Dynamics of biofilm formation under different nutrient levels and the effect on biofouling of a reverse osmosis membrane system

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\textit{Pseudomonas aeruginosa} PAO1 wild type and a mucoid derivative (FRD1) which over produces alginate were used to foul reverse osmosis (RO) membranes. When operated at a constant flux, biofilm formation on the RO membrane resulted in a slow rise in transmembrane pressure (TMP) of 22\% for the initial four days of operation, followed by a sharp increase of 159\% over the following two days. The initial slow increase in TMP was probably due to the formation of a biofilm on the membrane surface, which then accelerated the rate of biofouling through the effect of concentration polarization. At later stages of operation, most of the bacterial biomass consisted of dead cells. The amount of extracellular polymeric substances appeared to correlate positively with the number of dead cells. The results indicate that prolonging the initial stage of slow TMP increase and avoiding the latter stage of accelerated TMP increase would provide a sustainable operation of the RO system. These results suggest that nutrient limitation could reduce biofilm accumulation and delay the increase in TMP.

\textbf{Keywords:} reverse osmosis (RO); biofouling; biofilm formation; nutrient level; transmembrane pressure (TMP); extracellular polymeric substances (EPS)

Introduction

Reverse osmosis (RO) membranes are designed primarily for the retention of ions and are widely employed for water desalination or industrial water production. Membrane fouling, resulting in reduced efficiency and increased application of pressure to maintain flux and use of chemicals to clean membranes, is one of the key issues to be solved in the application of RO membranes (Kang & Cao 2012).

Fouling is defined as the accumulation of materials such as biological/organic, colloidal, particulate and crystalline matter on the surface of process equipment, in this case the surface of the membrane, resulting in deterioration of performance (Kucera 2010). 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). Biofilms are multicellular consortia of microorganisms embedded in extracellular polymeric substances (EPS) at close proximity to one another (Donlan 2002). EPS is typically composed of nucleic acids, polysaccharides, proteins, lipids and other biological macromolecules. The EPS provides a highly hydrated gel matrix in which microbial cells can establish a stable synergistic consortium (Lawrence et al. 2003).

The presence of a biofilm on the surface of an RO membrane results in higher transmembrane pressure (TMP), leading to a higher energy requirement and increased cost of operation (Flemming 1997). An extensive effort has been made in recent years to understand the nature of biofouling in RO systems. The degree of biofouling in such systems is determined by three basic fouling factors: the nature of the feed (eg nutrient level, salt concentration and microbial loading), the properties of the membrane (hydrophobic, hydrophilic, roughness and surface charge) and the hydrodynamic environment (eg flux and cross-flow velocity (CFV)) (Matin et al. 2011). The interactions between these parameters are complex and can determine the performance of the RO system. In previous work, biofouling experiments with RO systems were performed either with or without (shear-inducing) spacers under conditions of constant flux (Suwarno et al. 2012). The TMP initially showed a slow increase followed by an exponential increase in

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pressure (Suwarno et al. 2012), the latter of which has been termed the ‘TMP jump’. Similarly, when operated under conditions of constant pressure, Herzberg and Elimelech (2007) observed a decrease in flux. This consisted of a 3–4 h lag phase where the flux showed minimal decline, followed by a substantial flux decline (20%) within 15 h. This phenomenon of a TMP jump has also been associated with biofouling in membrane bioreactors (MBRs) (Cho & Fane 2002; Ognier et al. 2002; Zhang et al. 2006). However, to date, little is known about the mechanisms by which biofouling effects this abrupt increase in TMP in RO systems.

The objective of this study was to correlate biofilm development on RO membranes over time with the TMP jump under different nutrient conditions. Further, the relative contributions of bacterial cells (live and dead) and the biofilm matrix components (extracellular DNA (eDNA), polysaccharides and proteins) to the rise in TMP were determined by quantitative microscopic measurements of the biofilm and by chemical extraction. To further investigate the role of EPS in the increase in TMP, Pseudomonas aeruginosa PAO1 wild type (WT) and its mucoid mutant FRD1, which over produces alginate, were used to foul the membrane. The RO set-up 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 eDNA and polysaccharides in the EPS of the biofilms.

Materials and methods

Set-up and operation of the RO reactor

The RO pressure reactor was assembled as two cells in series as described previously (Suwarno et al. 2012). Each stainless steel RO cell had a flat plate geometry and flow channel sizes of 310 × 60 × 0.8 mm (L × W × H) with an effective area of 0.0186 m². 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 this tank at ∼25 ± 1 °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 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 metre (McMillan, model 114) and the conductivity was measured using a conductivity metre (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 metres (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, modelJX110A) to monitor the channel pressure.

RO membranes (DOW Filmtec, BW-30) were cut to size and soaked in MilliQ water for at least 12 h. The hydrated membranes were then sterilized in 70% ethanol (Merck) for 1.5 h followed by rinsing with MilliQ water. The membranes were compacted at a maximum flux, which was up to 651 m⁻² h⁻¹ (liters per meter squared per hour, LMH) overnight with MilliQ 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⁻¹) was added to the feed tank to a final concentration of 2 g l⁻¹. The system was allowed to mix for 1.5 h before a nutrient broth (NB) stock solution (5 g l⁻¹) was added to the feed tank to provide average nutrient concentrations of 0, 10, 15 or 20 mg l⁻¹ (20 mg l⁻¹ NB is equal to 6.5 mg l⁻¹ of total organic carbon (TOC)). 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 daily and the TOC was tested with a TOC analyser (Shimadzu, Model TOC-VWS). Bacterial numbers were determined by colony-forming unit (CFU) counts.

Bacterial fouling of membranes

To investigate biofilm development on RO membranes over time at different nutrient levels, and to correlate the effect of nutrient concentration with the rise in the TMP of the RO system, 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). WT P. aeruginosa (strain PAO1) or a mutant which over produces alginate (strain FRD1) (Mathee et al. 1999) were used to foul the RO membranes. The bacteria were grown in 300 ml batches in nutrient broth (NB) (5 g l⁻¹ NB, 2 g l⁻¹ NaCl, Difco NB-BD diagnostics) with agitation at 150 rpm at room temperature 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⁻¹ NaCl solution to an optical density 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 microilters (KAREL 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 a CFV of 0.17 m s⁻¹, giving an input load of 10⁵ CFU ml⁻¹. Experiments were conducted at a constant flux (35 LMH) and TMP was monitored continuously.

The effects of cell deposition and biofilm growth on changes in TMP were characterized under 7 conditions: (1) no bacteria and full nutrient supply (No PAO1 + 20 mg l⁻¹ NB); (2) continuous bacterial injections and full nutrient supply (PAO1 + 20 mg l⁻¹ NB); (3) continuous bacterial injections and 3/4 nutrient supply (PAO1 + 15 mg l⁻¹ NB); (4) continuous bacterial injection and 1/2 nutrient supply (PAO1 + 10 mg l⁻¹ NB); (5) continuous bacterial injection and no nutrient supply (PAO1 + 0 mg l⁻¹ NB); (6) bacterial injection for the initial 2 days and full nutrient supply (2 days PAO1 + 20 mg l⁻¹ NB); (7) continuous injection of P. aeruginosa FRD1 and full nutrient supply (FRD1 + 20 mg l⁻¹ NB).

Membrane autopsy

The fouled membranes were removed from the RO modules for examination at the conclusion of the experiment. Analyses included fluorescence staining and CLSM observation to quantify biofilm volumes, the percentages of live and dead cells, and volume of EPS. Viable bacterial counts were determined as were the concentrations of polysaccharide and protein extracted from the membrane surface.

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). eDNA was measured using the BOBO-3 stain (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 μl of SYTO 9 and 1.5 μl of PI in 1 ml Milli-Q water, respectively. The working solution was prepared by mixing 100 μl of SYTO 9 and 100 μl of PI stock solutions in 800 μl of 0.85% NaCl solution. For BOBO-3 staining, the working solution was prepared by mixing 2 μl of 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 of MilliQ water. The working solution was prepared by mixing 100 μl of stock solution in 900 μl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.1 mM CaCl₂, 0.1 mM MnCl₂ and pH 6.8).

The fouled membrane samples were soaked in the working solutions of the stains, and incubated for 1 h in the dark at room temperature and rinsed once with the corresponding buffer solution before microscopic observation using a ZEISS LSM710 CLSM (ZEISS, Germany), equipped with Argon laser at 488 nm and DPSS561-10 laser at 561 nm. Thresholding was fixed for all image stacks. Between 3 and 6, microscopic images were taken for each sample and used for quantification of biovolume (μm³ μm⁻²) using IMARIS software (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 colour and intensity. The function automatically calculated the biovolume using their proprietary algorithms. This essentially involved calculating the volume (μm³) of the voxels (volumetric pixels) belonging to each object with the total volume of the biofilm being the sum volume of all the objects. The biovolume (μm³ μm⁻²) was the total volume (μm³) per area (μm²).

Viable bacterial counts

Membranes were cut into 1 x 2 cm segments, soaked in 1 ml of 2 g l⁻¹ 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⁻¹ NB and 10 g l⁻¹ agar), incubated at 37 °C for 24 h.

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 were subsequently added. The solution was vortexed briefly and incubated at 4 °C for 3 h. Subsequently, the bacterial suspension was centrifuged at 14,000 g at 4 °C for 10 min, the supernatant was then transferred to clean 1.5 ml tubes and kept at −20 °C.
The polysaccharide content of the EPS was measured by the phenol-H\textsubscript{2}SO\textsubscript{4} method (Gerhardt et al. 1994). Briefly, 1 ml of 9\% phenol and 5 ml of concentrated H\textsubscript{2}SO\textsubscript{4} were added to 1 ml of the sample and 1 ml MilliQ water, and incubated at room temperature 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 the EPS was analysed using the bicinchoninic acid 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 was used to generate a standard curve for quantification of proteins.

**Results**

Live/dead staining results of the *P. aeruginosa* inoculum showed that ~98\% of the cells were alive. With an inoculum density of 10\(^8\) CFU ml\(^{-1}\), this indicated that there were ~10\(^3\) dead cells ml\(^{-1}\). The CFU counts of the bacterial suspension were stable over the 24 h period used for inoculation (Suwarno et al. 2012).

**Dynamics of biofilm formation**

**Increase in TMP**

The TMP profile in the absence of bacteria showed that between 3 and 6 days, the TMP had increased by 5 and 16\%, respectively (Figure 1). When bacteria were continuously injected into the system (PAO1 + 20 mg l\(^{-1}\) NB, Figure 1), the TMP increased substantially by day 6. The first stage was characterized by a slow increase in TMP (5, 8, 11 and 22\% on days 1–4, respectively), followed by a second stage where an abrupt rise in the TMP from 22 to 159\% was observed on days 5 and 6.

**Comparison of TMP and biofilm development**

Biofilm development progressed from individual cells (day 1, Figure 2(a)–(d)), to small, then large aggregates (day 2 and 3, Figure 2(e)–(l), respectively), at which time large microcolonies formed on the surface (day 5, Figure 2(q)–(t)) and finally, on day 6, a thick biofilm was observed that covered most of the membrane (Figure 2(u)–(x)). The biovolume of live and dead cells, biofilm thickness and CFUs were quantified for comparison with the TMP profile. The biofilm increased in volume from 0.07 to 1.39 μm\(^3\) μm\(^{-2}\) (days 1–4), and then showed a significant increase on days 5 and 6 for both live and dead cells (Figure 3(a)). The percentage of dead cells increased on days 5 and 6 (Figure 3(a)). This increase in biovolume was reflected in the increase in culturable cells, increasing from 7.06 × 10\(^6\) (day 4) to 3.17 × 10\(^7\) and 5.78 × 10\(^7\) CFU cm\(^{-2}\) on days 5 and 6, respectively (Figure 3(b)). The trend in CFU (Figure 3(b)) and the biovolume of live cells (Figure 3(a)) was similar, and those changes coincided with the abrupt rise in the TMP. The biofilm thickness (Table 1) increased from 7.60 to 21.00 μm over the 6 day period.

**Quantification of EPS**

The biovolumes calculated for polysaccharides and eDNA were equal to each other at all time points (Figure 3(c)). In comparison, the relative proportion of proteins extracted from the biofilm was higher on days 5 and 6 than that of the extracted polysaccharides (Figure 3(d)). The combined biovolumes of eDNA and polysaccharides were more than twofold higher on day 4 than on day 3, and by day 6 had increased twenty-eightfold compared to day 1. However, the polysaccharide content, as measured by the staining method, appeared to increase substantially from day 4 (Figure 3(c)), in contrast to the amount calculated by extraction (Figure 3(d)). To determine if there was a relationship between bacterial cells and the amount of EPS, the eDNA and polysaccharide biovolumes were normalized to the total biovolume of cells and the volume of live cells (Table 1). For days 1–5, the biovolume of the 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 decreased markedly, giving an EPS: cell ratio of less than 1 in all cases. This was surprising since the number of cells and the volume of the EPS constituents increased across all days.

**Effect of EPS on biofilm formation and fouling**

The TMP profiles for both WT PAO1 and an isogenic, mucoid mutant that overproduces alginate were identical.
Figure 2. CLSM 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, e, i, m, q, u) and Column 2 (b, f, j, n, r, v) are images of live/dead staining respectively, in which the live cells are green and the dead red. Column 3 (c, g, k, o, s, w) are images of eDNA stained with BOBO-3. Column 4 (d, h, l, p, t, x) are images of extracellular polysaccharides stained with ConA-FITC.
for the first 4 days of operation (Figure 4). However, the mucoid strain subsequently initiated an earlier TMP ‘jump’ than the WT PAO1 (day 5.5 vs. day 6; Figure 4). The total biovolume of the biofilm was 70% higher for PAO1 than for the mucoid strain (Figures 5(a), (b), (e), (f) and 6(a)), despite the latter reaching the maximum TMP 12 h earlier. Similarly, the number of CFUs (Figure 6(b)) was higher for PAO1 ($5.78 \times 10^7$ CFU cm$^{-2}$) than for the mucoid strain ($3.17 \times 10^7$ CFU cm$^{-2}$). On day 3 of the experiment, when the TMP profiles were identical, there was no difference in the biovolume of the biofilms formed by the two strains, although the PAO1 had 140% more CFU than the mucoid strain (Figure 6(a) and (b)). By contrast, biofilms of the mucoid strain had higher amounts of polysaccharides and eDNA during both the low and high TMP stages (Figure 6(c) and (d)). The protein concentration was similar for the two strains at the high TMP stage (Figure 6(d)). When the EPS components were normalized for total biofilm biovolume/cells, it was clear that the mucoid strain contained ~fourfold more of the EPS components than the WT strain (Table 2).
Effect of nutrient concentration on biofilm formation

There was a clear relationship between nutrient concentration in the feed (20, 15, 10 and 0 mg l$^{-1}$) and the increase in TMP (Figure 7). After 6 days at the highest nutrient concentration, 20 mg l$^{-1}$, the TMP had increased by 159%, while at 15 and 10 mg l$^{-1}$ the corresponding increase in TMP was 39 and 12%, respectively. In the absence of nutrient input, the increase in TMP was just 6%. It should be noted that while the lower nutrient resulted in lower TMP over the 6 days, the TMP was still observed to undergo the abrupt rise or ‘jump’ and reach maximum pressure, the difference being in the time it took; on day 6 for 20 mg l$^{-1}$ of NB and day 8 for 10 mg l$^{-1}$ of NB. When the injection of bacteria was stopped on day 2, the TMP profile was identical to the module with continuous injection of bacteria (Figure 7).

Quantitative analysis of the biofilms indicated that in the absence of added nutrient, after 3 days, the biovolumes for live and dead cells were 0.01 and 0.58 $\mu$m$^3$, respectively, with dead cells making up 98% of the total bacteria (data not shown). After 6 days, the biovolumes of live and dead cells were 0.16 and 1.18 $\mu$m$^3$, respectively, and the percentage of dead cells decreased to 88% (Figure 8(a)). At 10 mg l$^{-1}$ NB, after 6 days, the structure and the components of the biofilm (Figure 8 and Supplementary Figure S1) were similar to the 3-day old biofilms formed under the 20 mg l$^{-1}$ NB regime (Figure 2 and 3). [Supplementary material is available via a multimedia link on the online article webpage]. The rise in TMP for the two different biofilms, 12% on day 6 at 10 mg l$^{-1}$ and 11% on day 3 at 20 mg l$^{-1}$ (Figure 7), was also similar.

Discussion

Dynamics of biofilm formation and its effect on TMP rise

A minor rise in TMP was observed in the absence of bacteria (Figure 1). The RO set-up was open to the air and some bacteria may have entered the system and

![Figure 4](image-url) - TMP profiles of the RO system inoculated with *P. aeruginosa* WT and mucoid (FRD1) strains (PAO1 + 20 mg l$^{-1}$ NB; FRD1 + 20 mg l$^{-1}$ NB). The RO system was run using feed solution with 2 g l$^{-1}$ NaCl, at 35 LMH flux and a CFV of 0.17 m s$^{-1}$.

![Figure 5](image-url) - CLSM 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 and fluorescently stained and imaged. Column 1 (a, e) and Column 2 (b, f) are images of live/dead staining respectively, in which the live cells are green and the dead red. Column 3 (c, g) are images of eDNA stained with BOBO-3 and Column 4 (d, h) are images of extracellular polysaccharides stained with ConA-FITC.
fouled the membrane. CLSM observation (data not shown) showed that there was some biofilm present on the RO membrane (total biovolume 0.37 μm³/C²), which was only 5–10% of the biovolume observed when bacteria were injected) and this biofilm may have caused the increased TMP. It is also possible that NB and NaCl could have led to the increased TMP due to the effect of chemical fouling by NB components and concentration polarization (CP) (Suwarno et al. 2012). CP is the phenomenon in membrane operation where the solute concentration is higher in the boundary layer near to the membrane surface. CP occurs due to the retention of solutes by the membrane (Baker 2004). The effect of CP on the rise in TMP has been shown previously, where the flux was determined to account for the polarization effect of solutes (Mulder 1991). The presence of a biofilm layer on the membrane further increased the polarization effect and hence elevated the TMP (Herzberg & Elimelech 2007). Therefore, the increased TMP in the experiments without the addition of bacteria could be attributed either to the influence of CP, or to the airborne recruitment of fouling bacteria, or a combination of both. However, compared to the fouling observed when bacteria were injected into the system, the change in TMP without bacterial addition was minor. Therefore, over the period of the study, microbial fouling and biofilm

Table 2. The thickness and the biovolume ratio of EPS to bacterial cells for the biofilms formed by the WT PAO1 and FRD1.

| Condition       | PAO1  | FRD1  |
|-----------------|-------|-------|
| Thickness (μm)  | 21.00 | 19.00 |
| eDNA/live cells | 1.00  | 4.78  |
| eDNA/total cells| 0.46  | 1.77  |
| EPS/live cells  | 0.91  | 3.51  |
| EPS/total cells | 0.41  | 1.30  |

Figure 6. Characterization of the biofilms on the RO membranes. *P. aeruginosa* PAO1 and its mucoid mutant FRD1 were inoculated into the RO system and biofilm formation and TMP were monitored. Membranes were removed for autopsy on the third day and when the TMP maximum was reached. (a) The biovolume (μm³ μm⁻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 (μg cm⁻²) of extracellular polysaccharides and proteins determined by extraction. Bars represent standard errors (n = 3–6).
development was determined to be the primary contributor to deterioration of RO function, with the results indicating that there was a positive relationship between live bacterial cells and the abrupt rise in TMP. When the injection of bacteria was stopped after 2 days (Figure 7), 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 injected continuously. This would suggest that it is the development of the biofilm on the membrane, rather than recruitment of planktonic cells that led to the abrupt rise in TMP. This could be due to the production of EPS components by the bacteria or to the increase in cell number due to replication once attachment had begun and the biofilm community had started to form.

The TMP profile can be divided into two stages. The first stage was characterized by a slow increase in TMP, 22% in the initial 4 days, followed by a second stage where an abrupt rise in TMP or 'TMP jump' from 22 to 159% was observed over the next 2 days. The abrupt rise in TMP could be due to the production of EPS components by the bacteria or to the increase in cell number due to replication once attachment had begun and the biofilm community had started to form.

Figure 7. TMP profiles of the RO system run under different nutrient levels (20, 15, 10 and 0 mg l⁻¹ NB) with continuous injection of bacteria and at 20 mg l⁻¹ NB with injection of bacteria for the first 2 days only. The NaCl concentration in the feed solution was 2 g l⁻¹; the flux was 35 LMH and the CFV was 0.17 m s⁻¹. ‘X’ shows where the confocal image data (Supplementary Figure 1) were collected.

Figure 8. 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 on the sixth day. (a) The biovolume (µm³ µm⁻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 (µg cm⁻²) of EPS and proteins determined by extraction. Bars represent SEs (n=3–6).
in TMP coincided with the process of biofilm formation. The results presented in Figure 1, without bacterial injection, suggest that the initial slow increase in TMP may be the result of chemical fouling, i.e., from the NB and NaCl in the feed solution. This is supported by data that show this process to be indistinguishable from the potential effects of bacterial attachment, biofilm formation and EPS excretion (Figures 4, 7). An accelerated increase in TMP started from ~day 3, just prior to the point at which the abrupt rise occurred. Analysis of the biofilm at this stage suggests 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 covered the surface of the membrane more completely, and potentially became denser and less porous rather than increasing in absolute thickness. In the latter stage of the experiment, with high TMP, the proportion of dead cells was higher than the proportion live cells. Cell lysis would result in an increase in eDNA, which was observed, as well as in 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 & Elimelech 2007; Chong et al. 2008). The proposed scenario is consistent with results presented by Cho and Fane (2002) for an MBR, where biofouling became a “self-accelerating” process. Once the membrane was heavily fouled, leading to an abrupt rise in TMP, the biofilm mostly containing dead bacteria and EPS, was attached tightly to the membrane and was difficult to remove by any physical or chemical method. Hence, although biofouling cannot be eliminated completely, for sustainable operation prolonging the stage exhibiting a slow increase in TMP and avoiding the stage where TMP rises abruptly is essential.

Two methods were used to quantify the EPS in this study: chemical extraction analyses and staining with fluorescent dyes followed by imaging with CLSM. There is no universal chemical extraction method due to the complexity of the EPS. The bacterium used in this study, *P. aeruginosa* produces at least three distinct extracellular polysaccharides that contribute to biofilm development and architecture: alginate, Psl and Pel (Ryder et al. 2007). Alginate is composed of non-repeating 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 chemical extraction method and fluorescence staining may therefore detect different polysaccharides. In addition, the polysaccharide matrix can potentially undergo a sol-gel transition (Seviour et al. 2009), which may change diffusion properties and alter the TMP, without a concomitant increase in biovolume. Consequently, the two methods may give different concentrations of the polysaccharides, which highlights the importance of using multiple methods for EPS quantification.

**The importance of EPS**

Herzberg and Elimelech (2007) reported that both bacterial cells and EPS contribute to a decrease in RO performance. Bacterial cells on the membrane hinder the back diffusion of salt resulting in an elevated osmotic pressure (BEOP) on the membrane surface, and therefore a decrease in permeate flux (or an 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. Synthesis of alginate, one of the polysaccharide components of *P. aeruginosa* EPS, has been shown 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 twofold–sixfold higher levels of alginate (Mathee et al. 1999).

Fouling can be defined as the increase in 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, FRD1 and PAO1 strains showed the same rise in TMP (TMP maximum), but the FRD1 strain achieved maximum TMP within a shorter time frame (5.5 days for FRD1 vs. 6 days for PAO1). This difference is relevant and hence it is concluded that FRD1 had a greater fouling effect than PAO1. The biofilm formed by the mucoid FRD1 strain had fewer cells, as shown by live/dead staining and CFU counts, and more polysaccharide production than the WT PAO1. Therefore, it is possible that the increase in EPS may account for the greater fouling effect observed with the mutant strain. 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 in producing alginate (Silo-Suh et al. 2005). One benefit may be to increase the structural cohesion of the biofilm, which would presumably be an advantage in maintaining the biofilm under conditions of high shear. The data presented here support this hypothesis, where the EPS appeared to play an important role in biofilm development and the decreased performance of the RO system.
The effect of different nutrient levels

NB was added into the feed tank of the RO system to provide average nutrient concentrations of 0, 10, 15 or 20 mg l\(^{-1}\) (20 mg l\(^{-1}\) NB is equal to 6.5 mg l\(^{-1}\) TOC). In comparison, seawater coming into a desalination plant, eg a seawater RO plant, usually has a TOC of <3 mg l\(^{-1}\) with very low assimilable organic carbon (AOC) (Escobar et al. 2000). The purpose of using a higher nutrient load in the laboratory study was to accelerate the process of membrane biofouling so that replicated experiments could be performed within a realistic time frame. In addition, the flux of 35 LMH was \(~50\%\) higher than a typical RO plant, again to provide information within a reasonable time frame. As a result, the phenomenon of an abrupt and rapid rise in TMP observed in this study appeared after a relatively short period of operation (\(ca\) 6 days). However, qualitatively similar biofouling development for fluxes as low as 5 or 10 LMH have also been observed (data not shown). Therefore, these results are applicable to biofouling in an RO plant. Although the initial nutrient load in the feed to the RO plant may be lower, the TMP would eventually increase due to the high rejection properties of RO membranes leading to CP. The accumulation of nutrient on the surface of membranes over time would eventually reach a level sufficiently high to support biofilm development and accelerate growth. Conventional pretreatment processes, including microfiltration/ultrafiltration are not designed to target the removal of nutrients, therefore remaining nutrients can cause subsequent biofouling on the RO membrane. Thus, it is proposed that limiting the nutrient concentration in the influent may be one strategy to control the process of biofouling in RO plants.

The results from this study support this proposal. For example, it has been shown that a higher carbon concentration increased the rate and extent of biofilm accumulation (Peyton 1996). The work presented here is in agreement with Klahre and Flemming (2000) who observed that the addition of nitrogen and phosphorus to the phenonemon of an abrupt and rapid rise in TMP. However, qualitatively similar biofouling development for fluxes as low as 5 or 10 LMH have also been observed (data not shown). Therefore, these results are applicable to biofouling in an RO plant. Although the initial nutrient load in the feed to the RO plant may be lower, the TMP would eventually increase due to the high rejection properties of RO membranes leading to CP. The accumulation of nutrient on the surface of membranes over time would eventually reach a level sufficiently high to support biofilm development and accelerate growth. Conventional pretreatment processes, including microfiltration/ultrafiltration are not designed to target the removal of nutrients, therefore remaining nutrients can cause subsequent biofouling on the RO membrane. Thus, it is proposed that limiting the nutrient concentration in the influent may be one strategy to control the process of biofouling in RO plants.

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References

Baker RW. 2004. Membrane technology and applications. Chichester: John Wiley.

Cho BD, Fane AG. 2002. Fouling transients in nominally subcritical flux operation of a membrane bioreactor. J Membr Sci. 209:391–403.

Chong TH, Wong FS, Fane AG. 2008. The effect of imposed flux on biofouling in reverse osmosis: role of concentration polarisation and biofilm enhanced osmotic pressure phenomena. J Membr Sci. 325:840–850.

Davies DG, Chakrabarty AM, Geesey GG. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by Pseudomonas aeruginosa. Appl Environ Microbiol. 59:1181–1186.

Dewanti R, Wong ACL. 1995. Influence of culture conditions on biofilm formation by Escherichia coli O157:H7. Int J Food Microbiol. 26:147–164.

Donlan RM. 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis. 8:881–890.

Escarobar IC, Hong S, Randall AA. 2000. Removal of assimilable organic carbon and biodegradable dissolved organic carbon by reverse osmosis and nanofiltration membranes. J Membr Sci. 175:1–17.

Flemming H-C. 1997. Reverse osmosis membrane biofouling. Exp Therm Fluid Sci. 14:382–391.

Flemming H-C, Schaukel G, Griebe T, Schmitt J, Tamachkifornow A. 1997. Biofouling – the Achilles heel of membrane processes. Desalination. 113:215–225.

Gerhardt P, Murray RGE, Wood WA, Krieg NR. 1994. Methods for general and molecular bacteriology. Washington (DC): American Society for Microbiology.

Ghayeni SBS, Beaton PJ, Schneider RP, Fane AG. 1998. Adhesion of waste water bacteria to reverse osmosis membranes. J Membr Sci. 138:29–42.
Herzberg M, Elimelech M. 2007. Biofouling of reverse osmosis membranes: role of biofilm-enhanced osmotic pressure. J Membr Sci. 295:11–20.

Kang GD, Cao YM. 2012. Development of antifouling reverse osmosis membranes for water treatment: a review. Water Res. 46:584–600.

Klahre J, Flemming H-C. 2000. Monitoring of biofouling in papermill process waters. Water Res. 34:3657–3665.

Kucera J. 2010. Reverse osmosis: design, processes, and applications for engineers. Salem: Scivener Pub.

Lawrence JR, Swerhone GDW, Leppard GG, Araki T, Zhang X, West MM, Hitchcock AP. 2003. Scanning transmission x-ray, laser scanning, and transmission electron microscopy mapping of the exopolymeric matrix of microbial biofilms. Appl Environ Microbiol. 69:5543–5554.

Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JIA, Jensen P, Johnsen AH, Givskov M, Ohman DE, Soren M, et al. 1999. Mucoid conversion of Pseudomonas aeruginosa by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiology. 145:1349–1357.

Matin A, Khan Z, Zaidi SMJ, Boyce MC. 2011. Biofouling in reverse osmosis membranes for seawater desalination: phenomena and prevention. Desalination. 281:1–16.

Mulder M. 1991. Basic principles of membrane technology. Dordrecht: Kluver Academic.

Ognier S, Wisniewski C, Grasmick A. 2002. Membrane fouling during constant flux filtration in membrane bioreactors. Membr Technol. 147:6–10.

Peyton BM. 1996. Effects of shear stress and substrate loading rate on Pseudomonas aeruginosa biofilm thickness and density. Water Res. 30:29–36.

Ryder C, Byrd M, Wozniak DJ. 2007. Role of exopolysaccharides in Pseudomonas aeruginosa biofilm development. Curr Opin Microbiol. 10:644–648.

Schneider RP, Ferreira LM, Binder P, Bejarano EM, Goes KP, Slango E, Machado CR, Rosa GMZ. 2005. Dynamics of organic carbon and of bacterial populations in a conventional pretreatment train of a reverse osmosis unit experiencing severe biofouling. J Membr Sci. 266:18–29.

Seviour T, Pijuan M, Nicholson T, Keller J, Yuan Z. 2009. Gel-forming exopolysaccharides explain basic differences between structures of aerobic sludge granules and flocular sludges. Water Res. 43:4469–4478.

Silo-Suh L, Suh SI, Phabbs PV, Ohman DE. 2005. Adaptations of Pseudomonas aeruginosa to the cystic fibrosis lung environment can include deregulation of zwf, encoding glucose-6-phosphate dehydrogenase. J Bacteriol. 187:7561–7568.

Suwarno SR, Chen X, Chong TH, Puspitarsari VL, McDougald D, Cohen Y, Rice SA, Fane AG. 2012. The impact of flux and spacers on biofilm development on reverse osmosis membranes. J Membr Sci. 405–406:219–232.

Zhang J, Chua HC, Zhou J, Fane AG. 2006. Factors affecting the membrane performance in submerged membrane bioreactors. J Membr Sci. 284:54–66.