Biofouling, the combined effect of microorganism and biopolymer accumulation, significantly reduces the process efficiency of membrane bioreactors (MBRs). Here, four biofilm components, alpha-polysaccharides, beta-polysaccharides, proteins and microorganisms, were quantified in MBRs. The biomass of each component was positively correlated with the transmembrane pressure increase in MBRs. Proteins were the most abundant biopolymer in biofilms and showed the fastest rate of increase. The spatial distribution and co-localization analysis of the biofouling components indicated at least 60% of the extracellular polysaccharide (EPS) components were associated with the microbial cells when the transmembrane pressure (TMP) entered the jump phase, suggesting that the EPS components were either secreted by the biofilm cells or that the deposition of these components facilitated biofilm formation. It is suggested that biofilm formation and the accumulation of EPS are intrinsically coupled, resulting in biofouling and loss of system performance. Therefore, strategies that control biofilm formation on membranes may result in a significant improvement of MBR performance.

**Keywords:** biofouling; cake layer; biofilm; transmembrane pressure; co-localization; extracellular polymeric substances

### Introduction

The membrane bioreactor (MBR) is a wastewater treatment technology that combines, in one integrated system, an activated sludge biological degradation process with sludge-water separation by membrane filtration (Melin et al. 2006). MBRs have a number of advantages over conventional technologies, including reduced treatment space and sludge production, higher biomass concentration and a higher quality of effluent (Gander et al. 2000; Melin et al. 2006). The major hindrance in implementation of MBRs is membrane biofouling. Biofouling is the build-up of organic and biological cake-layers on the membranes, blocking the membrane pores (Baker & Dudley 1998). The biofouling layers increase the hydraulic resistance of the membrane, resulting in an increase in the transmembrane pressure (TMP) when operated at a constant flux, or a decreased flux when operated at a constant pressure (Sombatsompop et al. 2006). Therefore, it is necessary to investigate the mechanism of membrane biofouling, which will ultimately be informative for developing strategies to control membrane fouling in the MBR.

As a major component of membrane biofouling, biofilms, which are formed of a complex mixture of microbial cell clusters, polysaccharides and proteins, have been intensively investigated (Meng et al. 2007; Yang et al. 2011; Hwang et al. 2012). However, no consensus has been reached on their role in the biofouling process. For example, there are different types of polysaccharide; it has been shown that alpha-polysaccharides play an important function in the initial attachment of the biofilm onto the membranes, while beta-polysaccharides and proteins contribute to the construction of the mature biofilm structures (Chen, Lee, Yang et al. 2006). Polysaccharides have a higher correlation to membrane permeability than proteins, although proteins dominate biofouling components (Gao et al. 2013). Therefore, further work needs to be done to elucidate the specific contributions of the biopolymers and the bacteria involved in the fouling process.

Currently, two hypotheses have been established to describe the process of biofouling (Lee et al. 2008). One hypothesis is that the deposition of biofouling layers is a consequence of filtration of extracellular polysaccharides (EPS) and microbes in the water onto the membrane to
form a cake-layer. The alternative hypothesis is that microbes specifically attach onto the membrane to establish a biofilm and subsequently grow into EPS encased communities that ultimately block the membrane pores. The specific mechanism of fouling is important as it relates directly to the types of control strategy that may be the most effective. If the biocake and fouling are solely a consequence of passive migration of materials from the bulk phase onto the membrane, then strategies designed to reduce the deposition of those components, such as more frequent backwashing, may be best suited to control biofouling. Conversely, if active biofilm development and secretion of the associated EPS is the key to the fouling process, then strategies should ideally focus on reducing biofilm growth on the membrane surface. Image based analysis is particularly well suited to address these alternate hypotheses because it is, unlike more general extraction and chemical analysis approaches, able to spatially resolve the different biopolymers and the cells. Therefore, this study aimed to investigate the relationships between each of the EPS components and the microbial cells to define their role in the biofouling process.

Materials and methods

MBR system and operation

Two identical laboratory scale MBR systems were designed and operated in parallel to treat synthetic wastewater (Figure 1a). Each MBR was composed of an anoxic tank and an aerobic tank with a membrane module. The effective volumes of the anoxic tank and the aerobic tank were 2 L and 5 L, respectively. The ‘curtain’ style hollow fiber membrane modules were installed in the two MBRs and each membrane module had an area of 565 cm² (Figure 1b). The synthetic wastewater was composed of glucose (320 mg l⁻¹), beef extract (60 mg l⁻¹), peptone (80 mg l⁻¹), KH₂PO₄ (7 mg l⁻¹), MgSO₄•7H₂O (14 mg l⁻¹), FeSO₄•7H₂O (7.3 mg l⁻¹) and sodium acetate (90 mg l⁻¹). The total organic carbon (TOC) of synthetic wastewater was 200 mg l⁻¹. Activated sludge was collected from the Ulu Pandan wastewater treatment plant in Singapore and acclimated with synthetic wastewater for 60 d before the start of experiments.

The synthetic wastewater was fed into the anoxic and aerobic tanks and then recycled continuously from the aerobic tank to the anoxic tank (1.21 h⁻¹). The hydraulic retention time and sludge retention time for each MBR were maintained at ~10 h and 25 d, respectively for the two MBRs. The concentration of the mixed liquor suspended sludge (MLSS) was maintained at 3–5 g l⁻¹. Both MBRs were operated at room temperature (25–26°C). The MBR system was controlled by a computer with SCADA software (IFIX). The permeate pump speed was controlled to maintain a constant flux of 13–15 l m⁻² h⁻¹ (LMH). The parameters, such as membrane flux, TMP, pH, dissolved oxygen, and temperature were monitored and automatically recorded using a data logger and computer. The TOC of the influent and permeate was measured using a multi N/C® 2100s (AnalytikJena, Eisfeld, Germany).

Staining of biofilm samples

Three replicate membrane samples were collected for confocal laser scanning microscope (CLSM) analysis at different time points of operation and represent various stages of increasing TMP. The membrane samples were immersed in 20 μM SYTO 63 red fluorescent nucleic acid stain solution (Molecular Probes, Invitrogen, Grand Island, NE, USA) for 30 min to stain the microbial cells. The membranes were then soaked in 500 μl of fluorescein-5-isothiocyanate (FITC ‘Isomer 1’, Molecular Probes, Invitrogen) staining solution (1 mg ml⁻¹) for 1 h to stain the proteins. The membranes were subsequently immersed in freshly prepared 0.2 mg ml⁻¹ concanavalin A-tetramethylrhodamine (Molecular Probes, Invitrogen) staining solution for 30 min to stain the alpha-mannopyranosyl and alpha-glucopyranosyl sugar residues. Finally, membranes were stained with 1 g l⁻¹ of calcifluor white solution (Sigma, St Louis, MO, USA) for 30 min to label the beta-D-glucopyranose polysaccharides (Chen, Lee, Yang et al. 2006; Chen et al. 2007). After staining, the samples were immersed in phosphate buffered saline (PBS, pH 7.2) for 10 min twice to remove excess stain. All the staining procedures were performed at room temperature in the dark.

CLSM imaging

The stained hollow fiber membrane samples were put onto glass slides for imaging using an inverted CLSM (LSM 710, Carl Zeiss, Göttingen, Germany). The calcifluor white was excited at 405 nm and detected using the emission range of 410–480 nm. FITC stained samples were excited at 488 nm and observed at 500–540 nm for emission. The tetramethylrhodamine conjugated concanavalin A was detected via excitation at 543 nm and emission at 550–600 nm. The SYTO 63 was excited at 633 nm and captured at 650–700 nm (Chen et al. 2007). Triplicate samples were imaged and quantified for each time point. For each membrane sample, 3–5 images were captured randomly. Therefore, a total of 9–15 images were collected at each time point.

Analysis of the 3D images

Three dimensional reconstructions of the biofilms were produced using the software ZEN-2009 light edition
Figure 1. The MBR system used in this project. (a) A schematic view of the internal submerged MBR. (b) The configuration of the 'curtain' style HF membrane module.
(Carl Zeiss) by the ‘3D’ process. Images were processed using the ‘maximum intensity projection’ (MIP) to convert the 3D image into integrated 2D images. The MIP 2D images were split into different channels in the $xy$ direction to separately observe the differently stained components. The orthogonal projection was also performed to the 3D images by the Ortho process to convert the 3D image to 2D images in the $xy$, $xz$ and $yz$ directions.

The membrane surface coverage of the biofilm components was calculated using the MIP 2D images function of Image J Version 1.46 (http://imagej.net/). The images were first converted to 8-bit gray images through ‘image-type’ and were subsequently made into binary images through ‘process-make binary’. The images were adjusted using the ‘Filters’ process. Finally, the surface coverage of the individual biofilm components was calculated using the command of ‘Analyze particles’. The quantitative volume analysis of the 3D CLSM images was performed using Imaris Version 7.3.1 (Bitplane, Zurich, Switzerland). The biovolume of each channel in the 3D CLSM images was calculated by applying the ‘surface’ function to the 3D images and calculating the volume of surfaces. The co-localization analysis was performed using the Coloc function (Imaris). The Mander colocalization coefficient was calculated for the image data to determine correlations between biofilm components for each sample.

**EPS extraction and quantification**

The EPS in the activated sludge mixture was extracted based on the NaOH-formaldehyde protocol (Liu & Fang 2002). Sludge liquor (10 ml) was centrifuged at 4,000 $g$ at 4°C for 10 min and the supernatant was collected into a clean tube for quantification of the soluble EPS. To extract the bound EPS, the cell pellet was resuspended in 5 ml of 0.85% NaCl and 0.22% formaldehyde solution at 4°C for 1 h, after which 5 ml of 1 M NaOH were added and incubated at 4°C for 3 h. The sludge solution was centrifuged at 13,000 $g$ for 20 min. The supernatant solution containing the extracted, bound EPS was transferred to a clean tube. The soluble and bound EPS were both filtered through 0.45 μm membrane filters (PALL, Product No.: 4614). The concentration of carbohydrates in the soluble and bound EPS was determined by the phenol-sulfuric acid method using glucose to generate a standard curve (DuBois et al. 1956). The concentration of proteins in the EPS was quantified by the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard (Bradford 1976).

**Results**

**Two-stage TMP increasing curve in MBRs**

The MBR systems were operated at a constant flux of 13–15 LMH for ~115 d and the TMP was monitored continuously. It was observed that the two MBRs exhibited similar TMP profiles over the entire study period (Figure 2). Both were observed to operate at a steady TMP stage (3–15 kPa) for the first 80–87 d and a TMP jump stage (15–90 kPa) for the later 28–35 d, which has been reported previously (Tardieu et al. 1998; Cho & Fane 2002; Ognier et al. 2002). The two MBR modules differed slightly as they made the transition into the TMP jump phase. For MBR-1, the transition occurred after the first 82 d while for MBR-2, it occurred after 87 d. The average rate of TMP increase was 0.183 kPa d$^{-1}$ and 0.172 kPa d$^{-1}$ at the low TMP stage in MBR-1 and 2, respectively. In the TMP jump stage, the average rate of TMP increase was 2.143 kPa d$^{-1}$ for MBR-1 and 2.778 kPa d$^{-1}$ for MBR-2. Compared to steady stage, the rate of TMP increase was almost 15-fold faster in the TMP jump phase relative to the low TMP stage.

**Heterogeneous distribution of biofouling components on the membrane**

Four biofilm components, alpha-polysaccharides, beta-polysaccharides, proteins and microorganisms, were

![Figure 2](image-url). The TMP profiles and sampling time points for the two replicate MBRs. The TMP values are shown as triangles (MBR-1) and stars (MBR-2). The horizontal dashed line indicates the threshold TMP and the vertical dashed line separates the TMP into low TMP stage (<15 kPa) and the TMP jump stage (>15 kPa). The solid arrows show the time points for biofilm staining experiments and the dashed arrows show the time points for EPS extraction.
quantified in the biofilm across the different stages of MBR operation. These individual components were observed to be distributed heterogeneously on the membrane. When the MBR TMP was low (3–10 kPa), the four individual components were all non-uniformly distributed across the membrane as small clusters on the membranes in both MBRs (Figures S1–S4). [Supplementary information is available via a multimedia link on the online article webpage.] At 5 kPa, the surface coverage of these components on the membranes was 3.79 ± 1.65% (alpha-polysaccharides), 6.46 ± 2.31% (beta-polysaccharides), 2.55 ± 1.51% (proteins) and 1.18 ± 0.47% (microorganisms), respectively (Figure S5). As the experiment continued, the clusters became larger (in circumference and thickness) and covered more area of the membrane, where the surface coverage increased to 8.94 ± 2.9% (alpha-polysaccharides), 7.69 ± 4.42% (beta-polysaccharides), 20.02 ± 6.85% (proteins) and 4.82 ± 1.97% (microorganisms), respectively at 9 kPa (Figure S6). A thin, confluent mat of protein covered most of the membrane area at 10–15 kPa (Figure S1) while the alpha-polysaccharides, beta-polysaccharides and microorganisms established a confluent layer only after the TMP exceeded 15 kPa (Figures S2–S4). Thus, in contrast to the other three components, only the protein layer was established before the TMP jump. Examining the four components together, the biofilm was also distributed as small clusters on the membranes at TMP of 3–10 kPa in both MBRs (Figure 3) and the thickness ranged from 1–5 μm. When the TMP was 10–15 kPa, the size of the clusters (a combination of circumference and thickness) increased to 1–10 μm. At this time, some clusters appeared to merge together to form a continuous layer. During the TMP jump phase, 30–90 kPa, the biofilm almost completely covered the membrane surface and there was very little unfouled membrane. While uniformly spread across the surface at 30–90 kPa, the thickness of the biofilm was also heterogenous, varying from 5 to 20 μm. Therefore, the correlation with a complete surface coverage of the membrane by the proteins, rather than biofilm thickness, appeared to be important for the rapid deterioration of MBR function here.

**Correlation of biofouling components and TMP increases in MBR**

The biovolumes of the four biofilm components were quantified to determine their specific contribution to the TMP increase. The biovolumes, μm³, are presented as normalized against the membrane surface area, μm².

**Alpha-polysaccharides**

The biovolume of alpha-polysaccharides was observed to increase along the TMP rising profile. When the TMP was between 3 and 15 kPa, the biovolumes of the alpha-polysaccharides were 0.04–0.44 μm³ μm⁻² in MBR-1 and 0.06–0.25 μm³ μm⁻² in MBR-2. After the TMP increased to 15–30 kPa, the alpha-polysaccharides were observed to occupy 0.25–0.8 μm³ μm⁻² (MBR-1) and 0.25–0.42 μm³ μm⁻² (MBR-2). The biovolume of the alpha-polysaccharides increased substantially to 1.18–1.77 μm³ μm⁻² in MBR-1 and 0.47–1.47 μm³ μm⁻² in MBR-2 at the TMP of 60–90 kPa (Figure 4). The Pearson’s correlation coefficient (r) between the TMP increase and the accumulation of alpha-polysaccharide (Table 1) was 0.794 and 0.743 in MBR-1 and MBR-2, respectively, indicating that the TMP increase was moderately correlated with the increased biovolume of the alpha-polysaccharides.

**Beta-polysaccharides**

The biovolume of beta-polysaccharides also increased during the TMP increase. In MBR-1, the beta-polysaccharide volumes were 0.03–0.46 μm³ μm⁻² at 3–15 kPa, 0.33–0.5 μm³ μm⁻² at 15–30 kPa, 0.5–0.8 μm³ μm⁻² at 30–60 kPa and 0.8–2.2 μm³ μm⁻² at 60–90 kPa (Figure 4a). The Pearson’s correlation coefficient (r) for the accumulation of beta-polysaccharides and TMP increase was 0.94 (Table 1), indicating that the TMP rise correlated strongly with the increase in beta-polysaccharide biovolume. A similar pattern of increase was observed for MBR-2 (Figure 4b), where the Pearson’s correlation coefficient (r) was 0.656 (Table 1), which indicated a moderate correlation between the two factors. This was not as strong as observed for MBR-1. However, there is a significant positive correlation in the two separate MBRs between the TMP rise and beta-polysaccharide accumulation.

**Proteins**

The protein biovolumes were 0.026–0.63 μm³ μm⁻² in MBR-1 and 0.06–0.96 μm³ μm⁻² in MBR-2 when the TMP was between 3–15 kPa. At a TMP of 15–30 kPa, the protein volumes were 0.58–1.02 μm³ μm⁻² in MBR-1 and 0.82–0.96 μm³ μm⁻² in MBR-2. Further, the protein volumes increased to 1.53–1.69 μm³ μm⁻² in MBR-1 and 1.59 μm³ μm⁻² in MBR-2 when the TMP was 30–60 kPa, and 3.01 μm³ μm⁻² in MBR-1 and 2.62–2.65 μm³ μm⁻² in MBR-2 when the TMP jumped to ~ 90 kPa (Figure 4). The Pearson’s correlation coefficient (r) indicated a strong correlation between the protein accumulation and TMP rise, 0.952 (MBR-1) and 0.959 (MBR-2) (Table 1).

**Microorganisms**

At a TMP of 3–15 kPa, the microbial volume was 0.01–0.24 μm³ μm⁻² in MBR-1 and 0.04–0.28 μm³ μm⁻².
\( \mu \text{m}^{-2} \) in MBR-2. When the TMP increased to 15–60 kPa, the biovolumes for the microbes were 0.27–0.6 \( \mu \text{m}^3 \) \( \mu \text{m}^{-2} \) in MBR-1 and 0.4–0.97 \( \mu \text{m}^3 \) \( \mu \text{m}^{-2} \) in MBR-2. Finally, when the TMP reached the maximum level, 80–90 kPa, the microbial volume grew to 0.9 \( \mu \text{m}^3 \) \( \mu \text{m}^{-2} \) in MBR-1 and 1.37 \( \mu \text{m}^3 \) \( \mu \text{m}^{-2} \) in MBR-2 (Figure 4). The Pearson’s correlation coefficients \((r)\) between the TMP change and the increase of microbial volume were 0.921 for MBR-1 and 0.772 for MBR-2 (Table 1), which implied the TMP rise was strongly correlated to the number of microorganisms in MBR-1 and also had a moderate correlation to the microbial volume in MBR-2.

**Role of proteins in the TMP increase**

While there was a strong positive correlation between the accumulation of various biofilm components and the TMP rise in the MBRs, it remains unclear which of the components was the most important factor driving the sudden TMP jump. The TMP profile was divided into a steady low TMP stage and TMP jump stage separated by a threshold TMP of 15–20 kPa. Therefore, the changes in the biofouling components were examined in the two distinct TMP stages.

Proteins comprised the majority of the biofouling biomass during the TMP rise

In the initial low TMP stage for MBR-1 (3–6 kPa across the first 17 d), the alpha- and beta-polysaccharides were the dominant biofilm components on the membranes (32–63% and 21–60%, respectively), in comparison with the proteins and microbial cells, which had much lower abundance (5.1–7.6% and 2.1–8.5%, respectively). Over time and in particular when the TMP reached the transition phase (8–17 kPa, 24 d to 85 d), the proteins became the dominant components (24.3–53.4%), in comparison with the lower abundance for the alpha-polysaccharides (13.2–29.4%), beta-polysaccharides (9.8–26.5%) and microorganisms (9.2–14.3%). This trend was maintained during the high TMP stage (20–90 kPa), where the relative protein percentages remained largely unchanged (Figure 5a). The same trend was observed for MBR-2 (Figure 5b). It was noted that the protein became a major biofouling component before the TMP increased to the threshold 15–20 kPa; the relative abundance of protein increased from 5–14.1% at 3–5 kPa to 37.1–49.8% at 10–15 kPa. This was consistent with the above description of heterogeneous distribution of biofouling
components, where the thin, confluent protein mat was observed at 10–15 kPa (Figure S1) while the confluent organic layers of the three other components were established only after the TMP exceeded 15 kPa (Figures S2–S4).

It was observed that the biovolume of proteins increased faster than the alpha-polysaccharides, beta-polysaccharides and microbes. For MBR-1, the volume of proteins...
increased 6.5–13.6-fold and 22-fold when the TMP was ~10 kPa (69 d) and 17 kPa (85 d). In comparison, the volumes of the alpha-polysaccharides, beta-polysaccharides and microorganisms increased more slowly. When

the TMP was 17 kPa at 85 d, the biovolume of the alpha-polysaccharides, beta-polysaccharides and microorganisms had only increased 1.99-fold, 1-fold and 5.19-fold, respectively relative to the volume at 10 d

### Table 1. Correlation between the accumulation of biofilm components and TMP increase.

|                      | Alpha-polysaccharide (MBR-1, MBR-2) | Beta-polysaccharide (MBR-1, MBR-2) | Protein (MBR-1, MBR-2) | Microbes (MBR-1, MBR-2) | Biofilm (MBR-1, MBR-2) |
|----------------------|-------------------------------------|-------------------------------------|------------------------|-------------------------|------------------------|
| Pearson correlation (r) with TMP | 0.794, 0.743                       | 0.94, 0.656                        | 0.952, 0.959           | 0.921, 0.772            | 0.966, 0.903           |
| Significance (p <)   | 0.003, 0.009                        | 0.0001, 0.028                      | 0.0001, 0.0001         | 0.0001, 0.005           | 0.0001, 0.0001         |

*Correlation was significant at the 0.05 level (2-tailed).

#The biofilm represents the total biomass of the four components in the merged CLSM images.

**Figure 5.** The relative percentage of each biofilm component during operation of (a) MBR-1 and (b) MBR-2. The right y-axis shows the relative percentage of each biofouling component. The percentages of different components were calculated by dividing the volume of each component by the total volume of the four components. The left y-axis shows the TMP.
The same trend was observed for MBR-2, where the biovolumes of alpha-polysaccharides, beta-polysaccharides and microorganisms increased 2.99, 3.19 and 3.61-fold, respectively while the volume of proteins had increased 10.63-fold when the TMP rose to 18 kPa after 89 d (Figure 6a). Further, the protein volume increased 65-fold in MBR-1 and 34.34-fold in MBR-2 when the TMP jumped to 88 kPa. For the same samples, the alpha-polysaccharides, beta-polysaccharides and microorganisms increased only 9.33, 2.73 and 17.35-fold, respectively for MBR-1 and 3.41, 3.2 and 3.74-fold, respectively for MBR-2 (Figure 6). Therefore,
in both MBRs, the proteins increased the most as the TMP increased.

**Correlation of sludge EPS components and a TMP increase in MBR**

The data above present the relative abundance of different biopolymers in the biofilm. In particular, it is apparent from the data that the proteins were enriched in the biofilm relative to the cells and other biopolymers. One possible source of the proteins and polysaccharides is from the sludge or the bacteria in the sludge. Therefore, to determine if the enrichment of proteins in the biofilm could be due to a concomitant enrichment in the sludge and therefore deposition into the biofilm, the EPS components in activated sludge were quantified at different stages of operation. It should be noted that the goal was not to make a quantitative comparison of the EPS components in the biofilm and the sludge, but rather to determine the relative abundance of different polymers in the sludge for comparison with their relative abundance in the biofilm and how these ratios changed over time. As described above, the EPS in sludge was divided into two components, soluble EPS (sEPS) and bound EPS (bEPS), where the total carbohydrates (polysaccharides) and proteins were quantified.

For both MBRs, there were no significant changes in the protein or carbohydrate concentrations in the sEPS component of the sludge (Figure 7). Similarly, the protein concentrations did not vary in the bEPS across the TMP curve. In contrast, the concentrations of the carbohydrates in the bEPS increased at a steady rate for both systems, increasing from 60 μg ml⁻¹ at the low TMP stage to 120–160 μg ml⁻¹ at the high TMP stage (Figure 7). Despite showing an increase in concentration across the TMP profile, there was no significant correlation of the carbohydrate concentration in the bEPS and the TMP rise (Table 2).

**Relationships between the microbes and other biopolymers during biofouling**

To determine whether the distribution of EPS biopolymers was closely associated with the microbial cells, images were investigated by co-localization analysis (Figure 8) (Zinchuk & Zinchuk 2008; Dunn et al. 2011). Mander’s coefficient was used to represent the overlapping degree of the co-localized signals (Zinchuk & Zinchuk 2008). Mander’s coefficient ranges from 0–1, where 0 indicates that none of the pixels in the selected channel co-localize with another channel and 1 indicates that 100% of the pixels in the selected channel co-localize with the signals from the first channel.

At the TMP of 3–10 kPa, an average of 47.8 (±18.3)% of alpha-polysaccharides in biofouling colocalized with 78.8 (±12.6)% of microbial cells on membrane in the two MBRs (Table 3). Similarly, 41.3 (±13.7)% of beta-polysaccharides in biofouling colocalized with 81.3 (±16.6)% of microbial cells while 50.8 (±15.2)% of proteins colocalized with 69.2 (±23.7)% of microbial cells (Table 3). When the TMP increased to the threshold 15–20 kPa, more EPS components were found to colocalize to the microorganisms (68.5 (±15.9)% of alpha-polysaccharides vs 83.8 (±15.4)% microorganisms; 62.6 (±12.1)% of beta-polysaccharides vs 73.6 (±8.3)% microorganisms; 59.7 (±9.5)% of proteins vs 86.2 (±0.6)% microorganisms). When the TMP continued to rise to 60–90 kPa, the trend of colocalization between the EPS components and microorganisms was maintained on membrane, where there were 59 (±11.5)% of alpha-polysaccharides, 59.7 (±16.1)% of beta-polysaccharides and 57.1 (±15.2)% of proteins in biofouling colocalized with 90–97.3% of microorganisms. This suggested a strong overlap of the EPS components with the microbial cells on membrane.

**Discussion**

The biofouling phenomenon, leading to an increase in TMP and membrane resistance, is a crucial problem for the wider application of MBRs (Nguyen et al. 2012). In this study, the TMP increase occurred in two distinct phases, a steady, low TMP stage (3–15 kPa) and a rapid TMP increasing stage (15–90 kPa), which was consistent with previous reports (Ognier et al. 2002; Zhang et al. 2006; Guglielmi et al. 2007). In the rapidly increasing stage, the rate of TMP increase was almost 15-fold faster than in the steady phase. This rapid TMP increase has also been termed the TMP jump phase (Cho & Fane 2002). Therefore, the key question remains as to which biofouling component contributes most to the TMP jump resulting in decreased system performance.

**The distribution of biopolymers and cells on membranes**

The increase in TMP is in part a consequence of the increased resistance of the biocake or biofilm that forms on the membrane (Hwang et al. 2012). To determine the relationship between the TMP rise and the biofilm formed on the membranes, quantitative image analysis was performed. Here, image based analysis was used to spatially resolve and quantify the different biopolymers present in the biofilm. Other studies have used a combination of imaging and chemical analysis of the biofilm and both measures have given qualitatively similar results (Yun et al. 2006; Chen et al. 2013). In the present study image data were collected from multiple positions on the membrane surface, representing $1.64 \times 10^5 – 2.73 \times 10^5 \mu m^2$, which is greater than the minimum area
Figure 7. The concentration of carbohydrates and proteins in the sludge for (a) MBR-1 and (b) MBR-2, showing the TMP at each test point (▲), carbohydrates in the soluble EPS (sEPS) (□), carbohydrates in the bound EPS (○), proteins in sEPS (○) and proteins in bEPS (▲). The data points are the average values of EPS components in sludge. The error bars are the SEs of the mean (n = 3).

Table 2. The correlation between sludge EPS components and TMP increase.

|                     | Carbohydrates in sEPS (MBR-1, MBR-2) | Carbohydrates in bEPS (MBR-1, MBR-2) | Proteins in sEPS (MBR-1, MBR-2) | Proteins in bEPS (MBR-1, MBR-2) |
|---------------------|--------------------------------------|--------------------------------------|--------------------------------|--------------------------------|
| Pearson correlation | −0.311, 0.408                        | 0.573, 0.56                          | −0.43, 0.067                    | 0.025, −0.462                   |
| *Significance (p)   | 0.549, 0.422                         | 0.235, 0.248                         | 0.395, 0.9                      | 0.963, 0.356                    |

*Correlation was significant at the 0.05 level (2-tailed).
Figure 8. Co-localization analysis of the 3D image of the biofilm. (a) The 3D image of the biofilm at 5–10 kPa. (b–d) The co-localized pixels (white) are displayed between the alpha-polysaccharides (red) and microbes (purple), beta-polysaccharides (blue) and microbes (purple) and proteins (green) and microbes (purple). The scatter plot graphs next to the co-localized images in b, c and d show the intensity distribution of the pixels. Scale bars = 10 µm.
suggested \( (1 \times 10^5 \mu m^2) \) to be sufficient to represent the biofilm (Korber et al. 1993). Therefore, the image based data presented here are representative of biofilm on the membrane surface. It was observed that the individual biofouling components (alpha-polysaccharides, beta-polysaccharides, proteins and microorganisms) were all heterogeneously distributed across the membrane surface at both low and high TMP. This was particularly obvious for the low TMP stage, where the biofilm appeared as small clusters of cells and EPS. Over time, these small clusters increased in size and at the time when the TMP was at its highest, 60–90 kPa, covered most of the membrane surfaces. This heterogeneous distribution of biofouling clusters on the membrane has also been previously reported (Chen, Lee, & Tay 2006). It was also reported that while the total amount of EPS or polysaccharide differed only slightly between polysaccharide mutants of \( P. \ aeruginosa \), the spatial organization of the biofilm was clearly distinct and correlated with differences in TMP (Barnes et al. 2014). Thus the spatial organization of the biofilm played an important role in the fouling process. It has been proposed that the transition of TMP from a slow to a fast rate of increase, as observed during the TMP jump, may be a consequence of the heterogeneous distribution of the fouling layer (Cho & Fane 2002). The hypothesis is that some, but not all, parts of the membrane would become colonized or fouled and this would lead to a local reduction in permeability and flux for those regions. Therefore, when a MBR is operated at a constant average flux, this would require the flux through unfouled or less fouled portions of the membrane to increase to maintain that flux. Previous work has demonstrated that an increased flux is associated with an increased rise in the TMP (Le Clech et al. 2003). This process would lead to a self-accelerating process of fouling on the rest of the membrane, with a concomitant increase in the TMP required to push water across the less fouled portions of the membrane. In this way, the heterogeneous biofilm distribution observed here may lead to the rapid TMP jump observed.

While the accumulation of the biofilm on the membrane seems to be a driver of the increase in TMP, the biofilm is composed of many different macromolecules as well as cells and it remains possible that much of the fouling issue is specific to the accretion of a specific biofilm component, eg the EPS or cells. Previous studies have separately investigated the different biofilm components in an attempt to answer this question and most have concluded that EPS components are important for the TMP rise (Ye et al. 2005; Le-Clech et al. 2006). For example, it has been suggested that the polysaccharide component was a key factor in fouling of the membranes

| Time (d) | TMP (kPa) | Alpha-polysaccharides (αs) vs microbes (M) | Beta-polysaccharides (βs) vs microbes (M) | Proteins (Pr) vs microbes (M) |
|---------|-----------|----------------------------------------|----------------------------------------|-----------------------------|
|         |           | Mander_αs | Mander_M                  | Mander_βs | Mander_M | Mander_Pr | Mander_M |
| 17      | 8.2       | 0.600     | 0.598                    | 0.395     | 0.970    | 0.615     | 0.371    |
| 24      | 9.5       | 0.283     | 0.770                    | 0.267     | 0.589    | 0.376     | 0.385    |
| 60      | 9.7       | 0.244     | 0.813                    | 0.232     | 0.999    | 0.259     | 0.889    |
| 69      | 10.6      | 0.440     | 0.928                    | 0.349     | 0.808    | 0.515     | 0.853    |
| 88      | 20.5      | 0.729     | 0.926                    | 0.496     | 0.987    | 0.646     | 0.867    |
| 106     | 60.9      | 0.723     | 0.929                    | 0.747     | 0.819    | 0.692     | 0.868    |
| 115     | 89        | 0.511     | 0.999                    | 0.658     | 0.951    | 0.537     | 0.999    |

| Time (d) | TMP (kPa) | Alpha-polysaccharides (αs) vs microbes (M) | Beta-polysaccharides (βs) vs microbes (M) | Proteins (Pr) vs microbes (M) |
|---------|-----------|----------------------------------------|----------------------------------------|-----------------------------|
| 10      | 4.5       | 0.786     | 0.771                    | 0.725     | 0.949    | 0.756     | 0.691    |
| 17      | 6.5       | 0.411     | 0.587                    | 0.352     | 0.637    | 0.611     | 0.339    |
| 24      | 7.3       | 0.350     | 0.924                    | 0.401     | 0.668    | 0.362     | 0.738    |
| 60      | 7.8       | 0.385     | 0.710                    | 0.434     | 0.887    | 0.474     | 0.941    |
| 69      | 9.5       | 0.549     | 0.848                    | 0.477     | 0.632    | 0.463     | 0.849    |
| 88      | 16.3      | 0.525     | 0.683                    | 0.505     | 0.653    | 0.502     | 0.856    |
| 106     | 75.8      | 0.479     | 0.941                    | 0.419     | 0.806    | 0.370     | 0.949    |
| 115     | 89.4      | 0.647     | 1.000                    | 0.789     | 0.872    | 0.704     | 0.966    |
(Fonseca et al. 2007). Recently, it was reported that overproduction of the alginate polysaccharide was associated with an accelerated TMP jump (Chen et al. 2013). In contrast, a separate study indicated that proteins were the main EPS component (Meng et al. 2007). In this study, two types of polysaccharides (alpha and beta), proteins and microbial cells were all present in the biofilm at both low and high TMP stages in the MBRs. This was consistent with the previous publications (Chen, Lee, Yang et al. 2006; Chen et al. 2007), showing the alpha-polysaccharides, beta-polysaccharides, proteins and microorganisms were all involved in the biofouling process and should be considered together when the biofouling mechanisms are investigated. Furthermore, the alpha-polysaccharides, beta-polysaccharides, proteins and microorganisms were all positively correlated with the TMP increase in this study. This indicated that the combined accumulation effect of the individual biofouling components on the membrane may be the factor that led to the increased TMP. It remains possible that the fouling was associated with polysaccharides not detected by the two stains used here. If other polysaccharides were present that did not contain alpha-mannopyranosyl and alpha-glucopyranosyl residues, they would not be detected. Their influence may be to increase the biofilm matrix and hence could also impact the relative contribution of the biofilm to the TMP increase. However, if polysaccharides were present that were not detected here, significant voids in the composite images would be expected, where cells might seem to be isolated in space; such observations were not apparent here. The similar limitation applies to the chemical methods of EPS quantification. For example, the EPS extraction with ultrasonication methods may result in a higher yield of proteins (Yu et al. 2008) while EPS extraction with EDTA or formaldehyde plus NaOH may lead to the lower proportions of proteins and polysaccharides in EPS (Pan et al. 2010). EPS extracted using cation exchange resin contained more high molecular weight substances with aromatic and condensed structure but a relatively lower content of proteins to carbohydrates compared to those extracted by formaldehyde plus NaOH (Lee et al. 2013). Fulvic-like substances were detected only in the EPS extracted using the formaldehyde plus NaOH (Pan et al. 2010). Compared to the CLSM results, the most commonly used EPS extraction method, formaldehyde plus NaOH extracted only a limited portion of EPS (Liu & Fang 2002). Thus, methods for more general or the inclusion of additional stains for other polysaccharides could further improve understanding of their contribution to fouling.

The role of proteins in the TMP increase

While all four components were correlated with the TMP rise, proteins increased faster than the cells or either of the two types of polysaccharide monitored. Moreover, on a percentage basis relative to the total biomass biovolume, the proteins were present in the highest proportion at the threshold TMP stage and TMP jump phase. By comparison, the polysaccharides represented the highest proportion of the four components when the TMP was relatively stable and low and both the alpha- and beta-polysaccharides made relatively similar contributions. In comparison, it was also previously reported that the alpha-polysaccharides were the dominant EPS components while proteins and beta-polysaccharides were minor components during the initial stages of biofilm formation in MBRs (Chen, Lee, & Tay 2006). The higher protein biomass observed in this study was consistent with other published work showing that proteins accumulated preferentially in the biofilm (Tian et al. 2012). Indeed, in the present study the proteins formed a confluent, organic mat on the membrane earlier than the other components. Collectively, the data suggest that the production of proteins was more important than the two types of polysaccharides or the cells during the transition of from the low to high TMP stage under the conditions used for this study. This may also suggest that strategies that target proteins may be suitable for controlling the biofouling process in MBRs.

Is formation of the biocake layer an active or passive process?

Although the compositions of biofouling layers and their contributions to the TMP increase have been elucidated, the mechanisms by which these materials accumulate at the membrane surface are not yet clear. The cake-layer may originate in the sludge biomass, eg soluble EPS, and block the membrane pores as a consequence of deposition on the membrane (passive) or may be produced by the bacterial biofilm attached to the membrane (active) (Lee et al. 2008; Vyrides & Stuckey 2011). To address this question, the composition and distribution of biopolymers and microbial cells were investigated.

The results in this study showed that the biofouling layer had a different biopolymer composition compared to the bulk sludge. The alpha-polysaccharides and beta-polysaccharides dominated the biofouling biomass in the low pressure stage of MBR operation while the proteins comprised the majority of the biofilm biomass as the pressure was observed to increase. In contrast, the protein biomass as well as the polysaccharides in the soluble EPS remained relatively constant across all stages of
Figure 9. The correlated distribution of EPS components and microorganisms on a membrane. The 3D image of the biofilm at 5–10 kPa (a) was processed using the ‘maximum intensity projection’ function in ZEN-2009 light edition (Carl Zeiss) to convert images into 2D images (b). The biofouling components are composed of alpha-polysaccharides (red), beta-polysaccharides (blue), proteins (green) and microorganisms (purple).
MBR operation. This finding is supported by another study, where the biofilm exhibited a higher filtration resistance than the bulk sludge cake layer (Wang et al. 2007). The surface associated biofilm is likely to be the source of biopolymers responsible for the loss of MBR performance, although it remains possible that the biopolymers are deposited from the soluble phase onto the developing biofilm biomass.

The distribution of cells and biopolymers on the membrane was not homogenous. Initially, the biocake layer formed as clusters of cells and polymers which subsequently increased to cover the whole of the membrane surface. This was consistent with the typical biofilm formation and growth mode (Stoodley et al. 2001; Gu et al. 2013). Based on the colocalization analysis, ~60% of the EPS components co-localized with 80–90% of the microbial cells on the membrane after the TMP entered the jump stage. Further, the CLSM images showed that the most of non-colocalized EPS component was distributed surrounding the microbial cells as the microbial matrices (Figure 9). This was consistent with EPS production in biofilms and capsule production by single cells (Kreft & Wimpenny 2001). Based on the results presented here, it is speculated that most of the EPS components on the membrane may be the byproducts of microorganisms that colonize the membrane. Thus, biocake formation may be primarily the result of biofilm growth; this is consistent with previous studies (Miura et al. 2007; Al-Juboori & Yusaf 2012; Dreszer et al. 2013). This suggests that active biofilm formation may be an important contributor to the reduced MBR performance.

It has been shown that polysaccharides play an important role in establishment of the biofilm for Pseudomonas aeruginosa (Ghafoor et al. 2011), Staphylococcus epidermidis (Heilmann et al. 1996) and Escherichia coli (Wang et al. 2004). Therefore, it is possible that the polysaccharides, which were dominant in the early stage of membrane colonization, play similar roles here. By contrast, mature biofilms of a Pseudomonas spp. isolate were comprised of up to 50% protein by biomass while the polysaccharides only occupied 15% of the biofilm matrix (Baum et al. 2009). This phenomenon seems consistent with the observations presented here that the protein content of the biofilm also substantially increased over time. Therefore, the results presented here suggest that the key reason for membrane clogging is the growth of mature biofilm as opposed to the adsorption of sludge EPS. The sludge EPS may still play an important role in this process, for example by promoting the initial attachment of microorganisms to the membrane (Vu et al. 2009). In this study, the percentage of microorganism-colocalized EPS components increased from 40–50% at 3–10 kPa to 57–69% at 15–90 kPa. This is also an indication that the sludge EPS may play a more important role in establishing the early biofilm on membranes rather than the later mature biofilm. It should be noted that what has been studied here is the development of the microbial biofilm on the membranes in relation to the TMP change. Thus, the focus has been on the developmental stages. However, microbial attachment and biofilm development have been reported to be proceeded by, and dependent on, the formation of a chemical conditioning film (Siboni et al. 2007; Anand et al. 2014). The conditioning film may be proteins, sugars or other macromolecules in the environment that facilitate the formation of the biofilm and hence are likely to be important for the initial stage of biofilm initiation.

Based on the observations here and previously published work, the following proposed model for MBR membrane fouling is suggested, together with suggestions for future work to test the model predictions. New membranes accumulate a conditioning film, comprised of polysaccharides and proteins, which are predominantly deposited from the bulk phase of the sludge. In the model presented here, this occurs during the low pressure phase. Subsequently, the conditioned membrane is colonized by microorganisms that establish biofilms and produce EPS components necessary to firmly fix them on the membrane surface. As the biofilm grows, the polysaccharide content of the biofilm increases, either through continued production from the biofilm or further deposition from the bulk phase. However, the primary change in the biofilm is an increase in the protein content and this is associated with a change in permeation, leading to a rapid increase in TMP. Thus, it is proposed that the EPS is ultimately responsible for the reduction in water permeation, rather than the cells, but that the cells are important in the production of the EPS components. As a model, the above scheme lends itself to a range of experiments which can be used to determine if the conditioning film is essential for subsequent biofilm formation, and if so, which biopolymers contribute to the loss of performance. Key experiments would therefore purify polysaccharide and protein components from the bulk phase and use those to condition the membrane surface to determine whether this increases the subsequent biofilm attachment or development. Similarly, once a biofilm is established, experiments should be designed that can track the incorporation of soluble EPS components into the biofilm and quantify that relative to those components produced by the biofilm cells. Given that the type of polysaccharide has a significant impact on membrane performance, the polysaccharides should be purified and identified as well as characterized in terms of their physical properties. For example, the Pel and Psl polysaccharides of P. aeruginosa have distinct mechanical properties (Chew et al. 2014), which significantly impact the ability of P. aeruginosa to foul water purification membranes (Barnes et al. 2014).
Conclusions
This study shows that alpha-polysaccharides, beta-polysaccharides, proteins and microbial cells all accumulated on the membranes over time and were positively correlated to the TMP increase during MBR operation. While the polysaccharides may have played a role in initial biofilm development, the rapid and significant increase in protein biomass was associated with the rapid TMP rise and subsequent loss of MBR performance. The study suggests that active biofilm formation on membranes may contribute more to the loss of MBR performance than pressure-driven adsorption of sludge soluble EPS. Therefore, the prevention or reduction of biofilm formation on membranes in situ may represent a valuable strategy to enhance MBR performance. Further work aimed at reducing biofilm initiation or the growth of the biofilm on the membrane surface may yield invaluable strategies to control the problem of biofouling.

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