Effect of dead cells on biofouling in the reverse osmosis process
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ABSTRACT
Biofilm formation in membrane processes causes a flux decline, pressure drop increase, and other adverse effects. Understanding the mechanisms of fouling, control, and cleaning are important in order to resolve fouling problems. In this paper, the effect of cell viability on biofouling was studied via a feed water analysis and membrane autopsy. Pseudomonas aeruginosa PAO1 was used as the model bacteria. Biofouling tests were divided into two parts: live cells and dead cells (autoclaved cells). The feed water was periodically collected every 3 h to analyze the total direct counts (TDCs), heterotrophic plate counts (HPCs), and extracellular polymeric substances (EPS). A membrane autopsy was performed to characterize the fouled membrane through TDCs and EPS. When dead cells were inoculated into the feed water, low concentrations of TDCs and EPS were measured in both the feed water and fouled membrane. As a result, it was determined that initial flux decline by biofouling can be reduced if feedwater is disinfected before the reverse osmosis process.

INTRODUCTION
The desalination process using reverse osmosis (RO) membranes is one of the best technologies for solving global water scarcity problems. However, the productivity of RO processes is seriously hampered by fouling. Continuously accumulated foulants such as inorganics, organics, and microorganisms on the membrane surface reduce process performance, including an increase in the pressure drop, salt passage, and flux decline. Furthermore, biofouling has been related to an increase in energy requirements (Flemming et al. 1997; Hong & Elimelech 1997; Zhu & Elimelech 1997; Abd El Aleem et al. 1998; Vrouwenvelder et al. 1998; Hoek & Elimelech 2003; Xu et al. 2006; Herzberg & Elimelech 2007). As such, understanding and controlling biofouling are major tasks required to resolve these problems in membrane processes.

Among the variety of fouling types, biofouling caused by microorganisms remains difficult to control because even if 99.99% of microbes are removed through pretreatment, this very small amount of microbes can continue to grow on the membrane surface and cause biofouling (Flemming 2002). The major flux decline caused by biofouling is due to the fact that accumulated microbial products such as cells and extracellular polymeric substances (EPS) on the membrane surface block the reverse salt diffusion and then increase the external concentration polarization, which subsequently increases the osmotic pressure and reduces the permeate flux. These biofouling mechanisms are referred to as biofilm enhanced osmotic pressure (BEOP) (Herzberg & Elimelech 2007; Chong et al. 2008).

To date, various studies for understanding and preventing the biofouling problem have been undertaken (Matin et al. 2011). Pretreatments, biocide applications, modifications of the membrane surface and spacers, and membrane cleaning have been performed in attempts to reduce or prevent biofouling. Although many researchers have studied the mechanisms and control of biofouling,
however, there have been few papers published that further the understanding of biofouling effects incurred by dead cells. Typically, research concerning pretreatments and biocides that reduce biofouling has been performed without regard for the effect of dead cells on biofouling. Various disinfection techniques such as ozonation, chlorination, and UV irradiation directly attack the membrane surface of microbes as well as intracellular components that release proteins and lipid peroxides, and incur a change in cell permeability which has been identified in damaged cells (Young & Setlow 2003; Cho et al. 2010). Recently, Azami et al. (2011) studied the relationship between cell viability and EPS in a membrane bioreactor; their paper showed that the flux decreased according to an increase in the EPS concentration, caused by an increase in the concentration of dead cells.

The objectives of this paper are as follows: (1) to elucidate the biofouling mechanism of dead cells through an analysis of feed water and fouled membranes; and (2) to compare biofouling propensity due to cell viability in feed water.

MATERIALS AND METHODS

Preparation of feed water

*Pseudomonas aeruginosa* PAO1 GFP was selected as the model bacterium strain for biofouling in this study. The bacteria were incubated in a Luria-Bertani (LB) broth (Difco, USA) for 7 h, at which time a late exponential phase at 37 ± 1 °C was reached (Figure 1). The optical density was measured at the 600 nm wavelength using a UV-Vis spectrophotometer. As the optical density reached 1, pure cells were immediately harvested from the bacteria stock by centrifugation at 8,000 rpm for 10 min. After centrifugation, the supernatant was carefully discarded. Phosphate-buffered solution (PBS) was added to the remaining pellet of bacteria stock and was then centrifuged at 8,000 rpm for 10 min; this step was repeated three times to remove excess LB broth.

Dead cells were prepared by autoclaving at 121 °C for 15 min (Azami et al. 2011). To compare the effect of biofouling caused by live cells and dead cells, cells were respectively inoculated in feed water. For ease of reading, in this paper, live cell enriched feed water is referred to as LEW and dead cell enriched feed water is DEW.

Feed water was composed of PBS and 1:1,000 diluted LB broth as the enhanced biofouling condition. The components of 1 L PBS are as follows: 0.2 g KCl, 0.2 g KH₂PO₄, 8 g NaCl, 0.1 g MgCl₂.6H₂O, 1.15 g Na₂HPO₄.2H₂O, and 0.1 g CaCl₂ (Pasmore et al. 2001).

Laboratory scale RO system

A laboratory scale RO system was used for the biofouling tests. An RO membrane and spacer (RE8040-SHN, Woongjin Co., Korea) were used as received from Woongjin Co.; RE8040-SHN was used as the polyamide thin-film composite. An SEPA cell (GE Osmonics, USA), having an effective membrane area of approximately 139 cm², was used as the membrane test unit. The following conditions were applied to all biofouling tests: constant pressure of 800 psi, crossflow velocity of 1.2 L/min, temperature of 25 ± 1 °C, and feed water volume of 8 L. The permeate flux was measured using a flow meter and was automatically recorded on a computer.

Procedure of biofouling test

Prior to every biofouling test, the laboratory scale RO membrane system was cleaned using the following sequence of chemicals: 0.5% NaOCl, 5 mM EDTA at pH 11, 2 mM SDS at pH 11, and 70% ethanol (Herzberg & Elimelech...
After cleaning, the system was rinsed with deionized water. After the disinfection step, an RO membrane coupon that was soaked in deionized water for 1 day was installed in the SEPA cell.

The laboratory scale RO system was operated with 7 L deionized water for membrane compaction for 24 h. After compaction, concentrated PBS was added to the feed water, and the system was allowed to rest for 4 h to obtain a stabilized flux. After the stability step, live or dead cells were inoculated into the feed water. During the biofouling test, feed water was collected every 3 h; at the end of testing, the fouled membrane coupon was soaked in PBS.

**Analysis methods for feed water and membrane autopsy**

To elucidate the correlation between flux decline and biomass parameters, the feed water and fouled membrane were further analyzed. Samples collected from the feed water every 3 h were analyzed based on their total direct cell counts (TDCs), heterotrophic plate counts (HPCs), and EPS. The fouled membrane was also analyzed through TDCs and EPS.

TDCs were observed using confocal laser microscopy (CLSM; LSM5, Zeiss, Germany) with cells stained by 6-diamidino-2-phenylindole (DAPI). Specific methods for DAPI staining and counting of cells was described by Lee et al. (2010).

To analyze the metabolically active cells, the HPC method was applied; HPCs can indirectly represent the cell viability. For this analysis, feed water samples collected were diluted from $10^{-1}$ to $10^{-6}$. Then, 1 mL of the diluted samples was spread on an LB agar plate. All samples were subsequently incubated at 37 ± 1°C for 48 h. After incubation, the number of colonies on the LB agar plate was counted.

EPS contain organic compounds such as carbohydrates, proteins, humic acid, DNA, uronic acid, and inorganic components (Frølund et al. 1996; Liu & Fang 2002; Sheng et al. 2010). EPS were extracted using a modified method through the following steps (Liu & Fang 2002): (1) 2 mL NaOH was added to each 5 mL sample which were then incubated at 4°C for 3 h; (2) incubated samples were separated by centrifugation at 20,000 g for 20 min at 4°C; and (3) the supernatant was filtered through a 0.22 μm polyvinylidene difluoride (PVDF) membrane (Millipore, USA) to extract EPS. Carbohydrates were measured using a modified phenol sulfuric acid method (Dubois et al. 1956) at a wavelength of 490 nm. In brief, 0.6 mL of the sample solution and 0.3 mL of 5% phenol solution were mixed and vortex-stirred, 1.5 mL of sulfuric acid (95–97%) was directly injected in the samples which were then incubated for 30 min at room temperature. Glucose was used in the preparation of a calibration curve. Total proteins were analyzed through a microplate method using a bicinchoninic acid (BCA) protein assay kit (#23225, Pierce, USA); the protein concentration was calculated using a standard curve bovine serum albumin at a wavelength of 562 nm.

**RESULTS AND DISCUSSION**

**Analysis of feed water**

Live and dead cells were respectively inoculated in feed water to determine the initial flux decline according to the change of feed water component. Feed water was periodically sampled every 3 h during the biofouling test. The initial flux decline by biofouling of live or dead cells was elucidated through the change in total cells and EPS concentration.

To investigate the change of cells in feed water, TDCs and HPCs were measured; TDCs include live and dead cells, whereas HPCs can only measure active cells (live cells). Therefore, the microbial activity and viability can be predicted by the correlation of TDCs and HPCs.

Figure 2 shows the variation of total cells in the feed water. In the figure, LEW (live cells enriched feed water) refers to live cells inoculated in the feed water, whereas DEW (dead cells enriched feed water) refers to dead cells inoculated in the feed water.

The data at 3 h shows that fewer dead cells were attached to the RO membrane than live cells. The EPS of dead cells were scattered throughout the feed water due to autoclaving, whereas live cells were still attached to the cell surface (Sheng et al. 2010). Therefore, live cells could more easily attach to the RO membrane than dead cells.

After 6 h of operational time, most cells in the feed water were attached to the membrane surface. At this time, the TDCs concentration also increased; from our
results, it appears that there was a regrowth of bacteria, rather than a detachment of bacteria from the membrane surface (Figure 3). Supporting this consideration is that the growth curve of \textit{P. aeruginosa} PAO1 displayed a mid-exponential phase after 6 h.

**Change of EPS concentration**

In this paper, EPS primarily refers to carbohydrates and proteins, and omits other EPS. Figure 4 presents the variation of EPS concentration in the feed water, where it is seen that DEW has a higher initial amount of EPS than LEW. EPS, which surrounded the bacteria, were detached by autoclaving (Liu et al. 2007), and existed in the feed water. Therefore, DEW has a higher initial EPS concentration than LEW. However, the EPS concentration of DEW decreased with time because the EPS of DEW were consumed by the small amount of live cells (maximum $1.5 \times 10^5$ CFU/mL), which has a low growth potential (Figure 2).

The EPS concentration of LEW increased with time. EPS produced from live cells were significantly related to the bacteria growth phase because EPS were produced through bacteria metabolism. As such, bacteria in the exponential phase produce a large quantity of EPS (Jia et al. 1996). Therefore, as LEW initially contains live cells ($2.88 \times 10^7$ CFU/mL), EPS were produced through the growth of live cells, whereas DEW had a small initial amount of live cells ($6.43 \times 10^3$ CFU/mL) and did not produce EPS (Figure 3).

**Biofouling behavior according to cell viability**

Figure 5 shows the permeate flux decline relative to the cell viability. In the figure, the initial DEW permeate flux
(48 L/(m² h)) was lower than for LEW (53 L/(m² h)), due to the fact that DEW contained a higher EPS concentration (40 mg/L) than LEW (34 mg/L) due to EPS extracted from bacteria. The rate of DEW flux decline was slightly reduced up to 6 h; after this time, a relatively dramatic flux decline was observed. It would thus appear that the total cells and EPS increased in the feed water due to bacteria growth, after 6 h from the start time.

Although the initial LEW permeate flux (53 L/(m² h)) was higher than DEW (48 L/(m² h)), the LEW permeate flux sharply decreased with time. Because the EPS support the attachment with other materials (Simões et al. 2010), the LEW flux decreased as the EPS concentration increased according to the growth of live cells and the subsequent increase in attached cells on the membrane surface.

Membrane autopsy

The primary reason for flux decline in RO processes is due to the accumulation of foulants on the membrane surface (Hoek & Elimelech 2003; Chong et al. 2008). The cause of flux decline can be measured by analyzing fouled membranes. In this paper, EPS and TDCs were measured to determine the cause of flux decline and to elucidate the effect of dead cells on biofouling.

Figure 6(a) and (b) shows total cells attached and accumulated EPS on an RO membrane. When LEW was used as the feed water, accumulated total cells and EPS were higher than for DEW. Because LEW has higher HPCs than DEW, the total cells and EPS for LEW were increased due to the large amount of live cells.

Autopsy results display the same trend as the results of the feed water analysis. Thus, from both results, it is expected that initial biofouling could be reduced by using disinfected feed water.

CONCLUSIONS

Biofouling tests were performed to determine how much the initial flux decline depends on cell viability. DEW has low permeate flux from start time to 3 h because dead cells have a high initial EPS concentration in feed water. However, after 6 h, LEW has a high EPS concentration due to the growth of microbes. Therefore, in this paper, it was found that dead cell enriched feed water has a lower biofouling potential than when live cells are present.

The initial attachment of cells and EPS on the membrane surface according to cell viability was also observed. Note that the operation time of the biofouling test was relatively short because the flux decline in the initial stage was the primary focus of this study. As a subsequent investigation, biofouling tests will be conducted for a longer operational time to ensure biofilm formulation, along with

![Figure 6](https://iwaponline.com/ws/article-pdf/13/5/1396/415064/1396.pdf)
a more detailed membrane autopsy such as zeta potential, roughness, and contact angle. Furthermore, cleaning effects according to cell viability will be identified.

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