons were air dried. Subsequent measurements were taken using tapping mode with a scan rate of 0.4 Hz. A Silica Nitride cantilever (Mikromasch, Germany) was utilised with a specified spring constant of 0.5 N m\(^{-1}\) and a resonant frequency between 50-65 Hz. These measurements were performed in triplicate at random areas of 10 µm x 10 µm and 50 µm x 50 µm for all tested membrane samples. The samples were analysed for surface roughness and for the detection of surface topographical heterogeneities or defects. These defects are defined as irregularities on the membrane surface with depth and width greater than the nanometre size peaks and troughs of the membrane surface. Image analysis of the samples was performed using the programme Gwyddion (Necas et al., 2012). A mark or indentation was placed in the membrane centre, using a scalpel, and located on the AFM, using a microscope. Areas adjacent to this mark were measured and the samples were sent for SEM analysis.

2.2.7. Scanning Electron Microscopy

SEM was performed with a dual beam field emission scanning electron microscope (Hitachi Quanta 3D FEG Dual Beam FE-SEM) on the membrane sample, following adhesion testing in both flow cell and cross-flow system. When removed from the system, the MFS or flow cell devices were submerged and opened; this was in order to prevent bubbles. 1cm x 1cm coupons of the membranes were taken and immersed in a petri dish with a solution of 2.5% Glutaraldehyde for 24 hours to preserve bacterial cell shape and structure. A stepwise dehydration treatment of MilliQ water with increasing ethanol volumes was utilised according to Herzberg et al. (Herzberg & Elimelech, 2007). Following the drying, the coupons were adhered to SEM stubs, using a carbon adhesive and coated with a thin layer of gold (Eimtach K575K). Images were taken at an accelerating voltage of 5 kV, current of 5.92 pA and
magnifications of 1200x were taken. The samples were analysed using Image J® software.

Dry membrane samples used for SEM analysis were placed in SEM for analysis of surface heterogeneities. Each sample would be marked, as previously explained in Section 2.2.6. The area adjacent to the mark was analysed for surface defects similar to those measured with AFM and the results are compared to confirm that surface defects are visible when using SEM.

2.2.8. Statistical Analysis

In order to determine the variation in the quantity of counted *Ps. fluorescens* or *S. epidermidis* cells on two different membrane types (NF270 and BW30), the analysis of variance (ANOVA) was used; this was determined for different flow rates and permeate flux. Turkey’s test for pairwise comparisons assuming equal variance was performed with MINITAB v15.1 (Minitab Inc., State college, PA) at a 5% significance level. The error bars are standard error of mean.

2.3. Results and Discussion

2.3.1. Microbial Adhesion to Solvents

Microbial adhesion to solvents for *Ps. fluorescens* and *S. epidermidis*, as presented in Table 1, were found to have similar adhesion properties to those found in the literature (Dostálková et al., 2013; Lerebour et al., 2004).

<table>
<thead>
<tr>
<th>% Adhesion</th>
<th>Chloroform</th>
<th>Hexadecane</th>
<th>Decane</th>
<th>Ethyl Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ps. fluorescens</em></td>
<td>90.20 ± 1.53</td>
<td>44.89 ± 3.67</td>
<td>67.56±2.31</td>
<td>5.77 ± 0.39</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>71.96±4.08</td>
<td>32.07±4.91</td>
<td>49.25±5.60</td>
<td>10.66±2.53</td>
</tr>
</tbody>
</table>

*Table 1:* Affinity of *Ps. fluorescens* and *S. epidermidis* suspended in RW−C for the four solvents used in the MATS analysis.
Both *Ps. fluorescens* and *S. epidermidis* cells demonstrate a high affinity for chloroform, 90% and 72% respectively. This acidic solvent suggests that both have a strong electron acceptor character. This was compared to the solvent ethyl acetate, with a percentage adhesion of 5.77% for *Ps. fluorescens* and 10.66% for *S. epidermidis*, showing a low affinity for the electron donor. Nonpolar solvents hexadecane and decane show a low affinity for *Ps. fluorescens* and *S. epidermidis*, with *S. epidermidis* having the lowest affinity with 32% for hexadecane and 49% for decane. The high affinity to chloroform suggests that *Ps. fluorescens* has stronger electron donor characteristics than *S. epidermidis*, while the low affinity for nonpolar solvents indicate both bacterial cells are hydrophilic, with *Ps. fluorescens* being more hydrophilic than *S. epidermidis*.

2.3.2. Bacterial Zeta Potential

In order to characterise the physicochemical properties of *Ps. fluorescens* and *S. epidermidis* cells, the global surface charge was evaluated. The experiments were conducted in 0.001 M NaCl at pH 3, 7 and 9. Further, the experiments were performed in triplicate and error bars represent standard error of mean. Zeta potential measurements were implemented at pH values 3, 7 and 9, as presented in Figure 5.
Figure 5: Zeta potential of *Ps. fluorescens* and *S. epidermidis* at pH values of 3, 7 and 9.

As shown in figure 5 above, the increase in negative zeta potential was observed as pH increases from 3 to 7 and the global surface charge increases for both *Ps. fluorescens* and *S. epidermidis* cells. Zeta potential values for both *Ps. fluorescens* and *S. epidermidis* at pH 3 resulted in a minimum in global surface charge values of -8.7 and -0.9 (mV) respectively. Cell wall electronegativity was stable for *Ps. fluorescens* cells with values of -14.7 for a pH of 7 and -14.3 mV for a pH of 9. The *S. epidermidis* cells at pH 7 and 9 showed a similar trend with zeta potential values of from -10.1 to -9.6 mV at pH 7 and 9 respectively.

*Ps. fluorescens* and *S. epidermidis* were shown to be electronegative, which is comparable to those found in literature (Kiers *et al.*, 2001; Van Loosdrecht *et al.*, 1987). Tourney *et al.* (Tourney *et al.*, 2010) showed deprotonation of cell wall function groups with increasing pH, demonstrating an increase in zeta potential pending deprotonation of all functional groups. This behavioural contrast indicates that cell wall properties of *Ps. fluorescens* and *S. epidermidis* are distinct from each
other. The EM variations between the two strains suggest differing cell wall composition, which, therefore, demonstrates different adhesive properties.

2.3.3. Surface Roughness and Surface Heterogeneities

AFM was utilised to assess the surface characterization in terms of surface roughness, as displayed in Table 2. Values for Zeta Potential and Contact Angle were obtained from literature (Semião et al., 2014; Tang et al., 2007; Tu et al., 2011).

<table>
<thead>
<tr>
<th></th>
<th>$R_a$(nm)</th>
<th>$R_{ms}$(nm)</th>
<th>Contact Angle (°)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF270</td>
<td>16.9 ± 4.94</td>
<td>23.67 ± 5.8</td>
<td>8.4 ± 0.5</td>
<td>-24 (Tu et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Semião et al., 2014)</td>
</tr>
<tr>
<td>BW30</td>
<td>42.3 ± 5.5</td>
<td>53.9 ± 7.1</td>
<td>25.6 ± 0.8</td>
<td>-5.2 (Tang et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Semião et al., 2014)</td>
</tr>
</tbody>
</table>

Table 2: Mean Roughness, Contact Angle and Zeta Potential measurements for BW30 and NF270.

As can be seen in Table 2 the contact angle was slightly higher for the BW30, indicating a more hydrophobic membrane in comparison to the NF270. The NF270 had a higher negative charge with a zeta potential of -24 mV compared to the BW30, which had a lower zeta potential of -5.2 mV. In addition to the zeta potential, NF270 and BW30 membranes are hydrophilic and negatively charged; this could repel negatively charged bacteria, causing a reduction in attachment. As NF270 has higher negative charge of -24 mV in comparison to -5.2 mV of BW30, the NF270 membrane was expected to repel more cells.

The mean surface roughness values of BW30 and NF270 membranes agreed with roughness values found in literature (Hoek et al., 2003; Mänttäri et al., 2004).
Analysis of potential defect regions was performed using larger raster scanning areas of 50 µm x 50 µm on membranes. The larger raster scan measurements establish variation in roughness caused by the surface topographical heterogeneities. These are identified as large (50 µm x 50 µm) and small (10 µm x 10 µm) raster scans and demonstrate a variation in the roughness measurement (Boussu et al., 2005; Johnson et al., 2012). Differences in mean roughness can be caused by variability in the membrane batches employed in this study.

Figure 6, as shown hereunder, is the representative AFM micrographs of NF270 and BW30 membranes of small (10 µm x 10 µm) and large (50 µm x 50 µm) raster scanning areas.

![AFM images of NF270 and BW30 membranes](image)

**Figure 6:** AFM images of NF20 (A-C) and BW30 (B-D) membranes of 10µm×10µm (A, B) and 50µm×50µm (C, D) raster scanning areas.

Significant membrane topographical heterogeneities were detected on larger raster scans (Figure 6 C-D) which would not be observed in small raster cans (Figure 6 A-B). 10 images of surface topographical heterogeneities were obtained from area rasters of 50 µm x 50 µm, using AFM, and measured for their depth and width for both membranes; these measurements are depicted in Table 3. Similar widths of topographical heterogeneities were observed with the defect width measured at 10 ±
2.2 µm for NF270 and 12 ± 1.6 µm for the BW30. The depth of NF270 was higher at 1 ± 0.2 µm and 0.4 ± 0.1 µm for the BW30.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Surface Topographical Heterogeneities Width (µm)</th>
<th>Surface Topographical Heterogeneities Depth (µm)</th>
<th>Membrane Maximum Height (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF270</td>
<td>10 ± 2.2</td>
<td>1 ± 0.2</td>
<td>517.1 ± 98.6</td>
</tr>
<tr>
<td>BW30</td>
<td>12 ± 1.6</td>
<td>0.4 ± 0.1</td>
<td>342.5 ± 59.1</td>
</tr>
</tbody>
</table>

**Table 3**: Surface topographical heterogeneities size characterisation for BW30 and NF270 membranes.

The size of both *Ps. fluorescens* and *S. epidermidis* is approximately 1µm in width, which could comfortably attach within the surface topographical heterogeneities of the NF270 membrane with a depth of 1 ± 0.2 µm. This could potentially provide a protective area from shear stress, whereas the BW30 depth of 0.4 ± 0.1 µm was less than half of the width of both bacterial strains, which in turn would provide less sheltered areas from shear stress. Due to the accommodating size of the NF270, when compared to the BW30, this may present more favourable sites of adhesion under dynamic flow conditions.

2.3.4. Dynamic Initial Adhesion Assays

Dynamic adhesion assays were performed onto BW30 and NF270 membranes to assess the preference of bacterial adhesion to surface topographical heterogeneities. Two different flow rates of 22.2 mL min\(^{-1}\) and 66.6 mL min\(^{-1}\) were performed in order to provide two differing hydrodynamic conditions and to qualitatively and quantitatively assess the adhesion of *Ps. fluorescens* and *S. epidermidis* to the two membranes.
Shown in Table 4 are the Maximum cell loading ($q_{\text{max}}$) and adhesion velocity ($k_d$), which are calculated using Eq.2.4 and Eq.2.5 respectively. The experiments were performed in triplicate. A flow rate of 22.2mL min$^{-1}$ corresponds to a velocity of 0.012.m s$^{-1}$, a Re$_{dh}$ of 26.7 and a shear rate of 0.030 s$^{-1}$. In addition, a flow rate of 66.6 mL min$^{-1}$ corresponds to a velocity of 0.036.m s$^{-1}$, a Re$_{dh}$ of 80.3 and a shear rate of 0.092 s$^{-1}$.

<table>
<thead>
<tr>
<th></th>
<th>Estimated Maximum cell loading</th>
<th>Adhesion Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$q_{\text{max}}$ ($10^7$ cells cm$^{-2}$)</td>
<td>$k_d$ ($10^{-3}$ cm min$^{-1}$)</td>
</tr>
<tr>
<td></td>
<td>22.2 mL min$^{-1}$</td>
<td>66.6 mL min$^{-1}$</td>
</tr>
<tr>
<td><strong>Ps. fluorescens NF270</strong></td>
<td>0.17 ± 0.08</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td><strong>Ps. fluorescens BW30</strong></td>
<td>3.36 ± 1.5</td>
<td>2.79 ± 1.35</td>
</tr>
<tr>
<td><strong>S. epidermidis NF270</strong></td>
<td>0.57 ± 0.05</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td><strong>S. epidermidis BW30</strong></td>
<td>0.46 ± 0.05</td>
<td>0.18 ± 0.03</td>
</tr>
</tbody>
</table>

*Table 4:* Estimated maximum cell loading and deposition rate of *Ps. fluorescens* and *S. epidermidis* on NF270 and BW30 under 22.2 mL min$^{-1}$ and 66.6 mL min$^{-1}$ volumetric flow rate conditions.
Maximum cell loadings for *Ps. fluorescens* and *S. epidermidis* display no significant effect on the volumetric flow rates, with ANOVA values of p=0.3463 and p=0.292 for both bacteria respectively. Despite this, the membrane type displayed differences in adhesion. The maximum cell loadings saw a significant difference during adhesion of *Ps. fluorescens* (p=0.0001) but no significant difference for *S. epidermidis* (p=1.00). The significant difference in adhesion of *Ps. fluorescens* produced a 20-fold increase of the maximum cell loading for the rougher BW30 membrane, when compared to the smoother NF270. This indicates that the bacterial adhesion may be influenced by the membrane surface properties and the physico-chemical properties of the bacteria. This was shown in a study by Margalit *et al.* (Margalit *et al.*, 2013); in a model of a parallel plate flow chamber the bacterial-surface interactions influenced the bacterial deposition. These bacterial-surface interactions included the bacterial deposition, buoyancy and lift forces, and the predisposition of the adhesion depending on the strain of the bacteria selected and the adhesive surface.

In order to further elucidate the adhesion of the bacteria, the observed accumulation of bacterial cells onto membranes following 30 min adhesion experiments were examined. These were measured at different flow rates and are presented in Figure 7.

![Figure 7: Observed adhered *Ps. fluorescens* and *S. epidermidis* cells on NF270 and BW30 membranes following 30 min adhesion experiments.](image-url)
Bacterial adhesion of *Ps. fluorescens* to the rougher BW30 membrane surface displayed a 1 log increase of cells (10⁷ cells cm⁻²). In comparison, the smooth NF270 membrane surface led to a reduced number of *Ps. fluorescens* (10⁶ cells cm⁻²); moreover, no change in bacterial adhesion was observed with differing volumetric flow conditions. The differences in membrane properties, as previously discussed, may be the influencing factor in the distinct adhesion profiles of the bacterial strains. As the NF270 membrane was more hydrophilic and negatively charged, the lower bacterial adhesion was anticipated. In contrast, the BW30 saw greater bacterial adhesion due to its weaker hydrophilicity and charge.

However, unlike the *Ps. fluorescens*, the adhesion of *S. epidermidis* was not affected by the membrane surface properties. On both NF270 and BW30 membranes, *S. epidermidis* deposition revealed approximately 3 x 10⁶ cells cm⁻², regardless of the flow rate (p=1.0). This indicates that the differences in bacterial attachment may be influenced by the bacterial properties rather than the membrane properties. The differences in bacterial properties, such as the flagella possessed by *Ps. fluorescens*, may play an important role in the adhesion of the bacteria onto a rougher surface. The anchoring due to flagella may have resulted in the significantly greater adhesion observed on the BW30 membrane, which has greater surface features in comparison to NF270 (p<0.0001). By providing anchoring sites, the *Ps. fluorescens* can withstand any changes in flow conditions. Friedlander et al. (Friedlander et al., 2013) showed that swimming motility and the presence of flagella improved the initial bacterial attachment by furthering access to surfaces of adhesion. Furthermore, Friedlander et al. presented that *Staphylococcus* cells are hindered in mobility and adhesive properties by the presence of pili.

2.3.5. Scanning Electron Microscopy

Following dynamic adhesion assays, SEM analysis was performed to assess the influence of surface topographical heterogeneities on the adhesion of bacterial cells. As can be seen in Figure 8, the images collected were analysed for the fraction of surface topographical heterogeneities, known as defects. The bacterial cells within
defect areas were analysed and the mean fraction of total adhered bacterial cells within these defects for both bacterial strains were determined.

![Graph showing surface area coverage and fraction of adhered cells for NF270 and BW30 membranes.]

Figure 8: Surface area coverage (%) of *Ps. fluorescens* and *S. epidermidis* cells per cm² of BW30 and NF270 membrane defect area and the fraction of bacterial adhesion in defect areas. The fraction of adhered *Ps. fluorescens* cells (circle symbol) and *S. epidermidis* cells (triangle symbol) in the membrane topographical heterogeneities under dynamic conditions (66.6 mL min⁻¹) are represented as closed (black symbols). The fraction of bacterial adhesion in membrane topographical heterogeneities under permeate flux conditions at 8 bar and 0.66 L.min⁻¹ feed flow rate is symbolized with open (white) symbols.

As can be seen in Figure 8, the defect area of NF270 covered 7% of the total membrane surface, whereas defects covered 12% of the total surface area of the membrane surface. The fraction of defects of the BW30 (12%) was significantly higher when compared to the 7% coverage of the NF270 (p=0.025). The adhesion of bacteria within surface topographical heterogeneities for NF270 and BW30 produces 11% and 30% of the total bacterial counts respectively. Permeate flux conditions impacted *S. epidermidis* cells, when adhering to smoother NF270 membrane surface topographical heterogeneities, and resulted in a doubling from 15% to 30%. However, no changes of adhesion were observed for *Ps. fluorescens* on NF270, when subjected to the same conditions. Changes in adhesion of *Ps. fluorescens* for
the rougher BW30 membrane led to a lower fraction of adhered cells within surface topographical heterogeneities with the introduction of permeate flux. The influence of permeate flux on the fraction of adhered *S. epidermidis* cells to BW30, in contrast, displayed no significant changes.

Higher levels of adhesion without permeate flux of *Ps. fluorescens* could potentially be due to the motility and anchoring ability of the flagella, as previously discussed. This is in contrast to the *S. epidermidis*, which showed very little difference in adhesion onto BW30 with or without permeate flux conditions. The average bacterial dimensions which can be seen in Table 3 demonstrate the potential for the surface topographical heterogeneities to shield the cells from the hydrodynamic shear forces. This may, in some cases, contribute to the doubling of adhesion within these surface topographical heterogeneities, when permeate flux was introduced. Studies have shown that the formation of certain topographies on various different substrates promotes bacterial adhesion (Ghayeni *et al.*, 1998; Taylor *et al.*, 1998; Whitehead & Verran, 2006b). One such study by Lee *et al.* (Lee *et al.*, 2013) showed that the most prominent areas of bacterial adhesion were those of low shear stress. These areas of low shear stress may be provided by the topographical heterogeneities of the NF270 and BW30 membranes and, in turn, may serve as a protective niche in which bacterial cells may proliferate and form biofilms. This would expedite the development of biofilm formation after the cleaning of membrane surface, causing a rapid reduction of the membrane efficiency (Lee *et al.*, 2013).

As shown in the SEM images of *Ps. fluorescens* and *S. epidermidis*, cells adhered to both membranes in defect areas without permeate flux (Figure 9). These images illustrate that both membrane’s surface topographical heterogeneities appear as indentations in the membrane’s surface. Images of *Ps. fluorescens* and *S. epidermidis* bacteria demonstrate that these bacteria adhere within the topographical heterogeneities. However, there are several *S. epidermidis* bacteria on the edges of the BW30 (D) membrane rather than inside the topographical heterogeneity. Finally, an image of a *Ps. fluorescens* bacterium attached to the NF270 membrane surface (E) contain flagella, which may indicate that these appendages have an influence on *Ps. fluorescens* bacterial adhesion onto the membrane surface.
Figure 9: Representative SEM micrographs depicting the presence of bacterial cells within membrane surface topographical heterogeneities. The adhered *Ps. fluorescens* cells were observed on NF270 (A) and BW30 (B) membranes and adhered *S. epidermidis* cells were depicted on NF270(C) and BW30 (D) membranes. An additional image portrayed a close-up micrograph of adhered *Ps. fluorescens* cells with their flagellum on a NF270 membrane (E).
The angular orientation of adhered *Ps. fluorescens*, as exhibited in Figure 10, was investigated to determine whether adhesion onto the membrane surface with respect to the direction of flow was different in areas of surface topographical heterogeneities or homogenous areas.
Figure 10: Population distribution of the mean number of adhered *Ps. fluorescens* cells, based on the angle at which they adhere on the membrane in relation to the direction of the flow. The NF270 (A, C, E) and BW30 membranes (B, D, F) are shown at flow rates of 22.2 mL min\(^{-1}\) (A-B) and 66.6 mL min\(^{-1}\) (C-D) and under permeate flux NF270 (E) and BW30 (F). The angle of bacteria adhered to membrane without topographical heterogeneities is shown in white and the angle of bacteria within topographical heterogeneities is shown in grey; the accumulated white and grey areas are the total number of bacteria adhered to the membrane surface.
The investigation of the pattern of bacterial cells adhering to the membrane surface could clarify whether defects can provide shielding for shear stress. As the bacterial flagella of *Ps. fluorescens* can assist in the attachment during initial adhesion (Diaz *et al.*, 2010; Diaz *et al.*, 2011), this could result in the adhesion of bacteria in random orientations, as displayed in Figure 10. The orientation of the adhered *Ps. fluorescens* cells displays no dissimilarities for NF270 and BW30 membranes, with and without permeate flux conditions. There was no noticeable difference in the distribution from 0 to 90° for surface topographical heterogeneities and the homogeneous membrane surface. This may be due to the bacterial properties; however, further studies are required.

2.4. Conclusion

In order to determine the interactions of *Ps. fluorescens* and *S. epidermidis* to the membrane surfaces, the membrane’s surface topographical heterogeneities were investigated with and without flux conditions. The analysis was performed with a combination of AFM, SEM and fluorescent microscopy. Judging from AFM measurements of the surface topographical heterogeneities, the width and depth was larger than that of the tested cells, particularly the NF270. Permeate flux conditions were shown to significantly influence the adhesion patterns of the cells, with membrane properties and bacterial properties showing a significant effect on adhesion without the presence of permeate flux. Up to 30% of the fraction of adhered bacteria were found in surface topographical heterogeneities, with 13% of these topographical heterogeneities covering the surface area of the membrane. With the introduction of permeate flux, cell adhesion within surface topographical heterogeneities was dependent on the bacterial and membrane properties; this was further emphasised by the angular orientation during adhesion with permeate flux. The angle of attachment showed no distinguishable difference in the angle of attachment for surface topographical heterogeneities and homogenous membrane surface. Surface properties are a fundamental factor in bacterial adhesion. Moreover, surface topographical heterogeneities are an intrinsic part of this and provide niches protected from hydrodynamic shear stress. These, in turn, potentially provide areas that assist in the proliferation of bacteria and the growth of biofilm.
Analysis of vivo and in-vitro biofilm structure with varying nutrient and shear conditions

Abstract

The growth of biofilms on surfaces is a broad and complex process which has been the subject of many studies in recent years. The prediction of biofilm growth and behaviour requires further examination, particularly with the influence of nutrients and shear stress. As bacteria attach to surfaces in diverse environmental conditions, which contain a variety of different nutrient concentrations, studies on the effects on biofilm formation can pose a challenge to quantify the biofilms’ characteristics. Research carried out on nutrient concentration and shear stress has indicated that nutrient concentration may influence the biofilm architecture. This chapter will focus on investigating the effects of nutrient concentration and hydrodynamic shear stress on the structural formation of \textit{Ps. fluorescens} biofilm under two different dynamic conditions. Two different biofilm growth techniques were assessed for their structure; an air-liquid interface biofilm that was grown for 24 hours and a biofilm grown on glass in a flow cell, observed \textit{in situ}. For 48 hours, the air-liquid interface biofilm was assessed at two different nutrient dilution factors of 1:1 and 1:10. The flow-cell biofilms were subjected to the two different diluted nutrient factors and two different flow rates of 0.4 mLmin$^{-1}$ and 0.7mLmin$^{-1}$. The analysis of the biofilm structure was performed using CLSM. This process was carried out with biofilm staining to display cells and EPS for the 24-hour biofilm grown at the air-liquid interface. Analysis was carried out on the distribution of the cells and EPS throughout the air-liquid interface biofilms. The biofilm grown in an air-liquid interface resulted in a three-fold increase in the EPS biovolume, whereas no difference was noted for the low nutrient biovolume. The flow cell biofilm showed an increased biovolume for low nutrient and higher shear stress conditions, suggesting that harsher growth conditions of the biofilm result in greater biofilm development over the course of 48 hours.
3.1 Introduction

Pre-treated water may vary in terms of nutrient composition. Due to the different environments that the water is extracted from, a variation in bacterial attachment and biofilm growth may occur. The initial adhesion of the bacteria to the surface is an important step in the development of the biofilm. Research suggests that the effects of nutrient concentration continue to influence bacterial adhesion (Subramani & Hoek, 2008). Little is known, however, about the changes in the structure of the biofilm due to nutrients and shear stress in biofilm development. In order to achieve a full understanding of this biofilm growth on surfaces, further quantification of the biofilm behaviour is required. As stated previously, bacteria will attach to surfaces, where diverse environmental conditions exist. Therefore, studies on the formation of biofilm, subject to these conditions, continue to pose challenges when quantifying biofilms’ characteristics. The development of tools and techniques that are adaptable to these various environments provides further insight into biofilm formation and assists in the development of strategies pertinent to sustaining clean surfaces. Such techniques can be applied in developing novel cleaning methods and creation of antifouling surfaces. In using this approach, properties of the surface, such as roughness, surface charge and hydrophobicity, have been identified as parameters that influence the attachment of bacteria in addition to the response of the bacterial cell wall physico-chemical properties and structure (An & Friedman, 1998). The interaction between surface and bacteria influences an important process in the formation of biofilms. However, other environmental factors must also be considered, such as pH, temperature, chemicals, nutrient availability and shear force.

The initial adhesion of the bacteria to the surface is an important step in the development of the biofilm and the effects of nutrient concentration have been demonstrated to influence bacterial adhesion (Costerton et al., 1995; Rochex et al., 2007). Peyton et al. demonstrated the growth of Ps. aeruginosa in an annular reactor, which is a higher biofilm thickness, roughness, areal mass density and a substrate loading rate (Peyton, 1996). Studies under Moreira et al. showed that, under dynamic conditions with high shear stress, high biofilm development of E.coli occurred at low glucose concentrations of 0.25 gL\(^{-1}\) until 12 hours (Moreira et al.,
Despite this, little is known of the changes in architecture of the biofilm due to nutrient concentration.

The objective of this study was to investigate the effects of nutrient concentration and hydrodynamic shear stress on the structural formation of \textit{Ps. fluorescens} biofilm under dynamic conditions. The structural analysis was performed by CLSM with biofilm staining to quantify bacteria and EPS in the biofilm.

3.2 Methods

3.2.1 Bacterial Strains, Cultural Conditions and Preparation

The mCherry expressing \textit{Ps. fluorescens} PLC1701 (Lagendijk \textit{et al.}, 2010) was selected for the biofilm adhesion assays. The \textit{Ps. fluorescens} was stored at -80°C in King B (King \textit{et al.}, 1954) broth supplemented with 20% glycerol. The cultures were obtained by selecting a single colony grown on King B agar (Sigma Aldrich, Ireland) at 28°C and inoculating 100 mL King B broth supplemented at a final concentration of 10 µg.mL$^{-1}$ of gentamicin (Sigma Aldrich, Ireland). The inoculation medium was then incubated at 28°C with shaking at 75 rpm for 16 hours until an optical density (OD) of 0.8-1.0 at a wavelength of 600nm was obtained. The cultures were centrifuged (Eppendorf Centrifuge 5415C) at 7000 RPM for 10 min; subsequently, the supernatant was discarded and the bacterial pellet was re-suspended in King B.

3.2.2 Air-Liquid Interface Biofilm Growth

A \textit{Ps. fluorescens} biofilm was grown at an air-liquid interface in orbital agitation (Safari \textit{et al.}, 2014), as shown in Figure 11.
Figure 11: *Ps. fluorescens* biofilm grown at an air-liquid interface. Biofilms were grown on the coverslip at the interface between the air and King B, as shown in A. On the right is an image of a 24-hour *Ps. fluorescens* biofilm and below is the z-direction scanned during CLSM measurements as shown in B.

Coverslips of Borosilicate Glass 22mm × 22mm (VWR, Ireland) were placed lengthwise, using a tweezers, into falcon centrifuge tubes (VWR, Ireland). The supports for the coverslips were placed in the falcon tubes to prevent movement of the coverslip during orbital agitation. In order to ensure sterility, the falcon tubes containing coverslips were sealed with cotton wool and autoclaved. Following this, 3mL of King B of differing concentration was inserted into the sterile centrifuge tubes. The King B medium was measured at a pH of 7.4, using a Mettler Toledo pH meter (Mason Laboratories, Dublin). Two experiments were conducted at different dilution factors, one at 1:1 (referred to as high nutrient) King B, while a second experiment consisted of Grade 1 pure water (MilliQ water, Biopure 15 and Purelab...
flex 2, Veolia, Ireland), and King B at a dilution factor of 1:10 (referred as low nutrient). The 3mL of medium was supplemented with gentamicin (Sigma Aldrich, Ireland) at a final concentration of 10 µg.mL⁻¹. Each individual tube was inoculated with 5µl volume of the re-suspended overnight culture. The centrifuge tubes prepared, as shown in Figure 11, were incubated over a period of 24 hours, with an orbital agitation of 75 rpm and a temperature of 28°C. The coverslips were removed from the centrifuge tubes and gently rinsed in a sterile 0.1M NaCl solution to remove any nonadherent material from the biofilm surface.

3.2.3 Air-Liquid Interface Tube Biofilm Staining

For bacterial and EPS staining, Syto 9 (green nucleic acid stain: Molecular Probes) and Concanavalin A (Con A) staining protocol, in conjunction with a fluorophore (Alexa fluor 633) (Life Technologies™), were employed. Directly after the rinsing process, the biofilms were stained with Syto 9 at a final concentration of 3.5 µgml⁻¹. Stained biofilms were rinsed in a sterile 0.1M NaCl solution and subsequently stained with Con A-AlexaFluor633 at a final concentration of 200 µg ml⁻¹. Finally, the coverslip was rinsed before the CLSM analysis. The coverslips were placed within a phosphate buffered saline (PBS) solution (Sigma Aldrich, Ireland) that was also enclosed by a Nunc Lab-Tek II Chamber Slide (VWR, Ireland).

3.2.4 Dynamic Biofilm Growth

Flow cell systems allow for the direct measurement of a biofilm, using direct microscopic observation. The flow cells used were model BST 81 from Biosurface Technologies Corporation (Bozeman, MT, USA) and were set up as shown in Figure 12. This flow cell was used to examine the 48-hour growth of Ps. fluorescens biofilm on a cover slip using different nutrient concentrations.
King B was prepared in a 20L feed tank at two different dilution factors of 1:1 (high nutrient) and 1:10 (low nutrient). The flow cell system, with the exception of the waste tank, was autoclaved, which ensured sterility. In order to maintain temperature, the flow cell system was placed within an oven at 28°C and left for one hour to allow the feed tank (ThermoFisher, UK) temperature to achieve equilibrate. pH was checked using a Mettler Toledo pH meter (Mason Laboratories, Dublin) at both the three-way valve and at the waste tank, using a 50mL tube. The system was maintained at a pH of 7.4 until bacterial injection. Biofilm within the flow cell chamber was grown by injecting 5mL *Ps. fluorescens* into the three-way valve (Cole-Parmer, IL, USA). The bacteria was then allowed 1 hour to settle. The flow of the liquid through the chamber was controlled by pumping media through the silicone tubing (VWR, Ireland) into the flow chamber. A continuous flow of media through the flow cell chamber was maintained by a Watson-Marlow 205S peristaltic pump (OH, USA). After 48 hours, the King B media was replaced with a flow of PBS that was injected into the flow cell system, using the three-way valve for 15 minutes. The valves on both ends of the flow cell were closed and the flow cell was disconnected from the system at the point when these valves had been closed. Two different flow rates were used, one at 0.4 mLmin⁻¹ and one at 0.7mLmin⁻¹.
corresponding to a $Re_{th}$ of 0.42 and 0.85 respectively. The flow cell was then analysed by CLSM, using a custom-made holder, as shown in Figure 13.

![Flow cell in custom made holder](image)

**Figure 13:** Flow cell in custom made holder

### 3.2.5 Confocal Laser Scanning Microscopy

CLSM was performed using an Olympus FV1000 CLSM at the Live Cell Imaging core technology facility platform, Conway Institute, UCD. Both the air-liquid interface and the flow cell system experiments were repeated to provide biofilms from 3 independent inocula for both growth conditions, resulting in up to 9 different areas of biofilm. These were repeated for both stained and unstained biofilms. The two wavelengths were used for EPS and bacterial analysis Syto9 and Con A-AlexaFluor633, excited at 488 nm and 633 nm respectively. 3D projections were collected at a z-step of 1µm, using an Olympus UPL SAPO 10×/0.4 air objective for flow cell analysis and Olympus UPL SAPO 60×/1.2 for air-liquid interface. The biofilms’ structural quantification (biovolume, surface coverage, thickness, roughness and distribution profile) was performed using PHLIP MATLAB program developed by J. Xavier (http://phlip.sourceforge.net/phlip-ml), and images of coverage of EPS and bacteria were implemented using Image J from NIH (https://imagej.nih.gov/ij/).

The biovolume is the number of pixels occupied by biofilm in a stack multiplied by the product of the squared pixel size and the scanning step. The surface coverage is
calculated as the fraction of pixels in a cross-section image that is occupied by biofilm. In addition, the biofilm mean thickness is the height in the z-direction for every point on the xy plane, and the roughness is the standard deviation of the distribution [Mueller et al, 2006].

3.2.6 Statistical Analysis

Data presented are the mean ± standard error of the mean. Statistical analysis was performed by analysis of variance (ANOVA) in Tukey’s test for pairwise comparisons, using MINITAB v15.1 (Minitab Inc., State College, PA) at a 5% level of significance (p <0.05).

3.3 Results and Discussion

3.3.1 EPS and Bacterial Staining of Air-Liquid Interface Biofilm Growth

The biofilm analysis of Ps. fluorescens and EPS stained with Syto 9 (green nucleic acid stain: Molecular Probes) and Concanavalin A (Con A) respectively, as described in sections 3.2.2 and 3.2.3, is presented in Table 5. The biovolume, substratum coverage, mean thickness and biofilm roughness are separated into four different sections. Ps. fluorescens biovolume is the total volume of the bacteria within the biofilm and EPS sugar residue biovolume is the total volume of the EPS within the biofilm (Xavier et al., 2003). These sections, along with high and low nutrient dilution factors, are subcategorised into Syto 9 stained Ps. fluorescens bacteria and Con A stained EPS sugar residues.
Table 5: Biovolume, substratum coverage, mean thickness and biofilm roughness of 24-hour *Ps. fluorescens* biofilm grown at the air-liquid interface with low nutrients and high nutrients.

<table>
<thead>
<tr>
<th></th>
<th>Total Biovolume [µm$^3$]</th>
<th>Substratum Coverage (%)</th>
<th>Mean Thickness (µm)</th>
<th>Biofilm Roughness</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Nutrient <em>Ps.</em></td>
<td>56988 ± 14379</td>
<td>16.2 ± 2.9</td>
<td>9.0 ± 0.8</td>
<td>0.45 ± 0.029</td>
</tr>
<tr>
<td><em>fluorescens</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Nutrient EPS</td>
<td>68453 ± 12278</td>
<td>20.8 ± 3.5</td>
<td>10 ± 0.7</td>
<td>0.46 ± 0.053</td>
</tr>
<tr>
<td>sugar residues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Nutrient <em>Ps.</em></td>
<td>27593 ± 4714</td>
<td>10.1 ± 1.9</td>
<td>8.7 ± 0.8</td>
<td>0.34 ± 0.029</td>
</tr>
<tr>
<td><em>fluorescens</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Nutrient EPS</td>
<td>18463 ± 3129</td>
<td>9.3 ± 1.9</td>
<td>9.1 ± 1.2</td>
<td>0.35 ± 0.027</td>
</tr>
<tr>
<td>sugar residues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A two-fold difference in total cell biovolume was observed ($p = 0.004$) between biofilms grown under high nutrients and low nutrients conditions with values of 56988 ± 14379 µm$^3$ and 27593 ± 4714 µm$^3$ respectively. A significant difference ($p = 0.03$) in EPS production between the high and low nutrient was shown. With high nutrient content at a higher EPS total, biovolume was observed as 68453 ± 12278 µm$^3$. The low nutrient content then observed a value of 18463 ± 3129 µm$^3$. This three-fold increase in EPS production may be due to greater nutrient availability, which impacts the development of biofilm architecture. A decrease in the biofilms’ biovolume at the high nutrient concentration may be due to cell starvation. Sani *et al.* observed a drop in biomass of *E. coli* strains between 2-5 days of biofilm starvation, noting that the response of biofilms due to starvation are strain specific (Sani *et al.*, 2011) This response due to starvation may be because the biofilm becoming stressed. In a mixed species biofilm, Lee *et al.* noted that the biomass of a single species within this biofilm was significantly higher than that of the other species within the
same biofilm, when nutrient reduction occurred. They further postulated that this may be due to the competition within the biofilm between the different bacterial species (Lee et al., 2014). This indicates that different species react differently to nutrient concentration, depending on the environment in which it is grown.

Moreira et al. determined in a study that biofilm grown at low shear stress can be influenced by nutrient concentration (Moreira et al., 2015). Higher EPS production assists in the growth and proliferation of the attached bacteria (Flemming, 2002; Vanysacker et al., 2014). No significant difference was observed in the substratum coverage and thickness of high and low nutrient biofilm. Biofilm roughness, for both the bacteria and EPS, displays a significant difference in high and low nutrients (p = 0.04 and p = 0.048). As the shear stress is caused by the medium within the air-liquid interface of the biofilm, the roughness of the biofilm surface may be unexpectedly influenced due to erosion. The varying hydrodynamic shear forces at the interface may cause the removal of cells at the biofilm surface and change the biofilm characteristics.

The rinsing of the biofilm coverslips prior to the CLSM analysis may also effect the biofilm characteristics, in particular that of the biofilm surface and thickness. As the biofilm is grown in King B, there may be nonadherent debris on the biofilm surface, which can interfere with CLSM results. In oder to prevent detection of this debris, the biofilm is rinsed; this, however, may result in biofilm damage. The experiments are conducted in triplicate to reduce the damage measured by CLSM during rinsing, providing a more accurate description of the biofilm.

Figure 14 displays the EPS and bacteria of high nutrient and low nutrient biofilms. The glass surface was covered with microbial colonies, indicating that heterogeneous biofilm growth occurred. The higher nutrient biofilm exhibits larger clusters with EPS (red) production around regions of bacteria. Conversely, the low nutrient biofilm presented as distributed more with small colonies, which covered the majority of the surface. The red EPS regions coincide with existing regions of green Ps. fluorescens.
Figure 14: CLSM imaging of high nutrient *Ps. fluorescens* biofilm with bacteria stained with Syto 9 (green) and EPS stained with Con A (red). The biofilms were randomly selected.

The depth distribution profile of a biofilm’s characteristics is shown in Figure 15 below. The biofilm coverage was analysed in 1µm sections from the glass substrate to the top of the biofilm surface. Each 1µm section was 1µm of the biofilm depth. The high nutrient biofilm was analysed in 15 sections and the low nutrient biofilm was analysed in 13 sections. This was due to the variable thickness of the biofilm; the biofilm depth was normalised to compare the four different samples. Normalisation of the data was used to convert the different z values of the biofilm depth to a common scale. The equation used was as follows:

$$Z' = \frac{z-z_{min}}{z_{max}-z_{min}} \times 100$$  \[3.1\]

where the $z$ is the biofilm depth, $z_{min}$ is the minimum value of the biofilm depth and $z_{max}$ is the maximum value of the biofilm depth. The surface coverage (%) is the percentage of surface covering each individual 1µm section of the biofilm from the glass to the top of the biofilm. High nutrient and low nutrient surface coverage for bacteria and EPS is shown below in Figure 15. Errors of distribution profiles are the standard error of three randomly selected biofilms.
Figure 15: Distribution profile of biofilm surface coverage at 1µm biofilm depth increments for four 24-hour *Ps. fluorescens* biofilms with high and low nutrient factors and with surface coverage (%) shown for EPS and stained bacteria. High nutrient for stained bacteria and EPS are displayed in A and B respectively, and the bacterial growth with low nutrients as C and D respectively.

The lower bacterial and EPS surface coverage was found to be near the glass/biofilm interface and at the top of the biofilm (Mueller *et al*., 2006). This lower biofilm growth was observed by Möhle *et al* who determined that either the stain incubation time was not long enough or the laser of the CLSM was not able to scan through the dense biofilm (Möhle *et al*., 2007). As the substrate glass was in the 24-hour *Ps. fluorescens* biofilm experiment, the biofilm was scanned from bottom to top of the biofilm. This would suggest that the CLSM would be capable of penetrating to the required biofilm depth. Therefore, increasing the stain incubation time may result in deeper staining of the biofilm depth. The glass/biofilm interface near 100% of the normalised biofilm depth saw a reduction in bacteria and EPS. This may be due to older bacterial cells, which formed the initial structure of the biofilm, dying. As the biofilm grows, the nutrients must be transferred from the top to the initial layer. If
this was not adequately supplied while the biofilm thickness continues to increase, the initial biofilm layer may become starved of nutrient and die. This may be the resultant decease seen in all the distribution profiles. The highest peak of the distribution profile relating to the high nutrient factor was the EPS with a surface coverage peak of 26% and a bacterial surface coverage peak of 14%. When compared to the low nutrient factor, *Ps. fluorescens* substratum coverage was found to be 15.9% and EPS surface coverage of 11.7%. As there was very little difference between the peak surface coverage of the *Ps. fluorescens* regardless of nutrient factor, this correlates with the surface coverage found in Table 5. However, larger differences are found in the peak surface coverage of the EPS; this may be because of the significant differences in biovolume. Due to the increased EPS biovolume with higher nutrients, a greater surface coverage near the centre of the biofilm indicates that more EPS production in that region may be influenced by the nutrient availability.

Fagerlind *et al.* (Fagerlind *et al.*, 2012) developed a computational model to predict the cell death and dispersal within biofilms. They assessed the three factors which would govern the cell death: starvation, cell damage and cell viability. From this model, they determined that, as the biofilm grows, the nutrients from the bulk liquid diffuse through the biofilm. However, the thicker the biofilm grows, the less nutrients reach the bottom of the biofilm. This suggests a steep nutrient gradient, where the nutrients progress down through the biofilm and are absorbed by the biofilm’s top and middle sections, culminating in starvation of cell and subsequent cell death and detachment of the lower biofilm section. This model concurs with the lower surface coverage (%) of bacteria near the glass/cell interface, which shows a decrease in live bacteria, and may also be seen in the EPS production for the high and low nutrient biofilms. According to the model by Fagerlind *et al.*, a reduction in nutrient availability would begin to impact the biofilm growth near the centre of the biofilm at approximately 50% of the normalised biofilm depth. The higher nutrient biofilm produces more EPS than the lower nutrient biofilm, as discussed; this may be due to the nutrient gradient further limiting nutrient access to bacteria within the middle and lower sections of the biofilm, which was evident in the 11% substratum coverage peak of the low nutrient biofilm.
3.3.2 Dynamic Biofilm Growth

The analysis of 48-hour *Ps. fluorescens* biofilm, which was grown on glass in a flow cell with nutrient factors of 1:1 (High Nutrient) and 1:10 (Low Nutrient), is described in section 3.2.4. These two experiments were conducted at different flow rates of 0.4mLmin\(^{-1}\) and 0.7mLmin\(^{-1}\), which is presented in Figure 16.

![Figure 16: Biovolume, substratum coverage, mean thickness and biofilm roughness of 48-hour *Ps. fluorescens* biofilm grown in flow cell with low nutrients (green) and high nutrients (red), two different flow rates of 0.4mLmin\(^{-1}\) (low flow rate) and 0.7mLmin\(^{-1}\) (high flow rate).](image)

Initial comparisons of the *Ps. fluorescens* biofilm growth at high nutrient conditions with high and low flow rate show no significant difference in biovolume (p = 0.17),
substratum coverage (p = 0.20), thickness (p = 0.39) and roughness (p = 0.83). Further observations of the low nutrient conditions with high and low flow rate also show no significant difference in characteristics of biovolume (p = 0.14), thickness (p = 0.56) and roughness (p = 0.69). Substratum coverage showed a difference (p = 0.04) with 37.8 ± 6.6 at a high flow rate and 22.4 ± 1.8 at a low flow rate. The change in flow rate did not have an impact on the biofilm’s characteristics; this may be due to the low difference in the flow rates. It could be considered that the difference in substratum coverage could be due to the low nutrients: at a slightly higher flow rate, more nutrients would be provided to the biofilm. While no difference in bacterial growth was shown due to differences in shear stress, a significant difference for both flow rates between the high and the low nutrient factor was detected. For a low flow rate, the high nutrient content was at a lower total biovolume of 4296417 ± 440788 µm³ and a low nutrient content of 6480892 ± 366296 µm³. This is comparable to the high flow rate with high nutrient content *Ps. fluorescens* biofilm which also displayed a lower total biovolume of 3298928 ± 435886 µm³ and 8285581 ± 1079271 µm³ for a high flow rate and low nutrient content. Both high and low flow rates show a significant difference in biovolume due to nutrients (low, p = 0.008 and high, p = 0.005).

Surface topography of the glass is considered negligible; in industrial conditions, however, the membrane surface is a factor that must be considered. In Chapter 2, surface topographical heterogeneities are considered as areas of low shear stress, providing cover for the bacteria and subsequent biofilm. This low shear, when compared to the low shear of the flow cell, suggests that nutrient concentration has greater influence on bacterial adhesion than the shear stress. However, other influencing factors such as hydrophobicity of the membrane and bacteria are not considered in this chapter and may have a greater influence on bacterial adhesion and proliferation.

The biofilm thickness at low nutrients was approximately 92µm for a high flow rate and 91µm for a low flow rate. This was in contrast to the high nutrient grown biofilm with a thickness of around 10-20 µm less than the lower nutrient thickness, regardless of the flow rate. The biofilm roughness, however, showed no significant difference with the high flow rate corresponding to a p value of 0.238. The low flow rate differs in this regard with a significant difference between the two nutrient factors (p = 0.008).
Based on this, it can be determined that the *Ps. fluorescens* growth, with the total biovolume and surface coverage, was influenced by changes in nutrient availability, in particular at low flow rates in the case of substratum coverage. As previously discussed in section 3.3.1, the biofilm stress response to lower nutrient concentration differs to that of the air-liquid interface grown biofilm. This may be due to a number of factors; firstly, the flow cell biofilm is grown for a longer period of time, which may contribute to a greater biofilm production during a longer stressed response, as observed by Sani *et al.* (Sani *et al.*, 2011), secondly, the flow cell is an enclosed environment and biofilms are observed *in situ*, and, thirdly, there is no requirement to wash the biofilm and, therefore, no damage to the biofilm occurs.

The differences in biofilm growth become more prominent with lower nutrients. The 1:10 nutrient factor appears to produce a thicker biofilm, covering a greater surface, which, in turn, results in a greater total biovolume, when compared to the high nutrient biofilms.
Figure 17: Representative CLSM images of high nutrient (A-B) and low nutrient (C-D) *Ps. fluorescens* biofilms at high (A, C) and low flow rate (B, D). One representative biofilm for each nutrient factor and flow rate was chosen at random.

Figure 17 shows the biofilm growth of high nutrient and low nutrient *Ps. fluorescens* biofilms at high and low flow rate. The biofilms above show more surface coverage with lower nutrients, while higher nutrient biofilms are more clustered with less surface coverage. No differences in the flow rate were observed in the CLSM images. This corresponds with the graphs of Figure 16. In order to obtain a better image of the distribution of the bacteria throughout the biofilm, the distribution profile of each biofilm was examined.
Figure 18: Distribution profile of biofilm surface coverage at 1µm biofilm depth increments for four 24-hour *Ps. fluorescens* biofilms with high (High Nutrient) and low nutrient (Low Nutrient) factors at two different flow rates, one at 0.4 mLmin\(^{-1}\) (Low Flow Rate) and 0.7mLmin\(^{-1}\) (High Flow Rate). Errors of distribution profiles are the standard error of two randomly selected biofilms.

The distribution of bacteria throughout the biofilm in Figure 18 above displays the changes in surface coverage of the CLSM stack in the z-direction, shown in Figure 11, due to high and low nutrient factors. As a result of the variable thickness of the biofilm, the depth was normalised to compare the four different biofilms. Coverage of the biofilm was found at the glass/liquid interface, with 0% at the glass and 100% at the biofilm-liquid interface. The peaks of all distribution profiles were found to be between 30- 60%. High nutrient biofilms showed very little variation in bacterial attachment throughout each 1µm section of the biofilm. The *Ps. fluorescens* biofilm maintained a mean highest peak of approximately 17%, regardless of the flow rate. Both biofilms show a change in EPS and bacterial surface coverage between the
glass/biofilm interface and the biofilm/liquid interface. With regard to the glass/biofilm interface, the substratum coverage starts at approximately 7% for the high flow rate and at 2.7% for the lower flow rate. This suggests a greater quantity of the bacteria at the bottom of the flow cell with a higher flow rate.

The distribution throughout the low nutrient presented two distinct profiles. The mean peak of the low flow rate with low nutrient reached a maximum of 16% with small fluctuations in the overall bacterial growth. The maximum peak was distributed closer to the glass than the previously grown biofilms at 34% normalised biofilm depth. For the low nutrient, high flow rate there was a much larger difference in the biofilm distribution when compared to the other distribution profiles of around 16-17%. The peak substratum coverage indicated a maximum of 63% within the 30-60% normalised biofilm depth. The higher flow rate biofilm, unlike the high nutrient biofilms discussed, saw a different substratum coverage and bacterial survival at the glass/biofilm interface. The low flow rate resulted in a 14.6% substratum coverage, while the higher flow rate showed a substratum coverage of 4.6%. These differences in the two biofilms suggest different biofilm architecture.

For high nutrient grown biofilms, there were little discrepancies in the architecture due to a high or low flow rate. However, a noticeable difference in biofilm architecture was observed with low nutrient grown biofilm for both high and low flow rates. This would suggest that the slight differences in flow rate result in differences in the biofilm formation with lower nutrient availability. Rochex et al. noted that increasing shear stress slows down the biofilm maturation producing a younger biofilm, when growing biofilm in a Conical Couette–Taylor Reactor (CCTR) from paper machine process water. This was observed as a biofilm of greater biomass and thickness (Rochex et al., 2008). While this was not noticeable in the high nutrient grown biofilm, it may be due to the increased nutrient availability causing an increase in the biofilm development faster than the erosion and sloughing of biofilms caused by the shear stress (Duddu et al., 2009). For the low nutrient grown biofilms there was a three-fold peak difference in the biofilm centre, when comparing the high and low flow rates. The differences in development were in agreement with studies by Moreira et al., as previously discussed in section 3.1. There was an observed higher development of biofilm with dynamic conditions when subjected to conditions of high shear stress and low nutrient levels (Moreira et al., 2013).
3.4 Conclusion

In order to demonstrate the influence of nutrient concentration and shear stress on the structure of *Ps. fluorescens* biofilm, two experimental set-ups were utilised: air-liquid interface for 24-hour biofilm growth and flow cell at 48-hour biofilm growth. CLSM analysis of the 24-hour biofilm grown at the air-liquid interface showed a significant difference in the biovolume of the high and low nutrient grown biofilms. This may be due to a stress response as the lower nutrient biofilm produces less bacteria and EPS than that of the higher nutrient biofilm. A three-fold increase in EPS biovolume was noted for high nutrient availability. With the 48-hour growth of biofilm in the flow cell, the introduction of shear stress results in a noticeable increase in biovolume of low nutrient *Ps. fluorescens* biofilm at a high flow rate, contrary to what was observed with the air-liquid interface. This difference at low nutrient and higher flow rate may be a stress response due to the biofilm producing more EPS to protect itself from the combination of the higher shear and low nutrient availability, as there was no noticeable difference of biofilm grown in high nutrient and high shear conditions. This demonstrates that shear stress, combined with the stress response of low nutrient availability, promotes the biofilm growth in the *Ps. fluorescens* species. This indicates that nutrient concentration and shear stress are important factors in the propagation and proliferation of biofilms; this response, however, is limited to *Ps. fluorescens*. In order to further understand the biofilm growth due to nutrient concentration, an analysis of the adhesive characteristic and stiffness of the biofilm is required.
CHAPTER 4

Quantitative AFM analysis of biofilm formation with differing nutrient factors

Abstract

AFM indentation on biofilms is a well-established technique and employed to establish the mechanical properties. Using this technique, the indentation of biofilms results in force-distance curves from which the information on the biofilm properties, such as elasticity and adhesive force, can be obtained. This information is important in the further quantification and understanding of biofilm growth on surfaces. While the characterisation of biofilms is ongoing, it has been observed that, while bacteria develop in different environment conditions, stressors, such as pH, shear stress, antibiotics and nutrient availability, have resulted in complex responses in biofilm and are identified as parameters that influence biofilm development. For this reason, the quantification of biofilm, using AFM force-curves, has become essential in determining these biofilm characteristics. The changes in the mechanical properties of the developing biofilm due to nutrient availability is one such environmental stressor, which is the focus of this chapter. AFM was used to determine the adhesive and viscoelastic properties of the *Ps. fluorescens* air-interface grown biofilm for two different King B nutrient dilution factors, one at 1:1 (referred to as high nutrient) and another at 1:10 (referred to as low nutrient). The qualitative imaging of the biofilm distribution was performed using SEM. The analysis showed that the low nutrient biofilm had twice the Young’s modulus in comparison to the high nutrient biofilm, with values of 4.98 ± 0.02 kPa and 2.35 ± 0.08 kPa respectively. The decreased nutrient availability resulted in a higher adhesion force and work of adhesion with distributed colonies across the surface, when compared to the high nutrient biofilm. Overall, the high nutrient biofilm led to a reduction in the adhesive and elastic nature of the biofilm, with SEM imaging showing large clusters at the air-liquid interface.
4.1 Introduction

Bacterial adhesion onto substrates and subsequent biofilm formation are issues that can cause contamination to a surface interface. Once biofilm formation occurs, removal can be difficult and often damage the substrate surface (Ang et al., 2006; Varin et al., 2013; Vrouwenvelder et al., 1998). There are many factors which influence the biofilm growth and development, such as surface properties, physico-chemical properties, temperature, nutrient concentration and shear stress, to name a few (Dourou et al., 2011; Habimana et al., 2014; Jung et al., 2013). However, the underlying mechanisms of the biofilm development due to nutrient availability are not fully understood. Research into the biofilm and its reaction to nutrient concentration is required to further understand the fundamental principal behind biofilm growth to surfaces. Many environmental stressors, such as antibiotics and nutrient levels, result in unique biofilm architecture consisting of EPS secreted to protect cells that are established within the biofilm.

AFM is a useful tool to determine the adhesion forces of this biofilm, since the EPS secreted by the bacteria is a sticky substance and, thus, may be detected. While many studies focus on the adhesion of multiple cells (Myint et al., 2010; Semiao et al., 2013) and of biofilm growth (Flemming & Wingender, 2010), few observe the adhesion forces of biofilm due to nutrient concentration. AFM nanoindentation has advanced into a technique capable of providing adhesive and cohesive forces of both single cells and biofilm aggregates (Abe et al., 2011; Safari et al., 2014). The Hertz model (Hertz, 1882) is employed in the nanoindentation experiments to estimate the elastic modulus of the surface indented (Dufrène, 2015; Touhami et al., 2003). This well-established model provides an estimate of the elastic modulus from the area of non-adhesive contact of an indentation curve. In order to demonstrate the adhesive properties of a material, the retraction section of the indentation curve is analysed. The adhesive property is an important factor used to indicate the quantity of EPS produced by the biofilm (Beech et al., 2002; Méndez-Vilas et al., 2007). Razatos et al. demonstrated that adhesion forces of E. coli cell surface was effected by the polysaccharide produced (Razatos et al., 1998). Further studies by Fang et al. considered the differences in cell-tip adhesion force and cell-
cell adhesion force of sulphate, reducing bacteria. Bacterial adhesion on mica was also due to the EPS production of the bacteria (Fang et al., 2000).

As the differences in adhesive forces vary, the type of cantilever tip is an important component to consider, when performing nanoindentation. Standard cantilevers such as Si$_3$N$_4$ pyramidal tips have been widely used to determine the single point force of a biofilm (Fang et al., 2000; Wang et al., 2015). However, these cantilevers have an estimated tip radius, which, when entered into the Hertz model, can lead to differing results (Beech et al., 2002). Colloid cantilevers provide a larger and easier to measure tip, since microspheres of known radius are attached to a tip-less cantilever. In this study, nanoindentation is employed to investigate Ps. fluorescens biofilm formed at an air-liquid interface for 24 hours with two different nutrient concentrations. These biofilms were evaluated using a 10µm colloidal cantilever for their adhesive properties and an estimate of the elastic modulus is determined to assess the levels of EPS secreted in the biofilm matrix.

4.2 Methods

4.2.1 Bacterial Preparation

Ps. fluorescens PLC1701 (Lagendijk et al., 2010) was selected for the air-liquid interface biofilm assays. A single colony was selected from Ps. fluorescens stored at -80°C in King B (King et al., 1954) broth supplemented with 20% glycerol. This was then grown on King B agar at 28°C. Prior to the experiments, Ps. fluorescens were grown in King B medium supplemented at a final concentration of 10 µg.mL$^{-1}$ of gentamicin (Sigma Aldrich, Ireland) at 28 °C and 75 r.p.m. for 16 hours, reaching cell optical density of OD$_{600}$ 0.8-1.0. The bacteria were separated from the medium by centrifuge at 7000 r.p.m for 10 mins. The supernatant was discarded and the bacterial pellet was re-suspended in King B. The King B medium was measured at a pH of 7.4.
4.2.2 Air-Liquid Interface Biofilm

*Ps. fluorescens* biofilm was grown at an air-liquid interface in orbital agitation, as discussed in section 3.2.2 (Safari *et al.*, 2014). A coverslip of Borosilicate glass was inserted into a falcon centrifuge tube, and a 10mL pipette was inserted to prevent movement of the coverslip. The centrifuge tube was sealed using cotton wool and autoclaved to ensure sterility. Two different experiments were conducted, one with a King B dilution factor of 1:1 (high nutrient) and the other with a dilution factor of 1:10 (low nutrient). Following this, 3mL of King B of dilution factors 1:1 and 1:10 were inserted into the separate sterile centrifuge tubes. The 3mL of low or high nutrient King B was supplemented with gentamicin at a final concentration of 10 µg.mL⁻¹. Each centrifuge tube was inoculated with 5µl volume of the re-suspended overnight culture. These cultures were incubated at an orbital agitation of 75 rpm and temperature of 28°C for 24 hours. This produced a biofilm at the glass air-liquid interface, as outlined in yellow in Figure 19:

Figure 19: Air-liquid interface 24-hour grown biofilm. The fluorescent image was taken using a fluorescent microscope (Nikon, Japan).
4.2.3 Cantilever Materials and Preparation

Pyramidal cantilevers were not used since their tip can become blunt over several measurements, thus, resulting in reduced measurement quality (Bykov et al., 1998). The use of colloid probes is an adaptable way of measuring colloid-biofilm interactions with greater accuracy due to a larger, quantifiable tip (Beech et al., 2002). In principle, the creation of colloid cantilevers (Ducker et al., 1991) offers a technique capable of modifying the type of colloid material that is used and the colloid surface, providing a versatile platform for the quantification of adhesive forces (Beaussart et al., 2013; Urbiztondo et al., 2009).

AFM cantilevers were created by manually manipulating a 10µm silica sphere (Whitehouse Scientific, UK) onto a *NSC 12 E* tip-less cantilever, using an adhesive resin. In preparation for attaching the colloid to the probe, a glass slide (VWR, Ireland) was utilised to hold the cantilevers in place during colloid manipulation. Carbon tape (Agar Scientific, UK) was placed at the end and ¾ the length of the glass slide. A second glass slide was attached to the top with the end of the slide attached to the carbon tape at the ¾ length mark. Approximately 2cm of carbon tape was placed on the end of the slide. The *NSC 12 E* tip-less cantilever (MicroMasch, Lithuania) was placed protruding over the edge with the carbon tape holding it in place, as shown in Figure 20:

![Figure 20: Glass slide set-up for colloid and glue application.](image-url)
In preparation for colloid manipulation, a glass pipette was modified to be approximately 1mm in diameter. This was constructed by dipping the middle of the glass pipette into a blow torch flame. The pipette was gently pulled and rolled while pulling out of the flame; this was repeated until the diameter was reduced to approximately 1mm. Once the 1mm diameter was obtained, the two ends were pulled apart, creating two ends with reduced diameter. These ends were examined and any modified pipettes that were damaged or that possessed a larger diameter were removed.

On two separate glass slides, a small amount of silica beads were placed onto one of the glass slides, using a tweezers. On the other, a drop of UV curable epoxy resin (TE Connectivity Chemicals, USA) was mixed and placed on the other glass slide. The glass pipette with a 1mm diameter was attached to a micromanipulator DC-3K with a push button controller MS 314 (Märzhäuser Wetzlar GmbH & Co. KG, Germany). The glass pipette was manoeuvred into the edge of the epoxy resin and a small amount was retained on the pipette tip. Using the micromanipulator, the pipette tip was directed to the tip-less cantilever and small amounts of resin were transferred on contact. The pipette, which transferred the resin, was then replaced with a new pipette and a 10µm silica sphere MSS1-10 (Whitehouse Scientific, United Kingdom) was attached to the epoxy on the surface of the cantilever, using the same process. The colloidal probe was subsequently cured in an oven at 100°C for 1 hour. The cantilevers were then imaged for quality by SEM, and any visually damaged probes were discarded.

4.2.4 Atomic Force Microscopy and Nanoindentation

AFM was performed on biofilm to obtain the indentation and retraction curves to determine the elastic and adhesive properties. These force measurements were performed using an Asylum Research MFP-3D AFM (California, US) and Nikon Ti/E fluorescence microscope (Nikon, Japan), which were placed on a vibration table (TS-150, JRS Scientific Instruments, Switzerland), and enclosed in an acoustic isolation chamber. The Asylum Research software was employed during acquisition of force curves. For sample preparation, air-liquid interface biofilms were rinsed in a 0.1M NaCl solution and subsequently placed in the AFM holder containing PBS.
The experiments were performed in duplicate for each biofilm condition and biofilms remained in PBS solution, with ionic strength of 1mM, during measurement. The 10µm colloid cantilevers were prepared, as shown in section 4.2.3, and calibrated at 0.13N m⁻¹ at room temperature.

The elastic and adhesive properties were obtained by analysing the deformation of the cantilever observed by the Piezo displacement. Initial indentation was performed on a glass surface and immersed in PBS to determine the cantilever deflection sensitivity. This was subsequently subtracted from the total Piezo displacement during biofilm indentation (Hutter et al., 1993; Jones et al., 2002; Touhami et al., 2003) allowing for the calculation of the indentation depth. The deflection of the cantilever was used to calculate the force, using the following formula (Carl et al., 2008):

\[
\text{Force [N]} = \text{Spring Constant [N/m]} \times \text{Cantilever Sensitivity [m/V]} \\
\times \text{Deflection [V]}
\]

where the cantilever sensitivity is the position of the laser on the cantilever. The calibration was performed by the probe calibration within the Asylum Research MFP-3D AFM software (California, US), which utilises the thermal noise method (Hutter & Bechhoefer, 1993). An interval of 30 min was allowed, prior to force curve acquisition, to minimize the fluctuations caused by the thermal noise. Force curves were performed on biofilm at the air-liquid interface at various locations on the biofilm samples. These were performed in a two-dimensional array of 4 × 4 (16 force curves) over a 20 × 20 µm² area. At least 100 force curves measurements were obtained for each biofilm at a scan rate of 0.5 µms⁻¹ and force set point limit of 8-12nN. After each force map, the cantilever was tested on glass to ensure that no biofilm residue had attached. If tip contamination had occurred, the cantilever was rinsed in ethanol, then MilliQ water, and placed in a UV ozone cleaner (ProCleaner, Bioforce Nanosciences, Iowa, USA) for 15 mins.

Initial data-processing was performed with the Asylum Research MFP-3D AFM software by providing the force (nN) from the raw photodetector signal (voltage). The data was then converted, using a procedure file provided, into a format for Protein Folding and Nanoindentation Software (PUNIAS,
http://punias.free.fr/) analysis, using Sneddon’s modification for the Hertz model (Carl & Schillers, 2008). The contact point for the Hertz model was manually selected, which was chosen at the point at which the force increases from the zero value. The zero value is the position where the cantilever deflection was independent of the piezo position.

![Figure 21](image_url)

**Figure 21**: Sample force curve measured for 24-hour air-liquid interface biofilm, where $h_1$ is the intersection of the original contact point and the retraction curve and $h_2$ is the end-point of the adhesive energy marker on the x axis. Raw data was modified using PUNIAS software.

Figure 21 is an example of how a Force Curve is analysed. The viscoelastic behaviour of the EPS produced by the biofilm is observed by the hysteresis between the approach (red line) and the retraction (blue line) curve. The adhesive energy ($E_{adh}$) is the area (shown in grey) of the retraction curve. This area was calculated from the original contact point of 0 nm vertical deflection and is measured in attojoules (aJ). The adhesive force ($F_{adh}$) was calculated as the separation distance at the intersection of the original contact point and the retraction curve ($h_1$) and the end-point of the adhesive energy marker on the x axis ($h_2$). This was calculated using the following equation:

$$E_{adh} = -\int_{h_1}^{h_2} F_{adh}$$  \hspace{1cm} (4.2)
The equation for the Hertz model using a spherical tip utilised by the PUNIAS software is:

\[ F_{sphere} = \frac{4}{3} \frac{E}{(1 - \nu^2)} \sqrt{R} \delta^2 \]  \hspace{1cm} 4.3

where \( E \) is the Young's modulus, \( \nu \) is Poisson's ratio, \( R \) is the radius of the sphere and \( \delta \) is the sample deformation. The calculations were based on the assumption that a biofilm is an incompressible material composing of mainly water (up to 97%) (Characklis et al., 1990; Donlan, 2001). Due to the flow properties, the biofilm is considered a viscoelastic polymeric material. Dimitriadis et al. calculated that the Poisson value was 0.5 by computational methods (Dimitriadis et al., 2002). However, due to the change of the biofilm over time, the property can be described as between a rubber elasticity (0.5) to a glassy regime (0.33) (Domke et al., 1998; Laspidou et al., 2005; Liu et al., 2017). Graeves et al. performed a study into the Poisson’s ratio of various materials and concluded that most polymer materials tended to 0.5 due to a shift from elasticity to viscoelastic or time-dependent viscoplastic flow (Greaves et al., 2011). As the assumption was made that a biofilm is an incompressible material, the Poisson ratio of 0.5 was used.

In order to determine the Young’s modulus of the biofilm from the force curve, the equation is derived with respect to the sample deformation. This is shown as:

\[ F_{sphere}^2 = \left( \frac{4}{3} \frac{E}{(1 - \nu^2)} \sqrt{R} \right)^\frac{2}{3} \delta \]  \hspace{1cm} 4.4

The slope of the force curve measurement is taken as the difference in the deformation of the sphere and the sample. By manipulating equation 4.3 to show the difference between the sample deformation \( \delta \) and the sphere \( F_{sphere} \), the following equation is obtained:
\[
\left(4 \frac{E}{3(1 - \nu^2)} \sqrt{R}\right)^{\frac{2}{3}} = \frac{\Delta F_{\text{sphere}}}{\Delta \delta} = \text{Slope}
\]

From the previous equation, the Young's modulus can be calculated in relation to the slope (Carl & Schillers, 2008):

\[
E = \frac{3}{4} \left(\frac{\Delta F_{\text{sphere}}^{2/3}}{\Delta \delta}\right)^{\frac{2}{3}} \frac{1 - \nu^2}{\sqrt{R}} = \frac{3}{4} \text{slope}^{3/2} \frac{1 - \nu^2}{\sqrt{R}}
\]

This allows for easy calculation of the Young’s modulus from the force curve slope. The Young’s modulus was used to determine the tensile elasticity, while the adhesion force provides the attractive force between the colloid and biofilm. Finally, the work of adhesion was the energy required to separate the colloid from the biofilm surface. Force curves were performed on biofilms with low nutrient and high nutrient availability with the values of Young’s modulus, adhesive force and work of adhesion for each. Young’s modulus graphs were displayed using SigmaPlot 12.0 (Systat Software Inc.).

4.2.5 Confocal Laser Scanning Microscopy

In order to observe the 3D structure, CLSM images were acquired using an Olympus FV1000 CLSM (Live Cell Imaging core technology facility platform, Conway Institute, UCD). \textit{Ps. fluorescens} biofilm was grown for 24 hours at the air-liquid interface for both high and low nutrient availability. Biofilms were grown 3 times each, with different inocula and washed in a sterile 0.1M NaCl solution, once removed from the centrifuge tube. Each biofilm was stained with Syto9 and, subsequently, Con A-AlexaFluor633 for EPS and bacterial analysis. After a final rinsing, the biofilm was enclosed in a Nunc Lab-Tek II Chamber Slide. Up to 3 different areas of each biofilm were examined via CLSM. The wavelengths used for
Syto9 excitation were 488nm and 633nm for Con A-AlexaFluor633. The two wavelengths were used for EPS and bacterial analysis Syto9 and Con A-AlexaFluor633, excited at 488 nm and 633 nm respectively. A z-step of 1µm using an Olympus UPL SAPO 60×/1.2 was employed. The biofilms’ images of coverage of EPS and bacteria were implemented using Image J from NIH (https://imagej.nih.gov/ij/).

4.2.6 Scanning Electron Microscopy

SEM images were performed using a dual beam field-emission scanning electron microscope (Hitachi Quanta 3D FEG Dual Beam FE-SEM) at the UCD Nano-imaging and Materials Analysis Centre (NIMAC). SEM was used to analyse air-liquid interface biofilms grown for 24 hours with high and low nutrient availability. Grown biofilms were rinsed in 0.001 M NaCl to remove any excess cells. The biofilm was fixated in a solution containing 2.5% glutaraldehyde, 0.1 M sodium cacodylate, and 0.075% ruthenium red at pH 7.4 for 4 hours (Habimana et al., 2009). Following fixation, the biofilms were washed in 0.1M sodium cacodylate buffer. All samples underwent a step-wise dehydration process of exposing biofilm samples to MilliQ water with increased ethanol volumes of 10, 25, 50, 75, 90 and 100%, each at 10 min intervals according to Herzberg et al. (Herzberg & Elimelech, 2007). The samples were then exposed to 50%, then 100% hexamethyldisilane (Sigma, Ireland) and dried in air. Following drying, the coverslips with fixated biofilm were adhered to SEM aluminium stubs, using a carbon adhesive. The biofilm grown on coverslips samples were coated with a thin layer of gold (Eimtach K575K). Images were taken at an accelerating voltage of 5 kV, current of 5.92 pA and magnifications of 1200x were taken. The samples were analysed using Image J ® software.
4.3 Results and Discussion

4.3.1 Colloid Cantilever Evaluation and Maintenance

Before nanoindentation can be initiated, the colloid must be attached to the end of the cantilever. Fundamentally, the type of material which can be used to glue the colloid to the cantilever is unlimited. However, in the instance of colloid cantilevers, materials in the form of spherical particles with smooth surface are preferred, therefore, limiting the number of available materials. As the size, roughness and material of the colloid cantilever is easier to determine, when compared to the sharp tipped cantilever, this facilitates correlation with the Hertz model (Butt et al., 2005; Kappl et al., 2002). The colloid was selected as a 10µm diameter silica sphere. In order to ensure quality colloid cantilever production, the cantilevers were examined by SEM. The colloid cantilevers were placed on an aluminium stub, using carbon adhesive, and analysed at a voltage of 5kV, current of 5.92pA and up to 3500x magnification. In order to maintain cantilever condition, the gold coating process, typical of sample preparation discussed in Section 4.2.6, was disregarded. Any colloid cantilevers that were visually acceptable were subsequently calibrated and accessed by force curve on glass to ensure no damage had occurred during production. The force curves measurements were obtained for each biofilm in PBS. The force distance curve and an SEM image of a colloid cantilever with no visual defects are shown in Figure 22 below.

Figure 22: Force vs Distance curve for 10µm silica colloid cantilever on glass in PBS.
Any contamination or surface irregularities of the colloid cantilever under SEM resulted in the cantilever’s disposal. Up to 5 cantilevers of similar quality, as shown above in Figure 22, were produced. The diameter of these colloid sphere tips was measured by SEM; the average diameter was 9.81µm with a standard error of 0.16µm. This was found to be smaller than the 10µm sphere predicted. While SEM is a viable technique in the visual appraisal of the colloid, another aspect of the colloid cantilever properties is the surface roughness. Yang et al. measured the surface roughness and surface heterogeneities of glass colloid probes used in AFM adhesion measurements. They concluded that these probes had surface roughness with multiscale features, such as surface heterogeneities, which require a more effective adhesion model, including surface topographies. (Yang et al., 2007). However, by adding further measurements of topography to characterise the quality of the colloid cantilevers, damage may occur, resulting in greater defects of the colloid cantilever. As the topographical features of the silica bead vary, it is assumed the colloid is a smooth surface. The zeta potential of the colloid cantilever is taken from Wu et al. as -65Mv; in a low ionic 0.1Mm solution, and, therefore, electrostatic interactions based on the DLVO theory were considered (Wu et al., 2008). However, judging from the force curves observed in Figure 21 and further on in Figure 24, the long distance of the interactions of the force curves is greater than that of the electrostatic interaction forces, as observed by Dufrêne et al. and Bowen et al. (Dufrêne et al., 2017; Bowen, 1998). Therefore, these forces are considered negligible.

While 5 out of 13 colloid cantilevers were created that performed within the acceptable standard, several others were disposed of due to varying defects, such as damaged colloid, no colloid or glue contamination, as shown in Figure 23. After the calibration of colloid cantilevers, measurements were performed. As parts of the biofilm may have adhered after force measurements, several force curves were taken, indicating the colloid condition (Safari et al., 2014). Any failed colloid probes were cleaned and re-examined, using SEM to confirm removal of contamination.
4.3.2 Biofilm Nanoindentation

The adhesion force, work of adhesion and Young’s modulus of 24-hour \( Ps. \) \textit{fluorescens} air-liquid interface biofilm, grown with low and high nutrient availability, were characterised using AFM. According to these results, selected elasticity profiles were displayed (Fig 23), histograms of Young’s modulus distribution were plotted (Fig 24), and both adhesive force and work of adhesion were calculated (Table 6). Biofilms grown were analysed by CLSM and shown separately in section 3.3.1, which noted that, while there was no increase in biovolume, a three-fold increase in EPS biovolume was observed for high nutrient biofilms. Additionally, biofilm imaging displayed high nutrient biofilms in large clusters, while low nutrient biofilm developed smaller, distributed colonies.

The elastic profiles presented in Figure 24 show the force-indentation profiles of 24-hour grown biofilms with high nutrient availability, low nutrient availability and glass calibration curve. All force curves were performed in PBS with a set-point limit of 9-12nN. Based on the results, the high nutrient biofilm samples displayed a larger indentation depth \((0.20 \pm 0.08 \mu m)\), when compared to the smaller indentation depth of the low nutrient biofilm sample \((0.08 \pm 0.007 \mu m)\). This was less than 10% of the overall biofilm depth measured by CLSM (section 3.3.1), which is within the valid range for the Hertz model. The differences in biofilm force indentation curves indicate a stiffer biofilm sample surface with low nutrient availability. O’Toole \textit{et al.} determined that \( Ps. \) \textit{fluorescens} cells are influenced by the osmolarity of the medium, which would result in differences in biofilm development. They postulated that salt-mediated effects can result in ionic changes and, thus, biofilm changes; however,
osmolarity of the overall medium may be a vital indication of biofilm formation (O’Toole et al., 1998).

Research into biofilm development focuses on EPS matrix development in the formation of the biofilm architecture. Aimhou et al. determined the cohesive strength of 12-day biofilms, which correlated with polysaccharide concentrations and showed an increase in cohesive strength with biofilm depth (Ahimou et al., 2007). This cohesive strength may be due to the EPS composition. The low nutrient samples resulted in less indentation into *Ps. fluorescence* biofilms, which may be due to the continued EPS production similar in composition of the lower depths of biofilm. The lower nutrient levels of the biofilm may form a stiffer EPS matrix to protect the bacteria within, which would correspond with observations made using CLSM of reduced EPS, as observed in section 3.3.1. The low nutrient grown biofilm displayed less substantial EPS production; because of this, the AFM colloid tip was likely in contact with cells covered in a smaller quantity of EPS. In comparison, a greater indentation depth was observed with the high nutrient biofilm indicating a greater EPS layer that covered the biofilm surface.

![Elasticity profiles of selected force-indentation profiles of high nutrient (blue), low nutrient (red) and glass calibration curve (black).](image)

Figure 24: Elasticity profiles of selected force-indentation profiles of high nutrient (blue), low nutrient (red) and glass calibration curve (black).

The non-linear indentation curve of both biofilm samples may be due to sub-layers, as discussed by Safari et al. and suggested by both Stewart & Franklin and Habimana et al. (Habimana et al., 2009; Safari et al., 2014; Stewart & Franklin,
These sub-layers indicated heterogeneous chemical and physiological levels within the biofilm structure; however, the sudden non-linear indentation area may be caused by the deformation of the top EPS layer surface. As the colloid compresses the biofilm, the non-linear indentation may be a result in the buckling of the viscoelastic surface (Biot, 1964; Suleiman et al., 1981). Other forces can also interact with the initial surface contact, such as capillary forces (Alessandrini et al., 2005; Xu et al., 1998), which can be reduced. When performed in liquid, however, these may influence the initial contact. Dagang et al. showed that 96-hour static grown *Ps. fluorescens* biofilms influenced the cantilever probe by steric forces and hydrophobic interactions for more than 100nm prior to contact due to the biofilm EPS. These forces may continue to interact with the colloid cantilever indentation (Dagang et al., 2016).

The Young’s modulus of 24-hour *Ps. fluorescens* air-liquid interface biofilm, grown with low and high nutrient availability, is shown in Figure 25. As can be seen, Young’s modulus values were displayed by histogram because of the high variability of the measurements. This can be attributed to two causes: one is the difference in measurements across the biofilm surface, the other is the variability produced from the three repeat cell cultures performed for each experiment (Touhami et al., 2003). Moreover, the complete overlap of approach and retraction curve during nanoindentation may not occur as the biofilm can display a limited degree of plastic deformation (Zeng et al., 2014), which may result in higher elastic values. The low nutrient biofilm displayed a higher average elastic modulus of 4.98 ± 0.02 kPa compared to the lower average value of 2.35 ± 0.08 kPa for a high nutrient biofilm with standard error of the mean. The elastic modulus ranged from 3 to 9.5 kPa for low nutrient, whereas the elastic modulus ranged from <0.1 to 10.5 kPa. This shows that the low nutrient biofilm is twice as stiff as the high nutrient biofilm. The elastic modulus is higher than that of Zeng et al. who conducted nanoindentation on *Ps. fluorescens* biofilm using a 59.2 μm colloid cantilever, which produced a Young’s modulus of 0.10 ± 0.01 kPa (Zeng et al., 2014). This was at the lower end of the high nutrient biofilm, however, the size of the cantilever used and the process of biofilm growth may have influenced this lower value of the Young’s modulus.
Figure 25: Histogram of the Young’s Modulus (kPa) distribution of 24-hour grown *Ps. fluorescens* biofilm at low and high nutrients. (A) is the Young’s Modulus of high (blue) and low nutrients (yellow), (B) is the breakdown of the Young’s Modulus at high nutrients between 0 and 1 kPa, as highlighted in the red section of graph (A).
Higher levels of EPS were present in the high nutrient biofilm, as previously mentioned in section 3.3.1, and the EPS would result in a more elastic response of the biofilm. In contrast, the higher elastic modulus of the low nutrient biofilm could be due to the lower EPS produced, which would cause more rigid cells to be closer to the surface during nanoindentation. EPS production significantly alters the physical structure of the cell-substrate interface, which may result in a softer biofilm, while with less EPS coverage, higher cell-colloid interactions could occur. Safari *et al.* noted that *Ps. fluorescens* biofilm with the addition of Calcium ions produced higher EPS sugar residues in 48-hour air-liquid interface biofilms. However, biofilm elastic and mechanical properties were reduced arising in a reduced Young’s modulus (Safari *et al.*, 2014). This modification of biofilm formation suggests specific bacterial response depending on nutrient content. As discussed in section 1.3.3, Steinberger *et al.* observed that *Pseudomonas aeruginosa* cells, grown on membranes for 16 hours in static conditions, elongated, while a constant width was maintained under lower nutrient. They suggested that this elongation granted bacteria improved nutrient collection without changes in the ratio of surface to volume (Steinberger *et al.*, 2002). This elongation of the bacteria may result in indentation occurring on the bacterial surface for the low nutrient biofilm rather than on an EPS covered surface. Therefore, a higher elastic modulus may be caused by the reduced EPS and the bacterial starvation due to lower nutrient availability.

The average adhesive force and work of adhesion of 24-hour *Ps. fluorescens* air-liquid interface biofilm, grown with low and high nutrient availability, are shown in Table 6. From the results shown in this table, it can be determined that the low nutrient grown biofilm has a stickier surface with a 7-fold increase in the adhesive force (P < 0.001). Considering the Young’s modulus results (Figure 25), the low nutrient biofilm produces a hard and sticky biofilm surface, whereas the high nutrient biofilm produces a less sticky and soft surface. The low nutrient biofilm showed a significant difference in the work of adhesion (P < 0.001). For the adhesive properties, an increase in adhesion energy is typically associated with a greater attachment of EPS to the cantilever tip and to the increased volume of EPS (Auerbach *et al.*, 2000; Li *et al.*, 2004b; Safari *et al.*, 2014). This difference in adhesion forces is suggested to occur due to a stronger stretching of polyproteins (Marszalek *et al.*, 1999).
It has been shown that materials secreted in the EPS by different bacterium might vary in their mechanical properties, such as stickiness and viscosity, and that this EPS accumulation can result in a variation in measurement of elasticity (Oh \textit{et al.}, 2009; Volle \textit{et al.}, 2008b). Nutrients, however, may play an important role in the production of EPS, since, as the biofilm grows, the materials produced within the EPS and the amounts of EPS produced can determine the biofilm character (Vobèreková \textit{et al.}, 2016). This \textit{Ps. fluorescens} biofilm formed by the lower nutrient air-liquid interface suggests a stickier surface. Compared to that, however, from previous discussions of CLSM analysis, less EPS was produced, suggesting that, while the bacteria remain, the composition of the EPS produced on the surface of the biofilms is different. Francius \textit{et al.} researched the EPS coverage of \textit{Lactobacillus rhamnosus GG} cells. By comparing a wild type and a mutant strain with limited EPS production, they determined that the cells were covered in a smooth, ridge lattice of globular proteins, the roughness of which was on the nanometre scales, whereas the polysaccharide producing cells were rougher (Francius \textit{et al.}, 2008). As the low nutrient biofilm produced less EPS than the high nutrient, the cantilever may be in contact more with these globular proteins, resulting in the higher adhesive force.

Other properties to consider when discussing adhesive force of the biofilm are the physicochemical and mechanical properties of the colloid cantilever. Surface roughness has been shown to influence the adhesion of bacteria to the surface (Allen \textit{et al.}, 2015; Crawford \textit{et al.}, 2012; Palacio \textit{et al.}, 2012). While it is assumed that the

<table>
<thead>
<tr>
<th>Biofilm</th>
<th>Adhesion Force (nN)</th>
<th>Work of Adhesion (Aj)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High nutrients</td>
<td>0.16 ± 0.01</td>
<td>5.21 ± 0.60</td>
</tr>
<tr>
<td>Low nutrients</td>
<td>4.3 ± 0.16</td>
<td>185.48 ± 14.01</td>
</tr>
</tbody>
</table>

\textbf{Table 6:} Adhesion Force and Work of Adhesion 24-hour \textit{Ps. fluorescens} biofilm with low nutrients and high nutrients. Error is represented using Standard Error of the Mean.
colloid cantilever is smooth, nanofeatures on the surface and particularly large surface heterogeneities may result in further adhesion to the biofilm surface, and, therefore, cause a slightly increased adhesive response. The physiochemical properties of the colloid, while selected for being inert, may be modified during interaction, such as the attachment of EPS to the cantilever surface (Volle et al., 2008a). Although protocols were in place to ensure the optimum method of measurement, EPS can be pulled away from the biofilm and attached at the initial force curve measurement, causing a change in the force curve. As a result, the adhesive forces would be taken as an indicator that the low nutrient biofilm produces a stickier EPS matrix than that of the high nutrient biofilm.

4.3.3 Scanning Electron Microscopy

SEM images of 24-hour *Ps. fluorescens* air-liquid interface biofilm, grown with low and high nutrient availability, are shown in Figure 26. The higher nutrient biofilm shows a large line of thick biofilm; however, the lower nutrient displays a more dispersed biofilm. This corresponds with the CLSM experiments performed in Chapter 4, where larger clusters of biofilm are observed for high nutrient biofilms, when compared to lower nutrient biofilms, which contained dispersed colonies.
Figure 26: SEM images of 24-hour *Ps. fluorescens* biofilms grown by air-liquid interface. A and C are low nutrient, B and D are high nutrient.

4.3 Conclusion

The investigation of the influence of nutrient concentration on the mechanical properties of air-liquid interface *Ps. fluorescens* biofilms grown for 24-hours is shown at low and high nutrient availability. AFM analysis of the elastic and adhesive properties of the biofilms was performed using a constructed colloidal cantilever of 10µm. The AFM data of the air-liquid interface biofilms showed the low nutrient biofilm to have twice the elastic modulus in comparison to that of the high nutrient biofilm. While the low nutrient biofilm produced a stiffer biofilm, the Young’s modulus of the high nutrient biofilm formed two separate distributions: one between 0-0.7kPa and another between 0.9-10.5kPa. These may indicate multiple EPS
layering, however, further studies are required. The adhesive force of the biofilms exhibited a higher adhesion force and work of adhesion for the low nutrient biofilm. Overall, the AFM data showed an increased viscous effect and higher adhesion at the biofilm surface with lower nutrient availability. The SEM imaging revealed large areas of coverage for the high nutrient biofilm with smaller colonies for the low nutrient biofilms. The difference in biofilm distribution and the higher viscous surface may be due to the lower nutrient intake, and shear stress, thus, producing a more rigid EPS matrix to protect the cells.
CHAPTER 5

Conclusion and Future Work

The previous chapters of this thesis explored the impact of 3 different parameters: topographical features, shear stress and nutrient concentration on initial bacterial attachment and the subsequent biofilm development. The influence of the NF/RO surface topography on the initial attachment of bacterial cells is the focus of Chapter 1, whereas the influence of shear stress and nutrient concentration on the biofilm development is discussed throughout Chapters 2 and 3. This fundamental study covers several aspects; however, it encompasses only a small part of the overall subject prompting further research in the area.

5.1 Surface Topographical Heterogeneities and Initial Bacterial Adhesion

The initial adhesion of bacteria to NF270 and BW30 has been thoroughly studied with respect to its physicochemical and surface characteristics (Semião et al., 2014; Semiao et al., 2013). Nonetheless, there is limited research into the possible influence of initial bacterial adhesion onto NF270 and BW30 membrane topographical heterogeneities. This thesis demonstrates the presence of surface topographical heterogeneities, with dimensions large enough to accommodate the Ps. fluorescens and S. epidermidis cells utilised in Chapter 2. In order to further determine the adhesion preference of bacterial cells, a technique to measure the initial bacterial adhesion within these topographical heterogeneities was required.

Further research into other membranes surfaces is required in order to elucidate further the adhesion of bacteria onto NF and RO membranes, utilising the combination of SEM, AFM and fluorescent microscopy to identify membrane defects and the subsequent bacterial adhesion. The measurement of these topographical heterogeneities and subsequent bacterial adhesion can be used to identify membranes prone to surface damage, and further expand the supposition that topographical heterogeneities provide environmental niches ideal for attachment during the initial phase of biofilm development. This was observed in Chapter 2,
where up to 30% of the fraction of adhered bacteria onto a membrane surface were found in surface topographical heterogeneities. This large fraction of adhered bacteria suggests that these topographical heterogeneities play a role in bacterial adhesion site preference and, furthermore, may provide a niche in which the bacteria may proliferate without the influence of hydrodynamic conditions.

The presence of surface topographical heterogeneities on both RO and NF membranes may also impact the membrane efficiency on an industrial scale. These heterogeneities combined with spacers provide areas in which bacteria may adhere. These protect the bacteria from shear and, during the cleaning process, may introduce a recess, which is difficult for the chemical cleaning to reach, making the removal of bacteria from the membrane surface more difficult. This results in bacteria remaining on the surface and biofilm formation occurring at a faster rate than a new membrane. In addition, the cleaning of the membrane surface may cause damage during biofilm removal, increasing the number of topographical heterogeneities. This, in turn, results in an increase of bacterial adhesion onto the membrane topographical heterogeneities, further biofilm development and subsequent membrane damage.

A further study would be into the cause of surface topographical heterogeneities. While these may be an inherent defect caused by the manufacturing process or due to the membrane handling before experimentation, the identification and reduction of possible causes may reduce the number of recesses that provide protection from environmental stresses. The identification of topographical heterogeneities in this thesis can be employed in the study to reduce these features. Moreover, the measurement of membrane topographical heterogeneities after membrane cleaning processes could facilitate in identifying areas that may be more prone to bacterial adhesion. It can also identify surface changes, including the potential of creating membrane surface heterogeneities during the cleaning process. It should be conducted at minimisation of membrane surface topographical heterogeneities that may influence bacterial adhesion during membrane biofouling experimentation.
5.2 Nutrient Load and Shear Stress

Following the study into initial bacterial adhesion, Chapters 3 and 4 focused on the next stage of biofilm formation. The biofilms form in a variety of environmental conditions, with hydrodynamic and nutrient conditions as the two conditions selected for this study. During biofilm formation, these important factors may influence the growth and architecture of the biofilm. Chapter 3 implemented two experiments (flow-cell and air-liquid interface) to study the influence that nutrient availability and shear stress have on the biofilm growth and development, whereas, Chapter 4 studied the biofilm development dependence on nutrient availability, using the air-liquid interface experiment performed in Chapter 3. The biofilm grown was analysed using AFM nanoindentation, which is discussed in section 5.3.

Studies involving biofilm formation during dynamic conditions have shown an increase in the rate of biofilm growth (Moreira et al., 2015; Peyton, 1996). However, the time in which the biofilms are grown and the species that are used vary in each study, thus, making comparisons difficult. Further work may involve the use of different types of bacteria and over longer periods of time, allowing for further development. This would provide for more long-term observations of biofilm architecture.

Another study is the differing nutrients within the feed nutrient concentration that have been demonstrated to influence bacterial adhesion (Costerton et al., 1995; Rochex & Lebeault, 2007). This has resulted in additional studies into varying the nutrients available within the medium. By using the flow-cell technique in Chapter 3, more accurate biofilm architecture can be obtained by different nutrients; this technique eliminates the need to remove the biofilm from its environment and reduces any damage that may occur to the biofilm structure. Other factors, such as pH or temperature changes, were not explored during experimentation. These factors may influence biofilm growth and would be an additional avenue of study. Additionally, changes in shear stress over time could assist in the biofilm development, inducing the erosion and sloughing of biofilms (Duddu et al., 2009). By varying the shear stress of the experiment and viewing the biofilm grown in situ, a greater understanding of the changes in the biofilm architecture due to shear stress could be obtained without any damage caused by the removal from the system.
As discussed in Chapter 2, the surface topography plays an important role in bacterial adhesion. This can be further explored in the experiments developed in Chapter 3. A substrate of glass was utilised to remove the factor of surface topography from experimentation. However, with further studies, the use of different substrates could be included. A limit to this would be the requirement of a transparent substrate to allow for CLSM analysis, providing a restriction on the number of materials that could be employed.

A further limit to the flow-cell experiment in Chapter 3 was the flow rate due to feed tank load. As it was a non-circulating system, large amounts of the medium were required. Because of the required quantity, the flow rate was maintained in laminar flow. Further research into the expansion of this experiment to allow for a turbulent flow regime would provide a more comprehensive overview of the effects of flow-rate on the biofilm architecture. For a more complete study into the flow rate, the air-liquid interface experiment would require a computational model to be developed in order to determine the shear stress of liquid due to the orbital rotation.

As nutrient availability and shear stress are shown to influence the biofilm formation, this can be applied to industrial applications. Further research into other membranes surfaces is required in order to elucidate further the adhesion of bacteria onto NF and RO membranes, utilising the combination of SEM, AFM and fluorescent microscopy to identify membrane defects and the subsequent bacterial adhesion. By understanding the biofilm structure, cleaning techniques can be customised to more effectively remove the biofilm, with the possibility of reducing membrane damage during cleaning.

5.3 Biofilm Nanoindentation

As mentioned in section 5.2, changes in environmental conditions may cause changes in biofilm architecture. For nanoindentation, this may cause a stiffer or softer biofilm. In Chapter 4, the focus was the nanoindentation of a biofilm with differing nutrient concentration. Studies into biofilm growth due to pH, temperature, shear stress and the introduction of chemicals in order to assess the reactions of biofilm to these environmental changes would provide a more comprehensive analysis of biofilm structure.
Chapter 4 focused primarily on the mechanical properties of an air-liquid interface grown biofilm. The biofilm grown in a low nutrient environment produced a stiffer and stickier biofilm at the air-liquid interface, when compared to the higher nutrient grown environment. This may be a stress response of the biofilm, producing more EPS to protect the bacteria. Since EPS is a sticky substance, it is more difficult to remove and requires harsher chemical treatments, which results in difficulties to remove a biofilm grown in a low nutrient environment. By increasing the nutrient availability within the water system, the biofilm grown may be removed more easily, however, more research would be required.

The air-liquid interface experiment was chosen as further development of the flow-cell experiment and required the opening of the flow cell and subsequent biofilm damage to allow for nanoindentation. However, further studies into cell adhesion are necessary to understand bacterial adhesion onto various surfaces. Cell adhesion occurs when the cell binds to a substrate, which can be either another cell or a substrate. Single cell force spectroscopy is a technique that could analyse cell-substrate interactions. While many studies focus on the adhesion of multiple cells (Myint et al., 2010; Semiao et al., 2013) and of biofilm growth (Flemming & Wingender, 2010), research into single cell and its reaction to surfaces is required to further understand the fundamental principal behind bacterial adhesion to membrane surfaces, as outlined in Chapter 2.

5.4 Final Thoughts

This thesis is a fundamental study into bacterial adhesion and subsequent biofilm growth. While no approach was found to prevent or effectively remove bacterial adhesion and growth, further research is required to provide a much broader understanding in biofilm development. In this study, a small aspect of bacterial attachment and growth was developed to better comprehend this complex phenomenon, which is a stepping stone towards full understanding.

As summed up by Sun-Tzu in the Art of War (Tzu, 1963):

“If you know the enemy and know yourself, you need not fear the result of a hundred battles. If you know yourself but not the enemy, for every victory gained you will
also suffer a defeat. If you know neither the enemy nor yourself, you will succumb in every battle".
A. Nomenclature

A.1 List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>CFD</td>
<td>Computational Flow Dynamics</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin-Landau-Verwey-Overbeek (theory)</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
</tr>
<tr>
<td>MFS</td>
<td>Membrane fouling simulator</td>
</tr>
<tr>
<td>NF</td>
<td>Nanofiltration</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Re_{adh}</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>RW-C</td>
<td>Raw water medium without carbon</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>XDLVO</td>
<td>Extended - DLVO</td>
</tr>
</tbody>
</table>

A.2 List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>OD of bacterial suspension before mixing</td>
</tr>
<tr>
<td>A_{o}</td>
<td>OD of bacterial suspension after mixing</td>
</tr>
<tr>
<td>C_{0}</td>
<td>The initial bacterial suspension feed concentration.</td>
</tr>
<tr>
<td>E</td>
<td>The Young’s modulus</td>
</tr>
<tr>
<td>E_{adh}</td>
<td>The adhesive energy</td>
</tr>
<tr>
<td>F_{adh}</td>
<td>The adhesive force</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$h_1$</td>
<td>Separation distance at the intersection of the original contact point and retraction curve</td>
</tr>
<tr>
<td>$h_2$</td>
<td>The end-point of the adhesive energy marker on the x axis</td>
</tr>
<tr>
<td>$h_o$</td>
<td>Height of rectangular channel</td>
</tr>
<tr>
<td>$k_d$</td>
<td>The deposition rate of bacteria on membranes</td>
</tr>
<tr>
<td>$N$</td>
<td>Number of samples taken</td>
</tr>
<tr>
<td>$Q$</td>
<td>Volumetric flow rate</td>
</tr>
<tr>
<td>$q_{\text{max}}$</td>
<td>The maximum cell loading and the accumulation factor</td>
</tr>
<tr>
<td>$q(t)$</td>
<td>Bacterial loading as a function of time</td>
</tr>
<tr>
<td>$r$</td>
<td>The roughness value</td>
</tr>
<tr>
<td>$R$</td>
<td>The radius of the colloid sphere</td>
</tr>
<tr>
<td>$R_a$</td>
<td>Arithmetic average height or roughness average</td>
</tr>
<tr>
<td>$R_{\text{rms}}$</td>
<td>Root means squared roughness.</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
</tr>
<tr>
<td>$w_o$</td>
<td>Width of rectangular channel</td>
</tr>
<tr>
<td>$z$</td>
<td>Biofilm depth</td>
</tr>
<tr>
<td>$z_{\text{min}}$</td>
<td>Minimum value of the biofilm depth</td>
</tr>
<tr>
<td>$z_{\text{max}}$</td>
<td>Maximum value of the biofilm depth</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$</td>
<td>Accumulation factor, obtained by the exponential fit of the adhesion experimental data</td>
</tr>
<tr>
<td>$\Delta t$</td>
<td>The time difference between two time points</td>
</tr>
<tr>
<td>$\delta$</td>
<td>The sample deformation.</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Absolute viscosity</td>
</tr>
<tr>
<td>$\theta(t)$</td>
<td>The number of adhered cells over a time period</td>
</tr>
<tr>
<td>$\nu$</td>
<td>Poisson's ratio</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Wall shear rate</td>
</tr>
<tr>
<td>$\tau_w$</td>
<td>Shear stress</td>
</tr>
</tbody>
</table>
References


Diaz, C., Schilardi, P. L., Salvarezza, R. C., & Fernandez Lorenzo de Mele, M. (2011). Have flagella a preferred orientation during early stages of biofilm...


bacterial adhesion during NF filtration processes. Water Research, 47(8), 2909-2920.


