The role of biofilm formation in reverse osmosis membrane system performance and possible antifouling strategies

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**The Role of Biofilm Formation in Reverse Osmosis Membrane
System Performance and Possible Antifouling Strategies**

CHEN XI

School of Biological Sciences

2015

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System Performance and Possible Antifouling Strategies**

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School of Biological Sciences

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PRELIMINARY DATA

Biofilm Dispersal by Sodium Nitroprusside (SNP)

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ABBREVIATIONS

Abbreviation	Description
°C	Degrees Celsius
AHLs	Acylated homoserine lactones
AI	Autoinducer
AOC	Assimilable organic carbon
BCA	Bicinchoninic acid
BEOP	Biofilm-enhanced osmotic pressure
BSA	Bovine serum albumin
CA	Cellulose acetate
c-di-GMP	cyclic-di-GMP
CFD	Computational fluid dynamics
CFP	Cyan fluorescent protein
CFV	Cross-flow velocity
CFU	Colony forming unit
CLSM	Confocal laser scanning microscope
cm	centimeter
ConA	Concanavalin A
CP	Concentration polarization
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CTF	CTC formazan
d	day
DGC	Diguanylate cyclases
DO	Dissolved oxygen
DOTM	Direct observation through the membrane
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances

FCP	Feed channel pressure
g	g-force
g/L	gram per liter
h	hour
L	liter
LMH	liters per square meter per hour
LP	Low pressure
M	molar
MBR	Membrane bioreactor
MF	Microfiltration
MFS	Membrane fouling simulator
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
MP	Medium pressure
m/s	meters per second
NB	Nutrient broth
NF	Nanofiltration
NO	Nitric oxide
No.	Number
nm	nanometer
OD	Optical density
ΔP	Feed channel pressure drop
PBS	Phosphate buffered saline
PDE	Phosphodiesterases
PI	Propidium iodide
QS	Quorum sensing
RO	Reverse osmosis

---

rpm	revolutions per minute
RT	Room temperature
s	second
SDS	Sodium dodecyl sulfate
SNP	Sodium nitroprusside
SRB	Sulphate-reducing bacterium
SWM	Spiral wound membrane
TBE	Tris-Borate EDTA buffer
TE	Tris-EDTA
THM	Trihalo-methanes
TMP	Trans-membrane pressure
TMP_0	Initial tran-smembrane pressure or trans-membrane pressure at $t = 0$
ΔTMP	Trans-membrane pressure increase
TOC	Total organic carbon
μg	microgram
μL	microliter
μm	micrometer
UF	Ultrafiltration
UV	Ultraviolet
WR	Working reagent
WT	Wild type
w/v	weight per volume
YFP	Yellow fluorescent protein

ABSTRACT

In this study, confocal microscope observation coupled with the fluorescence staining was employed to monitor biofouling of Reverse Osmosis (RO) membranes using *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* as model fouling organisms. A range of parameters, including the presence of feed channel spacers, nutrient level, flux and cross-flow velocity were compared to determine their roles in biofouling. In addition, novel biofouling controls (UV pretreatment and NO addition) were tested to determine their efficacy as biofilm control agents. It was found that biofilm formation resulted in a slow rise in TMP and followed by a sharp increase, called the 'TMP jump'. The initial slow increase in TMP was most likely due to the formation of a biofilm on the membrane surface, which then accelerated the biofouling rate through cake-enhanced polarization of nutrients. It was observed that nutrient limitation slowed biofilm accumulation and delayed the increase in TMP. It was evident that the presence of the spacer improved the performance of the RO system. In the presence of the spacer, biofilm development was slower and the TMP showed a much slower rise. When a spacer was introduced into the membrane module, the biofilm primarily occurred on the membrane, not on the spacer. While the inclusion of spacers delayed fouling, a faster TMP rise was observed under conditions of higher flux and lower cross-flow velocity.

Two approaches were examined to control biofilm development and hence, to improve the performance of the RO system, UV pretreatment to kill the bacteria in the feed and induction of biofilm dispersal using Nitric Oxide (NO). UV pretreatment effectively inactivated bacteria in the feed. While UV treatment delayed biofilm formation and fouling, it did not prevent biofilm formation. Preliminary data showed that the NO donor, Sodium NitroPrusside (SNP) could disperse biofilms formed on RO membranes.

The results presented here suggest that improving hydrodynamics (e.g. reducing the flux, increasing cross-flow) in membrane modules in combination with limiting

nutrient concentrations in the feed as well as rationally-designed feed spacers could be used to control biofouling and improve system performance.

CHAPTER 1 Introduction

1.1 Problem Statement

It is well-known that the world is now facing a water scarcity problem – depletion of groundwater and surface water due to unbalanced water demand and supply (Fritzmman et al. 2007). Since the development of the first asymmetric cellulose acetate reverse osmosis (RO) membrane in the early 1960's by Loeb and Sourirajan, RO is widely used to convert seawater, brackish water and municipal wastewater to potable water (Loeb and Sourirajan 1962). This groundbreaking invention led to further development of RO membranes used for water production and reclamation. For example, the government of Singapore, in a joint project between the Public Utilities Board (PUB) and Ministry of Environment, has initiated application of the NEWater process since 1998 that transforms secondary effluent from an activated sludge treatment process, through a series of steps involving microfiltration (MF) / ultrafiltration (UF), reverse osmosis and ultraviolet (UV) disinfection, to yield high quality water (Public Utilities Board 2002). NEWater can be mixed and blended with reservoir water and then undergo conventional water treatment to produce drinking water. Now, the NEWater factories produce almost 500,000 m³/d of pure water, among which Changi NEWater factory, at 225,000 m³/d, is the third largest membrane plant in the world. It is inevitable that RO technology will play a crucial role in supplying water to water scarce countries. The pivotal advantage of the RO membrane is its uniqueness in transport properties such that water can pass through with little hindrance while presenting a virtually impermeable barrier to salts and other components. However, the major challenge in RO technology is the fouling of the membrane.

Fouling is defined as the accumulation of materials such as biological/organic, colloidal, particulate and crystalline matters on the surface of process equipment (Epstein 1983). Biofouling – a phenomena related to biological material development which forms a sticky layer on the membrane surface (Baker and Dudley 1998;

Flemming 1997), is probably the most prevalent and problematic type of fouling in a RO system. For example, from the autopsies of 150 membrane samples collected all over the world, it was observed that more than 50% of the deposits removed from the membrane surface were biological/organic foulants (van Hoof et al. 2002). Continuous biofouling problems in RO lead to higher energy input requirements as an effect of increased hydraulic resistance (R_f) and biofilm enhanced osmotic pressure (BEOP), lower quality of product water due to concentration polarization (CP) – increased concentration due to solutes accumulation on the membrane surface, and thus significant increase in both operating and maintenance costs. This problem has caused failure in many RO desalination plants and therefore needs to be rectified.

Many precautionary and preventative steps have been developed to combat biofouling, which include the use of biocides to eliminate the microorganisms at source and the use of cleaners to remove the biofilm. However, the former may actually break down the complex organic molecules into simple molecules that could act as carbon sources for the microorganisms (LeChevallier 1991). In addition, these biocides tend to be ineffective and there is a desire to reduce the use of toxic compounds from environmental perspective. And the latter may fail to completely remove the biofilm even though an improvement in permeability may be observed (McDonogh et al. 1994), due to the fact that the cleaners are not targeting the extracellular polymeric substances (EPS) which are the foundation of a biofilm (Flemming et al. 2000). Hence, new approaches must be developed to control biofilm development and this requires a detailed understanding of how biofilms form in the first place.

Biofouling is the formation of a biofilm on the RO membrane surface, which is developed through a series of successive events such as surface conditioning, transport, attachment, growth and detachment (Characklis 1990). Since the growth of the microorganisms is nutrient dependent, the higher concentration of nutrient at the membrane surface due to the CP effect not only supports the survival of the microorganisms, but could also accelerate the biofilm formation rate. This means by proper control of nutrient supply, biofouling in the RO system can be avoided or

inhibited. Unfortunately, there is a lack of information available that relates biofilm development to nutrient concentration, which needs to be validated.

A related topic of interest is the effect of feed channel spacers (as used in the spiral-wound module) on biofouling. It is generally recognized that spacers can improve mass transfer and therefore reduce the CP level, but its direct link to the biofouling process is not well studied. Previously, it has been shown in a biofouling study without feed channel spacers that RO biofouling was a flux-driven process where higher flux increased fouling rate (Chong et al. 2008a). It was also shown that biofouling caused the BEOP effect due to elevated CP of solutes at the membrane surface, and this resulted in a loss of driving force (Herzberg and Elimelech 2007). The biofouling and BEOP effect were more severe at higher flux and lower crossflow velocity operation. However, in another study (Vrouwenvelder et al. 2009a), it was suggested that flux did not affect fouling and biofouling was more severe when the crossflow velocity was higher. The biofilm was primarily found on the feed channel spacer and blocked the flow channel which further caused an increase of the pressure drop along the channel. These contradictory observations relating to the biofouling process in RO need to be systematically addressed as it is critical to understand the mechanism for sustainable operation of RO technology.

The application of biocides to control biofouling is currently the most popular strategy adopted in RO desalination plants. However, biofouling is still observed and overdosing of biocides like chlorine may cause unfavorable effects on the membrane. Therefore, finding an alternative strategy to control or minimize the biofouling problem in RO processes is crucial to provide effective prevention of biofouling and be safe for the membrane and the environment.

1.2 Objectives

Based on the problem statement above, the objectives of this PhD project were to understand the role of biofilm development in the performance of a high pressure RO membrane system and to use that information to develop and optimize technologies for biofouling control to enhance membrane performance.

1. Quantify the effect of nutrient concentration on biofilm development and RO membrane fouling.
2. Determine the effect of spacers and operational parameters on RO membrane fouling.
3. Evaluate methods for controlling biofouling on RO membranes.

CHAPTER 2 Literature Review

2.1 Fundamentals of Reverse Osmosis

Reverse Osmosis (RO) is a technique that uses membranes with specific properties that allow them to selectively separate solutes from water (Kucera 2010). As shown in Figure 2.1, membranes are generally classified as conventional filtration, microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and RO based on their pore size. Compared to conventional and many other membrane-based filtration techniques, RO provides the best filtration presently available in terms of water purity, rejecting most solids, bacteria, viruses, macromolecules and ions.

Figure 2.1 'Filtration Spectrum' showing the difference in rejection capabilities between reverse osmosis (RO) membranes and other membrane technologies and the separation difference from the conventional filtration (Figure adapted from Kucera 2010).

RO is the preferential technology for seawater desalination. In the late 1960's, researchers had successfully demonstrated the first RO asymmetric cellulose acetate (CA) membrane, which could reject salt and pass potable water (Loeb and Sourirajan 1962). The primary competing technology in desalination is thermal processing to evaporate off pure water, leaving solutes behind. However, RO is preferable due to its lower energy requirements, as it does not require heat. Recent desalination data show that of the existing 59.9 million m³/d of worldwide desalination capacity for water purification, 61.1% relies on RO technology (Pankratz 2010).

2.1.1 Principles of Reverse Osmosis

RO is capable of removing salt ions from water, which is based on the use of a semi-permeable membrane that allows water and small amounts of ions to pass, but rejects most of the dissolved solids (Kucera 2010). As shown in Figure 2.2, the osmosis process naturally happens where water from a diluted solution or pure water moves across a semi-permeable membrane into a counterpart with a higher solute concentration until the concentration reaches equilibrium on both sides. The osmosis process results in a difference in the water level between the two sides, where the side that originally contained the higher concentration solution will end up with more volume, resulting in a higher water level in the column. The deviation in the water level is in part limited by the weight of the water, which also imposes a force that limits osmosis and therefore, the combination of difference in solute concentrations and water pressure is used to define the osmotic pressure.

RO is the process by which an applied pressure, greater than the osmotic pressure, forces water to pass through the semi-permeable membrane against the concentration gradient in the direction reverse to that of osmosis. In this way, relatively pure water passes through the semi-permeable membrane from a solution of high solute concentration to the solution with the lower concentration of dissolved solids, resulting in an increase in the concentration of solutes on the solution to which the force is applied. Hence, the water in one side is purified or desalted, and the solids

in the other side are concentrated or dewatered (Figure 2.2).

Figure 2.2 The principles of osmosis and reverse osmosis (RO) (Figure adapted from www.aquatechnology.net/reverse_osmosis.html).

2.1.2 RO Membrane Modules

2.1.2.1 RO Membrane Materials

The performance of the RO system depends directly on the characteristics of the membrane material, which includes the chemical nature of the membrane polymer and the structure of the membrane (Kucera 2010). It is expected that RO membranes provide high retention and flux, in addition to high strength and durability, good capacity of antifouling as well.

Based on the types of polymer backbone, RO membranes are usually classified into two categories: cellulose acetate (CA) and polyamide. CA membranes, which were developed by Loeb and Sourirajan in 1962, were the first commercial RO membranes (Loeb and Sourirajan 1962; Loeb 1981). The structure of this type of membrane is asymmetric with a solute-rejecting layer on the top and a microporous support layer on the bottom. Both layers are made of CA. Later, polyamide membranes were developed due to their better performance at relatively lower operating pressures and higher salt rejection compared with CA membranes.

Currently, aromatic polyamide membranes are the most popular RO membranes in use.

2.1.2.2 Feed Channel Spacers

In the RO spiral wound membrane (SWM) modules, the spacers are for keeping the membrane sheets apart, as well as to improve the hydrodynamic conditions (Da Costa et al. 1991; Geraldles et al. 2002a; Schwinge et al. 2004). There are many types of feed-channel spacers present, but the most commonly used ones are the ladder- and diamond-types, which are distinguished by the orientation of the spacers relative to the flow direction (Figure 2.3). Feed-channel spacers are usually made of plastic polypropylene (PP).

Figure 2.3 Feed-channel spacers. A. ladder-type; B. diamond-type. The arrow indicates the direction of flow of water across the spacer.

An extensive effort has been made to understand the feed-channel spacers with respect to RO performance. On one hand, their presence can enhance flux by promoting eddies. This suggestion is supported by data collected from long channel RO membrane cells where the presence of a spacer inserted into the feed-channel lessened the concentration polarization (CP) due to the enhanced turbulence (Mo and Ng 2010). On the other hand, the presence of the spacer may enhance the biofilm formation and decrease the performance of the RO system. Huang et al. (Huang et al. 2010) developed a new high-pressure optical membrane module with direct

microscopic observation system to visualize deposition of bacteria onto RO membranes with spacers. The results showed that the presence of spacer enhanced the initial attachment of the bacteria on the membrane surface compared to comparable conditions with no spacer. Vrouwenvelder et al. combined the methods of experimental investigation (Vrouwenvelder et al. 2009a) and computational fluid dynamics (CFD) (Vrouwenvelder et al. 2010) and reported that the majority of the biofilm biomass grew on the spacer rather than on the membrane and that the spacers increased the pressure loss along the flow channel. The suggestion that the spacers contribute to fouling is also supported by data showing that biofouling can be prevented by improved spacer geometry (e.g. thickness, orientation) and spacers coated with biocides such as organic (e.g. polydopamine) (Miller et al. 2012) or inorganic (e.g. silver, copper or gold) (Araujo et al. 2012) compounds also improved membrane performance.

2.1.2.3 RO Membrane Modules

Four types of RO membrane modules are available: spiral wound, hollow fiber, tubular, as well as plate and frame. The spiral wound module, as shown in Figure 2.4, is the most commonly used configuration in the desalination and reclamation industry due to its large surface to volume ratio and hence its productivity per unit volume is high. It is also well balanced between providing fouling resistance and being easy to clean.

2.1.3 Performance of RO Systems

1. Recovery

Recovery is the proportion of the influent that can be reclaimed as permeate (Kucera 2010), and it is calculated by the equation shown as follow:

$$\% \text{ Recovery} = (\text{permeate flow} / \text{feed flow}) \times 100$$

Higher recovery of purified water, on one hand, results in a less concentrated solution, however, it also results in lower quality permeate and accelerates fouling of

Figure 2.4 Cut-away view of a multi-leaf spiral wound module (Figure adapted from Wagner 2001).

the membranes, which is not optimal. Trades-off between these two aspects must be taken into account. Generally, most RO systems appear to operate in the range of 50% to 85% recovery for the entire systems, with 75% is designed for the majority of RO systems composed of multiple modules, while the recoveries fluctuate from about 10% to 15% for a single module in a system (Kucera 2010).

2. Rejection

Rejection is the proportion of an inflowing component retained by membrane (Kucera 2010). For instance, 97% sodium rejection means that the membrane retains 97% of the inflowing sodium, and that 3% of the influent sodium can pass through the membrane into the permeate. Rejection of a given component is calculated by the following equation:

$$\% \text{ Rejection} = [(C_f - C_p) / C_f] \times 100$$

where: C_f = inflowing concentration of a provided component

C_p = permeate concentration of the same component

Rejection is combined property of the membrane and the influent component. For a given component, rejection is determined by the characteristics of ionic charges of this component, degree of dissociation, molecular weight, polarity, degree of hydration, degree of molecular branching etc.

3. Flux

Flux is defined as the rate of a fluid volumetrically flowing through a certain area (Kucera 2010), and in the case of RO systems, the referred fluid is water and the given area is that of the RO membrane. Flux is measured as liters of water per square meter of membrane area per hour (liters/m²/h, LMH). The flux in a RO system is also dependent on the net applied pressure used, which forces the water across the membrane against the concentration gradient:

$$J=K(\Delta P-\Delta \pi)$$

where: J = water flux

K = water transport coefficient factor (related to the permeability and thickness of the membrane active layer)

ΔP = pressure deviation across the membrane

$\Delta \pi$ = osmotic pressure deviation across the membrane

The water transport coefficient, which is a particular to the specific membrane active layer (e.g. cellulose acetate vs. polyamide), varies directly with temperature and pH. Operational data shows that higher flux leads to accelerated membrane fouling and therefore, there is a trade-off between water yield and membrane fouling that must be considered when choosing the flux rate for a system.

4. Dead-end and cross-flow filtration

Dead-end filtration involves the entire fluid passing through the membrane, leaving the particles larger than the pore size on the membrane (Kucera 2010). Dead-end filtration is a batch process and has one influent stream producing one effluent stream (Figure 2.5). The filter of dead-end filtration will accumulate particles and eventually become clogged such that water can no longer pass through. In this

case, they have to be taken off-line and cleaned or replaced. The use of a syringe with a filter membrane is an example using dead-end filtration in the laboratory.

Figure 2.5 Dead-end filtration has one influent stream producing one effluent stream (Figure adapted from Kucera 2010).

In cross-flow filtration, feed water flows tangentially over the membrane surface, creating a pressure across the membrane (Kucera 2010), as a result of which, some water and dissolved solids can pass through the membrane while the remaining water and dissolved solids continue to flow over the membrane. Hence, cross-flow filtration has one influent stream but produces two effluent streams known as the permeate (clean water) and the concentrate (Figure 2.6).

Figure 2.6 Cross-flow filtration has one influent stream but produces two effluent streams (Figure adapted from Kucera 2010).

2.1.4 Concentration Polarization (CP)

When the membrane rejects the solute, the solute concentration close to the membrane surface (C_s) increases and is higher than that in the bulk feed solution (C_b). This is due to the fact that the convective flow of the solute to the membrane surface must be balanced by the back diffusion of solute to the bulk feed solution. This build-up in concentration in this boundary layer region is referred to as concentration polarization (CP) (Figure 2.7).

Figure 2.7 Concentration polarization (CP), where C_b is the solute concentration in the bulk feed solution and C_s is that at the membrane surface (Figure adapted from Kucera 2010).

CP is not desirable in the RO systems for three main reasons. Firstly, higher solute concentration leads to the increased osmotic pressure close to the membrane surface, and flux will thus decline unless additional pressure is applied to compensate for the increased osmotic pressure. In addition, as more solutes accumulate at the membrane surface, the solute rejection is reduced since the driving force for solute transport is the concentration gradient across the membrane. The third impact is the accelerated fouling, which is induced either by the formation of insoluble salts from

accumulated solutes when the solubility limit is exceeded, or as a consequence of higher nutrient concentrations at the membrane surface that will attract more microorganisms to the membrane surface and subsequently form biofilms.

2.1.5 Pressure Drop

1. Trans-membrane pressure (TMP) drop and feed channel pressure (FCP) drop

Membrane fouling will increase the pressure drop, which is divided into two phenomena: the trans-membrane pressure (TMP) drop and the feed channel pressure (FCP) drop (Figure 2.8) (Vrouwenvelder et al. 2009a). The TMP drop is the change in pressure between the feed and permeate lines, showing the fractional resistance over the membrane; while FCP drop is the counterpart between the feed and brine lines, the pressure difference between the inlet and outlet of the membrane chamber.

Figure 2.8 Pressure drops in membrane based filtration showing the trans-membrane pressure (TMP) drop and feed channel pressure (FCP) drop (Figure adapted from Vrouwenvelder et al. 2009a).

2. Constant flux and constant trans-membrane pressure (TMP) operation

As a consequence of fouling, trans-membrane pressure (TMP) increases under constant flux operation and flux drops during constant pressure operation (Ridgway 1991). Most RO plants tend to operate at fixed production rate, which requires fixed

flux. In this operation, when the membrane is fouled, a higher TMP must be applied to compensate for fouling through adjusting the pump rate to supply higher feed pressure. As soon as the pump reaches the maximum rate capacity, the permeate flux declines due to fouling, which is now equivalent to fixed TMP operation. For those RO systems that do operate using a fixed pressure, the system tends to have a high initial flux, which in turn leads to a rapid build-up of the deposit layer at the membrane surface (Chong et al. 2008a). Thus, fouling causes a drop in the flux, which in turn diminishes fouling until a steady state is reached. The TMP and flux profiles of the above two operation modes are illustrated in Figure 2.9.

Figure 2.9 Illustration of trans-membrane pressure (TMP) and flux profiles for constant flux operation (A) and constant pressure operation (B) (Figure adapted from Chong 2008).

Despite the differences in fouling patterns, the majority of experimental studies investigating RO fouling have been performed under fixed pressure (TMP) conditions. However, most full scale plants operate under fixed flux and hence laboratory studies should focus on the latter for relevance. Ideally, the fouling process under fixed flux and fixed pressure operations would be compared as they may give different fouling dynamics and mechanisms.

2.2 Biofouling

In membrane technology, the most important types of fouling known include crystalline fouling, organic fouling, particle and colloid fouling, as well as

microbiological fouling (Geesey et al. 1994). The former three types of fouling are relatively easily managed by the appropriate pretreatment of the water. Biofouling of a surface, however, is difficult to control by simply reducing the existence of microorganisms in the aquatic phase, due to the fact that microorganisms can proliferate when they have appropriate nutrients. The beginning phase of biofouling involves bacterial attachment and is likely to infer a similar membrane-foulant interactions as other kinds of fouling. However, in later phases, biofouling reveals special characteristics which include extracellular polymeric substances (EPS) production and self-proliferation (Ridgway 1991). Even in systems with low concentrations of nutrients, the formation of biofilms appears to be an adaptation employed by microorganisms that allows them to survive and scavenge nutrients (Marshall 1985).

2.2.1 Main Causes of Membrane Biofouling

The existence of microorganisms and nutrients in the feed water are the two main causes for membrane biofouling. Microorganisms can be found everywhere in all types of water sources. The amount of 10^3 - 10^4 CFU/mL microorganisms was observed in the Persian gulf (Saeed et al. 2001) and the Caribbean Sea (Winters 1997). More microorganisms as high as 10^7 CFU/mL can be detected in domestic and industrial wastewater (Friedler et al. 2008). In addition to the raw water, microorganisms may also come from the inner wall of the membrane system piping where the biofilms have already formed on (Flemming 1997).

Nutrient availability is the other main cause of membrane biofouling since none of the real-world membrane system is sterile. The conventional pretreatment methods can only kill or remove one part of the microorganisms. Chlorination was reported to reduce the bacteria to 10^2 CFU/mL (Fujiwara and Matsuyama 2008). About 2 log reduction could be obtained by MF (Sadr Ghayeni et al. 1998b). UV could substantially reduce bacterial numbers in the raw seawater by around 2 log (from 10^4 to 10^2 CFU/mL) prior to the NF-SWRO stage in a pilot scale plant (Munshi et al. 2005). And the process media filtration-cartridge filter-UV disinfection may reduce

the bacteria from 10^4 to 10^2 CFU/mL and further 1 log reduction is obtained by the NF (Munshi et al. 2005). Even the bacteria concentration is as low as 10^2 CFU/mL, membrane biofouling is still observed due to the existence of nutrients. The key role of nutrients on biofouling has been studied in non-membrane systems (Peyton 1996; Klahre and Flemming 2000) and also RO systems (Hu et al. 2005; Hijnen et al. 2006; Hijnen et al. 2009; Masse et al. 2010; Chon et al. 2013).

2.2.2 Dynamics of Biofilm Formation

The well accepted definition for a biofilm is microbial organisms, attached onto a substratum and embedded in a matrix of extracellular polymeric substances (EPS), which allows for the development and formation of an independent ecosystem that could be homeostatically regulated (Percival 2000). Biofilms usually exhibit a gel-like consistency, which is related to the highly hydrated gel polymer matrix surrounding the biofilm, and is typical of a hydrogel (Flemming 2011). The matrix is composed of the following components (Geesey et al. 1994): (1) water, 75% to 95% of wet weight; (2) EPS: slime or organic glue, excreted by the microorganisms, 75% to 95% of dry weight; (3) microorganisms; (4) entrapped particles; (5) absorbed, dissolved substances. The EPS acts as a molecular sieve or ion exchanger that can absorb organic and inorganic molecules from the water. The biofilm composition may vary greatly, depending on the microorganisms involved, the composition of the raw water the temperature and the hydrodynamic conditions. However, most biofilms have the common characteristics of high water content and their gel-like consistency (Ridgway et al. 1985).

The advantages of sessile growth versus the planktonic state include (Percival 2000): (1) the expression of different but beneficial genes; (2) different growth rates which are believed to enhance antimicrobial resistance (Fletcher 1991); (3) increased production of EPS; (4) enhanced access to limited nutrients; (5) close proximity to cells so that they may be in mutualistic or synergistic association; (6) protection to a high degree from various antimicrobial assaults, which include biocides, antibiotics, antibodies and microbial predators.

Biofilm formation (Figure 2.10) is a complex process, but generally, it is described as consisting of 5 developmental stages: (1) formation of a conditioning film on the surface; (2) those events that bring the microorganisms to the close proximity to the surface; (3) adhesion, which is initially reversible and becomes irreversible once cells begin to secrete EPS components; (4) growth and proliferation of microorganisms within the colonized surface, formation of microcolonies followed by; (5) detachment or dispersal.

Figure 2.10 Diagram to show biofilm formation. The different colored circles show different types of microorganisms (Figure adapted from Percival 2000).

2.2.2.1 Development of the Conditioning Film

It seems to be generally accepted that adhesion of bacteria to a surface occurs immediately upon immersion of an unexposed, clean surface into a liquid containing organic substances and microbial cells. Studies indicate that conditioning of surfaces occurs within 15 min of exposure to a fluid and may have a thickness between 30 and 80 nm (Byers 1987).

While a clean surface may initially be either negatively or positively charged, after being exposed to fluids, most surfaces become negatively charged (Neihof and Loeb 1972; Neihof and Loeb 1974). It is now generally acknowledged that these conditioning chemicals can interact with surface appendages of microorganisms, including pili, fimbriae, glycocalyx and EPS (Vesper and Bauer 1986) to facilitate

microbial adhesion. Certain surface appendages are able to mediate some of the initial interactions with the conditioned surface. It has been suggested that the roles the conditioning film plays in microbial adhesion are to: (1) modify the physicochemical characteristics of the substratum; (2) function to concentrate nutrients at the surface; (3) adsorb and detoxify inhibitory substances dissolved in the aqueous medium; (4) concentrate and supply essential metal trace elements (Chamberlain 1992).

In a membrane system, once the feed water is in touch with the membrane, the organic substances in the feed immediately attach onto the surface of the membrane, which is chemically conditioned (Flemming 1997; Flemming 2011). This conditioning film offers the bacteria the initial active layer of the membrane to adhere. There is a 'transient association' between the bacteria and the conditioning layer, which is useful for the bacteria to slow down the motility and find a suitable place to adhere (Watnick and Kolter 2000). The success of the bacteria adhesion is affected by the membrane characteristics, in which easily adhesion is observed on hydrophobic and rough membranes (Sadr Ghayeni et al. 1998a). For example, the membranes fouled by the nutrients are rougher and more negatively charged, which would enhance the adhesion of the bacteria onto the membrane surface (Subramani et al. 2009).

Transparent exopolymer particles (TEP) have been described as a class of particulate acidic polysaccharides, which are large, transparent organic particles and quite common in natural water sources (Alldredge et al. 1993). TEP can be regarded as a kind of suspended EPS which are present in the form of discrete particles instead of surface-attached or dissolved EPS (Berman 2010; Berman 2013). Recently, TEP are considered as a potential foulant in membrane biofouling especially at the early stage of biofilm development on the membrane due to their wide existence in water (Bar-Zeev et al. 2012). As surface-active acidic polysaccharides, TEP can attach onto or be easily adsorbed by other solid surfaces including membranes due to their high stickiness and form the conditioning film. They can also serve as a nutrient source for the microbial growth in water. The TEP-associated membrane biofouling has been

reported in membrane filtration of seawater, surface water and wastewater (Villacorte et al. 2010; Valladares Linares et al. 2012; Van Nevel et al. 2012; Wu et al. 2012). This poses a new challenge on how to efficiently remove TEP at the pretreatment stage in order to prevent TEP-induced membrane biofouling.

2.2.2.2 Transport Mechanisms Involved in Adhesion of Microorganisms

The mechanisms of transport of microbial cells to a surface can be explained by a number of fluid dynamic processes: (1) mass transport, which is greatly influenced by the mixing in the bulk fluid and related to water flow rate, which is laminar or turbulent; (2) thermal effects: Brownian motion, molecular diffusion; and (3) gravity effects: differential settling, sedimentation (Characklis 1981). In static environments, adhesion is favored by many factors (Marshall et al. 1971). However, microbial motility, such as swimming is thought to increase the chances of microbial adhesion (Fletcher 1977; Marmur and Ruckenstein 1986).

2.2.2.3 Reversible and Irreversible Adhesion

After the surface has been conditioned and microorganisms have been transported through the boundary layer, microbial adhesion may subsequently occur. Studies on microbial adhesion show that adhesion occurs in two steps, reversible and irreversible adhesion (Zobell 1943). Reversible adhesion is generally acknowledged as initial contact and interaction of microorganisms with a substratum, where they still exhibit Brownian motion and can be easily removed (Rittmann 1982). In contrast, irreversible adhesion results in bacteria being permanently attached to a surface, which may be facilitated by the EPS components (Vigeant 2002). A three-step hypothesis for microbial adhesion has been proposed based on the distance between the microbial cells and the surface (Busscher and Weerkamp 1987). Van der Waals forces are found to operate at a distance larger than 50 nm away from the surface. While van der Waals forces and electrostatic interactions take place, which are related to reversible and irreversible adhesion, at a distance of 10 to 20 nm away from the surface. When the distance is no more than 1.5 nm, van der Waals, electrostatic and

specific (e.g. the hydrophobic groups associated with bacterial surface appendages) (Ridgway et al. 1985) interactions take place between the microorganisms and the surface, resulting in irreversible binding.

2.2.2.4 Microcolony and Biofilm Formation

After attachment to the surface, the microorganisms begin to grow and produce EPS, which is accumulated as a hydrated matrix in which the microorganisms become embedded (Percival 2000). Subsequently, the microorganisms are immobilized as a biofilm and depend on nutrients either from the bulk phase or from other organisms co-existing within the biofilm. Biofilms typically are composed of many different species of bacteria and can also include higher organisms entrapped within the biofilm matrix. These mixed microbial communities are tightly packed or dense and this feature facilitates interactions between cells, either those of the same organism (self) or with other organisms (non-self). Biofilms formation may facilitate close functional interaction, through which metabolites could be exchanged at cluster boundaries, which would allow the cohabitating bacteria to cooperate and thus enhance their growth potential (Blenkinsopp and Costerton 1991). Furthermore, specific functional microorganisms may create favorable living conditions that subsequently allow other functional groups to grow within the biofilm community. Microbial communities within biofilms are believed to be complex on both levels of taxonomy and function; there exists the probability that synergistic interaction occurs among different organisms. It could shield the microorganisms from disturbances of outside surroundings.

As biofilms develop, gradients occur, including oxygen, pH and nutrient gradients (Connell and Slatyer 1977), and as a consequence the bacteria within the biofilms from the outside of the biofilm to the interior, would experience different physiological conditions and hence the population would not be homogenous. Generally, when the thickness of the biofilm beyond a range, the base may become anaerobic, which would require cells at the base to change how they generate energy, e.g. to undergo anaerobic respiration or fermentative metabolism (Pfennig 1984).

2.2.2.5 Detachment from the Biofilm

As for all sessile organisms, at some stage during the biofilm life-cycle, bacteria must be able to disperse from the biofilm to colonize new habitats, reproduce and generate a new biofilm. While there are some forms of detachment which are passive, such as the erosion or sloughing of the biofilm by hydrodynamic shear (Stoodley et al. 2001; Liu and Tay 2002). It has also been demonstrated that there are active mechanisms that control the precise timing of biofilm dispersal. The processes to trigger biofilm dispersal include EPS matrix breakdown mediated by enzymes (Kaplan et al. 2003), cell loosened by surfactants (Davey et al. 2003) and signaling molecules production, which induce the dispersal response. Physiologically, dispersal is thought to be triggered by unfavorable conditions within the biofilm, such as nutrient limitation, oxygen lackage, temperature changes, waste product accumulation and low levels of nitric oxide (NO) (McDougald et al. 2012).

Given that dispersal is an active process, which is regulated as a consequence of environmental cues, then it stands to reason that it should be possible to identify those regulatory systems and the associated, downstream effector that control biofilm disassembly. One of the general themes that has emerged is the central role of the intracellular second messenger cyclic di-GMP (c-di-GMP) in regulating the switch between planktonic and biofilm growth (Schleheck et al. 2009). Indeed, c-di-GMP has been linked to biofilm formation and dispersal in a wide range of bacteria including *P. aeruginosa* and its general role in controlling this switch make it an interesting target for biofilm control. The signaling molecule nitric oxide (NO) was previously found to trigger biofilm dispersal in *P. aeruginosa* at low, nontoxic concentrations (Barraud et al. 2006) (this would be further discussed in Section 2.2.6.2 below for NO). C-di-GMP signaling is also involved NO mediated dispersal of *P. aeruginosa* (Barraud et al. 2006). Cell density-dependent regulation may induce microorganisms to detach from a biofilm through expressing matrix-degrading enzymes when the cell density reaches a certain level in biofilm microcolonies. Collectively, these observations suggest that the systems that regulate detachment events in microbial

biofilms represent opportunities to discover new approaches to control biofouling.

2.2.3 Extracellular Polymeric Substances (EPS)

Biofilms are multicellular consortia of microorganisms embedded in extracellular polymeric substances (EPS) in close proximity to one another (Donlan 2002). The EPS provides a highly hydrated gel matrix in which microbial cells can establish a stable synergistic consortium (Lawrence et al. 2003). The importance of EPS is very crucial in the process of biofilm development, which is emphasized here. EPS is typically composed of nucleic acids, polysaccharides, proteins, lipids and other biological macromolecules (Lawrence et al. 2003). However, the composition of biofilm EPS is highly dynamic and can vary, depending on many biological and nonbiological parameters.

2.2.3.1 Compositions of EPS

1. Polysaccharides

Extracellular polysaccharides are the most extensively studied component of the biofilm matrix and are often considered to be the most abundant component of EPS in biofilms (Christensen 1989). Depending on the type of extracellular polysaccharides, the carbohydrate monomers, glycosidic linkages and degree of substitution can vary extensively. Many extracellular polysaccharides are homopolysaccharides, which are based on repeating units of a single sugar, e.g. cellulose is made up of repeating glucose units (Rehm and Valla 1997). Heteropolysaccharides generally consist of repeating blocks or units of different carbohydrates, e.g. alginate is comprised of repeating units of β -D-mannuronate and α -L-guluronate (Rehm and Valla 1997).

Pseudomonas aeruginosa, one of the best studied, model biofilm bacteria, generates at least three distinct types of extracellular polysaccharides: alginate, Pel and Psl, which are important for laboratory biofilm development and structure (Ryder et al. 2007). Alginate is an unbranched, heteropolysaccharide composed of residues of β -D-mannuronate and α -L-guluronate linked at 1,4-uronic organization. Mucoid strains of *P. aeruginosa* overproduce alginate, which is also usually as a result of

mutated gene encoding the σ -factor AlgU negative regulator (MucA) (Ohman and Chakrabarty 1981), such as in the mutant FRD1 (MucA22). Alginate overproduction has been linked to exposure to ethanol, starvation and increased osmolarity (Govan and Deretic 1996). Alginate has been shown to be involved in initial adhesion of biofilm formation and physical maintenance of mature biofilms (Ryder et al. 2007). Mucoid isolates are commonly isolated from the lungs of infected cystic fibrosis patients and it has been hypothesized that the alginate matrix is important either in adaptation to the lung environment or for protection from the host immune response (Pedersen et al. 1992). Pel is abundant in glucose although the structure is currently not known, and Psl is composed of repeating pentasaccharide unit, which contains D-mannose, D-glucose and L-rhamnose (Jackson et al. 2004; Byrd et al. 2009). Yang et al. (2011) identified conditions in which *P. aeruginosa* PAO1 biofilms utilize Pel for greater structural stability in formation of microcolonies. Psl is extremely important for initial adherence of sessile cells to both biotic and abiotic substrates (Ma et al. 2006; Byrd et al. 2009; Byrd et al. 2010). Mature biofilms also take advantage of Psl for aiding structural stability and architecture (Ma et al. 2009). Ongoing investigations are focused on the role of both forms of Psl and its impact on single cells or biofilm communities (Mann and Wozniak 2012).

2. Proteins

Another major component of the EPS matrix is protein, which can come from both living and dead cells. Some of the proteins found in the matrix include lytic enzymes, produced by bacteria, which are capable of degrading biopolymers. Such enzymes include lyases and polysaccharases, which degrade polysaccharides. Some such enzymes are involved in extracellular polysaccharide biosynthesis, where the enzyme processes the growing polymer and can regulate chain length (Ghannoum and O'Toole 2004). The second role of such enzymes is in extracellular polysaccharide utilization, in which, enzymatic activity is used to break down extracellular polysaccharide polymers for use as carbon and energy substrates for growth (Ghannoum and O'Toole 2004). This suggests that the extracellular polysaccharides

can serve as carbon storage when the bacteria experience conditions of poor nutrient access. In addition, such enzymes, which are responsible for the degradation of EPS matrix, may facilitate dispersal of bacteria from biofilms (Flemming and Wingender 2010). The inclusion of enzymes in the EPS indicates that the EPS is an active part of the biofilm, capable of mediating biological transformations important for the encased cells.

3. Nucleic acids

Extracellular DNA (eDNA) has also been demonstrated to be a significant component of EPS. Initially, it was assumed that most of the eDNA was from lysed cells and had no specific function. However, it was recently suggested that during the initial period of biofilm formation by *P. aeruginosa*, eDNA acts as a structural component of the EPS matrix (Whitchurch et al. 2002). For example, the addition of DNase was shown to inhibit the development of biofilms by *P. aeruginosa* (Whitchurch et al. 2002). The DNA may be derived from membrane vesicles secreted by *P. aeruginosa*, as well as from lysed cells (Whitchurch et al. 2002). Another study provided evidence that in later stages of *P. aeruginosa* biofilm development significant amounts of DNA may be released into the EPS matrix through programmed cell lysis (Webb et al. 2003). While it seems clear that eDNA plays an important role in biofilm development, the exact role of eDNA in biofilm development is not yet known. One interesting concept that has not been rigorously tested is that the high eDNA content of the EPS matrix and high local cell density may play a role in horizontal gene transfer, however, this remains to be determined.

2.2.3.2 Functions of EPS

In biofilms, some general functions have been attributed to EPS such as keeping the bacteria cells together in a three-dimensional, gel-like network, adhesion to surfaces, the establishment of infections and protection of bacteria from stresses (summarized in Table 2.1).

Table 2.1 Proposed functions of EPS in the biofilms (Flemming and Wingender 2010)

Function	Relevance
Adhesion to surfaces	Initial step in colonization by planktonic cells to surfaces, whole biofilms attached to surfaces in long term
Aggregation of bacterial cells	Bridge between cells, adherent agent for multi-bacterial populations, developing high cell densities; formation of flocs, granules and biofilms, cause of biofouling and biocorrosion events
Structural elements of biofilms	Determination of mechanical stability of biofilms and the three-dimensional, gel-like network shape of EPS structure
Retention of water	Preventing desiccation under water-deficient conditions
Defensive barrier	Withstanding nonspecific and specific host defenses, resistance to certain biocides including disinfectants and antibiotics, resistance to EPS-lysing enzymes, protection for anaerobic bacteria from harmful effects of oxygen and against being grazed by protozoa
Organic compounds adsorption	Scavenging, adsorption and digestion of nutrients from the surrounding environments; nutrient removal for wastewater treatment
Sorption of inorganic ions	Promotion of polysaccharide gel formation by bridging between the EPS and the ions, ion exchange; accumulation of toxic metal ions (detoxification)
Enzymatic activity	Shortening the chain length, change of matrix structure and stability, formation of pores and channels, dispersal of biofilm cells by degrading structural EPS, degradation of extracellular macromolecules for nutrient usage, lysed cells can be used by the remaining population
Nutrient source	Source of C, N, P

Cell-cell recognition	Cell-cell communication via quorum sensing (QS) signals, specific recognition by lectins, horizontal gene transfer, symbiotic relationships with plants or animals
Redox activity	Electron donor or acceptor
Export of cell components	Release of intracellular materials as a result of metabolic turnover
Interaction of polysaccharides with enzymes	Retention, protection and stabilization of secreted enzymes
Gradient formation	Oxygen, pH, redox potential, ionic strength gradients

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2.2.4 Impacts of Biofouling on RO

Biofouling in RO systems leads to higher energy requirements due to the effect of resistance caused by biofilm formation on the membrane, which reduces the efficiency of water passage across the membrane. Biofilm formation is also associated with a loss of the effective driving force due to higher solute (salt) concentrations on the membrane surface as a result of enhanced concentration polarization (CP), which is identified as biofilm-enhanced osmotic pressure (BEOP) (Herzberg and Elimelech 2007). In addition, biofouling can cause an increase in the channel pressure drop (ΔP) (Vrouwenvelder et al. 2009a). In BEOP, the biofilm represents as an ‘unstirred’ layer on the membrane surface, where the retained solutes have to diffuse through the deposit in order to back transport to the bulk fluid. A reduction in the effective diffusion means an increase in the CP. For a constant flux operation, the presence of a biofilm on the surface of an RO membrane results in higher trans-membrane pressure (TMP), leading to a higher energy requirement and increased cost of operation (Flemming 1997).

Besides the system performance lose, biofouling may also lead to impacts of inappropriate treatment methods. To combat the biofilm problem, many different cleaning protocols and compounds have been used, most of which are toxic or

hazardous chemicals (e.g. chlorine or hypochlorite). However, these biocides tend to be ineffective and there is a desire to reduce the use of toxic compounds from environmental perspective.

2.2.5 Factors Affecting Biofouling in RO

An extensive effort has been made in recent years to understand the nature of biofouling in RO systems. The degree of biofouling in RO systems is determined by three basic fouling factors, the nature of the feed (e.g. microbial loading, nutrient level, salt concentration), the membrane properties (e.g. hydrophobic, hydrophilic, roughness, surface charge) and the hydrodynamic environment (e.g. flux, cross-flow velocity) (Matin et al. 2011). The interactions between these parameters are complex and can determine the performance of the RO system.

The main water sources for RO process are seawater and brackish water, which accounts for approximately 62% and 20%, respectively (Pankratz 2010). As stated above in Section 2.2.1, the existence of microorganisms and nutrients in the feed water are the two main causes for membrane biofouling. In addition to these, the presence of other foulants in the feed water may increase the significance of biofouling. For example, the existence of transparent exopolymer particles (TEP) (shown in Section 2.2.2.1) have been considered to be “major initiators” of membrane biofouling (Berman et al. 2011; Berman and Holenberg 2005).

It is well-known that the quality of the feed water is influenced significantly by the pretreatment and control methods. Generally, the target of pretreatment is to reduce the foulants in the feed water before entering the membrane set-ups (Sutskover-Gutman and Hasson 2010). The degree of membrane biofouling is dependent on the extent of pretreatment. Adequate pretreatment would improve the performance of the RO system and prolong the life span of the membrane modules. The common pretreatment methods for RO contain screening, chemical conditioning, sedimentation and filtration, followed by the chlorination (Voutchkov and Semiat 2008). Details of biofouling control are illustrated in Section 2.2.6.

The RO module consists of membranes, feed channel spacers, permeate

separators and the collective tube. The performance of the RO system depends directly on the characteristics of the membrane material. Based on the types of polymer backbone, RO membranes are usually classified into two categories: cellulose acetate (CA) and polyamide. Among them, the most popular one is the polyamide membrane which contains a crosslinked polyamide as the active selective layer on the top and a polysulfone support layer on the bottom. In order to improve the membrane performance, surface modification is tried recently to modify the hydrophobicity/hydrophilicity, reduce the roughness and change the surface charge (Flemming 2011). In the RO spiral wound membrane (SWM) modules, the spacers are for keeping the membrane sheets apart, as well as to improve the hydrodynamic conditions (Schwinge et al. 2004; Da Gerales et al. 2002a; Costa et al. 1991). Although not as popular as membrane modification, investigation of possible modification to the feed channel spacer is getting more attention in recent years (Araújo et al. 2012; Miller et al. 2012; Ronen et al. 2012).

In addition, modification of operation of the RO system, e.g. altered flux and cross-flow velocity (CFV) to slow or reduce fouling has also been investigated and while this approach has led to some improvements. The relationship between flux and membrane fouling had been studied by some researchers. While most of the studies focus on the colloidal fouling in microfiltration (MF) or ultrafiltration (UF) processes. The transition from a colloidal suspension to a more dense gel is dependent on the flux, in which higher flux decrease the time required for the transition to a dense gel (Sim et al. 2014). From another study with particles (Neal et al. 2003), it would be anticipated that the effects of flux on fouling would follow similar trends (fouling increasing with flux). One study involved in biofouling in RO, it was reported that biofouling was manifested as a rise in trans-membrane pressure (TMP) and surface loading, increased with imposed flux (Chong et al. 2008a). However, the data published by Vrouwenvelder et al. (2009a; 2009b) showed contrary results in an RO system with feed channel spacers, which showed that the impact of flux was not apparent towards biofouling. These contradictory observations relating to the biofouling process in RO need to be systematically addressed as it is critical to

understand the mechanism for sustainable operation of RO technology.

There is a conception ‘critical flux’, generally defined as the flux level above which particle and colloid deposition occurs on the membrane surface. A widely accepted recommendation to reduce fouling is by holding flux below the so called “critical flux” (Diez et al. 2014). In biofouling aspect, it is reported that stable flux can be achieved by operating below the critical flux in a forward osmosis (FO) membrane process fouled by microalgae (Zou et al. 2013). While in some other studies, it was observed that the critical flux concept is not a suitable approach to control biofouling of spiral wound RO membranes (Vrouwenvelder et al. 2009c). Even under the operation of the sub-critical flux the fouling still could not be completely prevented when a hollow fiber membrane module was submerged in the yeast suspension (Li et al. 2012).

Cross-flow velocity (CFV) is another important factor in flow hydrodynamics inside the feed channel (Winters 1997). In general, cross-flow filtration can help to prevent the membrane from fouling, which in theory is a continuous process, where the flow generates a shear force that keeps the membrane surface free of foulants. While the debate still exists for the effect of CFV on fouling in RO membrane systems. Chong et al. (2008a) reported that biofouling reduced with CFV in an RO system. Further, Wicaksanaa et al. (2012) also demonstrated that lower biofouling rate was achieved at higher CFV in a microfiltration process. From other studies with colloids (Chong et al. 2008b) and particles (Neal et al. 2003), it would be anticipated that fouling would reduced by decreasing with cross-flow. The results support the BEOP model where the fouling rate increased with decreasing CFV through the reduction in the effective mass transfer. However, recent published data by Vrouwenvelder et al. (Bucs et al. 2014; Vrouwenvelder et al. 2009a; Vrouwenvelder et al. 2009b) showed contrary results in an RO system with feed channel spacers. Increased crossflow was shown to lead to a higher ΔP rise due to a higher supply of nutrients.

2.2.6 Control of Biofouling

Conceptually, strategies to ensure that biofouling does not impact the optimal, long-term membrane performance are based on prevention of fouling or the removal of biofilms from the membrane once it is fouled. The prevention of fouling essentially requires sterilization of the inflowing water (not feasibly practical), the continuous addition of inhibitors such as biocides at high concentrations or the addition of compounds that hinder microbial adsorption. Biofilm removal requires the application of compounds that lead to the detachment or dispersal of the pre-established biofilm through mechanical or chemical techniques. There are currently a number of approaches (National Research Council. 2008) that are used to control biofilm formation on RO membrane systems, which have their individual advantages. While effective to some degree, none of the methods prevent biofouling or remove established biofilms completely and hence additional approaches are needed to improve the long-term operation of RO systems for water production.

2.2.6.1 Conventional Treatment Methods

The most commonly used oxidant for biofouling control is chlorine or hypochlorite. The major obstacle to the use of chlorine for biofilm control in RO systems is that it is an oxidant, which can cause significant damage to the thin-film RO membranes commonly made from polyamide materials (National Research Council. 2008). Hence, the use of such oxidants requires them to be removed before entering RO membrane modules, which is typically achieved by the addition of reducing agents, such as sodium bisulfite. This results in the water entering the RO modules not containing any residual biocide and allows for bacteria that have not been killed prior to entering the modules to subsequently grow and form biofilms on the membranes.

Ozone and ultraviolet (UV) treatment are considered as potential alternatives to chlorine-based control of biofilm forming bacteria in the feed water. Ozone is a much more effective disinfectant than UV, but it may damage the oxidant-sensitive membranes, while UV treatment does not (Glaser et al. 1983).

1. Chlorination

Within potable water systems, chlorine is one of the most generally used antimicrobial agents used, which has two effects on bacteria (Bitton 1994). First, chlorine disintegrates the bacterial cell membrane, which permeabilizes the membrane, resulting in leakage of proteins, DNA and RNA from the cell. Secondly, chlorine directly damages nucleic acids and enzymes through oxidation. The activity of chlorine is not bacteria specific and hence can have similar effects on mammalian cells and can also result in the formation of toxic by-products, trihalo-methanes (THMs) (Walker et al. 2000) And when chlorine is used as pretreatment in membrane technology, dechlorination is necessary because the existence of chlorine can damage the membrane. As a consequence, there is significant interest in the development of alternative disinfectants as a primary biocide in potable water systems.

Chloramines have been proposed as one of the alternatives to chlorine due to the concerns about public health because of the production of THMs. However, the general use of chloramines in potable water systems and for controlling biofilms is handicapped because of the concern that it leads to generation of nitrites at low concentrations (O'Neill et al. 1997). This may result in the product water not meeting nitrite standards for the potable water, which is unacceptable as it is well acknowledged that nitrites have important consequences on public health.

Chlorine dioxide is a strong oxidant formed by the combination of chlorine and sodium chlorine and effectively inactivates a wide range of bacteria and viruses over a broad pH range (Tanner 1989). However, it is unable to maintain an effective residual through the whole circulation system. However, it is superior to chlorine in the fact that it will not interact with precursors forming THMs.

2. Ozonation

Ozone is a more powerful oxidizing reagent than hypochlorous acid. Ozone has not only been acknowledged to disrupt permeability of microorganisms' membranes, lower the activity of the enzymes and also damage DNA (Ishizaki et al. 1984), and

has also been shown to inactivate viruses through nucleic acid damage (Roy et al. 1981). Although ozone does not form THMs, ozone rapidly decays in aqueous environments, decomposing back to oxygen. Therefore, ozone is typically added to raw water and filtered for primary disinfection, followed by chloramine addition afterwards as disinfectant for the distribution system.

3. Ultraviolet (UV) Light

The ultraviolet (UV) method of disinfection involves exposure of water to light from a mercury vapor arc lamp that emits UV (Walker et al. 2000). UV works well against viruses and bacteria in that it causes thymine dimerization, which blocks DNA replication (Walker et al. 2000). UV disinfection is mainly advantageous in its capability to inactivate microorganisms in the aquatic phase leaving behind no residuals (Walker et al. 2000). However, the lack of residual is also a disadvantage since there is no downstream protection against microbial growth once the UV-treated water leaves the UV unit. The microorganisms that were not killed by the UV radiation will grow and foul downstream equipment. UV technology is also constrained by other limitations such as a high energy requirement and a high capital investment.

Two types of UV lamps are commonly used. Low pressure (LP) UV emits monochromatic wavelength at 253.7 nm, and medium pressure (MP) UV provides broad polychromatic output from 220 to 300 nm (Wolfe 1990). UV disinfection can be used to control the inflowing source water such that supplying in sterilizing water and preventing biofilm development. It has been reported that UV pretreatment is a promising option for combating biofouling of RO membranes and enhancing the performance of the trains (Harif et al. 2011). However, much research is warranted to better understand the extent of its effects on preventing biofouling in membrane applications for the control of biofilm development in RO systems.

2.2.6.2 Future Methods

As indicated above, microorganisms are much less sensitive to biocides when

they grow as a biofilm than when they are in the free-living, planktonic phase. This effect is mediated by producing EPS, changing gene expression, e.g. to induce genes involved in resistance and slowing down growth rate for some of the cells within the biofilm. Due to the dilemma that traditional biocides cannot eradicate biofilms completely and the general desire to use nontoxic compounds, there is a strong interest to develop novel methods that could be effective in the control of membrane fouling.

1. Enzymes

Enzymes can degrade specific macromolecules involved in maintaining the biofilm at a surface, including (Walker et al. 2000; Brisou 1995): (1) microbial adhesins; (2) the polysaccharides in EPS. Due to the heterogeneity of the EPS present in the biofilm matrix, a mixture of different enzymes would be necessary for a sufficient removal of the biofilm (Chan and Wong 2010). Enzymes and detergents have also been used in combination to improve disinfectant efficacy.

2. Natural Antifoulants

Biofouling is not only a problem for industrial processes, but also affects living systems. This is particularly true for sessile marine organisms such as algae that are exposed to high numbers of bacteria present in the water column. As a consequence of this pressure to resist fouling, algae have evolved a range of strategies to avoid being overgrown by fouling organisms. These include the shedding of tissue, excess mucus production and secondary metabolites that are antifouling (Fusetani 2004).

One of the best examples of antifouling compounds from the higher organisms is the production of halogenated furanones, a group of compounds, which have various activities with broad spectra against multicellular behaviors among bacteria. They were found from a type of marine red algae, *Delisea pulchra* (de Nys et al. 1993). More than 30 furanones are generated by *D. pulchra* that are secreted onto the surface of the plant and act to defend it against being colonized by both prokaryotic and eukaryotic microorganisms (de Nys et al. 1993). Based on the discovery of furanones,

efforts have been made to artificially synthesize the natural compounds as well as synthetic analogues (Manny et al. 1997).

Several studies have shown the inhibitory impact of furanones on biofilms formed by *E. coli* (Ren et al. 2001), *Bacillus subtilis* (Ren et al. 2002), *P. aeruginosa* (Hentzer et al. 2002), *Streptococcus mutans* (He et al. 2012) as well as many others. It has been reported that the furanones specifically target and interfere with quorum sensing (QS) systems in *P. aeruginosa* PAO1, to control biofilm formation and virulence factor expression (Hentzer et al. 2003). QS is a term to describe the phenomenon that microorganisms communicate via producing and receiving chemical cues, called autoinducers (AIs) (McDougald et al. 2007). Bacterial cells generate and excrete AIs, and as the concentration reaches a threshold, the whole bacterial population modifies gene expression to simultaneously display the same phenotype at the population level. Several different QS systems have been found present in bacteria. In general, the N-acylated homoserine lactone (AHL) system is found in Gram-negative bacteria and the auto-inducer 2 (AI-2) system in both Gram-positive and Gram-negative bacteria (Rice et al. 2005). Halogenated furanones can interrupt QS systems in bacteria by acting as agonistic or antagonistic agents. Because of the structural similarity between furanones and the QS signal molecules, a hypothesis that furanones may interrupt the QS circuits had been proposed (Givskov et al. 1996). QS is based on competitive inhibition of autoinducer binding to the receptor. Furanones appear to mediate their effects on biofilm development and virulence factor expression at concentrations that are not inhibitory to growth, which is distinct from the biocidal activity of antibiotic compounds such as chlorine. This finding implies that furanones could be used for combating bacterial biofilms.

For example, halogenated furanones significantly reduced *P. aeruginosa* PAO1 drug resistance and enhanced its antibiotic susceptibility to tobramycin and SDS (Hentzer et al. 2003). Corrosion inhibition by furanones was confirmed via the study that the mild steel was protected against the corrosion induced by the sulphate-reducing bacterium (SRB), *Desulfotomaculum orienti* (Ren and Wood 2004). The use of covalently bound furanones coated on biomaterials or membranes has been

investigated. Bacterial load of *Staphylococcus epidermidis* was significantly reduced on furanone-coated materials (Baveja et al. 2004; Hume et al. 2004). This indicates that there is a potential application of furanone-coated materials to reduce medical device-associated infections and there is the potential that this strategy could be applied to control membrane biofouling as well.

3. Nitric Oxide (NO)

Nitric oxide (NO) is a water-soluble, free radical gas that can diffuse in biological systems (Schmidt et al. 2004). It has not only been shown to be important in physiological signaling in eukaryotic biology studies, including humans, but it has also been shown that NO is the signaling trigger controlling the switch between planktonic and biofilm growth. In the biofilm, when NO accumulates, as a by-product of the denitrification pathway, which happens when the biofilm becomes limited for oxygen, the biofilm is induced to disperse back into planktonic cells and operates at concentrations that are non-toxic to bacteria (Barraud et al. 2006). A *ΔnirS* mutant strain of *P. aeruginosa*, which cannot make NO, did not undergo dispersal (Barraud et al. 2006). Based on this, it may be possible to artificially induce biofilm dispersal by adding NO exogenously. There are a range of compounds, called NO donors, that have been developed for human health applications, where they are used to control heart rate and blood pressure (Lin et al. 1983). Such compounds can also be used to deliver NO to bacterial biofilms to induce dispersal. For example, the compound sodium nitroprusside (SNP) was shown to induce biofilm dispersal at concentrations as low as 25-500 nM. NO can has been shown to not only disperse single species biofilms formed under laboratory conditions, but can also disperse biofilms formed by multiple species from water treatment and distribution systems (Barraud et al. 2009a). A preliminary study on the mechanism on NO-induced dispersal showed that in *P. aeruginosa*, addition of NO stimulated phosphodiesterase (PDE) activity, followed by induction of accelerated degradation of the secondary messenger cyclic di-GMP (c-di-GMP) which is known to regulate the switch between planktonic and biofilm growth (Barraud et al. 2009b).

In addition to inducing dispersal, the addition of NO has been shown to reduce the high level of antimicrobial resistance associated with biofilm formation. For example, *P.aeruginosa* biofilms that were first exposed to NO were almost completely eradicated by the subsequent addition of antimicrobial compounds (Barraud et al. 2006). Thus, the combined exposure of a biofilm to NO along with the traditional antimicrobials may offer novel strategies to control a range of medically and industrially relevant biofilms. To date, the use of NO has not been implemented in a high pressure membrane system.

From the economic aspects, chemical control (chlorination) is relatively cheap and hence the most popular method in water disinfection. Next, the conventional filtration is also economic and easy to install and operate. Membrane filtration (MF/UF) is considered to spend more, while better quality of treated water is obtained. UV is relatively expensive than any other method, which may be used more and more widely due to its environmental friendly method.

2.3 Summary

A literature review on the basic fundamentals of reverse osmosis, the principles of biofouling and various methods or techniques to control the biofouling process are reported in this chapter.

Reverse osmosis is a relative novel and advanced technique for water purification. However, its usage is limited seriously by fouling, especially by biofouling. To combat biofouling, it is essential to understand the formation process of the biofilms on the RO membranes in the first place. So far, there are some debates on the influences of the operating conditions (without vs. with feed channel spacer, different nutrient levels, different flux and cross-flow velocity) on biofilm development and TMP increase. Therefore, the assessment of the roles of the above conditions on biofouling is critical. The researchers had discovered that some compounds could be used to control biofouling in RO. Unfortunately, previous methods are not suitable for the RO process in long term. They may damage the membranes or pollute the environment. Thus, there is an increasing request to develop

new techniques, such as UV pretreatment, quorum sensing inhibitors or biofilm dispersal agents to control biofouling.

2.4 Research Gaps

From the studies in the literature review stated above, it is clear that biofouling, typically the result of microbial growth or biofilm formation on the membrane, is probably the most prevalent and problematic type of fouling in an RO system (Flemming 1997). Because of that, new approaches must be developed to control biofilm development and this requires a detailed understanding of how biofilms form in the first place.

It is correct that biofouling is a mixed species biofilm problem and ideally such communities would be used for fouling studies. However, there are few or no examples of mixed species cultures at the level of high diversity observed in RO membrane biofilms, that can be shown to reproducibly form biofilms in a controlled fashion. Therefore, *Pseudomonas aeruginosa* was used as a mono-species biofilm culture. This is appropriate as there are multiple publications on *P. aeruginosa* fouling of membranes, which allows for relative comparison, because *P. aeruginosa* is found in membrane fouling communities and because we have access to a range of mutants, e.g. the alginate over producing mutant, that allow for detailed studies of specific genes or gene products on membrane biofouling. In this study, for example, to more clearly delineate the role of polysaccharides in RO performance, *P. aeruginosa* PAO1 wild type (WT) and its mucoid mutant FRD1, which over produces alginate, were used to foul the membrane.

An extensive effort has been made in recent years to develop the technologies to understand the nature of biofouling in RO. Among them, confocal laser scanning microscope (CLSM) allows detailed, non-destructive and three-dimensional examination of thick microbial samples. Fluorochromes are stains or probes that are added to cells to obtain a fluorescent signal. The fluorescent nature of the compounds greatly facilitates their use in studying bacteria associated with optically nontransparent surfaces (Maukonen et al. 2000). In this study, RO set-up was

operated at constant flux and biofouling was measured by the increase of TMP and by membrane autopsy. Fouled membranes were stained with different types of fluorescent dyes. *Ex situ* observation with CLSM was conducted to visualize the architecture of the biofilms, as well as for quantification of EPS.

RO biofouling is a problem of biofilm development. Prior to this study, there have been limited studies focusing on biofilm characterization at different stages of biofouling in RO. It was reported that in an membrane bioreactor (MBR) process, the main factors that accelerate biofouling were the soluble microbial products (SMP) combined with EPS, in which the rapid rise of SMP triggered a sudden increase in the TMP, while the accumulation of EPS caused the sustained rise in TMP (Gao et al. 2013). And in another MBR system, it was shown that two mechanisms of inhomogeneous pore loss and changes in percolation due to EPS accumulation were responsible for the steep TMP rise (Hwang et al. 2012). However, so far, most of the studies involved in ‘TMP jump’ are concentrated on MBR, further study is called for in RO.

Most RO biofouling studies were done in laboratory-scale flat sheet units without the involvement of feed channel spacers. While the negative impact of biofilm development on the membrane is obvious, the contribution of feed spacer to the biofouling problem has not been well understood. Flux and cross-flow velocity (CFV) have been shown to be the most important hydrodynamic parameters in RO process. It is true that there is abundant literature on critical flux which shows fouling can be controlled by limiting flux. However, some published data by Vrouwenvelder et al. (2009a, 2009b) showed contrary results in an RO system. These work showed that the impact of flux was not apparent towards biofouling. These contradictory observations relating to the biofouling process in RO need to be systematically addressed as it is critical to understand the mechanism for sustainable operation of RO technology. Therefore, in this study, an approach to understand the impact of different hydrodynamic conditions (flux and crossflow velocity) on biofouling in RO spacer-filled channels was investigated.

The common pretreatment methods for controlling RO biofouling is chemical

addition (chlorine). This method is well-known to cause negative effects on membrane and the environment as stated above in Section 2.2.6. Based on this issue, the application of ultraviolet (UV) as a pretreatment, as well as NO addition to disperse the pre-established biofilms were investigated in this study.

CHAPTER 3 Dynamics of Biofilm Formation under Different Nutrient Levels and the Effect on Trans-membrane Pressure (TMP)¹

3.1 Introduction

An extensive effort has been made in recent years to understand the nature of biofouling in RO systems. The degree of biofouling in RO systems is determined by three basic fouling factors, the nature of the feed (e.g. nutrient level, salt concentration, microbial loading), the membrane properties (hydrophobic, hydrophilic, roughness, surface charge) and the hydrodynamic environment (e.g. flux, cross-flow velocity) (Matin et al. 2011). The interactions between these parameters are complex and can determine the performance of the RO system. In the process of this study, it was observed that RO biofouling experiments performed under conditions of constant flux, the TMP initially showed a slow increase followed by an exponential increase in pressure that has been termed ‘TMP jump’. Similarly, when operated under conditions of constant pressure, Herzberg and Elimelech (2007) observed a decrease in flux, which consisted of a 3-4 h lag phase where the flux showed minimal decline, followed by a drastic flux decline (20%) within 15 h. This phenomenon of TMP jump has also been associated with biofouling in membrane bioreactors (MBRs) (Zhang et al. 2006; Cho and Fane 2002; Ognier et al. 2005). However, to date, little is known about the mechanisms by which biofouling influences the TMP jump in RO systems.

The objective of this study was to correlate biofilm development on RO membranes over time under different nutrient conditions with the TMP jump. Further, the relative contributions of bacterial cells (live and dead) and the biofilm matrix components (extracellular DNA, polysaccharides and proteins) to the TMP rise were

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¹ This study was undertaken in collaboration with Stanislaus Raditya Suwarno from the Singapore Membrane Technology Centre (SMTC) who operated the lab-scale reverse osmosis (RO) experimental system, recorded the running parameters and passed the RO membrane samples to me. I developed the hypotheses, designed the experiments, optimized the methodology, imaged and quantified the biofilms on the membranes, analyzed the data and interpreted the phenomena, and took sole responsibility for the presentation of the results here.

determined by quantitative microscopic measurements of the biofilm and by chemical extraction. To further investigate the role of extracellular polymeric substances (EPS) in the TMP rise, *Pseudomonas aeruginosa* PAO1 wild type (WT) and its mucoid mutant FRD1, which over produces alginate, were used to foul the membrane. The RO system was operated at constant flux and the increase in TMP was monitored continuously to evaluate performance. *Ex situ* observation by confocal laser scanning microscopy (CLSM) was also conducted to visualize the architecture, as well as for quantification of extracellular DNA (eDNA) and polysaccharides in the EPS of the biofilms.

3.2 Materials and Methods

3.2.1 RO Set-up and Operation

The RO set-up (Figure 3.1) was assembled as two cells in series. Each stainless steel RO cell had a flat plate geometry and flow channel sizes of $310 \times 60 \times 0.8$ mm (L $\times$ W $\times$ H) with an effective area of 0.0186 m^2 . The design and operation provided conditions that simulate typical large-scale RO processes. The feed tank was equipped with a stirrer (IKA, model Eurostar) and had a capacity of 10 L. A chiller (Polyscience, model 9612AA2P) was used to circulate cooling water through a coil to maintain the feed solution in the feed tank at approximately $25 \pm 1^\circ\text{C}$. To simulate RO process operation, the feed solution was pumped at high pressure through the test cells at a specified flow velocity, and a small fraction of the feed permeated through the membranes as water flux at a controlled rate. A high pressure pump (CATPUMP, model 227) was used to deliver the feed solution, while system pressure and cross-flow velocity (CFV) were controlled using a back pressure regulator (Swagelok, model KBP) and a flow control valve (Cole Parmer, model CP-32505-40), respectively. The feed flow rate was monitored with a turbine flow meter (McMillan, model 114) and the conductivity was measured using a conductivity meter (Mettler Toledo, model Seven Multi). A mass-flow controller (Brooks Instrument, model 5882) was installed on the permeate side of each cross-flow cell to maintain the amount of permeate withdrawn. Permeate quality was monitored by conductivity meters

(Rosemount Analytical, model Solu Comp II) and the pressure of feed and permeate streams were monitored by pressure transducers (Bourdon Haenni, model E913). Each RO cell was equipped with differential pressure transmitters (Yokogawa, model JX110A) to monitor the channel pressure.

RO membranes (DOW Filmtec, BW-30) were cut to size and soaked in Milli-Q water for at least 12 h. The hydrated membranes were then sterilized in 70% ethanol (Merck) for 1.5 h and then rinsed with Milli-Q water. The membranes were compacted at a maximum flux, which was up to 65 liters/m²/h (LMH) overnight with Milli-Q water until a stable flux was achieved. Following compaction, the flux was set to the desired value (35 LMH) and a NaCl stock solution (200 g/L) was added to the feed tank to a final concentration of 2 g/L. The system was allowed to mix for 1.5 h before a nutrient broth (NB) stock solution (5 g/L) was added into the feed tank to provide average nutrient concentrations of 0, 10, 15 or 20 mg/L (20 mg/L NB equals to 6.5 mg/L total organic carbon) accordingly. The system was allowed to mix for a further 1.5 h prior to the start of the experiment. The feed solution was replenished twice per day and the total organic carbon (TOC) was tested with TOC analyzer (Shimadzu, Model TOC-VWS) and bacteria numbers were determined by colony forming unit (CFU) counts.

3.2.2 Bacterial Fouling of Membranes

P. aeruginosa PAO1 WT or its mutant FRD1 (Mathee et al. 1999) was used to foul the RO membranes. Bacteria were grown in 300 mL batches in NB (5 g/L NB, 2 g/L NaCl, Difco NB-BD diagnostics) with shaking at 150 rpm at room temperature (RT) for 24 h. The bacterial cells were subsequently harvested by centrifugation at 4000 g for 20 min. The pellet was resuspended in sterile, 2 g/L NaCl solution to an optical density (OD) of 0.05 at 600 nm using a spectrophotometer (Shimadzu, model UV1800). The stability of the bacterial suspension was detected by viable bacteria counts. Samples were taken periodically from the bacterial suspension within 24 h period. Between 3 and 6 samples were taken and used for quantification of CFUs.

The bacterial suspension was injected into the system before the feed solution

entered the RO cells using an injection pump (ELDEX, model 5979-Optos Pump 2HM). The RO system was operated in fully-recycled mode where the bypass, concentrate and permeate flows were returned to the feed tank. Therefore, a set of microfilters (KAREI, 5 and 0.2 μm for concentrate and 0.2 μm for bypass) were installed downstream of the pressure cell to prevent excess bacteria from entering the feed tank and turning the feed tank into an ‘active bioreactor’. Bacteria were continuously injected into the flow line at a dilution rate of 1:500 based on CFV (0.17 m/s). Experiments were conducted at constant flux (35 LMH) and TMP was monitored continuously.

Figure 3.1 Diagram of the RO set-up consisting of two RO cells in series. P-1, P-2, P-3, P-4 and P-5 are pressure transducers.

3.2.3 Membrane Autopsy

The fouled membranes were removed from the RO modules for autopsy, which included fluorescence staining and CLSM observation to quantify biofilm volumes, percentages of live and dead cells, and volume of EPS. Viable bacterial counts were determined as well as polysaccharide and protein concentrations extracted from the

membrane surface.

3.2.3.1 Fluorescence Staining and CLSM Observation

Fluorescence staining and CLSM were used to observe the architecture and components of the biofilms on the RO membranes *ex situ* using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, product #L7012), which utilizes mixtures of SYTO 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide (PI). Extracellular DNA was measured using BOBO-3 (Molecular Probes, product #B3586) and the abundance of extracellular polysaccharides associated with the biofilm was determined by ConA-FITC (Sigma, product #C7642) staining. All fluorescent dyes were used according to the manufacturers' specifications with minor modifications. Tables 3.1 and 3.2 show the fluorescent characteristics and the staining conditions of the dyes used in this study.

Membranes were first cut into small coupons that were either 1.5×2 cm or 0.5×2 cm, which were soaked in 50 mL or 1.5 mL tubes containing 10 mL or 1 mL of the working solution for the dye, and then incubated under appropriate conditions (Table 3.2). After incubation, the membrane samples were rinsed with the corresponding buffer solution (Table 3.2) before placing on a glass slide under a coverslip (VFM coverslips 24×50 mm, CellPath, product #SAH-2450-03A). The coupons of 1.5×2 cm size was inlaid into the plastic frame glued onto the glass slide (VFM singlefrost 1.0-1.2 mm, CellPath, product #MAG-1000-03T) and observed using 10 $\times$ objective. The coupons, 0.5×2 cm, were cut and inlaid into the well of the 8-well glass slide (6 mm, Thermo Scientific) and observed using 63 $\times$ objective. The plastic frame of the well was used prevent the coverslip from compressing the biofilm on the membrane.

Table 3.1 The fluorescent dyes used to stain the biofilms on the RO membranes and their fluorescent characteristics.

Dye	Max. excitation wavelength (nm)	Max. emission wavelength (nm)	Fluorescence color	Staining targets
SYTO 9	480	500	Green	Live cells
PI	490	635	Red	Dead cells
BOBO-3	570	602	Red	eDNA
ConA-FITC	494	518	Green	Extracellular polysccharides

Table 3.2 The fluorescent dyes used to stain the biofilms on the RO membranes and their staining conditions.

Dye	Stock solution	Buffer solution	Working solution	Working condition
SYTO 9	1.5 μ L SYTO 9 in 1 mL Milli-Q water	0.85% NaCl solution	100 μ L SYTO 9 stock solution and 100 μ L PI stock solution in 800 μ L in the dark for 1 h	Incubate at room temperature(RT)
PI	1.5 μ L PI in 1 mL Milli-Q water		0.85% NaCl solution	
BOBO-3	----	TE buffer or TBE buffer	2 μ L BOBO-3 stock solution in 1 mL TE buffer or TBE buffer	
ConA-FITC	1 mg ConA-FITC powder in 1 mL Milli-Q water	PBS	100 μ L ConA-FITC or ConA-TRITC stock solution in 900 μ L PBS	

Note:TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0;

TBE buffer: 45 mM Tris-borate, 1 mM EDTA, pH 8.0;

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.1 mM CaCl₂, 0.1 mM MnCl₂, pH 6.8

Microscopic observation and image acquisition of biofilms were performed using a ZEISS LSM710 confocal laser scanning microscope (ZEISS, Germany). Images were captured using ZEISS LSM710 confocal microscope bundled program ZEN 2009. Thresholding was fixed for all image stacks. Between 3 and 6 microscopic images were taken for each sample and used for quantification of biovolume ($\mu\text{m}^3/\mu\text{m}^2$) using IMARIS (version 7.4.0, Bitplane, Switzerland). CLSM was used to capture 3D images, which were imported into IMARIS, where the 'Surfaces' function was used to reconstruct a new surface for each object based on the color and the intensity. The function automatically calculated the biovolume using their proprietary algorithms. This involved calculating the volume (μm^3) of the voxels belonging to each object and then the total volume of the biofilm was the sum volume of all the objects. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) was the total volume (μm^3) per area (μm^2).

3.2.3.2 Viable Bacterial Counts

Membranes were cut into 1×2 cm segments, soaked in 1 mL of 2 g/L NaCl solution in a 1.5 mL tube containing 20 glass beads (1.5 mm diameter). The tubes were shaken using a vortex (Biocote, model SA8) at 2500 rpm for 3 min. The bacterial suspension was serially diluted and CFUs were determined using nutrient agar (8 g/L NB and 10 g/L agar), incubated at 37°C for 24 h. Between 3 and 6 segments were taken for each membrane sample and used for quantification of CFUs.

3.2.3.3 EPS Extraction and Quantification

A volume of 700 μL of the bacterial suspension was transferred into clean 1.5 mL tubes containing 6 μL of 37% formaldehyde, vortexed briefly, incubated at 4°C for 1 h and 400 μL of 1 M NaOH was added. The solution was vortexed briefly and incubated at 4°C for 3 h. Subsequently, the bacterial suspension was centrifuged at 14000 g at 4°C for 10 min, the supernatant was transferred to clean 1.5 mL tubes and kept at -20°C. Between 3 and 6 segments were taken for each membrane sample and used for quantification of EPS.

The polysaccharide content of the EPS was measured by the phenol-H₂SO₄ method (Gerhardt et al. 1994). Briefly, 1 mL of 9% phenol and 5 mL of concentrated H₂SO₄ was added to 1 mL of the sample along with 1 mL Milli-Q water and incubated at room temperature (RT) for 30 min. The absorbance of the solution was measured at 492 nm (Shimadzu, model UV1800). Glucose was used to generate a standard curve for quantification of the polysaccharides.

The protein content of EPS was analyzed using the Bicinchoninic Acid (BCA) Assay Kit (Pierce, product #23225). A 25 µL volume of the sample was mixed with 200 µL of working reagent in 96-well microplates, incubated at 37°C for 2 h and the absorbance was measured at 562 nm using a microplate reader (TECAN infinite M200 pro, Austria). Bovine serum albumin (BSA) was used to generate a protein standard curve.

3.3 Results

To investigate biofilm development on RO membranes over time, a model bacterium, *P. aeruginosa* was used as the biofouling agent. *P. aeruginosa* is ubiquitous in soil and water and has been isolated from biofilms of RO membranes originating from pretreated secondary effluents (Ghayeni et al. 1998). In this study, it was used to foul the RO membranes. The stability of the bacterial suspension was detected by viable bacteria counts. Samples were taken periodically from the bacterial suspension within 24 h period. Experiments were repeated 3 to 6 times and the values are shown in Figure 3.2. The log CFU counts of the bacterial suspension were stable between 7.5 and 8. Because the bacterial suspension was continuously injected into the flow line at a dilution rate of 1:500 based on CFV (0.17 m/s), giving an input load of about 10⁵ CFU/mL. The Live/Dead staining results of the inoculum showed that around 98% of the cells were alive. This indicates that there were approximately 10³ dead cells/mL in the feed solution.

Figure 3.2 Stability of the bacterial suspension as determined by viable bacteria counts. Bars represent standard errors (n=3-6, which is the number of replicates).

The effects of cell deposition and biofilm growth on changes in TMP were characterized under 7 conditions: (i) no bacteria and full nutrient supply (No PAO1 + 20 mg/L NB); (ii) continuous bacterial injection and full nutrient supply (PAO1 + 20 mg/L NB); (iii) continuous bacterial injection and 3/4 nutrient supply (PAO1 + 15 mg/L NB); (iv) continuous bacterial injection and 1/2 nutrient supply (PAO1 + 10 mg/L NB); (v) continuous bacterial injection and no nutrient supply (PAO1 + 0 mg/L NB); (vi) bacterial injection for the initial 2 d and full nutrient supply (2 days PAO1 + 20 mg/L NB); (vii) continuous *P. aeruginosa* FRD1 injection and full nutrient supply (FRD1 + 20 mg/L NB). Continuous injection of the bacteria, as described in Section 3.2.2, was achieved by pumping the bacterial suspension into the system before the feed solution entered the RO cells at a dilution rate of 1:500 based on CFV (0.17 m/s).

3.3.1 Dynamics of Biofilm Formation

3.3.1.1 TMP Rise

The TMP profile in the absence of bacteria showed that after 3 and 6 d, the TMP increased by 5% and 16%, respectively (Figure 3.3). When bacteria were continuously injected into the system ('PAO1 + 20 mg/L NB', Figure 3.3), the TMP

increased dramatically by day 6. The first stage was characterized by a slow TMP increase (5%, 8%, 11% and 22% on days 1-4, respectively), followed by a second stage where an abrupt ‘TMP jump’ from 22% to 159% was observed on days 5 and 6.

Figure 3.3 TMP profiles of the RO system under different conditions: No PAO1 + 20 mg/L NB; PAO1 + 20 mg/L NB. The RO system was run using a feed solution with 2 g/L NaCl, at 35 LMH flux and a cross-flow velocity (CFV) of 0.17 m/s.

3.3.1.2 Comparison of TMP Rise and Biofilm Development

To determine if a correlation existed between the TMP rise and the number of bacterial cells in the biofilm, EPS or total biofilm biovolume, *P. aeruginosa*PAO1 was injected into the RO system and biofilm development was monitored until the TMP reached its maximum. Experiments were terminated after 1, 2, 3, 4, 5 and 6 d and membranes were autopsied at the end of each experiment. The biofilm development progressed from individual cells (day 1), to small aggregates which grew into large aggregates (day 2 and 3), at which time large microcolonies formed on the surface (day 5) and finally, on day 6, a thick biofilm was observed that covered most of the membrane (Figure 3.4). The biovolume of live and dead cells, biofilm thickness and CFUs were quantified for comparison to the TMP profile. The biofilm increased

in volume from 0.07 to 1.39 $\mu\text{m}^3/\mu\text{m}^2$ (days 1-4) and then showed a significant increase on days 5 and 6 for both live and dead cells (Figure 3.5A). The percentage of dead cells was observed to increase on days 5 and 6 (Figure 3.5A). This increase in biovolume was reflected in the increase in the number of culturable cells observed, increasing from 7.06×10^6 (day 4) to 3.17×10^7 and 5.78×10^7 CFU/cm² respectively on days 5 and 6 (Figure 3.5B) at which time the TMP jump was observed. The trend for CFU (Figure 3.5B) and the biovolume of live cells (Figure 3.5A) seemed quite similar and those changes correlated with the TMP jump. Therefore, there may be a relationship between the number of live cells and the TMP jump. The biofilm thickness (Table 3.3) increased from 7.60 to 21.0 μm over the 6-day period.

3.3.1.3 EPS Quantification

One of the key questions to be addressed was the relative contribution of the biofilm EPS to the decline in RO performance. Therefore, eDNA, polysaccharides and proteins, three of the main components of EPS, were also quantified. Surprisingly, the biovolumes calculated for polysaccharides and eDNA were equal to each other at all time points (Figure 3.5C). In comparison, the relative proportion of proteins extracted from the biofilm was higher on days 5 and 6 than the extracted polysaccharides (Figure 3.5D). The combined biovolumes of eDNA and polysaccharides were more than 2 fold higher on day 4 than on day 3 and by day 6 had increased 28 fold compared to day 1 (Figure 3.5C). However, the polysaccharide content, as measured by the staining method, appeared to increase dramatically from day 4 (Figure 3.5C), in contrast to the amount calculated by extraction (Figure 3.5D). To determine if there was a relationship between bacterial cells and the amount of EPS, eDNA and polysaccharide biovolumes were normalized to the total biovolume of cells and the volume of live cells (Table 3.3). For days 1-5, the biovolume of EPS components was always higher than the volume of cells in the biofilm. However, on day 6, when the TMP had reached its maximum, the amount of EPS material decreased significantly, where the ratio was less than 1 in all cases. This was surprising since the number of cells and volume of the EPS constituents increased

across all days.

Figure 3.4

Figure 3.4 (previous page) Confocal microscopic images of biofilm development on the RO membranes. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes were removed for autopsy daily and fluorescently stained and imaged. Column 1 (A, B, C, D, E, F) are Live/Dead stained biofilms, in which the live cells are green and the dead red. The magnification is 60× and the flow direction is from right to left. Column 2 (G, H, I, J, K, L) are images of Live/Dead staining and the magnification is 630×. Column 3 (M, N, O, P, Q, R) are images of eDNA stained with BOBO-3. Column 4 (S, T, U, V, W, X) are images of extracellular polysaccharides stained with ConA-FITC.

3.3.2 Effect of EPS on Biofilm Formation and Fouling

To more clearly delineate the role of polysaccharides in RO performance, biofilm formation and TMP profiles of the wild-type PAO1 and an isogenic, mucoid mutant that overproduces alginate were compared (Mathee et al. 1999; Pham et al. 2004). The TMP profiles for both strains were identical for the first 4 d of operation (Figure 3.6). However, the mucoid strain subsequently showed an earlier TMP jump than the wild-type PAO1 (day 5.5 vs. day 6; Figure 3.6). Interestingly, the total biofilm biovolume was 70% higher for PAO1 than for the mucoid strain (Figure 3.7 and 3.8A), despite the latter reaching the maximum TMP 12 h earlier. Similarly, the number of CFUs (Figure 3.8B) was higher for PAO1 (5.78×10^7 CFU/cm²) than for the mucoid strain (3.17×10^7 CFU/cm²). On day 3, when the TMP profiles were identical, there was no difference in the biofilm biovolume between the strains although the PAO1 was determined to have 140% more CFUs than the mucoid strain (Figure 3.8A, B). By contrast, biofilms of the mucoid strain were determined to have higher amounts of polysaccharides and eDNA at both the low and high TMP stages (Figure 3.8C, D). The protein concentration was similar for the two strains at the high TMP stage (Figure 3.8D). When the EPS components were normalized for total biofilm biovolume/cells, it was clear that the mucoid strain contained about 4 fold more of the EPS components than the wild type strain (Table 3.4). Thus, the mucoid strain produced a biofilm with less bacterial cells and more EPS.

Figure 3.5 Characterization of the biofilms on the RO membranes. *P. aeruginosa* PAO1 was inoculated into the RO system and biofilm formation and TMP were monitored. Membranes were removed for autopsy daily and the biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of live and dead cells was calculated using IMARIS (A) and culturable bacteria counts (B) were compared to the TMP profile. The biovolume of eDNA and extracellular polysaccharides stained with BOBO-3 and ConA-FITC, respectively, was calculated using IMARIS (C) and the amount ($\mu\text{g}/\text{cm}^2$) of extracellular polysaccharides and proteins determined by extraction (D) was compared to the TMP profile. Bars represent standard errors ($n=3-6$, which is the number of different images for A and C, or the number of different membrane segments in B and D).

Table 3.3 The thickness and the biovolume ratio of EPS to bacteria cells for the biofilms of ‘PAO1 + 20 mg/L NB’.

Days	1	2	3	4	5	6
Thickness (μm)	7.60 $\pm$ 1.52	12.00 $\pm$ 0.82	13.83 $\pm$ 3.25	15.25 $\pm$ 0.96	17.00 $\pm$ 1.73	21.00 $\pm$ 1.90
eDNA/Live cells	4.68 $\pm$ 0.16	1.09 $\pm$ 0.46	2.11 $\pm$ 0.34	3.64 $\pm$ 1.16	2.93 $\pm$ 0.21	1.00 $\pm$ 0.11
eDNA/Total cells	3.39 $\pm$ 0.27	0.75 $\pm$ 0.39	1.34 $\pm$ 0.30	2.28 $\pm$ 0.62	1.30 $\pm$ 0.01	0.46 $\pm$ 0.01
Extracellular						
polysaccharides	1.42 $\pm$ 0.06	3.20 $\pm$ 0.73	1.57 $\pm$ 0.14	2.89 $\pm$ 1.80	2.75 $\pm$ 0.32	0.91 $\pm$ 0.10
/Live cells						
Extracellular						
polysaccharides	1.03 $\pm$ 0.09	2.22 $\pm$ 0.73	1.00 $\pm$ 0.22	1.81 $\pm$ 1.09	1.23 $\pm$ 0.07	0.41 $\pm$ 0.01
/Total cells						

Figure 3.6 TMP profiles of the RO system inoculated with *P. aeruginosa* PAO1 WT and mucoid (FRD1) strains (PAO1 + 20 mg/L NB; FRD1 + 20 mg/L NB). The RO system was operated using feed solution with 2 g/L NaCl, at 35 LMH flux and a cross-flow velocity (CFV) of 0.17 m/s.

Figure 3.7 Confocal microscopic images of the biofilms of the WT and mucoid *P. aeruginosa* strains on the RO membranes. *P. aeruginosa* PAO1 and its mucoid mutant FRD1 were inoculated into the RO system and allowed to form biofilms. At the time point that the TMP maximum was reached, membranes were removed for autopsy, fluorescently stained and imaged. Column 1 (A, B) are Live/Dead stained biofilms, in which the live cells are green and the dead red. The magnification is 60 $\times$ and the flow direction is from right to left. Column 2 (C, D) are images of Live/Dead staining and the magnification is 630 $\times$. Column 3 (E, F) presents images of eDNA stained with BOBO-3. Column 4 (G, H) is images of extracellular polysaccharides stained with ConA-FITC.

3.3.3 Effect of Nutrient Concentration on Biofilm Formation

To determine the role of nutrients in biofilm development and performance of the RO system, biofilms were formed using *P. aeruginosa* where the nutrient concentrations were varied (20, 15, 10 and 0 mg/L; Figure 3.9). There was a clear relationship between nutrient concentration in the feed and the TMP increase, where it was observed after 6 d that at the highest nutrient concentration, 20 mg/L, the TMP had increased by 159%, while at 15 and 10 mg/L the TMP had only increased by 39% and 12%, respectively. In the absence of nutrient input, the TMP increased by 6%. It should be noted that while the lower nutrient resulted in lower TMP over the 6 d, the

Figure 3.8 Characterization of the biofilms on the RO membranes. *P. aeruginosa* PAO1 and its mucoid mutant FRD1 were inoculated into the RO system and allowed to form biofilms. Membranes were removed for autopsy on day 3 and when the TMP maximum was reached which differed for the two strains. (A) The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of total cells calculated using IMARIS, (B) the viable bacteria counts, (C) the biovolume of eDNA and extracellular polysaccharides stained with BOBO-3 and ConA-FITC, respectively, which was calculated using IMARIS, (D) the amount ($\mu\text{g}/\text{cm}^2$) of extracellular polysaccharides and proteins determined by extraction. Bars represent standard errors ($n=3-6$, which is the number of different images for A and C, or the number of different membrane segments in B and D).

Table 3.4 The thickness and the biovolume ratio of EPS to bacteria cells for the biofilms formed by the wild-type PAO1 and FRD1 strains.

Bacterial strain	PAO1	FRD1
Thickness (μm)	21.00 $\pm$ 1.90	19.00 $\pm$ 1.73
eDNA/ Live cells	1.00 $\pm$ 0.19	4.78 $\pm$ 0.51
eDNA/Total cells	0.46 $\pm$ 0.09	1.77 $\pm$ 0.09
Extracellular polysaccharides/ Live cells	0.91 $\pm$ 0.04	3.51 $\pm$ 0.55
Extracellular polysaccharides/Total cells	0.41 $\pm$ 0.02	1.30 $\pm$ 0.16

TMP was still observed to undergo the ‘jump’ and reach maximum pressure, although there was a dramatic difference the time it took, e.g. on day 6 for 20 mg/L and day 8 for 10 mg/L. Surprisingly, when the injection of bacteria was stopped on day 2, the TMP profile was identical to the module with continuous injection of bacteria (Figure 3.9).

Quantitative analysis of the biofilms indicated that in the absence of added nutrient, after 3 d, the biovolumes for live and dead cells were 0.01 and 0.58 $\mu\text{m}^3/\mu\text{m}^2$, respectively, with dead cells making up 98% of the total bacteria (data not shown). After 6 d, the biovolumes of live and dead cells were 0.16 and 1.18 $\mu\text{m}^3/\mu\text{m}^2$, respectively, and the percentage of dead cells decreased to 88% (Figure 3.11A). At 10 mg/L NB, after 6 d, the structure and the components of the biofilm (Figure 3.10 and 3.11) were quite similar to the 3-day old biofilms formed with 20 mg/L NB (Figure 3.3, 3.4 and 3.5). Interestingly, the TMP rise for the two different biofilms, 12% on day 6 at 10 mg/L and 11% on day 3 at 20 mg/L (Figure 3.9), was also similar suggesting a strong correlation between the amount and composition of the biofilm and the increase in pressure.

Figure 3.9 TMP profiles of the RO system run under different nutrient levels (20, 15, 10 and 0 mg/L NB) with continuous injection of bacteria and at 20 mg/L NB with injection of bacteria for the first 2 d only. The NaCl concentration in the feed solution was 2 g/L, the flux was 35 LMH and the cross-flow velocity (CFV) was 0.17 m/s. ‘□’ shows the time points at which the confocal image data (Figure 3.10) was collected.

3.4 Discussion

3.4.1 Dynamics of Biofilm Formation and its Effect on TMP Rise

From the results shown here, a minor TMP rise was observed in the absence of injected bacteria (Figure 3.3). The RO set-up was open to the air and some bacteria may have entered the system and fouled the membrane. CLSM observation showed that there was some biofilm present (total biovolume $0.37 \mu\text{m}^3/\mu\text{m}^2$ which is only 5% to 10% of the biovolume observed when bacteria were injected) on the RO membrane (data not shown) and this biofilm may have led to some of the increase of the TMP. It is also possible that NB and NaCl could lead to the TMP increase due to the effect of chemical fouling by NB components and concentration polarization (CP). The CP effect on TMP rise has been shown previously, where an imposed flux was

Figure 3.10 Confocal microscopic images of the biofilms on the RO membranes. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time point of 6 d, membranes were removed for autopsy and fluorescently stained and imaged. Column 1 (A, B, C, D, E) are Live/Dead stained biofilms, in which the live cells are green and the dead red. The magnification was 60× and the flow direction was from right to left. Column 2 (F, G, H, I, J) is images of Live/Dead staining and the magnification was 630×. Column 3 (K, L, M, N, O) is images of eDNA stained with BOBO-3. Column 4 (P, Q, R, S, T) is images of extracellular polysaccharides stained with ConA-FITC.

Figure 3.11 Characterization of the biofilms on the RO membranes. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes were removed for autopsy on day 6. (A) The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of live and dead cells and the thickness of the biofilms were calculated using IMARIS, (B) the viable bacteria counts, (C) the biovolume of eDNA and extracellular polysaccharides stained with BOBO-3 and ConA-FITC, respectively, which was calculated using IMARIS, (D) the amount ($\mu\text{g}/\text{cm}^2$) of extracellular polysaccharides and proteins determined by extraction. Bars represent standard errors ($n=3-6$, which is the number of different images for A and C, or the number of different membrane segments in B and D).

determined to account for the polarization effect of solutes (Mulder 1991). The presence of a biofilm layer on the membrane further increases the polarization effect and hence an elevated TMP will be observed (Herzberg and Elimelech 2007).

Therefore, the increase in TMP for the experiments without the addition of bacteria could be attributed to both effects. However, compared to the fouling observed when bacteria were injected into the system where the fouling was high, the change in TMP without bacterial addition was insignificant. Therefore, over the period of the study, microbial fouling and biofilm development was determined to be the primary contributor to deterioration of the RO function.

Interestingly, when the injection of bacteria was stopped after 2 d (Figure 3.9), there was no difference in the TMP profile and most of the biofilm parameters, e.g. thickness, CFU counts and polysaccharide content, were identical to the condition when bacteria were continuously injected. This would suggest that it is the development of the biofilm on the membrane, rather than recruitment of planktonic cells that leads to a TMP jump and this could be due to the production of EPS components by the bacteria or the increase in cell number due to replication, once they have attached and begun to form the biofilm community.

The TMP profile can be divided into 2 stages. The first stage was characterized by a slow TMP increase of 22% for the initial 4 d, followed by a second stage where an abrupt 'TMP jump' from 22% to 159% was observed over the next 2 d. The TMP jump was observed to correlate well with the process of biofilm formation. Based on the results presented in Figure 3.3, without bacterial injection, it is possible that the slow increase stage is the result of chemical fouling, e.g. from the NB and NaCl in the feed solution. This is supported by our data which show that this process is indistinguishable from the potential effects of bacterial attachment, biofilm formation and EPS excretion. The accelerated increase stage started from about day 3, just prior to the TMP jump point. Analysis of the biofilm at the jump stage suggested that there was an exponential increase in the biovolume of the biofilm as well as in CFUs, in contrast to the thickness, which appeared to increase in a quasi-linear fashion. The interpretation of these data is that the biofilm more completely covers the surface of the membrane and potentially becomes denser and less porous rather than increasing in absolute thickness. At the high TMP stage, a higher proportion of dead than live cells was observed. Cell lysis would result in an increase in eDNA, which was

observed, as well as other cellular components. These changes to the structure and composition of the biofilm would lead to rapid changes in both resistance and biofilm-enhanced osmotic pressure (BEOP) (Herzberg and Elimelech 2007; Chong et al 2008a). This scenario is consistent with results presented by Cho and Fane (2002) for an MBR, where biofouling becomes a ‘self-accelerating’ process. It was also reported recently that in an MBR process, the main factors that accelerate biofouling were the soluble microbial products (SMP) combined with EPS, in which the rapid rise of SMP triggered a sudden increase in the TMP, while the accumulation of EPS caused the sustained rise in TMP (Gao et al. 2013, Li et al. 2013). And in another MBR system, it was shown that two mechanisms of inhomogeneous pore loss and changes in percolation due to EPS accumulation were responsible for the steep TMP rise (Hwang et al. 2012). Overall, at this stage, the biofilm, mostly containing dead bacteria and EPS, is attached tightly to the membrane and is difficult to remove by any physical or chemical method. Hence, although biofouling cannot be eliminated completely, for sustainable operation, prolonging the slow increase stage and avoiding the accelerated increase stage is essential in real RO plants.

It should be noted that two methods had been used here to quantify the EPS: chemical extraction analyses, as well as staining with fluorescent dyes and imaging with CLSM. For the chemical method, there is currently no universal EPS quantification method due to the complexity of the EPS. For the staining method, the bacterium used in this study was *P. aeruginosa*, which produces at least 3 distinct extracellular polysaccharides that contribute to biofilm development and architecture: alginate, Psl and Pel (Ryder et al. 2007). Alginate is composed of nonrepetitive monomers of β -1,4 linked L-guluronic and D-mannuronic acids. Psl is a mannose-rich and galactose-rich polysaccharide. Pel is a glucose-rich matrix polysaccharide polymer. ConA can specifically bind to glycoproteins containing α -D-mannose or α -D-glucose and therefore should bind to alginate, Psl and Pel polysaccharides. The two methods may detect different polysaccharides. In addition, the polysaccharide matrix can potentially undergo a sol-gel transition (Seviour et al. 2009), which may changes its diffusion properties, and hence alter the TMP, without a concomitant

increase in biovolume. For these reasons, the two methods may give different concentrations for the polysaccharides, which highlights the importance of using multiple methods for EPS quantification, as was done here.

3.4.2 The Importance of EPS

Herzberg and Elimelech (2007) reported that both the bacterial cells and EPS are important for the decrease in RO performance. Bacterial cells on the membrane hinder the back diffusion of salt, which results in elevated osmotic pressure (BEOP) on the membrane surface, and therefore a decrease in permeate flux (or increase in TMP at fixed flux) and salt rejection. EPS contributes to the decline in membrane water performance by increasing the hydraulic resistance to permeate flow.

In this study, *P. aeruginosa* was selected to be the biofouling agent. The EPS in *P. aeruginosa* biofilms may consist of at least 3 different types of polysaccharides. Synthesis of alginate, one of the polysaccharide components of *P. aeruginosa* EPS, was shown previously to be up-regulated upon contact of the cells with a surface (Davies et al. 1993). In order to test the effect of EPS on biofilm formation and the performance of RO, *P. aeruginosa* strain FRD1, which overproduces alginate, was inoculated into the RO system. In liquid culture, the mucoid FRD1 strain, when compared to the parent strain PAO1, has been reported to exhibit no detectable differences in growth rate but to produce 2-6 fold higher levels of alginate (Mathee et al. 1999).

Fouling can be defined as the increase of TMP over time during the filtration process at constant flux. Therefore, a higher TMP within the same time frame or the same TMP within shorter time frame represents more severe fouling. In this study, the FRD1 and PAO1 strains showed the same TMP rise (TMP maximum), but the FRD1 strain achieved this maximum TMP within shorter time frame (5.5 d for FRD1 vs. 6 d for PAO1). This difference is not insignificant and hence, FRD1 had a greater fouling effect compared to PAO1. The biofilm formed by the mucoid FRD1 strain was observed to have fewer cells, as shown by Live/Dead staining and CFU counts, and more polysaccharide production than the wild-type PAO1. Therefore, it is possible

that the increase in EPS may account for the greater fouling effect observed here. The production of alginate is an energy-intensive process that diverts carbon sources from being utilized for energy and growth towards alginate production and therefore, there must be a significant benefit to biofilm cells to produce alginate (Silo-Suh et al. 2005). One of the benefits may be to increase the structural cohesion of the biofilm, which would presumably be an important factor in maintaining the biofilm under conditions of high shear. Our data presented here support this hypothesis, where the EPS appears to play an important role in biofilm development and decreased performance of the RO system.

3.4.3 The Effect of Different Nutrient Levels

In this study, NB was added into the feed tank of the RO system to provide average nutrient concentrations of 0, 10, 15 or 20 mg/L (20 mg/L NB equals to 6.5 mg/L total organic carbon) accordingly. In comparison, seawater coming into a desalination plant, e.g. seawater RO plant usually has a TOC of less than 3 mg/L with very low assimilable organic carbon (AOC) (Escobar et al. 2000; Schneider et al. 2005). The purpose of using higher nutrient load in this laboratory study was to accelerate the process of membrane biofouling so that experiments could be performed in a realistic time frame to allow for replication of results. In addition, the flux of 35 LMH was about 50% higher than a typical RO plant, again to provide information in a reasonable time frame. It should be noted that we have observed qualitatively similar biofouling development for fluxes as low as 5 or 10 LMH (data not shown). As a result, the TMP jump phenomenon observed in this study appeared after a relatively short period of operation. However, we believe the results obtained can be applied to explain the biofouling process in an actual RO plant. Although the initial nutrient load in the feed to the RO plant may be lower, it would eventually increase due to the high rejection properties of RO membranes leading to the effect of CP. The accumulation of nutrient on the surface of membrane overtime would eventually reach a level sufficiently high to support the biofilm development and accelerate the growth. It should be noted that conventional pretreatment processes,

including microfiltration/ultrafiltration are not designed to target the removal of nutrient, therefore the 'residual' nutrient can cause subsequent biofouling on the RO membrane.

The results presented here suggest that one possible approach to controlling biofilm development and fouling in RO systems is to limit the nutrient concentration in the influent. For example, it has been shown that an increase in carbon concentration increased the rate and extent of biofilm accumulation (Peyton 1996). This study is in agreement with Klahre and Flemming (2000) who observed that the addition of nitrogen and phosphorus in paper mill process water promoted biofilm formation. EPS production is also influenced by the nutrient conditions, where thicker extracellular matrix and stable biofilm formation developed in minimal salts medium supplemented with glucose (Dewanti and Wong 1995). The results presented here show a clear relationship between biofilm development, TMP rise and nutrient concentration in the feed water. While this is intrinsically logical, it should be remembered that these experiments used a continuous feed, which should provide fresh nutrients to the biofilm. The decrease in biofilm formation at the lower nutrient concentrations suggest that even at 15 mg/L, the biofilm is nutrient limited, which subsequently restricted growth. A study by Schneider et al. (2005) suggested that reducing biofouling in RO systems is largely dependent upon reducing the AOC. This supports the suggestion that pretreatment should remove nutrients and therefore control biofilm growth in RO systems (Flemming et al. 1997). While the data here show that a reduction in nutrient concentration can lead to a significant improvement of system operation, it will not prevent biofilm development nor will it prevent the eventual TMP jump. This is because the system continuously delivers nutrients, albeit at less than optimum concentrations, which the bacteria are prepared to scavenge and use for biomass development.

3.5 Summary

1. Biofilm formation on the RO membrane operated at constant flux resulted in a slow rise in TMP of 22% for the initial 4 d of operation and followed by a sharp increase of

159% over a period of 2 d. The 'TMP jump' was observed to correlate well with the process of biofilm formation. The initial slow increase in TMP was most likely due to the formation of a biofilm on the membrane surface, which then accelerated the biofouling rate through cake-enhanced polarization of nutrients.

2. The EPS appeared to play an important role in biofilm development and decreased the performance of the RO system.

3. When the RO system was operated using different concentrations of nutrients in the feed water, it was observed that nutrient limitation slowed biofilm accumulation and delayed the increase in TMP.

CHAPTER 4 The Impact of Feed Channel Spacers on Biofilm Development on RO Membranes²

4.1 Introduction

In RO spiral wound membrane (SWM) modules, spacers are essential for keeping the membrane leaves apart. Feed channel spacers can enhance flux by increasing the mass transfer due to eddy promotion in the flow (Schwinge et al. 2004). However, the disadvantage of spacers is that they increase the feed channel pressure (FCP) loss since a spacer is an obstacle to flow through the channel (Schwinge et al. 2004). Vrouwenvelder et al. (2009a) suggested that biofouling is mainly the problem of the feed channel spacer because when the spacer was present, the feed channel pressure drop was much higher than when the spacer was absent. It was suggested that improving the design of the feed channel spacer and optimizing the operation conditions of the RO system may be practical approaches to control biofouling (Vrouwenvelder et al. 2009a). However, systematic studies into the influence of feed channel spacer on biofouling are lacking.

The main objective of this study was to determine the influence of the feed channel spacer on trans-membrane pressure (TMP) and on the process of biofilm development in an RO membrane system without vs. with a spacer. The experiments were conducted at constant flux. The effects of biofouling were observed by increase in TMP, this was compared with the results of autopsies of both membranes and spacers. *Ex situ* examination of fouled membranes and spacers by CLSM provided information on temporal and spatial development of the biofilms. To reconcile these observations with other studies that emphasize the role of spacer fouling, the scenarios likely to lead to predominance of membrane or spacer fouling are discussed.

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² This study was undertaken in collaboration with Stanislaus Raditya Suwarno from the Singapore Membrane Technology Centre (SMTTC) who operated the lab-scale reverse osmosis (RO) experimental system, recorded the running parameters and passed the RO membrane samples to me. I developed the hypotheses, designed the experiments, optimized the methodology, imaged and quantified the biofilms on the membranes, analyzed the data and interpreted the phenomena, and took sole responsibility for the presentation of the results here.

4.2 Materials and Methods

4.2.1 RO Set-up and Operation

The RO set-up (see Chapter 3, Figure 3.1) was assembled as two cells in series. Each stainless steel RO cell had a flat plate geometry and flow channel sizes of $310 \times 60 \times 0.8$ mm (L $\times$ W $\times$ H) with an effective area of 0.0186 m^2 . The feed channel spacer effect was investigated where the spacer was put into RO cell 1 and compared to RO cell 2 (without spacer). The effect of the spacers was determined in two orientations that reflected the two most common spacer designs, diamond-type and ladder-type. The design and operation provided conditions that simulate typical large-scale RO processes. The feed tank was equipped with a stirrer (IKA, model Eurostar) and had a capacity of 10 L. A chiller (Polyscience, model 9612AA2P) was used to circulate cooling water through a coil to maintain the feed solution in the feed tank at approximately $25 \pm 1^\circ\text{C}$. To simulate RO process operation, the feed solution was pumped at high pressure through the test cells at a specified flow velocity, and a small fraction of the feed permeated through the membranes as water flux at a controlled rate. A high pressure pump (CATPUMP, model 227) was used to deliver the feed solution, while system pressure and cross-flow velocity (CFV) were controlled using a back pressure regulator (Swagelok, model KBP) and a flow control valve (Cole Parmer, model CP-32505-40), respectively. The feed flow rate was monitored with a turbine flow meter (McMillan, model 114) and the conductivity was measured using a conductivity meter (Mettler Toledo, model Seven Multi). A mass-flow controller (Brooks Instrument, model 5882) was installed on the permeate side of each cross-flow cell to maintain the amount of permeate withdrawn. Permeate quality was monitored by conductivity meters (Rosemount Analytical, model Solu Comp II) and the pressure of feed and permeate streams were monitored by pressure transducers (Bourdon Haenni, model E913). Each RO cell was equipped with differential pressure transmitters (Yokogawa, model JX110A) to monitor the channel pressure.

RO membranes (DOW Filmtec, BW-30) and spacers (Hydraunautics, LFC-1,

Figure 4.1) were cut to size and soaked in Milli-Q water for at least 12 h. The hydrated membranes and spacers were then sterilized in 70% ethanol (Merck) for 1.5 h and then rinsed with Milli-Q water. The membranes and spacers were compacted at a maximum flux, which was up to 65 liters/m²/h (LMH) overnight with Milli-Q water until a stable flux was achieved. Following compaction, the flux was set to the desired value (20 or 35 LMH) and a NaCl stock solution (200 g/L) was added to the feed tank to a final concentration of 4 g/L. The system was allowed to mix for 1.5 h before a nutrient broth (NB) stock solution (5 g/L) was added into the feed tank to provide average nutrient concentration of 20 mg/L (20 mg/L NB equals to 6.5 mg/L total organic carbon) accordingly. The system was allowed to mix for a further 1.5 h prior to the start of the experiment. The feed solution was replenished twice per day and the total organic carbon (TOC) was tested with TOC analyzer (Shimadzu, Model TOC-VWS) and bacteria numbers were determined by colony forming unit (CFU) counts.

4.2.2 Bacterial Fouling of Membranes

P. aeruginosa PAO1 was used to foul the RO membranes. Bacteria were grown in 300 mL batches in NB (5 g/L NB, 2 g/L NaCl, Difco NB-BD diagnostics) with shaking at 150 rpm at room temperature (RT) for 24 h. The bacterial cells were subsequently harvested by centrifugation at 4000 g for 20 min. The pellet was resuspended in sterile, 4 g/L NaCl solution to an optical density (OD) of 0.05 at 600 nm using a spectrophotometer (Shimadzu, model UV1800).

The bacterial suspension was injected into the system before the feed solution entered the RO cells using an injection pump (ELDEX, model 5979-Optos Pump 2HM). The RO system was operated in fully-recycled mode where the bypass, concentrate and permeate flows were returned to the feed tank. Therefore, a set of microfilters (KAREI, 5 and 0.2 µm for concentrate and 0.2 µm for bypass) were installed downstream of the pressure cell to prevent excess bacteria from entering the feed tank and turning the feed tank into an 'active bioreactor'. Experiments were initiated by continuous injection of the bacterial suspension into the flow line at a

Figure 4.1 Images of the feed channel spacer used in this study. (A) top view of a membrane spacer; scale bar, 10 mm; (B) cross-sectional view; scale bar, 250 μm ; (C) and (D) $l_m=2.5$ mm, $d_f=0.36$ mm, $\theta=90^\circ$.

dilution rate of 1:500 based on CFV (0.17 m/s), giving an input load of about 10^5 CFU/mL. Experiments were conducted at constant flux (20 or 35 LMH) and TMP was monitored continuously. The biofilm was allowed to develop in the RO cell for 3, 6 and 10 d.

4.2.3 Membrane and Spacer Autopsy

The fouled membranes and spacers were removed from the RO modules for autopsy, which included fluorescence staining and CLSM observation, as well as viable bacteria counts. The two edges of 1.5 cm of the membrane and spacer sheets were cut off and removed. The rest of the membrane was cut into 28, 1 cm segments

from the inlet to outlet (Figure 4.2). Segments NO. 17-18 were stained by CTC and photos were taken with a camera (SONY, NEX-5) to show the biofilm in large scale. Segments NO.19-20 (named Point A) were stained by Live/Dead stains and observed by CLSM (described by [12]). Points NO. 3, 13 and 23 were used to obtain CFUs.

Figure 4.2 The top-down schematic of the RO membrane used in this study. Point ‘CTC’ (segments NO. 17-18) on the membranes and spacers are selected to be stained by CTC and photos are taken with a camera. Point A (segments NO. 19-20) was selected to be stained using the Live/Dead reagents and observed by CLSM. Points 3, 13 and 23 were used for the viable bacteria counts. The flow direction was from right to left as indicated by the arrow.

4.2.3.1 Macroscopic Observation

Membrane and spacer samples used for macroscopic observation were obtained from the same location in all of the experiments (Segments NO. 17-18, Point ‘CTC’ in Figure 4.2). Biofilms were stained with CTC (5-cyano-2, 3-ditolyl tetrazolium chloride, Polysciences, product #19292) to visualize respiration active bacterial cells. Briefly, a CTC stock solution was prepared at the concentration of 50 mM in Milli-Q water and diluted with NB medium (5 g/L NB, 2 g/L NaCl) to the final concentration of 4 mM for the working solution. The membrane and spacer samples were incubated in CTC working solution at room temperature (RT) in the dark for 2 h and then rinsed once with Milli-Q water. Macroscopic observation and photo acquisition of the biofilms were performed using a digital camera (SONY, NEX-5).

4.2.3.2 CLSM Observation and Image Stitching

Membrane and spacer samples used for microscopic analysis were obtained from the same location in all of the experiments (Segments NO. 19-20, Point A in Figure 4.2). The architecture and components of the biofilms on the RO membranes and spacers were observed *ex situ* using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, product #L7012) according to the manufacturers' specifications with minor modifications. Briefly, SYTO 9 and PI stock solutions were prepared by adding 1.5 μL SYTO 9 and 1.5 μL PI in 1 mL Milli-Q water and diluted with 0.85% NaCl solution for 10 times to be the working solution. Membrane and spacer samples were cut into coupons of 1.5×2 cm, soaked in 50 mL tubes containing 10 mL working solution, incubated at room temperature (RT) in the dark for 1 h. After incubation, the membrane and spacer samples were rinsed with 0.85% NaCl solution before placing on the glass slide under coverslip (VFM coverslips 24×50 mm, CellPath, product#SAH-2450-03A). The coupons were inlaid into the plastic frame glued onto the glass slide (VFM singlefrost 1.0-1.2 mm, CellPath, product#MAG-1000-03T), which was used to prevent the coverslip from compressing the biofilm on the membrane and spacer.

Microscopic observation and image acquisition of biofilms were performed using a ZEISS LSM710 confocal laser scanning microscope (ZEISS, Germany). Images were captured using ZEISS LSM710 confocal microscope bundled program ZEN 2009. Thresholding was fixed for all image stacks. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of the biofilms was quantified using IMARIS (version 7.4.0, Bitplane, Switzerland). CLSM was used to capture 3D images, which were imported into IMARIS, where the 'Surfaces' function was used to reconstruct a new surface for each object based on the color and the intensity. The function automatically calculated the biovolume using their proprietary algorithms. This essentially involved calculating the volume (μm^3) of the voxels belonging to each object and then the total volume of the biofilm was the sum volume of all the objects. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) was the total volume (μm^3) per area (μm^2).

Because the viewing area at the magnification used in this study (10 $\times$ objective

and 0.6× zoom) was around 1.4×1.4 mm, it was not possible to image a whole square of the spacer in one field of view. Therefore, several microscopic images were taken at specific locations around the spacer filaments and the centre of the square and then combined together. For the diamond-oriented spacer, each image was obtained from 13 images captured by the confocal microscope at the lowest magnification, and used 9 images to capture the square for ladder-oriented spacer. The images were stitched together using Microsoft Office (USA).

(A)

(B)

Figure 4.3 Configuration of image stitching to produce whole spacer square information. (A) diamond-type spacer, (B) ladder-type spacer. The flow direction is from right to left.

4.2.3.3 Viable Bacteria Counts

Membrane and spacer samples NO. 3, 13 and 23 (Figure 4.2) were cut into 1×2 cm segments, soaked in 1 mL of 4 g/L NaCl solution in a 1.5 mL tube containing 20 glass beads (1.5 mm diameter). The tubes were shaken using a vortex (Biocote, model SA8) at 2500 rpm for 3 min. The bacterial suspension was serially diluted and CFUs were determined using nutrient agar (8 g/L NB and 10 g/L agar), incubated at 37°C for 24 h. Between 3 and 6 segments were taken for each membrane or spacer sample

and used for quantification of CFUs.

4.3 Results

The impact of feed channel spacers on RO membrane biofouling during the filtration process was evaluated by conducting experiments under 4 conditions: (i) without spacer at flux of 20 LMH; (ii) with diamond-type spacer at flux of 20 LMH; (iii) with diamond-type spacer at flux of 35 LMH; (iv) with ladder-type spacer at flux of 35 LMH. Experiments were conducted at constant flux and biofouling was indicated by the increase of TMP. The development of biofilm inside the spacer-filled membrane module was observed by autopsy of the membrane and spacer, which was comprised of macroscopic observation, confocal visualization and CFU counts.

4.3.1 Biofilm Development on the RO Membrane without Spacer

Confocal microscopic images showed the flow patterns and biofilm structure on the RO membranes without spacer at a constant flux of 20 LMH. Under low magnification (Figure 4.4A, B and C), the biofilms appeared to form as lines along the axis of the flow. At higher magnification (Figure 4.4D, E and F), after 3 d, very small microcolonies were observed. After 6 d, there were clusters or larger microcolonies formed with open areas surrounding those microcolonies. In addition, the microcolonies appeared to consist of mostly live cells while dead cells were more dispersed. And after 10 d, a very dense layer, mostly consisting of dead cells, was observed. The highest live cell biovolume was observed after 6 d, while more dead cells and total cells were observed after 10 d (Figure 4.5). Figure 4.4 also shows the TMP rise when the RO system as operated without spacer at flux of 20 LMH. After 10 d, the TMP increased by 24%. And the TMP increased steadily with time characterized by a slow initial TMP rise stage (from day 0 to day 7) followed by an accelerated TMP increase stage (from day 7 to day 10).

Figure 4.4 Confocal microscopic images and the TMP profile of biofilm development on the RO membranes without spacer at flux of 20 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time points of 3, 6 and 10 d, membranes were removed for autopsy, fluorescently stained with Live/Dead stains and imaged, in which the live cells are green and the dead red. For A, B and C, the magnification is 60 $\times$, the flow direction is from right to left and the scale bar is 200 μm . For D, E and F, the magnification is 630 $\times$ and the scale bar is 20 μm . The top panel shows biofilm development; the bottom panel shows the TMP comparison between without and with diamond-type spacer at flux of 20 LMH.

Figure 4.5 The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of live and dead cells of the biofilms on the RO membranes without spacer at flux of 20 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes were removed for autopsy on days 3, 6 and 10 and the biovolume was calculated using IMARIS.

4.3.2 Biofilm Development on the RO Membrane with Diamond-type Spacer

The development of the biofilm in the spacer-filled channel was observed *ex situ* via macroscopic and microscopic methods. For macroscopic observations, the biofilms on the membranes and spacers were stained with the CTC dye, which produces insoluble, fluorescent deposits in actively respiring cells. Macroscopically, this results in a biofilm that has a red colour (Figure 4.6). Biofilms that formed on the RO membranes with diamond-type spacer at flux of 35 LMH appeared to have an uneven appearance. While similar biofilm patterns were observed within each spacer square. When the experiments were conducted for a longer amount of time, it appears that the biofilms became thicker and denser as can be seen from the greater red color intensity.

Figure 4.6 Macroscopic images of biofilm development on the RO membranes with diamond-type spacer at flux of 35 LMH. (A) 0 d, (B) 3 d, (C) 6 d, (D) 10 d. Biofilms stained with CTC dye and images taken with a camera. The flow direction is from right to left.

Confocal microscopic images (Figure 4.7) showed that after 3 d, the biofilm growth was primarily on the membrane and preferentially aggregated on the two sides of the attached spacer filaments. There was almost no biofilm on the spacer filaments. After 6 d, the biofilm on the membrane became denser and the biomass started to appear on the spacer filaments. After 10 d, much more biomass had accumulated on both the membrane and spacer. The biofilm coverage on the membranes were 30%, 73% and 84% (Table 4.1), and the TMP increased by 10%, 15% and 40% (Figure 4.7) on days 3, 6 and 10, respectively.

The biofilms on the RO membranes with the spacer in the same orientation at flux of 20 LMH were also investigated. The confocal microscopic images (Figure 4.7) show that the biofilm formation process and structure were similar as observed when the flux was 35 LMH, but with less overall biofilm biomass accumulation. Similarly to the 35 LMH condition, the biofilm also appeared to initiate on the membrane, but lower biofilm coverage on the membrane was observed (19%, 27% and 67% on days 3, 6 and 10, respectively) (Table 4.1). The TMP increased by only 7%, 11% and 18% after 3, 6 and 10 d, respectively (Figure 4.7).

Figure 4.7 Confocal microscopic images and the TMP profiles of biofilm development on the RO membranes with diamond-type spacer at fluxes of 20 and 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time points of 3, 6 and 10 d, membranes and spacers were removed for autopsy, fluorescently stained with the Live/Dead stains and imaged. The flow direction was from right to left. Scale bar, 1 mm. The top panel shows biofilm development at flux of 35 LMH; the middle panel shows the TMP profiles of the RO system at fluxes of 20 and 35 LMH; the bottom panel shows biofilm development at flux of 20 LMH.

4.3.3 Biofilm Development on the RO Membrane with Ladder-type Spacer

To study the impact of spacer orientation, the diamond-type spacer was rotated 45° to reflect a ladder-type spacer. The filaments which were in contact with the membrane surface were oriented parallel to the cross flow and the filaments which were raised off the surface were oriented perpendicular to the flow. In this orientation, the feed water would flow underneath the perpendicular filaments. The experiments were performed at a flux of 35 LMH.

Similar to what was observed when using the diamond-type spacer, biofilm formation appeared to start on the membrane, specifically in the areas near to the attached filaments (Figure 4.8 and 4.9). In contrast to the results from the diamond orientation, where the biofilm appeared to form on the downstream side of the spacer filament, biofilms appeared to primarily form upstream of the spacer when the spacers were in the ladder orientation (Figure 4.8 and 4.9). These results were clear in both the macroscopic observations using CTC staining (Figure 4.8) as well as in the confocal microscopic imaging (Figure 4.9). The biofilm coverage was 88% on day 10 (Table 4.1). No significant difference in coverage for the two orientations of the spacers was observed. Relative to the diamond orientation, when the spacer was in the ladder orientation, the fouling phenomenon seemed to occur faster as shown by the TMP rise, which were up to 12%, 20% and 52% after 3, 6 and 10 d, respectively (Figure 4.9).

Biofilm biomass on the RO membranes and spacers was calculated as biovolume ($\mu\text{m}^3/\mu\text{m}^2$) using IMARIS. After 10 d, the biovolumes were 21.77 and 19.62 $\mu\text{m}^3/\mu\text{m}^2$ for the biofilms on the membranes without spacer and with diamond-type spacer at flux of 20 LMH, respectively (Figure 4.10). This suggests that at the same flux there is no obvious difference in the biofilm biovolumes on the membranes irrespective of whether the spacer is present or absent. The biovolumes on the membranes with diamond-type spacer at flux of 35 LMH increased from 9.69, 36.06 to 41.49 $\mu\text{m}^3/\mu\text{m}^2$ from days 3 and 6 to day 10 (Figure 4.10A). Similarly, for the biovolumes of the biofilms on the spacers, values increased from 0.10, 5.09 to 7.56 $\mu\text{m}^3/\mu\text{m}^2$ (Figure 4.10B). While at flux of 35 LMH, the fouling was 2-3 fold greater for both the

membrane and the spacer than at 20 LMH (Figure 4.10). The biovolumes on the membranes and the ladder-type spacers at flux of 35 LMH were 45.44 and 6.14 $\mu\text{m}^3/\mu\text{m}^2$ after 10 d (Figure 4.10). These values suggest that the biofilm biovolumes are not different for the two orientations of the spacers at the same flux. Irrespective of the flux and the spacer orientation, the biovolume ratio of the biofilms on the membranes to on the spacers ranged from 5 to 40. For example, after 3 d, the biofilm biovolumes on the membrane and diamond-type spacer at flux of 20 LMH were 9.50 and 0.24 $\mu\text{m}^3/\mu\text{m}^2$ (Figure 4.10), respectively, and the ratio was around 40. This indicates substantially more biomass is associated with the membranes than the spacers.

Figure 4.8 Macroscopic images of biofilm development on the RO membranes with ladder-type spacer at flux of 35 LMH. (A) 3 d, (B) 6 d, (C) 10 d. Biofilms stained with CTC dye and images taken with a camera. The flow direction is from right to left.

Figure 4.9 Confocal microscopic images and the TMP profiles of biofilms development on the RO membranes with ladder-type spacer at flux of 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time points of 3, 6 and 10 d, membranes and spacers were removed for autopsy and fluorescently stained with Live/Dead stains and imaged. The flow direction is from right to left. Scale bar, 1 mm. The top panel shows biofilm development; the bottom panel shows the TMP profiles of the RO system with ladder-type and diamond-type spacers at flux of 35 LMH.

Table 4.1 Biofilm coverage on the RO membranes with diamond-type and ladder-type spacers at fluxes of 20 and 35 LMH.

Spacer	Flux (LMH)	Coverage (d)		
		3	6	10
Diamond-type	20	19%	27%	67%
	35	30%	73%	84%
Ladder-type	35	13%	50%	88%

Figure 4.10 The biovolumes ($\mu\text{m}^3/\mu\text{m}^2$) of total cells of the biofilms on the RO membranes (A) and spacers (B) under different conditions: without spacer at flux of 20 LMH; with diamond-type spacer at flux of 20 LMH; with diamond-type spacer at flux of 35 LMH and with ladder-type spacer at flux of 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 3, 6 and 10 and the biovolume was calculated using IMARIS.

The CFUs per square centimeter of the biofilm on both the membrane and spacer was measured. There were 2.17×10^7 , 4.19×10^7 and 1.11×10^8 CFU/cm² on the membranes without spacer after 3, 6 and 10 d, respectively (Figure 4.11A). These values were higher than the CFUs observed on the diamond-type spacer at the same flux, 20 LMH.

The number of viable bacteria on the membranes with the diamond-type spacer at a flux of 35 LMH increased from 5.94×10^6 CFU/cm² on day 3 to 2.08×10^8 CFU/cm² on day 10, while on the spacer there were 2.28×10^5 CFU/cm² on day 3 and 4.22×10^6 CFU/cm² on day 10, approximately a 1-2 log increase for both populations (Figure 4.11). This suggests that the increase in CFU per unit area was similar for both the membrane and spacer and that the number of the viable bacteria on the membrane was typically about 1-2 log higher than on the spacer. That indicates the biomass mainly accumulated on the membrane, not the spacer. The CFU numbers on

the membranes and diamond-type spacers at flux of 20 LMH showed the similar trends as 35 LMH, where the biomass increased similarly on both the membrane and spacer, approximately 1 log here as opposed to about 2 log for 35 LMH (Figure 4.11). The biofilms on the membranes and ladder-type spacers of 3, 6 and 10 d at flux of 35 LMH were also investigated (Figure 4.11). The viable bacteria counts on both the membranes and spacers increased with time. The number of the viable bacteria attached on the spacer was also not nearly as on the membrane. For example, after 3 d, the CFU numbers on the membrane and the diamond-type spacer at flux of 20 LMH were 4.44×10^6 and 1.25×10^5 CFU/cm² (Figure 4.11), respectively, which was more than 1 log higher on the membrane.

Figure 4.11 The viable bacteria counts (CFU/cm²) of the biofilms on the RO membranes (A) and spacers (B) under different conditions: without spacer at flux of 20 LMH; with diamond-type spacer at flux of 20 LMH; with diamond-type spacer at flux of 35 LMH and with ladder-type spacer at flux of 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 3, 6 and 10 and the viable bacteria counts were determined based on the CFU numbers. Bars represent standard errors (n=3-6, which are the number of different membrane/spacer segments).

4.4 Discussion

4.4.1 Biofilm Development in the Absence of Spacers

The influence of feed channel spacers on TMP development and biofilm formation was studied. Experiments were operated without and with diamond-type spacer at flux of 20 LMH. Until 7 d, the TMP profiles, 13% increase, were similar for both conditions. This suggests that the rate of initial bacterial attachment and biofilm growth were rather similar regardless of the use of spacer or that the amount of biofilm formed does not influence the TMP rise. However, after 10 d, the TMP increased by 24% in the absence of the spacer compared to only 18% when spacers were present. This TMP increase might have been caused by an enhanced supply of nutrients to the biofilms. It is known that biofilm formation causes a biofilm-enhanced osmotic pressure (BEOP) effect of solutes (including nutrients) and this promotes the biofilm to reach mature stage faster. The use of spacer may result in a reduction of concentration polarization (CP) and BEOP and therefore prevent the acceleration of biofilm development on the membranes, resulting in a linear TMP until the end of the experiment on 10 d. This indicates that the presence of the spacer improved the performance of the RO system.

Confocal microscopic images show the differences of the flow patterns and biofilm structure on the RO membranes between without and with spacer. The 10× objective images show that the biofilms produced on the membranes without spacer had a more uniform coverage and the biofilms appear to have developed ridges that run parallel to the feed. In contrast, when a spacer was introduced into the membrane module, the biofilms appeared to have a less even appearance and formed in different areas of the membrane surrounded by the spacer. The 63× objective images provide more detailed information of biofilm development on the membranes without spacer. Three-day old biofilms were comprised of small colonies and the majority of the bacteria were alive as indicated by Live/Dead staining. Dead cells could also be observed at this stage. After 6 d, the colonies grew larger which may be due to a combination of clonal growth and recruitment of bacteria from the planktonic phase to the growing microcolonies. No increase in dead cells was observed at day 6 and the live-total ratio was 0.64. Severe fouling was observed after 10 d. The former bacterial

colonies were almost completely covered by a thick layer of dead cells. The live-total ratio after 10 d had dropped to 0.08.

4.4.2 Biofilm Development in Spacer-filled Channels

Experiments were also conducted using a diamond-type spacer at flux of 20 and 35 LMH. Macroscopic images show that the biofilms were not equally distributed across the membrane area. Microscopic images further illustrated that more dense biofilms were observed behind the spacer filament crossings and on the two sides of the attached spacer filaments. Significantly, less biofilm was found under the detached filaments. The possible reason for this observation may be due to the existence of the local shear forces around the spacer elements (Vrouwenvelder et al. 2010). The spacers are three-dimensional structures where some filaments sit flat on the membrane and others do not, medium can flow unimpeded over the top of or under the filaments but is blocked by the spacer crossings. The areas where the filaments do not sit flat on the membrane may experience the most shear since the liquid can flow under those, between the spacer filament and the membrane surface. In contrast, there is likely to be less shear on the two sides of the attached spacer filaments and the least behind the crossings which contact the membrane surface since the liquid must flow over and around those, resulting in areas of low or no liquid flow and hence, less shear.

In the spacer-filled channels, the biofilm appeared to start on the membrane surface. Subsequently, the biofilm grew and covered more area of the membrane. The biofilm also started to cover the surface of the spacer at the later stages of the experiment, but was always significantly lower than on the membrane. Thus, it appears that biofouling in RO system is mainly a membrane problem and is not primarily associated with the spacer. These results differ from those of Vrouwenvelder et al. (2009a), who reported that biofouling was a feed spacer problem because the feed channel pressure (FCP) drop was mainly caused by the feed spacer. The reason for differences in these conclusions may be because the experiment conditions of the two studies are quite different. The RO systems used in the work by

Vrouwenvelder et al. (2009a) were low pressure (120 kPa) systems, fed with low salinity water (tap water) and run at a flux of 0.

Experiments conducted at lower flux, 20 LMH, were performed here to examine the impact of flux on biofilm development. Compared with the higher flux (35 LMH), the TMP rise was less severe, in particular after 10 d of operation. Interestingly, at lower flux, the maximum biofilm coverage was 67%, which was similar to 84% at the higher flux. Despite the relatively similar coverage of biofilm, there was a striking difference in the TMP rise, 18% compared to 40% for the low vs. high TMP. The relationship between biofilm coverage and TMP rise is therefore not quite clear. Change of flux, however, did not affect the overall pattern of the biofilm inside the spacer-filled channel. Operation at a lower flux resulted in less biofilm biomass for the same experimental duration. The work presented here supports previous studies that suggested that biofouling is a flux-driven process (Chong et al. 2008a).

4.4.3 Effect of Spacer Orientation

The performance of the RO systems with feed channel spacers show a complicated dependence on the angle between the filaments and the angle of the spacer filaments towards the main flow direction (Schwinge et al. 2004). In this study, experiments on spacer orientation were conducted by rotating the diamond-type spacer by 45°, forming a shape similar to the ladder-type spacer. The attached filaments were parallel to the cross flow, while the detached filaments were perpendicular to the cross flow.

TMP profiles of the first 3 d were similar for diamond-type and ladder-type spacers. Subsequently, for the ladder-type spacer, the TMP rose faster and after 10 d the increase was nearly 52%, while for the diamond-type spacer, it increased by around 40%. The 3-day confocal image showed that the biofilm started near the parallel spacer filaments and on the downstream areas of the spacer square. The 10-day confocal image showed that the area in the centre of the spacer square was completely covered by the biofilm. The similarities between the biofilms grown in the channels filled with diamond-type and ladder-type spacers were that they both

initiated on the membrane areas near the attached spacer filaments, which presumably have low flow or shear, and the main biofilm biomass accumulated on the membrane, not on the spacer. Although the ladder-type spacer gave a faster increase in TMP, the biofilm coverage and volume were not significantly greater than that for the diamond-type. This may be due to subtle differences in biofilm structure and distribution, not evident by comparing relative biofilm biomass.

In colloidal fouling, different orientations of spacers provide different fouling behavior and patterns. Neal et al. (2003) studied the effect of spacer orientation on particle deposition using the method of direct observation through the membrane (DOTM). For 45° flow angle (diamond shaped), they observed deposition starting from the centre of the cell, due to a confluence effect because of two flows coming together from either side of the node. For the 90° flow angle (parallel shaped), they observed a clear area directly behind the filament, which they ascribe to the recirculation of flows in that region. For the 0° flow angle, they observed a less defined deposition pattern with particles found primarily along the side filaments. In this study, it was clear that the diamond-type (angle of 45°) spacer performed better in terms of controlling fouling than the ladder-type (angle of 90°).

4.5 Summary

1. In the presence of the spacer, the biofilm development on the membrane was lower and the TMP showed a much slower rise compared to a membrane without spacer. This indicates that the presence of the spacer improved the performance of the RO system.
2. When a spacer was introduced into the membrane module, the biofilm primarily occurred on the membrane, not on the spacer.
3. The diamond-type (angle of 45°) spacer performed better in terms of controlling biofouling than the ladder-type (angle of 90°).

CHAPTER 5 The Impact of Flux, Cross-flow Velocity (CFV) and Nutrient Level on Biofilm Development in Spacer-filled Channels³

5.1 Introduction

Insight into the factors influencing the development of biofilm and pressure drop increase is needed to develop Reverse Osmosis (RO) membrane systems, which are less susceptible to biofouling. Chong et al. (2008a) reported that biofouling was manifested as a rise in trans-membrane pressure (TMP) and surface loading, increased with imposed flux and reduced with cross-flow velocity (CFV). The role of flux was attributed to the enhancement of concentration polarization (CP), which contributes to biofilm-enhanced osmotic pressure (BEOP) and nutrients that facilitate biofilm growth. However, this study of the effect of flux on biofouling was in a RO cell without flow channel spacers. From other studies with colloids (Chong et al. 2008b) and particles (Neal et al. 2003), it would be anticipated that fouling would be reduced by the presence of spacers and that the effects of flux and cross-flow on fouling would follow similar trends (fouling increasing with flux and decreasing with cross-flow).

However, the data published by Vrouwenvelder et al. (2009a; 2009b) showed contrary results in an RO system with feed channel spacers. These works showed that biofilms grew primarily on the spacers in the form of streamers and the impact of flux was not apparent towards biofouling. Furthermore, increased crossflow was shown to lead to a higher ΔP rise due to a higher supply of nutrients. These contradictory observations relating to the biofouling process in RO need to be systematically addressed as it is critical to understand the mechanism for sustainable operation of RO technology. As shown in Chapter 4, under conditions using flux, the biofilm was

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³ This study was undertaken in collaboration with Stanislaus Raditya Suwarno from the Singapore Membrane Technology Centre (SMTTC) who operated the lab-scale reverse osmosis (RO) experimental system, recorded the running parameters and passed the RO membrane samples to me. I developed the hypotheses, designed the experiments, optimized the methodology, imaged and quantified the biofilms on the membranes, analyzed the data and interpreted the phenomena, and took sole responsibility for the presentation of the results here.

observed to form primarily on the membrane rather than on the spacer and that the presence of the spacer reduced the TMP rise.

Biofilm formation is also affected by nutrient concentration. Restricting the content of biodegradable compounds in the feed water by sand filtration pretreatment has been reported to lower biomass accumulation in a membrane test cell (Griebe T and Flemming 1998). Parallel test rig studies with spiral wound membrane modules operated with feed water with and without addition of a biodegradable compound showed that substrate dosage stimulated the development of biofouling (Vrouwenvelder et al. 2009b), supporting the findings of Griebe and Flemming (1998). Thicker biofilms were observed under high nutrient concentration conditions (Peyton 1996). Despite extensive biofilm literature, systematic studies on the influence of nutrient concentration on the development of biofouling in spacer-filled channels of the RO systems are lacking.

The hydrodynamic conditions and the supply of nutrients are important elements for biofilm development and both elements govern the development of biofilms on both the membrane and the spacer, influencing biofilm develop through changes in shear forces as well as the amount of nutrient delivered to the developing biofilm. Therefore, the first objective of this study was to investigate the role of flux and cross-flow velocity (CFV) on biofilm development in spacer-filled channels. The second objective was to determine the impact of nutrient concentration in the feed water on biofouling in the RO system. The experiments were conducted at constant flux with diamond-type spacers. The effects of biofouling were observed by increase in TMP and autopsy of both membranes and spacers with analysis of viable bacteria counts and EPS. *Ex situ* examination of fouled membranes and spacers by CLSM provided information on temporal development and spatial distribution of the biofilms.

5.2 Materials and Methods

5.2.1 RO Set-up and Operation

The RO set-up (Figure 3.1) was assembled as two cells in series. Each stainless

steel RO cell had a flat plate geometry and flow channel sizes of $310 \times 60 \times 0.8$ mm (L $\times$ W $\times$ H) with an effective area of 0.0186 m^2 . The feed channel spacer effect was investigated where the spacer was put into RO cell 1 and compared to RO cell 2 (without spacer). A diamond-type spacer was used to investigate the effect of flux, CFV and nutrient level on biofilm formation and fouling. The design and operation provided conditions that simulate typical large-scale RO processes. The feed tank was equipped with a stirrer (IKA, model Eurostar) and had a capacity of 10 L. A chiller (Polyscience, model 9612AA2P) was used to circulate cooling water through a coil to maintain the feed solution in the feed tank at approximately $25 \pm 1^\circ\text{C}$. To simulate RO process operation, the feed solution was pumped at high pressure through the test cells at a specified flow velocity, and a small fraction of the feed permeated through the membranes as water flux at a controlled rate. A high pressure pump (CATPUMP, model 227) was used to deliver the feed solution, while system pressure and cross-flow velocity (CFV) were controlled using a back pressure regulator (Swagelok, model KBP) and a flow control valve (Cole Parmer, model CP-32505-40), respectively. The feed flow rate was monitored with a turbine flow meter (McMillan, model 114) and the conductivity was measured using a conductivity meter (Mettler Toledo, model Seven Multi). A mass-flow controller (Brooks Instrument, model 5882) was installed on the permeate side of each cross-flow cell to maintain the amount of permeate withdrawn. Permeate quality was monitored by conductivity meters (Rosemount Analytical, model Solu Comp II) and the pressure of feed and permeate streams were monitored by pressure transducers (Bourdon Haenni, model E913). Each RO cell was equipped with differential pressure transmitters (Yokogawa, model JX110A) to monitor the channel pressure.

RO membranes (DOW Filmtec, BW-30) and spacers (Hydraunautics, LFC-1) were cut to size and soaked in Milli-Q water for at least 12 h. The hydrated membranes and spacers were then sterilized in 70% ethanol (Merck) for 1.5 h and then rinsed with Milli-Q water. The membranes and spacers were compacted at a maximum flux, which was up to $65 \text{ liters/m}^2/\text{h}$ (LMH) overnight with Milli-Q water until a stable flux was achieved. Following compaction, the flux was set to the desired

value (5, 10, 15 or 20 LMH) and a NaCl stock solution (200 g/L) was added to the feed tank to a final concentration of 4 g/L. The system was allowed to mix for 1.5 h before a nutrient broth (NB) stock solution (5 g/L) was added into the feed tank to provide average nutrient concentration of 10 or 20 mg/L (20 mg/L NB equals to 6.5 mg/L total organic carbon) accordingly. The system was allowed to mix for a further 1.5 h prior to the start of the experiment. The feed solution was replenished twice per day and the total organic carbon (TOC) was tested with TOC analyzer (Shimadzu, Model TOC-VWS) and bacteria numbers were determined by colony forming unit (CFU) counts.

5.2.2 Bacterial Fouling of Membranes

P. aeruginosa PAO1 was used to foul the RO membranes. Bacteria were grown in 300 mL batches in NB (5 g/L NB, 2 g/L NaCl, Difco NB-BD diagnostics) with shaking at 150 rpm at room temperature (RT) for 24 h. The bacterial cells were subsequently harvested by centrifugation at 4000 g for 20 min. The pellet was resuspended in sterile, 4 g/L NaCl solution to an optical density (OD) of 0.05 at 600 nm using a spectrophotometer (Shimadzu, model UV1800).

The bacterial suspension was injected into the system before the feed solution entered the RO cells using an injection pump (ELDEX, model 5979-Optos Pump 2HM). The RO system was operated in fully-recycled mode where the bypass, concentrate and permeate flows were returned to the feed tank. Therefore, a set of microfilters (KAREI, 5 and 0.2 μm for concentrate and 0.2 μm for bypass) were installed downstream of the pressure cell to prevent excess bacteria from entering the feed tank and turning the feed tank into an 'active bioreactor'. Experiments were initiated by continuous injection of the bacterial suspension into the flow line at a dilution rate of 1:500 based on CFV (0.10, 0.17 or 0.34 m/s), giving an input load of about 10^5 CFU/mL. Experiments were conducted at constant flux (5, 10, 15 or 20 LMH) and TMP was monitored continuously. The biofilm was allowed to develop in the RO cell for 6 and 10 d.

5.2.3 Membrane and Spacer Autopsy

The fouled membranes and spacers were removed from the RO modules for autopsy, which included fluorescence staining and CLSM observation to quantify biofilm volumes and percentages of live and dead cells. Viable bacteria counts were determined as well as polysaccharide and protein concentrations extracted from the membrane and spacer surface. The two edges of 1.5 cm of the membrane and spacer sheets were cut off and removed. The rest was cut into 28, 1 cm segments from the inlet to outlet (Figure 4.2). Segments NO. 17-18 were stained by CTC and photos were taken with a stereomicroscope (OLYMPUS SZ61) to show the biofilm in large scale. Segments NO.19-20 (named Point A) were stained by Live/Dead stains and observed by CLSM (described below). Segments NO. 3, 13 and 23 were used to obtain CFUs and the contents of polysaccharides and proteins in the EPS.

5.2.3.1 Macroscopic Observation

Membrane and spacer samples used for macroscopic observation were obtained from the same location in all of the experiments (Segments NO. 17-18, Point ‘CTC’ in Figure 4.2). Biofilms were stained with CTC (5-cyano-2,3-ditolyl tetrazolium chloride, Polysciences, product #19292) to visualize respiration active bacterial cells. Briefly, CTC stock solution was prepared at the concentration of 50 mM in Milli-Q water and diluted with NB medium (5 g/L NB, 2 g/L NaCl) to the final concentration of 4 mM to be the working solution. The membrane and spacer samples were incubated in CTC working solution at room temperature in the dark for 2 h and then rinsed once with Milli-Q water. Macroscopic observation and photo acquisition of biofilms were performed using a stereomicroscope (OLYMPUS SZ61).

5.2.3.2 CLSM Observation and Image Stitching

Membrane and spacer samples used for microscopic analysis were obtained from the same location in all of the experiments (Segments NO. 19-20, Point A in Figure 4.2). The architecture and components of the biofilms on the RO membranes and spacers were observed *ex situ* using the LIVE/DEAD BacLight Bacterial Viability Kit

(Molecular Probes, product #L7012) according to the manufacturers' specifications with minor modifications. Briefly, SYTO 9 and PI stock solutions were prepared by adding 1.5 μL SYTO 9 and 1.5 μL PI in 1 mL Milli-Q water and diluted with 0.85% NaCl solution for 10 times to be the working solution. Membrane and spacer samples were cut into coupons of 1.5×2 cm, soaked in 50 mL tubes containing 10 mL working solution, incubated at room temperature (RT) in the dark for 1 h. After incubation, the membrane and spacer samples were rinsed with 0.85% NaCl solution before placing on the glass slide under coverslip (VFM coverslips 24×50 mm, CellPath, product#SAH-2450-03A). The coupons were inlaid into the plastic frame glued onto the glass slide (VFM singlefrost 1.0-1.2 mm, CellPath, product#MAG-1000-03T), which was used to prevent the coverslip from compressing the biofilm on the membrane and spacer.

Microscopic observation and image acquisition of biofilms were performed using a ZEISS LSM710 confocal laser scanning microscope (ZEISS, Germany). Images were captured using ZEISS LSM710 confocal microscope bundled program ZEN 2009. Thresholding was fixed for all image stacks. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of the biofilms was quantified using IMARIS (version 7.4.0, Bitplane, Switzerland). CLSM was used to capture 3D images, which were imported into IMARIS, where the 'Surfaces' function was used to reconstruct a new surface for each object based on the color and the intensity. The function automatically calculated the biovolume using their proprietary algorithms. This essentially involved calculating the volume (μm^3) of the voxels belonging to each object and then the total volume of the biofilm was the sum volume of all the objects. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) was the total volume (μm^3) per area (μm^2).

Because the viewing area at the magnification used in this study ($10\times$ objective and $0.6\times$ zoom) was around 1.4×1.4 mm, which meant that individual image could not show the whole square of the spacer. Therefore, for the diamond-oriented spacer, 13 microscopic images were taken at specific locations around the spacer filaments and the centre of the square and then combined together. The images were stitched together as shown in Figure 4.3 using Microsoft Office Visio 2007 (Microsoft, USA).

5.2.3.3 Viable Bacteria Counts

Membrane and spacer samples of NO. 3, 13 and 23 (Figure 4.2) were cut into 1 × 2 cm segments, soaked in 1 mL of 2 g/L NaCl solution in a 1.5 mL tube containing 20 glass beads (1.5 mm diameter). The tubes were shaken using a vortex (Biocote, model SA8) at 2500 rpm for 3 min. The bacterial suspension was serially diluted and CFUs were determined using nutrient agar (8 g/L NB and 10 g/L agar), incubated at 37°C for 24 h. Between 3 and 6 segments were taken for each membrane or spacer sample and used for quantification of CFUs.

5.2.3.4 EPS Extraction and Quantification

A volume of 700 µL of the bacterial suspension was transferred into clean 1.5 mL tubes containing 6 µL 37% formaldehyde, vortexed briefly, incubated at 4°C for 1 h and 400 µL of 1 M NaOH was added. The solution was vortexed briefly and incubated at 4°C for 3 h. Subsequently, the bacterial suspension was centrifuged at 14000 g at 4°C for 10 min, the supernatant was transferred to clean 1.5 mL tubes and kept at -20°C. Between 3 and 6 segments were taken for each membrane or spacer sample and used for quantification of EPS.

The polysaccharide content of the EPS was measured by the phenol-H₂SO₄ method (Gerhardt et al. 1994). Briefly, 1 mL of 9% phenol and 5 mL of concentrated H₂SO₄ were added to 1 mL of the sample and 1 mL Milli-Q water and incubated at room temperature (RT) for 30 min. The absorbance of the solution was measured at 492 nm (Shimadzu, model UV1800). Glucose was used to generate a standard curve for quantification of the polysaccharides.

The protein content of EPS was analyzed using the Bicinchoninic Acid (BCA) Assay Kit (Pierce, product #23225). A 25 µL volume of the sample was mixed with 200 µL of Working reagent (WR) in 96-well microplates, incubated at 37°C for 2 h and the absorbance was measured at 562 nm using a microplate reader (TECAN infinite M200 pro, Austria). Bovine serum albumin (BSA) was used to generate a protein standard curve.

5.3 Results

5.3.1 Effect of Flux on Biofilm Development in Spacer-filled Channels

Chong et al. (2008a) have shown previously that RO membrane biofouling is a flux-driven process where higher flux increases the TMP rise. Further, results presented in Chapter 4 showed that biofilm formation initiated on the membrane rather than the spacer and that the presence of the spacer improved RO operation. One key question that remains is to be determined is if the change in TMP at different fluxes can be correlated with the change in biofilm biomass on the membrane or the spacer. To quantify the changes in flux and to correlate those with biofilm formation on RO membranes, the RO set-up was operated at different fluxes (5, 10, 15 and 20 LMH) and biofilm development was quantified at different time points.

Four separate experiments were conducted at fluxes of 5, 10, 15 and 20 LMH while superficial crossflow velocity (CFV) was fixed at 0.17 m/s. The values of 5, 10, 15 and 20 LMH were obtained from the current study, and were compared with the data obtained in Chapter 4 for 35 LMH. The TMP profiles (Figure 5.1) show that after 6 d, the TMP increased by 4%, 5%, 7%, 12% and 14% for fluxes of 5, 10, 15, 20 and 35 LMH, respectively. After 10 d, the TMP increased by 6%, 8%, 12%, 17% and 40%, respectively. The increase of TMP was linear except when the flux was 35 LMH. The TMP profile of 35 LMH experienced a slow increase stage until 8 d (21%), followed by an accelerated increase stage until 10 d (40%). The TMP profiles of 5 to 20 LMH may experience TMP jump if the test duration expanded long enough. From the TMP data, it was shown that higher flux was associated with a faster increase in pressure drop.

Figure 5.1 TMP profiles of the RO system with diamond-type spacer at different fluxes: 5, 10, 15, 20 and 35 LMH. The RO system was run using feed solution with 4 g/L NaCl, 20 mg/L NB at cross-flow velocity (CFV) of 0.17 m/s. The values of 5 to 20 LMH were obtained from the current study, while the data of 35 LMH were taken from the previous study reported in Chapter 4.

Autopsies of the membranes and spacers at the end of the experiments showed that there were some similarities and also some differences in biofilm development at different fluxes. Both macroscopic (Figure 5.2) and confocal (Figure 5.3) images of the membranes and spacers showed that biofilm coverage was non-uniform, with less or no biomass forming in the areas where spacer filaments were raised up from the membrane surface, where higher shear is expected to be experienced by bacteria on the membrane surface. In addition, the amount of live cells (shown in green colour) for both membrane and spacer biofilms was found to be significantly higher than dead cells (shown in red) for all test conditions. Additionally, the process of biofilm formation and the structure of the biofilm on the membranes were similar for all the fluxes, except that there was more biofilm biomass as the flux increased.

Figure 5.2 Macroscopic images of biofilm development on the RO membranes with diamond-type spacers at different fluxes: 5, 10, 15 and 20 LMH. Biofilms stained with CTC dye and images taken with a stereomicroscope. The magnification was 6.7×. The flow direction was from right to left.

Figure 5.3 Confocal microscopic images of biofilm development on the RO membranes with diamond-type spacers at different fluxes: 5, 10, 15 and 20 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time points of 6 and 10 d, membranes and spacers were removed for autopsy and fluorescently stained with Live/Dead stains and imaged, in which the live cells are green and the dead red. The flow direction was from right to left. Scale bar, 1 mm.

The total biovolume of the biofilms on the membranes (Figure 5.3) were quantified (Figure 5.4A and B) and the biofilm biomass was observed to increase

steadily as the flux increased. For example, after 10 d, the biofilm biovolume at 35 LMH was more than 5 fold compared to that of biofilm formed at 5 LMH. There was a less clear relationship for the biofilm biomass formed on the spacers on day 6, where the biovolumes formed at fluxes of 5 to 35 LMH were in the range of 2.74 to 5.79 $\mu\text{m}^3/\mu\text{m}^2$ (Figure 5.4C). On day 10, the relationship between biofilm biomass and the flux conditions was more closely correlated (Figure 5.4D). On the membrane surface, the amount of biofilm increased with flux. While on the spacer, there was no clear difference in the amount of biofilm observed at the different fluxes tested here.

The biovolume ratio of the biofilms on the spacer to on the membrane (Figure 5.5) showed a clear decreasing trend with the increase of flux. For both 6 and 10 d, the ratio decreased from 0.43 at 5 LMH to 0.12 at 35 LMH. It should be pointed out that for all conditions, the ratio of biofilm on the spacer vs. the membrane was less than 0.5, indicating that the biofilm forms preferentially on the membrane for all conditions. It should be noted that as the flux decreased, there was an increasing amount of biomass on the spacer and hence, at very low fluxes or no flux, the amount of biofilm formed on the spacer may be greater than that observed on the membrane.

CFUs per square centimeter of the biofilm on both the membrane and the spacer (Figure 5.6) were quantified and showed a similar trend as the biovolume of live cells (Figure 5.4). For example, after 10 d, the number of the viable bacteria on the membrane of 35 LMH was typically about 2 log higher than that of the biofilm formed at 5 LMH, while only 1 log higher on the spacer (Figure 5.6).

To further quantify the biofilm and its relationship with the TMP rise, extracellular polysaccharides (Figure 5.7A and B) and proteins (Figure 5.7C and D), which are part of the EPS matrix were also quantified. Quantification of the polysaccharides and proteins from the membranes showed a similar increase in concentration as the image and CFU data as the flux was increased. In contrast, the relationship of these EPS components extracted from the spacer material was less clear with respect to the increased TMP. For example, after 10 d, the polysaccharide contents on the spacers were approximately 25 $\mu\text{g}/\text{cm}^2$ for all fluxes (Figure 5.7B). Thus, the data presented here suggests that there is a direct relationship between flux

and the accumulation of biofilm on the membranes, as measured by multiple parameters, including imaging, CFUs and extraction of EPS components.

Figure 5.4 The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of live and dead cells of the biofilms on the RO membranes and spacers at different fluxes: 5, 10, 15, 20 and 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the biovolume was calculated using IMARIS. (A) on the membranes after 6 d, (B) on the membranes after 10 d, (C) on the spacers after 6 d, (D) on the spacers after 10 d. The values of 5 to 20 LMH were obtained from the current study, while the data of 35 LMH were taken from the previous study reported in Chapter 4.

Figure 5.5 The biovolume ratio of the biofilms on the spacers to on the membranes at different fluxes: 5, 10, 15, 20 and 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the biovolume was calculated using IMARIS. The values of 5 to 20 LMH were obtained from the current study, while the data of 35 LMH were taken from the previous study reported in Chapter 4.

Figure 5.6 The viable bacteria counts (CFU/cm²) of the biofilms on the RO membranes (A) and spacers (B) at different fluxes: 5, 10, 15, 20 and 35 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the viable bacteria counts were determined based on the CFU numbers. Bars represent standard errors (n=3-6, which are the number of different membrane/spacer segments tested). The values for 5 to 20 LMH were obtained from the current study, while the data for 35 LMH were taken from the previous study reported in Chapter 4.

Figure 5.7 The amount ($\mu\text{g}/\text{cm}^2$) of extracellular polysaccharides and proteins determined by extraction on the RO membranes and spacers at different fluxes: 5, 10, 15 and 20 LMH. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10. (A) polysaccharides on the membranes, (B) polysaccharides on the spacers, (C) proteins on the membranes, (D) proteins on the spacers. Bars represent standard errors (n=3-6, which are the number of different membrane or spacer segments tested).

5.3.2 Effect of Cross-flow Velocity (CFV) on Biofilm Development in Spacer-filled Channels

Cross-flow velocity (CFV) is another important factor to influence the extent of biofouling. Three different CFVs, 0.10, 0.17, 0.34 m/s at constant flux (15 LMH),

were tested to determine the impact of CFV on TMP rise (Figure 5.8). Over a 10 d period, the percent TMP increase was 11% and 9% for 0.10 and 0.17 m/s, respectively. A dramatic reduction in the percent TMP rise, 4%, was achieved at a cross-flow of 0.34 m/s, indicating that a faster TMP rise occurred at the lower CFV.

Figure 5.8 TMP profiles of the RO system with diamond-type spacer at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. The RO system was run using feed solution with 4 g/L NaCl, 20 mg/L NB at flux of 15 LMH.

Both the macroscopic observation with CTC staining (Figure 5.9) and confocal visualization with Live/Dead staining (Figure 5.10) showed that there was less biofilm accumulation on the membranes as the CFV increased. From the CTC images (Figure 5.9), it was observed that at a CFV of 0.10, there was some thick biofilm on the spacer and some streamers hanging off the spacer filaments. As the CFV increased, the biomass on the spacer clearly decreased and there were no streamers. It also appears that the biofilm was tightly bound to the spacer and was more compact as the CFV increased.

Similar to the experiments using different fluxes described above, autopsies of the membranes and spacers showed that the biofilms were not uniformly distributed over the surface of the membranes or the spacers at different CFVs (Figure 5.10). The

amount of live cells was found to be significantly higher at the CFVs of 0.17 and 0.34 m/s (94% live cells, Figure 5.11). At the lowest CFV of 0.10 m/s, the percentage of live cells was less, 68% (Figure 5.11).

Figure 5.9 Macroscopic images of biofilm development on the RO membranes with diamond-type spacer at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. Biofilms stained with CTC dye and images taken with a stereomicroscope. The magnification was 6.7 $\times$. The flow direction was from right to left.

Figure 5.10 Confocal microscopic images of biofilm development on the RO membranes with diamond-type spacer at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time points of 6 and 10 d, membranes and spacers were removed for autopsy and fluorescently stained with Live/Dead stains and imaged, in which the live cells are green and the dead red. The flow direction was from right to left. Scale bar, 1 mm.

After 10 d, the total biovolume of the bacteria cells decreased from $28.53 \mu\text{m}^3/\mu\text{m}^2$ at CFV of 0.10 m/s to $16.51 \mu\text{m}^3/\mu\text{m}^2$ at 0.34 m/s (Figure 5.11B). The biovolume of dead cells decreased dramatically from $9.23 \mu\text{m}^3/\mu\text{m}^2$ at 0.10 m/s to $1.04 \mu\text{m}^3/\mu\text{m}^2$ at 0.34 m/s (Figure 5.11B). There was no clear trend for the total biovolume of the bacteria cells on the spacers with the increase of CFV (Figure 5.11C, D). However, after 10 d, the biovolume of the dead cells on the spacers increased from 0.69 to $2.99 \mu\text{m}^3/\mu\text{m}^2$ when CFV increased from 0.10 to 0.34 m/s (Figure 5.11D),

where the trend was inverse of that on the membranes.

The biovolume ratio of the biofilms on the spacers to on the membranes (Figure 5.12) showed an increasing trend with the increase of CFV for all the sampling time points. For example, after 10 d, the ratio increased from 0.12 at CFV of 0.10 m/s to 0.33 at 0.34 m/s, which meant the biofilm on the spacer took up about 11% of the total biomass at lowest CFV, while about 25% at highest CFV. This indicates that relatively more biofilm biomass was observed on the spacers than the membranes with the increase in CFV.

The results of viable bacteria counts (Figure 5.13) and EPS quantification (Figure 5.14) supported the staining measurements. The number of viable bacteria and the amount of EPS of the biofilms on the membranes were higher than on the spacers for all the CFV conditions. The CFU numbers on the membranes were 1-2 log higher than on the spacers (Figure 5.13). And it appeared that as CFV increased, the amount of biofilm on the membrane decreased, but no obvious difference in total biofilm on the spacer was observed for the different CFVs tested. For example, after 6 d, the numbers of viable bacterial cells on the membranes decreased from 4.28×10^7 to 9.50×10^5 CFU/cm² when CFV increased from 0.10 to 0.34 m/s, while the CFU numbers on the spacers fluctuated in a small range between 4.72×10^5 and 1.06×10^6 CFU/cm².

5.3.3 Effect of Nutrient Level on Biofilm Development in Spacer-filled Channels

To examine the role of nutrients on biofilm development and TMP, an experiment was conducted using a NB concentration of 10 mg/L. The flux and CFV were set at 35 LMH and 0.17 m/s, respectively. The data were then compared to the previous experimental data reported in Chapter 4, which was performed at same flux and CFV with diamond-type spacer but double the amount of NB concentration (20 mg/L). A distinct reduction in the TMP rise was observed when the nutrient concentration was 10 mg/L compared to 20 mg/L (Figure 5.15). After 6 d, the TMP increased by 9% and 14% for NB of 10 and 20 mg/L, respectively. After 10 d, the TMP increased by 18% and 40%, for NB concentrations of 10 and 20 mg/L

respectively. The TMP profile of 10 mg/L was almost linear, while the TMP profile of 20 mg/L increased slowly in the initial 8 d, followed by an exponential stage in the next 2 d.

Figure 5.11 The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of live and dead cells of the biofilms on the RO membranes and spacers at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the biovolume was calculated using IMARIS. (A) on the membranes after 6 d, (B) on the membranes after 10 d, (C) on the spacers after 6 d, (D) on the spacers after 10 d.

Figure 5.12 The biovolume ratio of the biofilms on the spacers to on the membranes at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the biovolume was calculated using IMARIS.

Figure 5.13 The viable bacteria counts (CFU/cm²) of the biofilms on the RO membranes (A) and spacers (B) at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the viable bacteria counts were determined based on the CFU numbers. Bars represent standard errors (n=3-6, which are the number of different membrane/spacer segments tested).

Figure 5.14 The amount ($\mu\text{g}/\text{cm}^2$) of extracellular polysaccharides and proteins determined by extraction on the RO membranes and spacers at different cross-flow velocities (CFVs): 0.10, 0.17 and 0.34 m/s. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10. (A) polysaccharides on the membranes, (B) polysaccharides on the spacers, (C) proteins on the membranes, (D) proteins on the spacers. Bars represent standard errors ($n=3-6$, which are the number of different membrane/spacer segments tested).

Figure 5.15 TMP profiles of the RO system with diamond-type spacer at different nutrient levels: 20 mg/L NB; 10 mg/L NB. The RO system was run using feed solution with 4 g/L NaCl at flux of 35 LMH and cross-flow velocity (CFV) of 0.17 m/s. The values for 10 mg/L NB were obtained from the current study, while the data for 20 mg/L NB were taken from the previous study reported in Chapter 4.

The confocal microscopic images show that there was less accumulation of the biofilm attached on both the membranes and spacers at NB of 10 mg/L and this was observed on both days 6 and 10 (Figure 5.16). This observation was further confirmed by the biovolume calculations (Figure 5.17) and the viable bacteria counts (Figure 5.18). For example, after 6 d, the biovolumes of the biofilms on the membranes were 8.78 and 51.51 $\mu\text{m}^3/\mu\text{m}^2$ at NB concentrations of 10 and 20 mg/L, respectively. This represents 83% less biofilm at the lower NB concentration. The biovolumes on the spacers showed similar trend, where there was an increase from 0.69 to 5.79 $\mu\text{m}^3/\mu\text{m}^2$ for the NB concentrations changed from low to high level.

Figure 5.16 Confocal microscopic images of biofilm development on the RO membranes with diamond-type spacer at different nutrient levels: 20 mg/L NB; 10 mg/L NB. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. At the time points of days 6 and 10, membranes and spacers were removed for autopsy and fluorescently stained with Live/Dead stains and imaged. The flow direction was from right to left. Scale bar, 1 mm. The values for 10 mg/L NB were obtained from the current study, while the data for 20 mg/L NB were taken from the previous study reported in Chapter 4.

Figure 5.17 The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of live and dead cells of the biofilms on the RO membranes and spacers at different nutrient levels: 20 mg/L NB; 10 mg/L NB. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the biovolume was calculated using IMARIS. (A) on the membranes after 6 d, (B) on the membranes after 10 d, (C) on the spacers after 6 d, (D) on the spacers after 10 d. The values for 10 mg/L NB were obtained from the current study, while the data for 20 mg/L NB were taken from the previous study reported in Chapter 4.

Figure 5.18 The viable bacteria counts (CFU/cm²) of the biofilms on the RO membranes (A) and spacers (B) at different nutrient levels: 20 mg/L NB; 10 mg/L NB. *P. aeruginosa* PAO1 was inoculated into the RO system and allowed to form biofilms. Membranes and spacers were removed for autopsy on days 6 and 10 and the viable bacteria counts were determined based on the CFU numbers. Bars represent standard errors (n=3-6, which are the number of different membrane or spacer segments). The data for 10 mg/L NB were obtained from the current study, while the data for 20 mg/L NB were taken from the previous study reported in Chapter 4.

5.4 Discussion

From the results above, the TMP profiles, image data and quantification of CFUs as well as EPS components correlated well with each other. A low pressure drop corresponded with low biomass while a high pressure drop corresponded with high levels of biomass accumulation. As was observed in Chapter 4, the biomass on both the membranes and the spacers increased with time. Similarly, higher amounts of biomass were found on the membranes compared to on the spacers.

5.4.1 Impact of Flux towards Biofouling

Flux is one of the most important parameters thought to influence the extent of biofouling of RO membranes (Winters 1997). From both the TMP profiles and biomass observations, a lower TMP rise and less biofouling were observed when the

flux was lower. Thus, the results obtained here support previous work by Chong et al. (2008a), where it was shown that the growth of the biofilm at constant flux following initial bacteria colonization of the membrane surface increased with flux. However, a study on the relation between biofouling and membrane flux in spiral wound RO membranes showed that biofouling occurred irrespective of the actual flux (Vrouwenvelder et al. 2009c). The reason for the differences in conclusions may be because the experimental conditions of the two studies are quite different. The RO systems used in the study reported by Vrouwenvelder et al. (2009a) were low pressure (120 kPa) systems, fed with low salinity water (tap water) and only the feed channel pressure (FCP) drop was used to measure the biofouling. Therefore, under those conditions, the BEOP effect would not be obvious even if the biofilm formed in the feed channel.

It is known that biofilm growth is nutrient dependent, where the nutrient availability on the membrane surface is controlled by the magnitude of CP or BEOP, which is driven by flux. Flux induces convective transport of bacteria, nutrients and solutes to the membrane surface, resulting in biofilm development on the membrane. Therefore, operation at higher flux is subject to a more severe biofouling.

Another objective of the experiments undertaken here was to investigate the relative extent of membrane fouling and spacer fouling at different fluxes. The results show that greater amounts of biomass was found on the membranes at higher flux operation, and the difference became more obvious at the longest duration observed, 10 d. However, the amount of biomass on the spacers showed no clear pattern of change that correlated with flux. The biovolume ratio of the biofilms on the spacers to on the membranes also supported this point, which reduced with flux. This would indicate that higher flux leads to an increase in biovolume on the membranes, without a concomitant increase of biovolume on the spacers. It also suggests that lower flux resulted in relatively more biofilm on the spacers compared to the membranes. In all cases, the majority of the biofilm was always found on the membranes. For example, even the flux was as low as 5 LMH, up to 70% of the biofilm was attached on the membranes, not the spacers. It is interesting to note that in the experiments conducted

by Vrouwenvelder et al. (2009a) were performed under conditions without permeate production, e.g. Flux of 0. The observation from that work that the biofilm formed predominantly on the spacer, is supported by data presented in this chapter, where the ratio of biofilm on the spacer vs. the membrane increased with decreasing flux.

The term "critical flux", defined as the flux at which the rate of deposition of foulants on the membrane become zero, has been applied to describe colloidal silica fouling in the RO process (Chong et al. 2008b). However, based on the results obtained here, this concept does not appear to be applicable to biofilm mediated fouling. For example, even when the flux was lowered to 5 LMH, the TMP was observed to rise and biofilm formation was still observed. This hypothesis was supported by the study of Vrouwenvelder et al. (2009c), where it was observed that the critical flux concept is not a suitable approach to control biofouling of spiral wound RO membranes. It has been shown in many studies that total elimination of biofouling problem is effectively impossible (Flemming 1997; Baker and Dudley 1998).

5.4.2 Impact of Cross-flow Velocity (CFV) towards Biofouling

Cross-flow velocity (CFV) is another important factor in flow hydrodynamics inside the feed channel (Winters 1997). The debate also exists for the effect of CFV on fouling in RO membrane systems. In colloidal fouling, higher CFV induces higher shear rate on the membrane wall, overcomes CP or BEOP and reduces particles deposition (Chong et al. 2008b) and similar behavior is observed in biofouling (Chong et al. 2008a). In this report, the CEOP effect, as deduced from the transient CP data, was found to be more severe at low CFV conditions. Successes from RO plants operated at higher CFV have also been documented (Winters 1997). However, Vrouwenvelder et al. (2009c) showed that higher CFV increases the substrate load and accelerates biofouling in the study without permeate production. In their study, a higher linear flow velocity resulted in a higher initial pressure drop in addition to a more rapid and greater pressure drop increase and biomass accumulation due to more nutrient supply. These experiments here compared the impact of CFV in biofouling

studies with diamond-type spacer. TMP rise was slower with increased CFV (Figure 5.8). This is in good agreement with the previous cross-flow studies without feed channel spacers (Chong et al. 2008a). The results support the BEOP model where the fouling rate increased with decreasing CFV through the reduction in the effective mass transfer.

Two interesting phenomena were observed during this study. One was the biovolume of dead cells on the membranes decreased dramatically with the increase of CFV. However, the trend of the dead cells biovolume on the spacers was reverse of that on the membranes, which was increased with CFV. So far, the reason is unknown. It was reported by Vrouwenvelder et al. (2009a, 2009b) that with the increase of CFV, a higher supply of nutrients was achieved. This may be the reason why less dead cells were observed on the membrane with increasing CFV, which could obtain more organics and oxygen from the feed. The other was that the biovolume ratio of the biofilms on the spacers to on the membranes showed a clear increasing trend with CFV. This suggests that higher CFV leads to relatively more biofilm on the spacers, which may increase ΔP .

In summary, it would be valuable to consider hydrodynamics (e.g. flux, cross-flow velocity) in the spacer studies where the spacer will affect the liquid flow in the feed channel as well as in the open spaces of the spacer. Reducing flux can decrease biofouling and subsequently improve the performance of the RO system. However, in real RO plants, water production must be ensured. So, the optimization of the flux is necessary to balance biofouling limitation and water production. Similarly, increasing CFV also can decrease biofouling and delay TMP rise. However, to use an increased CFV would require a more powerful pump to increase the flow rate, which requires more energy and cost.

Both a reduction in flux and an increase in CFV can lead to relatively more biofilm on the spacers, which may increase the channel pressure drop which was proposed by Vrouwenvelder et al. (2009a) as the key indicator of RO biofouling. In practice, feed pump pressure has to be increased to maintain product flow, and is required to overcome both ΔP (channel fouling) and TMP rise (membrane fouling).

The relative contributions of ΔP and TMP are likely to be application and feed water dependent. Based on observations from the Water Factory 21 (Daugherty et al. 2006), when the feed pump pressure increased by about 9 bar and the ΔP increased by about 2 bar, so the TMP rise was the major contribution in this application. In this study with feed spacers, the general observation was that ΔP increase was relatively minor (around 1 kPa, data not shown) over the test period. The changes of TMP values were much more noticeable and increased by 3 bars (20 LMH) and 6 bars (35 LMH) after 10 d. However, the relative magnitudes of ΔP and TMP are dependent on the length of the flow channel. The test cell length in this study was 0.32 m, whereas a multi-module pressure vessel could be 6-8 m long, i.e. 18-25 times longer. This implies a vessel ΔP of 0.20-0.25 bar. Although it is still much less than the TMP rises observed of 3-6 bars. While more energy is required to overcome the increase of ΔP . Thus, it would be of particular interest to compare the micro-hydraulics, in the context of the spacers, with the deposition of the biofilm. Optimizing the flux and CFV in membrane modules in combination with advanced feed spacers may be an elegant approach to control biofouling.

5.4.3 Impact of Nutrient Level towards Biofouling

It was reported in Chapter 3 that in the absence of a spacer, when the NB concentration was 20 mg/L, the TMP jump occurred after 4 d and the TMP maximum was reached on day 6. If the NB was reduced to half, both the TMP jump point and the maximum were delayed for 2 d. When the spacer was included, as shown in this chapter, at NB concentration of 20 mg/L, the TMP jump was observed after 8 d. While the TMP profile was linear at a NB concentration of 10 mg/L until 10 d. Thus, under both conditions, with or without the spacer, the reduction of nutrient loading was observed to improve RO performance. These results on the effect of the spacer in slowing biofilm accumulation and TMP jump are supported by results presented in Chapter 4 where it was also observed that the presence of the spacer limits the biofilm formation and improves the performance of the RO system. Thus, the application of a spacer can work in conjunction with nutrient limitation to control biofouling in RO

system. It is known that the existence of the spacer generates local cross-flow velocity variations and induces turbulent flow conditions. Pereira et al. (Pereira et al. 2002) reported that thinner biofilms were found during studies on *Pseudomonas fluorescens* biofilms grown under turbulent flow conditions compared with laminar flow conditions. It would be valuable to extend the experiment at 10 mg/L NB with the spacer to determine if the TMP jump eventually occurs or if the TMP rise maintains its linear progression.

It has been shown in many studies that total elimination of biofouling problem is effectively impossible (Flemming 1997; Baker and Dudley 1998). This is caused by the nature of microorganisms where they can start from a very small number and proliferate and eventually develop a gel-like layer of mature biofilm. Current efforts to control biofouling of spiral wound membranes focus on pretreatment to remove nutrients in the influent which, based on data presented here, would slow the process of biofilm formation and TMP jump. The additional inclusion of the spacers plus nutrient limitation would improve RO system performance over time.

5.5 Summary

1. A faster TMP rise and more biofouling were observed when the flux was higher.
2. Irrespective of the specific flux, the majority of the biofilm biomass was always found on the membrane, not on the spacer. Furthermore, higher flux led to an increase in biofilm biomass on the membranes, without a concomitant increase on the spacers.
3. The fouling rate increased with decreasing CFV and higher CFV led to relatively more biofilm on the spacers.
4. The reduction of nutrient loading was observed to improve RO performance.
5. Nutrient limitation, improving hydrodynamics (e.g. reducing the flux, increasing cross-flow) in membrane modules in combination with rationally-designed feed spacers might be an elegant approach to control biofouling.

CHAPTER 6 Biofouling Control by UV Pretreatment⁴

6.1 Introduction

In recent years, membrane technology in water treatment has significantly improved. The most problematic challenge of membrane application to be addressed is biofouling, which is the issue of biofilms formed on the surface of the membranes (Flemming 1997). It has been reported that the initial deposition of bacteria is faster with a higher bacterial level in the feed water and that the faster deposition of bacteria is directly correlated with the fouling rate of the membrane system (Munshi 2001). So one of the methods to control biofouling is to minimize the total number of foulant microorganisms prior to entering the membrane module. The number of microorganisms has been traditionally controlled by the introduction of chemicals and/or usage of media filtration (microfiltration/ultrafiltration). However, these treatments have some disadvantages and are not fully effective. Chemical (e.g. chlorine) addition may have negative impacts on the membrane, such as membrane oxidation and damage, and may also produce unwanted by-products including trihalo-methanes (THMs). Media filtration has relatively low efficiency and surviving bacteria eventually produce mature biofilms in membrane modules. Despite intensive efforts, no technology has been found to be able to inhibit the development of biofouling completely (Marconnet et al. 2011). To avoid the above mentioned problems caused by chemicals and filtration, UV irradiation has been proposed as an alternative way to inactivate microorganisms from the feed water of the membrane unit (Marconnet et al. 2011).

The bactericidal action of UV radiation has been known for at least 135 years. In

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⁴ This study was undertaken in collaboration with Stanislaus Raditya Suwarno from the Singapore Membrane Technology Centre (SMTTC) who operated the lab-scale reverse osmosis (RO) experimental system, recorded the running parameters and passed the RO membrane samples to me. I developed the hypotheses, designed the experiments, optimized the methodology, imaged and quantified the biofilms on the membranes, analyzed the data and interpreted the phenomena, and took sole responsibility for the presentation of the results here.

1877, Downes and Blunt demonstrated that sunlight killed bacteria and showed that the action was associated chiefly with the short wavelength component of the radiation (Koller 1939). There are two types of UV lamps commonly used for disinfection, low pressure (LP)-UV lamps produced a narrow band of 254 nm wavelength, which is the optimal wavelength for disinfection and ozone destruction, as well as medium pressure (MP)-UV lamps emitted wide spectrum wavelength from 185 to 300 nm (Wolfe 1990).

To date, UV exposure has not been widely implemented as a membrane pretreatment to control microbial numbers in the influent water partly due to its perceived high cost. Indeed, there are relatively few studies on the use of UV for membrane pretreatment. It has been reported that UV irradiation prevented MF membrane fouling by reducing the microorganism concentration in the feed (Otaki et al. 1998; Huang et al. 2001). UV irradiation was also an efficient NF pretreatment to reduce NF membrane biofouling (Marconnet et al. 2011). In another study, applying UV irradiation ahead of NF membranes was successful in controlling biofouling problems in RO desalination process, which was downstream from the NF microfilters (Munshi et al. 2005). In addition, Harif et al. (2011) also demonstrated that MP-UV pretreatment prolonged the RO performance.

The objectives of this study were to determine the effect of UV pretreatment on biofouling control in RO system and to compare different types of UV lamps, as well as different UV dosages achieved by flowrate variation through the UV chamber at fixed intensity.

6.2 Materials and Methods

6.2.1 RO Set-up and Operation

The RO set-up (Figure 6.1) was assembled as two cells in parallel. Each stainless steel RO cell had a flat plate geometry and flow channel sizes of $310 \times 60 \times 0.8$ mm (L $\times$ W $\times$ H) with an effective area of 0.0186 m^2 . The first unit of RO (RO 1) was placed before the UV chamber where the flow was not exposed to the UV irradiation, hereby labeled as “No UV”. The second unit of RO (RO 2) was placed after the UV

chamber where the flow was exposed to UV irradiation and served as “With UV”. The UV dosage was controlled by varying the flowrate through the UV chamber using a variable speed pump (Hydra-Cell, Model D10E). There are two points (point A before UV and point B after UV) that allow feed sampling to test the efficacy of the UV exposure. The experiments were conducted without spacers.

There were two types of UV lamps used in this study. One was a low pressure UV lamp (LP, monochromatic, 253.7 nm) and the other was a medium pressure UV lamp (MP, polychromatic, 220-300 nm), provided by Trojan, Canada. The effectiveness of UV disinfection depends on the UV dosage, which is the product of lamp intensity and exposure time. UV dosage is the reciprocal of the exposure time, given the constant lamp intensity. In a flow channel, the exposure time could be translated into flow rate of the feed water where the lower the flow rate the higher the UV dosage [expressed as a dose in lumens (L) $\times$ time (min)]. In this study, the flow rates for MP-UV were 1.5, 5 and 10 L/min and for LP-UV was 6 L/min.

The design and operation provided conditions that simulate typical large-scale RO processes. The feed tank was equipped with a stirrer (IKA, model Eurostar) and had a capacity of 20 L. A chiller (Polyscience, model 9612AA2P) was used to circulate cooling water through a coil to maintain the feed solution in the feed tank at approximately $25 \pm 1^\circ\text{C}$. To simulate RO process operation, the feed solution was pumped at high pressure through the test cells at a specified flow velocity, and a small fraction of the feed permeated through the membranes as water flux at a controlled rate. A high pressure pump (CATPUMP, model 227) was used to deliver the feed solution, while system pressure and cross-flow velocity (CFV) were controlled using a back pressure regulator (Swagelok, model KBP) and a flow control valve (Cole Parmer, model CP-32505-40), respectively. The feed flow rate was monitored with a turbine flow meter (McMillan, model 114) and the conductivity was measured using a conductivity meter (Mettler Toledo, model Seven Multi). A mass-flow controller (Brooks Instrument, model 5882) was installed on the permeate side of each cross-flow cell to maintain the amount of permeate withdrawn. Permeate quality was monitored by conductivity meters (Rosemount Analytical, model Solu Comp II) and

the pressure of feed and permeate streams were monitored by pressure transducers (Bourdon Haenni, model E913). Each RO cell was equipped with differential pressure transmitters (Yokogawa, model JX110A) to monitor the channel pressure.

RO membranes (DOW Filmtec, BW-30) were cut to size and soaked in Milli-Q water for at least 12 h. The hydrated membranes were then sterilized in 70% ethanol (Merck) for 1.5 h and then rinsed with Milli-Q water. The membranes were compacted at a maximum flux, which was up to 65 liters/m²/hour (LMH) overnight with Milli-Q water until a stable flux was achieved. Following compaction, the flux was set to the desired value (35 LMH) and a NaCl stock solution (200 g/L) was added to the feed tank to a final concentration of 2 g/L. The system was allowed to mix for 1.5 h before a nutrient broth (NB) stock solution (5 g/L) was added into the feed tank to provide average nutrient concentrations of 20 mg/L (equals to 6.5 mg/L total organic carbon) accordingly. The system was allowed to mix for a further 1.5 h prior to the start of the experiment. The feed solution was replenished twice per day and the total organic carbon (TOC) was tested with TOC analyzer (Shimadzu, Model TOC-VWS) and bacteria numbers were determined by colony forming unit (CFU) counts.

6.2.2 Bacterial Fouling of Membranes

Pseudomonas fluorescens was used to foul the RO membranes. Bacteria were grown in 300 mL batches in NB (5 g/L NB, 2 g/L NaCl, Difco NB-BD diagnostics) with shaking at 150 rpm at room temperature (RT) for 24 h. The bacterial cells were subsequently harvested by centrifugation at 4000 g for 20 min. The pellet was resuspended in sterile, 2 g/L NaCl solution to an optical density (OD) of 0.05 at 600 nm using a spectrophotometer (Shimadzu, model UV1800).

The bacterial suspension was injected into the system before the UV chamber via an injection pump (ELDEX, model 5979-OptosPump 2HM). The RO system was operated in fully-recycled mode where the bypass, concentrate and permeate flows were returned to the feed tank. Therefore, microfilters were installed in the flow line to prevent excess bacteria from entering the feed tank and turning the feed tank into

an “active bioreactor”. This was achieved by using 5 and 0.2 μm filters (KAREI) on the UV return, RO bypass and RO concentrate flows and 0.2 μm filters (KAREI) on the UV bypass flow. Experiments were initiated by continuous injection of the bacterial suspension into the flow line at a dilution rate of 1:500 based on CFV (0.17 m/s), giving an input load of about 10^5 CFU/mL. Experiments were conducted at constant flux (35 LMH) and TMP was monitored continuously.

Figure 6.1 Simplified diagram of RO set-up consisting of two RO cells in parallel used for UV pretreatment experiments. A and B indicate the feed sampling points before and after UV equipment, respectively.

6.2.3 Membrane Autopsy

The fouled membranes were removed from the RO modules for autopsy, which included fluorescence staining and CLSM observation to quantify biofilm volumes, percentages of live and dead cells, and volume of EPS. Viable bacteria counts were also determined by performing plate counts.

6.2.3.1 Fluorescence Staining and CLSM Observation

Fluorescence staining and CLSM were used to observe the architecture and

components of the biofilms on the RO membranes *ex situ* using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, product #L7012). Extracellular DNA (eDNA) was measured using BOBO-3 (Molecular Probes, product #B3586) and the abundance of extracellular polysaccharides associated with the biofilm was determined by ConA-FITC (Sigma, product #C7642) staining. All fluorescent dyes were used according to the manufacturers' specifications with the following modifications. For Live/Dead staining, stock solutions were prepared by mixing 1.5 μ L SYTO 9 and 1.5 μ L PI in 1 mL Milli-Q water, respectively. The working solution was prepared by mixing 100 μ L SYTO 9 and 100 μ L PI stock solutions in 800 μ L of 0.85% NaCl solution. For BOBO-3 staining, the working solution was prepared by mixing 2 μ L stock solution in 1 mL TE (10 mM Tris, 1 mM EDTA, pH 8.0) or TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) buffer. For ConA-FITC staining, the stock solution was prepared by mixing 1 mg in 1 mL Milli-Q water. The working solution was prepared by mixing 100 μ L stock solution in 900 μ L PBS (137 mM NaCl, 2.7mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.1 mM CaCl₂, 0.1 mM MnCl₂, pH 6.8).

The membrane was first cut into small coupons that were either, 1.5 $\times$ 2 cm or 0.5 $\times$ 2 cm, which were soaked in 50 mL or 1.5 mL tubes containing 10 mL or 1 mL of the working solution for the dye, and then incubated under appropriate conditions (Table 3.2). After incubation, the membrane samples were rinsed with the corresponding buffer solution (Table 3.2) before placing on the glass slide under a coverslip (VFM coverslips 24 $\times$ 50 mm, CellPath, product#SAH-2450-03A). The coupons of 1.5 $\times$ 2 cm size was inlaid into the plastic frame glued onto the glass slide (VFM singlefrost 1.0-1.2 mm, CellPath, product#MAG-1000-03T) and observed using a 10 $\times$ objective. The coupons of 0.5 $\times$ 2 cm size was cut and inlaid into the well of the 8-well glass slide (6 mm, Thermo Scientific) and observed using a 63 $\times$ objective. The plastic frame or the well was used to prevent the coverslip from compressing the biofilm on the membrane.

Microscopic observation and image acquisition of biofilms were performed using a ZEISS LSM710 confocal laser scanning microscope (ZEISS, Germany). Images were captured using ZEISS LSM710 confocal microscope bundled program

ZEN 2009. Thresholding was fixed for all image stacks. Between 3 and 6 microscopic images were taken for each sample and used for quantification of biovolume ($\mu\text{m}^3/\mu\text{m}^2$) using IMARIS (version 7.4.0, Bitplane, Switzerland). CLSM was used to capture 3D images, which were imported into IMARIS, where the ‘Surfaces’ function was used to reconstruct a new surface for each object based on the color and the intensity. The function automatically calculated the biovolume using their proprietary algorithms. This essentially involved calculating the volume (μm^3) of the voxels belonging to each object and then the total volume of the biofilm was the sum volume of all the objects. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) was the total volume (μm^3) per area (μm^2).

6.2.3.2 Viable Bacterial Counts

Membranes were cut into 1×2 cm segments, soaked in 1 mL of 2 g/L NaCl solution in a 1.5 mL tube containing 20 glass beads (1.5 mm diameter). The tubes were shaken using a vortex (Biocote, model SA8) at 2500 rpm for 3 min. The bacterial suspension was serially diluted and CFUs were determined using nutrient agar (8 g/L NB and 10 g/L agar), incubated at 37°C for 24 h. Between 3 and 6 segments were taken for each membrane sample and used for quantification of CFUs.

6.3 Results

6.3.1 Impact of UV Pretreatment on Feed Bacteria Inactivation

UV effectiveness for bacterial disinfection was tested by taking samples from points “A” and “B” (Figure 6.1) for viable bacteria counts before and after the UV equipment. MP-UV at 1.5, 5 and 10 L/min gave viable bacteria reductions of 3.5, 3.3 and 2.9 log, respectively. A reduction by 3.4 log was achieved for LP-UV at 6 L/min. It is important to note that despite these reductions, UV treatment did not sterilize the feed and hence, there were a minimum of 1.5 log still present.

6.3.2 Impact of UV Pretreatment on TMP Increase

The performance of the RO pretreated with different UV lamps at different flow

rates is shown in Figure 6.2. The overall performance was evaluated based on the time taken for the TMP to increase by 30% (1.3 TMP_0) and is summarized in Table 6.1. Generally, the data showed an improvement in RO performance with the presence of UV irradiation by comparing the time taken to reach 1.3 TMP_0 for “No UV” and “With UV”. Only a marginal delay of 2.0% was achieved by MP-UV at a flow rate of 10 L/min. A greater improvement in performance was achieved at lower flow rates. The TMP rise was delayed by 7.9% for MP-UV at 5 L/min and 11.1% at lowest flow rate of 1.5 L/min. The most significant delay of 17.5% was achieved for LP-UV at 6 L/min. It is interesting to note that for relatively similar flow rates, 5 L/min and 6 L/min, the LP-UV exposure performed better than the MP-UV lamp, even though they show similar log reductions of the bacteria in the feed solution (3.3 log and 3.4 log, respectively).

6.3.3 Impact of UV Pretreatment on Biofilm Formation

The confocal images of the Live/Dead stained biofilms (Figure 6.3) showed that the structure of the biofilms on the RO membranes was similar for samples with and without UV pretreatment. The biofilms appeared to form lines that matched the flow direction when viewed at low magnification. Higher resolution images showed that the live cells (green) tended to aggregate together and the dead cells (red) were randomly distributed around the membrane surface. This would suggest that the dead cells from the feed water may randomly deposit on the membrane surface and that the clusters of live cells probably reflect where live cells deposited and subsequently had grown to form colonies.

The biovolumes of the biofilms formed on the membranes were quantified using IMARIS (Table 6.2). The results indicated that LP-UV pretreatment at flow rate of 6 L/min had the greatest effect on reducing live cells as well as total biofilm, with 84% and 73% reduction, respectively. At a similar flow rate, 5 L/min, for MP-UV, live cells were reduced by 70% and the total biofilm decreased by 26%. Interestingly, unlike the LP-UV treatment at 6 L/min, the MP-UV treatment at similar flow rate also

Figure 6.2 TMP profiles of the RO system without and with UV pretreatment. (A) MP-UV at flow rate of 1.5 L/min, (B) MP-UV at flow rate of 5 L/min, (C) MP-UV at flow rate of 10 L/min, (D) LP-UV at flow rate of 6 L/min.

Table 6.1 Effectiveness of UV pretreatment on the performance of the RO system evaluated by the time needed to reach 1.3 TMP rise.

UV lamp	Flow rate (L/min)	Time to reach 1.3 TMP ₀ (d)		TMP delay
		Control	UV	
MP	1.5	6.3	7.0	11.1%
	5	6.3	6.8	7.9%
	10	5.0	5.1	2.0%
LP	6	6.3	7.4	17.5%

showed less difference in the TMP profile compared to the control, supporting the hypothesis that the amount of biofilm is linked to the TMP rise. However, when the flow rate was decreased to 1.5 L/min, it was observed that the biovolume of live cells in the biofilms on the RO membranes was reduced by 78% and the total biofilm was reduced by 44% compared to the control. When using the MP-UV at 10 L/min, there was a 21% decrease in live cells, and a 19% increase in total biofilm.

Because EPS is important in the formation of biofilms, EPS, both eDNA and polysaccharides, was measured by fluorescence staining and CSLM observation for both the UV pretreated and control biofilms (Figure 6.3). The results of the biovolume of the EPS (Table 6.3) in the biofilms on the RO membranes showed that eDNA reduced by 53%, 43% and 36% for MP-UV at flow rates of 1.5, 5 and 10 L/min, respectively, but there was almost no reduction for LP-UV at 6 L/min. It is interesting to note that the reduction in eDNA does not correlate with the differences in TMP for the UV treatments. There was only a minor reduction for MP-UV for the biovolume of extracellular polysaccharides. This would suggest that the polysaccharide components are not correlated with the changes in TMP observed above.

The effect of UV pretreatment on viable bacteria of the biofilms were quantified by CFU counts (Table 6.4). It was observed that MP-UV at 1.5 L/min showed the greatest reduction in viable cells (74%) while the other pretreatments had negligible effects on CFU counts. Especially, using MP-UV at 10 L/min showed an increase in CFUs (from 6.39×10^6 to 6.67×10^6 CFU/cm²). LP-UV pretreatment at 6 L/min reduced cultivable cells by 33% which was approximately half the reduction in viable or total cells measured by confocal microscopy (Table 6.2). Since they were within an order of magnitude and hence the biofilms are different in terms of CFUs.

Figure 6.3 Confocal microscopic images of the biofilms on the RO membranes without and with MP-UV pretreatment at 1.5 L/min. Row 1 are images of Live/Dead staining, in which the live cells are green and the dead red. The magnification was 10 $\times$ and 0.6 $\times$ zoom and the flow direction was from right to left. Row 2 are images of Live/Dead staining and the magnification is 63 $\times$. Row 3 are the images of BOBO-3 staining, in which the eDNA is stained to red color. Row 4 are the images of ConA-FITC staining, in which the extracellular polysaccharides are stained to green color.

Table 6.2 The biovolume of the bacteria cells of the biofilms on the RO membranes without and with UV pretreatment.

UV lamp	Flow rate (L/min)	Control/ UV	Biovolume of live cells ($\mu\text{m}^3/\mu\text{m}^2$)	Reduction (%)	Biovolume of total cells ($\mu\text{m}^3/\mu\text{m}^2$)	Reduction (%)
MP	1.5	Control	0.18±0.05	77.78%	0.59±0.34	44.07%
		UV	0.04±0.01		0.33±0.15	
	5	Control	0.23±0.06	69.57%	0.34±0.10	26.47%
		UV	0.07±0.03		0.25±0.16	
	10	Control	0.19±0.09	21.05%	0.53±0.34	-18.87%
		UV	0.15±0.02		0.63±0.23	
LP	6	Control	0.38±0.10	84.21%	0.94±0.25	73.40%
		UV	0.06±0.01		0.25±0.04	

Table 6.3 The biovolume of EPS of the biofilms on the RO membranes without and with UV pretreatment.

UV lamp	Flow rate (L/min)	Control/ UV	Biovolume of eDNA ($\mu\text{m}^3/\mu\text{m}^2$)	Reduction (%)	Biovolume of polysaccharides ($\mu\text{m}^3/\mu\text{m}^2$)	Reduction (%)
MP	1.5	Control	2.51±1.45	52.99%	4.54±1.66	15.42%
		UV	1.18±0.49		3.84±0.20	
	5	Control	0.14±0.06	42.86%	2.11±0.13	0.47%
		UV	0.08±0.02		2.10±0.45	
	10	Control	0.11±0.05	36.36%	4.63±1.04	-6.05%
		UV	0.07±0.03		4.91±0.32	
LP	6	Control	1.02±0.29	0.98%	4.09±0.28	59.66%
		UV	1.01±0.39		1.65±0.67	

Table 6.4 Viable bacteria counts of the biofilms on the RO membranes without and with UV pretreatment.

UV lamp	Flow rate (L/min)	Control/UV	Number of viable bacteria (CFU/cm ²)	Reduction (%)
MP	1.5	Control	$7.50 \times 10^6 \pm 3.33 \times 10^6$	74.13%
		UV	$1.94 \times 10^6 \pm 5.87 \times 10^5$	
	5	Control	$6.61 \times 10^6 \pm 2.56 \times 10^6$	-2.57%
		UV	$6.78 \times 10^6 \pm 1.67 \times 10^6$	
	10	Control	$6.39 \times 10^6 \pm 2.55 \times 10^5$	-4.38%
		UV	$6.67 \times 10^6 \pm 1.65 \times 10^5$	
LP	6	Control	$2.89 \times 10^7 \pm 3.81 \times 10^6$	32.87%
		UV	$1.94 \times 10^7 \pm 2.54 \times 10^6$	

6.4 Discussion

6.4.1 Impact of UV Pretreatment on Feed Bacteria Inactivation

The performance of the UV lamps was tested by their ability to inactivate the planktonic bacteria in the feed water. Bacterial concentration in the feed before being exposed to the UV was around 10^5 CFU/mL. From the results above, it was clearly shown that UV pretreatment was effective to inactivate the bacteria in the feed, where there was approximately a 3 log reduction in CFUs depending on the UV dosage used. This is comparable to the results from another study, showing that UV could substantially reduce bacterial numbers in the raw seawater by around 2 log (from 10^4 to 10^2 CFU/mL) prior to the NF-SWRO stage in a pilot scale plant (Munshi et al. 2005).

6.4.2 Impact of UV Pretreatment on Biofouling Control

In this study, the impact of UV pretreatment for controlling RO biofouling was achieved with approximately 2.0%-17.5% delay in TMP rise. The overall results for membrane autopsy suggest that significantly less bacteria were found on the RO

membranes which received UV treated water. The differences in biofilm EPS between the two membranes were notable, in favor of UV pretreatment, yet less significant. It may be suggested that pretreatment disinfection using UV is a promising option for combating biofouling of RO membranes and prolonging operation of the systems.

In this study, the reduction in CFUs in the planktonic phase also appeared to be reflected in reductions in biofilm biomass for live cells as well as the viable cell counts. Thus, the quantitative biofilm biomass data for live cells and the CFU data seem to be well correlated. However, in contrast to the 3 log reduction of planktonic cells, the best reduction in biofilm biomass was observed to be only an 84% reduction in biomass when LP-UV was used at 6 L/min. It should be pointed out that the UV pretreatment was only used on the planktonic bacteria in the feed solution and was not applied to the biofilm on the membrane. These data would suggest that a significant reduction in planktonic cells does not eliminate biofilm development, but rather can slow this process, presumably by reducing the number of bacteria settling on the membrane. Thus, while there was a 3 log reduction in planktonic cells, there were still 10^2 CFU/mL which could contribute to biofilm development. Interestingly, when bacteria were injected into the RO membrane system for the first 2 d only, there was no difference in biofilm development compared to continuous injection (Chapter 3). Thus, biofilm development in the RO system may be primarily a function of initial attachment of bacteria and subsequently may be driven by the growth of those attached cells into mature biofilms.

6.4.3 Effect of UV Lamps and Dosages

The impact of UV pretreatment on biofouling control is UV lamp and dosage dependent. In this study, for MP-UV, when the flow rate was adjusted from 1.5 L/min to 5 and 10 L/min, the TMP rise delay decreased and more biofilm was observed. It is because the faster the flow rate the lower the UV dose, which may fail to inactivate of the bacteria in the feed.

When MP-UV was switched to LP-UV, the results indicated that LP-UV pretreatment at flow rate of 6 L/min had the greatest effect on biofouling control. At a

similar flow rate, 5 L/min, for MP-UV, less impact was obtained. While in recent years, the use of MP-UV lamps, vs. traditional LP-UV lamps, has become more popular, in terms of the degree of photoreactivation for the same germicidal UV dose. MP-UV lamps emit polychromatic light comprising UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (190-290 nm) wavelength ranges. It is thought that the additional wavelengths emitted affect other biological molecules, not only nucleic acids, hence leading to a greater inactivation impact. Moreover, the sensitivity of microorganisms can be wavelength dependent and also may depend on width of the light spectrum emitted by a source. However, the LP-UV lamps emit a monochromatic light at 254 nm, which is assumed to be the spectral sensitivity peaks for most of the microorganisms (Guo et al. 2009). Whether or not MP exposure is better, is not a simple question. In this study, the experiment results showed that at a similar flow rate, the MP-UV was less efficient than the LP-UV. In a practical application, the selection of the UV lamps depends on the effectiveness requested and the cost budget. MP-UV lamps usually operate at higher temperature and produce higher germicidal output compared to LP-UV. Therefore, MP-UV lamps consume more energy and are more suitable for bigger systems (Jin et al. 2006).

6.5 Summary

1. UV pretreatment was effective to inactivate the bacteria in the feed from around 10^5 to 10^2 CFU/mL.
2. The impact of UV pretreatment for controlling RO biofouling was notable, yet less significant than inactivating the planktonic bacteria in the feed. These indicate that a significant reduction in planktonic cells does not eliminate biofilm development, but rather can slow this process.
3. The impact of UV pretreatment on biofouling control is UV lamp and dosage dependent. The faster the flow rate the lower the UV dose, which may fail to inactivate of the bacteria in the feed. At a similar flow rate for LP-UV, more impact was obtained than MP-UV.

CHAPTER 7 Conclusions and Future Work

Reverse osmosis (RO) has become more attractive as the solution to the water scarcity problem in recent years and it is the most common method for seawater desalination and wastewater reclamation. However, its success has been hindered by biofouling where biofilm grows inside the membrane modules. Biofouling has been traditionally described as biofilm development on the membrane surface and leads to an increase in the required pressure. Hence, the objectives of this study were to investigate in detail the process of biofilm development in RO systems and to test novel biofouling controls. The findings described in this thesis provide new insights into the process of biofilm formation on RO membrane and its role in RO performance, as well as possible novel antifouling strategies. In addition, this work demonstrates, for the first time, the relationship between biofilm formation and ‘TMP jump’ in RO and biofilm structure, based on high resolution imaging, on RO membrane with different types of feed channel spacers.

7.1 Conclusions

In this study, two lab-scale experimental systems were used. One set-up was equipped with two flat sheet pressure cells in series in which *Pseudomonas aeruginosa* was injected to foul the membrane. In the other system, the pressure cells were arranged in parallel and *Pseudomonas fluorescens* was used to test the effect of UV pretreatment on membrane biofouling. The experiments were performed under conditions of constant flux and biofouling of the membrane was measured through the increase of TMP and membrane autopsy. Fluorescence staining and confocal microscope observation were conducted to analyze the biofilm *ex situ*.

Generally, biofouling is a mixed species biofilm problem and ideally such communities would be used for fouling studies. However, there are few or no examples of mixed species cultures at the level of high diversity observed in RO membrane biofilms that can be shown to reproducibly form biofilms in a controlled

fashion. Therefore, in this study, *Pseudomonas aeruginosa* was used as a mono-species biofilm culture. This is appropriate as there are multiple publications on *P. aeruginosa* fouling of membranes, which allows for relative comparison, because *P. aeruginosa* is found in membrane fouling communities and because we have access to a range of mutants, e.g. the alginate over producing mutant, that allow for detailed studies of specific genes or gene products on membrane fouling.

It should be noted that the results are presented from single experiments with technical replicates. Experiments were performed in parallel and hence comparable. The issue of replication is indeed an important one, especially in comparing results where the TMP differences appear to be small. Ideally, the experiments would be repeated multiple times. When the TMP profile of the RO system fouled with *P. aeruginosa* was compared in repeated experiments, and it was determined that differences in the time to reach the maximum TMP varied by only 6%. Therefore, differences in time to reach the maximum TMP >6% would be considered to be experimentally relevant (data not shown). Given time constraints, and the low amount of experimental variation, replicate experiments were not performed.

7.1.1 Biofouling Monitoring

An extensive effort has been made to develop the technologies to better understand biofouling in RO systems. One method which has been adopted the non-invasive technique initially developed by Fane and co-workers for colloidal fouling observation is called direct observation through the membrane (DOTM) (Li and Fane 2000; Mores and Davis 2001; Li et al. 2003; Huang et al. 2010). This technique allows online, non-destructive observation of fouling. However, it is only suitable to observe the optically transparent membranes since the fouling process on the feed-side membrane surface must be viewed from the permeate side through the membrane. Hence, it is suitable to observe biofouling only for the initial deposition stage. Another fouling detector called membrane fouling simulator (MFS) was developed to monitor biofouling in low pressure RO or NF filtration system (Vrouwenvelder et al. 2006). This device appears to be an effective early warning

sensor of biofouling by monitoring increased tangential pressure drop through the system. But the pressure limitation of MFS, which is only suitable to monitor low pressure membrane systems, limits its practical application. Confocal laser scanning microscopy (CLSM) allows detailed, three-dimensional examination of thick microbial samples. Fluorochromes are stains or probes that are added to cells to obtain a fluorescent signal, which facilitates their use in studying bacteria associated with optically nontransparent surfaces (Maukonen et al. 2000). For example, SYTO 9/PI was successfully used to stain the biofilms on the RO membranes to show the relative abundance of live and dead cells, as well as the overall structure of the biofilms (Kim et al. 2011).

In the current study, systematic approaches were developed to study biofouling in a laboratory scale RO system. The results illustrate that both biological methods (confocal microscopic observation, viable bacteria counts and EPS extraction) and engineering approaches (TMP profile and changes in flux as well as cross-flow velocity) can be combined to investigate biofouling on the RO membranes.

7.1.2 Biofilm Formation on RO Membrane and its Role in RO Performance

In Chapter 3, the aim of the dynamic study of biofilm formation was to correlate biofilm development on RO membranes over time under different nutrient conditions with the ‘TMP jump’. *P. aeruginosa* PAO1 wild type (WT) and a mucoid derivative FRD1 over producing alginate were used to foul RO membranes. Biofilm formation on the RO membrane operated at constant flux resulted in a slow rise in TMP of 22% for the initial 4 d of operation and followed by a sharp increase of 159% over a period of 2 d. The initial slow increase in TMP was most likely due to the formation of a biofilm on the membrane surface, which then accelerated the biofouling rate through cake-enhanced polarization of nutrients. At late stages of operation, most of the bacterial biomass consisted of dead cells. The amount of extracellular polymeric substances (EPS) appeared to positively correlate with the number of dead cells. Furthermore, the mucoid strain contained more of the EPS components than the wild type strain. It is hypothesized that this increase in EPS components may have been a

significant contributing factor to the faster 'TMP jump' for the mucoid strain compared to the wild type PAO1 (day 5.5 vs. day 6). When the RO system was operated using different concentrations of nutrients in the feed water, it was observed that nutrient limitation slowed biofilm accumulation and delayed the increase in TMP.

Flemming (1997) reported that biofilm formation in RO system has been schematically divided into 3 phases: induction phase, logarithmical accumulation phase and plateau phase (Figure 7.1). The induction phase is attributed to the initial adhesion of the bacteria from the feed, proliferation and colonization, which is possibly the most important phase for biofouling prevention. In the logarithmical accumulation phase, the growth of bacteria on the membrane surface contributes more to biofilm formation than does the recruitment of bacteria from the feed. The third phase is called the plateau phase, which is described as the balance of biofilm growth and cell detachment. In this model, biofouling was defined as biofilm development resulting in an unacceptable degree (the threshold of interference in Figure 7.1) of system performance loss. As demonstrated in this study (Chapter 3, 4 and 5), irrespective of whether the feed channel spacer was present or not, the TMP profile of the RO system could be divided into 2 stages, a slow initial TMP rise stage followed by an accelerated TMP increase stage, which correlated well with the process of biofilm formation. The biofilm development progressed from individual cells, to small then large microcolonies, and finally a thick biofilm mainly consisting of dead cells. It was shown through this work that the biofilm biomass may mainly come from the reproduction of the bacteria initially attached on the membrane surface, rather than recruitment from the feed planktonic phase (Chapter 3). The results of this study are supported by the findings from Flemming (1997) mentioned above. The slow TMP rise stage followed by the accelerated stage in this study is similar to the induction phase and the logarithmical accumulation phase, respectively, which is characterized by accelerated reproduction of the attached bacteria due to concentrated nutrient supply on the membrane surface. And the inset (the beginning of the induction phase, which is very short) in Figure 7.1 represents the usually rapid chemical conditioning of RO membrane, bacteria adhesion, primary colonization,

followed by a primary plateau. However, the plateau phase was not observed in this study. The reason may be because the experiments were stopped when the TMP reached the maximum. The data presented in this thesis support the notion that bacterial fouling is linked to loss of membrane performance, the “threshold of interference”. In the work presented in this thesis, the jump phase most closely resembles the “threshold of interference”.

Figure 7.1 Time-dependent development of biofilm accumulation (Figure adapted from Flemming 1997).

In Chapter 4 and 5, a series of experiments were undertaken to determine the relative influence of the membrane and the spacer in the establishment of a biofilm and the increase in TMP. The results showed that, in the presence of the spacer, the biofilm primarily occurred on the membrane and the main biofilm biomass accumulated on the membrane, not on the spacer. The presence of the spacer also affected the performance of the RO system, in which the biofilm development on the membrane was lower and the TMP showed a much slower rise compared to a membrane without spacer. To compare operational parameters with performance and biofilm formation, the operational parameters of the RO system, e.g. flux, cross-flow velocity (CFV) etc., were varied. The results indicated that a faster TMP rise was observed under conditions of higher flux and lower cross-flow. Thus, improving hydrodynamics (e.g. reducing the flux, increasing cross-flow) in membrane modules

in combination with rationally-designed feed spacers might be an elegant approach to control biofouling.

When the spacers were inserted into the RO modules, the biofilms were observed to be unevenly distributed, which may have been due to local cross-flow variation compared with the biofilms without spacer. The different spacer orientations affected the structure of the biofilms. There is ongoing debate among biofouling researchers on the effect of spacer on the performance of RO. Overall, the findings presented in Chapter 4 and 5 suggest that biofouling in RO system was mainly a membrane problem as the biomass predominantly accumulated on the membrane, and not on the spacer. It has been shown in many studies that presence of spacer increases the mass transfer coefficient, hence reduces the concentration polarization in the boundary layer and slower fouling is observed. It should be noted that the configuration (e.g. orientation) of the spacer and the operation conditions (e.g. flux, cross-flow) used in the experiments affected the proportion of fouling on the membrane and spacer. The effect of spacer orientation on membrane fouling performance may be described by the flow patterns caused by each arrangement. Ladder type spacer used in this study caused increased mass transfer due to increased flow rate especially under the detached tranverse filaments. However, ladder type configuration reduces the formation of eddies, which is crucial for reducing the boundary layer thickness and increasing the mass transfer (Schwinge et al. 2004). Da Costa et al. (1994) showed in their model that the highest mass transfer is achieved at hydrodynamic angle of 90° (diamond type spacers). A higher mass transfer coefficient causes lower concentration polarization and therefore less fouling is expected with diamond type spacers compared to ladder type spacer. This would suggest that further research into the design of spacers may yield benefits in terms of reduced fouling and hence improved RO operation. Recent published data in reverse osmosis systems showed that different commercial spacers were studied by a mathematical model, which indicated that the feed channel pressure drop increase caused by biofilm formation can be reduced by using thicker spacer geometry (Bucs et al. 2014). If the spacers were designed to be sinusoidal, the results showed that the more tortuous geometries (higher amplitude

and shorter wavelength) induced greater local fluid velocity, decreased concentration polarization and greater flux (Xie et al. 2014). And another study showed that the spacers which were polymerized with organo-selenium showed much better biofilm inhibition ability (Vercellino et al. 2013).

In the process of biofilm study, additional questions were raised based on the finding to date. The first question is the relative contribution of reproduction and recruitment to the biofilm and their relationship to fouling, which speaks to the relative importance of removing bacteria from the feed water as opposed to stopping the growth of bacteria which have settled onto the membrane. Experiments designed to improve our understanding on this issue were designed using *P. aeruginosa* PAO1 tagged with yellow fluorescent protein (YFP) which was injected into the RO set-up for 2 d, followed by the bacteria with cyan fluorescent protein (CFP) until the TMP reached maximum. Interestingly, there was no detectable fluorescence from either population injected into the RO system. The reason may be because the environment (e.g. high pressure, high salt concentration) of the RO system prevents the bacteria from expressing the fluorescent protein. Further work would be necessary to understand the failure of the fluorescent proteins to be detected which would facilitate the development of methods to address the relative importance of bacterial recruitment vs. cell division in the fouling of membranes.

In this study, it was found that the number of dead cells exceeded the number of live cells from the 'TMP jump point' onwards and most of the bacteria were dead when the TMP reached maximum. Thus, it would be of particular interest to understand the cause of cell death in the biofilm at this stage of development. While the cause is currently unknown, there are several hypotheses. One possible cause of cell death is limitation for specific nutrients such as carbon or dissolved oxygen (DO), along with high salt concentration and high pressure in the RO system. While the growth of *P. aeruginosa* was tested at NaCl concentrations ranging from 0 to 20 g/L and there was no significant change in growth rate for any of the conditions tested (data not shown). Alternatively, external agents, such as bacteriophage may induce cell lysis. For example, in *P. aeruginosa*, the filamentous phage Pf4 exists as a

prophage, which was observed to switch from a non-lytic, to a superinfective, lytic form, the timing of which correlated with the occurrence of biofilm killing (Rice et al. 2009). Further studies would be essential to determine the relative contributions of these various factors in the death of bacteria in the RO system. If the specific conditions can be identified, then it may be possible to induce cell death in the biofilm to control fouling on the membranes.

It has been recognized that both the bacterial cells and EPS contributed to the decrease of the RO performance (Herzberg and Elimelech 2007). It has been suggested that biofilm formation on the membrane may enhance the osmotic pressure by hindering the back diffusion of salt and similarly the EPS may contribute to the decrease in flux by increasing hydraulic resistance to permeate flow. The results presented in this study showed that the mucoid strain of *P. aeruginosa* showed a slightly faster TMP rise. It is interesting to note that biofilms of the mucoid strain had a higher EPS to cell ratio than the wild-type biofilm which suggests that the EPS component of the biofilm is important for the fouling process. However, given that the difference in TMP was only slight (half a day faster than the wild-type) it remains to be determined if the TMP rise is a function of overall cell number or EPS concentration. It should also be noted that, as indicated in Chapter 2, *P. aeruginosa* produces three different types of polysaccharide matrix material which are important for biofilm formation and only the effect of over production of alginate (mucoid strain) was tested here. Therefore, the role of the Pel and Psl polysaccharides should also be investigated.

7.1.3 Possible Antifouling Strategies

The strategies that are usually employed to minimize the effect of biofouling include adequate feed pretreatment (microfiltration or ultrafiltration pretreatment) to remove the bacteria; biocide dosage or UV irradiation to inactivate the bacteria; nutrient removal via sand filtration, membrane treatment (disinfection resistant and low fouling), module modification (feed channel spacer adaptation) and optimization of hydrodynamic operation (flux, cross-flow), as well as chemical cleaning of the

membrane to remove biofilm biomass. The present study provided insights not only into feed pretreatments of nutrient reduction and UV irradiation to limit biofouling, but also into spacer application and operational parameters optimization.

Pretreatment is one of the most critical processes for successful operation of RO from previous experience since it may reduce bacteria and substances that may cause membrane biofouling. The results from the current study show that limiting nutrients may be a strategy to control biofilm development and hence to improve RO function (Chapter 3). For example, restricting the content of biodegradable compounds in the feed water by sand filtration pretreatment has been reported to lower biomass accumulation in a membrane test cell (Griebe and Flemming 1998). However, the results were clear that nutrient reduction will not prevent biofilm formation nor will it prevent the eventual TMP jump, but rather it will delay the process. Thus, a solution to membrane fouling may require a combination of approaches, of which nutrient limitation is only one component approach.

In Chapter 6, it was demonstrated that UV pretreatment reduced the viable bacteria concentration by around 3 log (from 10^5 to 10^2 CFU/mL) in the feed and subsequently reduced RO membrane fouling. However, in contrast to the dramatic reduction of planktonic cells, the reduction in biofilm biomass was observed to be much less. Pretreatment of the feed water with UV at flow rates of 1.5, 5 and 10 L/min led to reductions in TMP increase which were 11.1%, 7.9% and 2.0% respectively for medium pressure UV (polychromatic, 220-300 nm). When the feed water was exposed to low pressure UV (monochromatic, 253.7 nm) at 6 L/min the TMP was reduced by 17.5% relative to the untreated control. Based on these results, it was concluded that UV pretreatment had some effect on reducing the TMP rise, but did not prevent the 'TMP jump'. This may be due to the ability of bacteria to survive or reactivate in the presence of available nutrients in the feed. There is also the issue that UV cannot leave any residual in the water to kill/control bacteria. And it has been reported that UV exposure can degrade macromolecular organic substances into small molecules, which are much easier to be absorb by microorganisms. Furthermore, when bacteria are inactivated by UV, they may lyse and release nutrients available to

the surviving population. Alternatively, membrane fouling, even when UV pretreatment is used, may be the result of a few viable cells attaching to the membrane surface and developing into mature structures through cell division. This is supported by the observation that the TMP rise was identical for both continuous injection of bacteria or when bacteria were injected for only the first 2 d of operation (Figure 3.9). The attached cells would benefit not only from the nutrients in the feed water, which would no longer be removed by the planktonic cells, but would also benefit from the nutrients released by the lysed cells. Thus, the UV pretreatment alone may not completely inhibit biofilm formation and the associated TMP rise.

Ridgway was the first author to point out that adaptation of feed channel spacers and hydrodynamics may be an approach to control membrane biofouling (Vrouwenvelder et al. 2009a). The results presented in Chapter 4 and 5, strongly suggest that the presence of the spacer improves the performance of the RO system. The spacer generates local cross-flow variations, thereby reduces concentration polarization (CP) and biofouling on the membrane (Schwinge et al. 2004; Mo and Ng 2010). The ladder-type spacer was observed to allow for biomass growth compared to the diamond-type and this implies that spacer design is important for control of membrane fouling. Studies of the hydrodynamics around spacers may yield novel spacer designs that can help to increase local shear on the biofilm and hence reduce biofilm formation.

It is clear that RO biofouling is operation dependent. Flux plays an important role in membrane biofouling as it affects the initial attachment of bacteria and subsequently influences the accumulation of solutes (and nutrient) which results in CP. Cross-flow velocity (CFV) plays an important role in membrane biofouling as it affects the shear stress on the membrane surface and overcomes the effect of CP (Chong et al. 2008a; 2008b). Membrane biofouling was more pronounced at higher flux and lower crossflow (Chapter 5). It would be beneficial to test different hydrodynamic conditions to control biofouling and which may subsequently prolong membrane life and save costs. Maintaining a balanced and low flux among different membrane elements is important. Similarly, cross-flow must be kept reasonably high

to provide enough shear stress and reduces biofouling.

Additionally, NO has been previously shown to induce bacterial dispersal through the intracellular second messenger, cyclic-di-GMP. In this thesis, preliminary testing of NO treatment *ex situ* showed that biofilm dispersal was higher after soaking the biofilm in a solution containing the NO donor, Sodium NitroPrusside (SNP). The most effective concentrations of SNP for the *ex situ* tests was found to be 0.5-1 μM , in which 17% and 70% of the viable cells were removed, respectively. These results will be used to determine if NO addition can reduce biofilm formation and TMP rise in the RO set-up *in situ*.

In conclusion, from this PhD project, although biofouling cannot be eliminated completely, biofouling control can be achieved by a combination of pretreatment to limit nutrient level and microbial loading, application of reasonably designed spacers, as well as optimization of hydrodynamic parameters, including flux and CFV. The findings presented here can potentially serve as a basis for further detailed investigation into the structure and function of biofilm formation in RO membrane systems, paving the way for new approaches to better control biofouling and improve the performance of the RO systems.

7.2 Future Work

Areas of potential and future research are listed below:

1. The use of confocal microscope is proven to be a good method for understanding the development of biofilms in the membrane module. However, this method currently requires membrane autopsy which is a destructive method. Therefore, incorporation of the confocal microscope into the RO flat sheet test cell system would allow non-destructive on-line observation of biofilm development inside the RO spacer-filled channels.
2. It should be noted that two methods had been used here to quantify the EPS: chemical extraction analyses, as well as staining with fluorescent dyes and imaging with CLSM. The two methods may detect different polysaccharides and may give different concentrations for the polysaccharides, which highlights the importance of

using multiple methods for EPS quantification in the future, such as Thermogravimetric Analysis (TGA), Mass Spectrometry (MS) and Infrared Spectroscopy (IR). In this study, it should also be noted that, as indicated in Chapter 2, *P. aeruginosa* produces three different types of polysaccharide matrix material which are important for biofilm formation and only the effect of over production of alginate (mucoid strain) was tested here. Therefore, the role of the Pel and Psl polysaccharides should also be investigated.

3. Biofilm development is initially influenced by the surface conditioning which induces bacterial attachment onto the membrane surface. The nature of the components contributing to the conditioning layer is not well understood. It has been proposed recently that transparent exopolymeric substances (TEP) present in the feed water are the “major initiators” in membrane biofouling. An investigation of the role of TEP particles in RO biofouling would provide a better understanding of the initial biofilm development. If TEP is confirmed to be a “major initiator”, methods for TEP minimization would be important.

4. Further work would be necessary to understand the relative contributions of bacteria reproduction and recruitment to the biofilm and their relationship with biofouling.

5. It would be of particular interest to understand the cause of cell death in the biofilm at ‘TMP jump point’. So far, most of the studies involved in TMP jump are concentrated on MBR, further study is called for in RO.

6. The effectiveness of UV as a pretreatment method in controlling biofouling in RO is limited due to unavailability of a “residual” effect from the UV lamp. As a result, reduction of bacterial cells does not provide long term reduction in TMP rise. The availability of nutrients in the feed water and possible bacteria reactivation are the causes for this. Therefore, future development in UV pretreatment could evaluate its combination with low doses of chemical biocides to provide the residual effect to suppress the growth of surviving bacteria.

7. The offline results of the SNP treatment were very encouraging. Future experiments would be designed to determine if NO addition can reduce biofilm

formation and TMP rise in the RO set-up *in situ*.

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PRELIMINARY DATA

Biofilm Dispersal by Sodium Nitroprusside (SNP)⁵

P.1 Introduction

The application of biocides to control biofouling is currently the most popular strategy adopted in RO desalination plants. However, biofouling is still observed. Therefore, this study aimed to deliver membrane-friendly and more effective biofouling control options for long term RO operations.

It has been reported (Barraud et al. 2006) that nitric oxide (NO), used widely as a signaling molecule in biological systems, caused dispersal of *P. aeruginosa* biofilm bacteria and dispersal was induced with low, sublethal concentrations (25 to 500 nM) of the NO donor sodium nitroprusside (SNP). And recent publication showed that when exposed to NO, dispersal of the *Pseudomonas aeruginos* PDO300 mutant biofilm was 25% lower than the wild-type, whilst dispersal of the alginate deficient mutant was 11% greater (Barnes et al. 2014). Strategies to induce biofilm dispersal are of interest due to their potential to prevent biofilms and biofilm-related fouling in RO. A preliminary experiment with NO treatment was performed offline. The purpose of the offline treatment was to find out the ability of NO to disperse pre-established biofilms on the RO membranes, as well as to find out the appropriate NO donor (SNP) concentration, which is not toxic to the bacteria but yet provides effective dispersal. The pre-established *P. aeruginosa* biofilms on the RO membranes were removed from the RO modules and autopsied. CLSM observation and viable bacteria counts were conducted to compare the differences between SNP untreated and treated membranes.

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⁵ This study was undertaken in collaboration with Stanislaus Raditya Suwarno from the Singapore Membrane Technology Centre (SMTC) who operated the lab-scale reverse osmosis (RO) experimental system, recorded the running parameters and passed the RO membrane samples to me. I developed the hypotheses, designed the experiments, optimized the methodology, imaged and quantified the biofilms on the membranes, analyzed the data and interpreted the phenomena, and took sole responsibility for the presentation of the results here.

P.2 Materials and Methods

The RO set-up consisting of two RO cells in series was used in this study. The details of the RO set-up and operation, as well as bacterial fouling of membranes are presented in Chapter 3. Briefly, the NaCl concentration of the feed solution was 2 g/L and the NB was 20 mg/L. The RO system was run at constant flux (35 LMH), the CFV was 0.17 m/s. *P. aeruginosa* PAO1 was inoculated and fouled the RO membrane.

Biofilm dispersal experiments using NO were done *ex situ*. SNP (Sigma, product #71778) was used as the model NO donor. Membrane samples, which had been fouled with *P. aeruginosa* PAO1, were taken from the RO series rig. SNP was dissolved in NaCl solution (0.85%) to achieve different concentrations of SNP solution (0.5 and 1 μ M) that were used to treat the biofilms. Membrane sheets were cut into small segments, then soaked in the SNP solution and incubated at room temperature (RT) in the dark for 2 h. As a control, an equivalent volume of the NaCl solution (0.85%) was added into the untreated control samples. After the treatment, all of the membrane samples were taken from the tubes and then analyzed for their biological contents. The details of Live/Dead staining and CLSM observation, as well as viable bacteria counts are presented in Chapter 3.

P.3 Results and Discussion

The Live/Dead staining results showed that the number of the live cells (green) decreased after SNP treatment and there was no significant change in the number of dead cells (red) (Figure P.1).

The CFU results showed that the reduction percentage of viable bacteria in the biofilms after SNP treatment was concentration dependent. For the concentrations of 0.5 and 1 μ M, about 17% and 70% of the viable cells were removed, respectively (Table P.1).

Figure P.1 Confocal microscopic images of the biofilms on the RO membranes without (A) and with (B) SNP treatment.

Table P.1 The biovolume ratio of the live cells to total cells and viable bacteria counts of the biofilms on the RO membranes without and with SNP treatment.

SNP concentration	0.5 μM		1.0 μM	
	Control	With SNP	Control	With SNP
Live cells/Total cells	42.01%	21.82%	68.78%	45.84%
Viable bacteria (CFU/cm ²)	2.00×10^7	1.67×10^7	2.17×10^7	6.67×10^6
Reduction	16.50%		69.26%	

The offline results of the SNP treatment were very encouraging. Future experiments would be designed to optimize the treatment conditions (e.g. concentration, test duration). Online tests will be conducted after the suitable concentration and test duration are confirmed in further offline experiments. Previous studies (Barraud et al. 2006) have shown that NO treatment increased the susceptibility of the bacteria to antimicrobials. Therefore, different types of antimicrobials would be added to the system in combination with NO donor. The objective would be to determine whether NO can enhance the efficacy of the antimicrobial compounds in the removal of the pre-established biofilms in RO system.

From the results in Chapter 3, NO treatment can be carried out for younger biofilms (before TMP jump), which contain a higher percentage of live cells.

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