Rapid novel test for the determination of biofouling potential on reverse osmosis membranes

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ABSTRACT

A novel method was proposed to determine biofouling potential by direct analysis of a reverse osmosis (RO) membrane through fluorescence intensity analysis of biofilm formed on the membrane surface, thereby incorporating fouling tendencies of both feedwater and membrane. Evaluation of the biofouling potential on the RO membrane was done by accelerated biofilm formation through soaking of membranes in high biofouling potential waters obtained by adding microorganisms and glucose in test waters. The biofilm formed on the soaked membrane was quantified by fluorescence intensity microplate analysis. The soaking method’s capability in detecting biofilm formation was confirmed when percentage coverage obtained through fluorescence microscopy and intensity values exhibited a linear correlation ($R^2 = 0.96$). Continuous cross-flow experiments confirmed the ability and reliability of the soaking method in giving biofouling potential on RO membranes when a good correlation ($R^2 = 0.87$) between intensity values of biofilms formed on the membrane during soaking and filtration conditions was obtained. Applicability of the test developed was shown when three commercially available polyamide (PA) RO membranes were assessed for biofouling potential. This new method can also be applied for the determination of biofouling potential in water with more than 3.6 mg L$^{-1}$ easily degradable organic carbon.

Key words | biofouling potential, fluorescence intensity, microplate analysis, percentage coverage, reverse osmosis membranes

INTRODUCTION

Microorganisms are ubiquitous in the environment and every operational plant possesses biofilm from the start of operation and the extent to which it grows and proliferates determines its adverse effect on the reverse osmosis (RO) process (Flemming 2002). Biofouling, unlike the other types of membrane fouling, can only be prevented and controlled with the proper selection of anti-fouling strategies (Pandey et al. 2012). Because of the diverse areas of research in RO membrane technology directed towards a better understanding, prevention, and control of biofouling, suitable methods of analysis that detect the propensity for biofouling is a key ingredient. Determining biofouling potential would specifically help in designing cost-effective RO processes, especially in the treatment of waters with high fouling potential, such as secondary treated effluent municipal waters (Pandey et al. 2012) and industrial wastewaters (Ridgway et al. 1985).

Existing biofouling potential tests involve the use of chemical and biological quality parameters of the water as indicators of biofouling. However, even when silt density index or turbidity values are within or below the allowable limits (Schneider et al. 2005; Huang et al. 2013), fouling still occurred. Since the presence of major bacterial groups in the raw source water or the pretreated RO feed does not necessarily indicate the formation of such bacterial groups on the RO membrane as biofilm (Khan et al. 2015), determination of biofouling potential based on the formed biofilm on the RO membrane is of greater merit in depicting actual biofouling tendencies. A microbiology-based assay which is recently developed assessed the early stage bacterial attachment of Klebsiella oxytoca to RO and nanofiltration membranes and showed the importance of membrane-bacterial interaction as a basis for evaluating
the susceptibility of membranes to bacterial attachment, subsequently to biofilm growth (Lutskiy et al. 2015) and, in the case of filtration processes, biofouling. Removing the attached biofilm from the membrane and ensuring its quantitative transfer for bacterial analysis is an added step that needs to be optimized (Lutskiy et al. 2015); thus direct evaluation of biofilm formed on the membrane with easy and rapid fluorescence analysis is an adequate way to determine the biofouling potential of the membrane. The novelty of this biofouling potential indicator method lies in the direct analysis of the biofilm formed on the membrane used, which to our knowledge does not currently exist. In this study, accelerated biofilm formation on the membrane was achieved by using high biofouling potential water through addition of microorganisms and glucose. This study aims to develop a rapid and reliable biofouling potential test that will be highly useful in determining biofouling potential of membranes being developed or improved as well as commercial RO membranes used for treatment. Moreover, the method developed in this study can also be applied in evaluating biofouling potential of waters with relatively high biofouling potential.

This method involved soaking a small piece of the RO membrane in the test water, and the amount of biofilm formed after soaking, considered to be a measure of biofouling potential, is determined by staining the soaked membranes with the green fluorescent dye SYTO 9. The amount of biofilm formed on the membrane surface is quantified in terms of fluorescence intensity measured by the fluorescence microplate reader. Epifluorescence microscopy is an established technology for viewing biofilms on surfaces but is limited to the analysis of very thin biofilms (McFeters et al. 1997) and as such is employed in this study to confirm the applicability of the soaking method for biofilm formation by comparing percentage coverage obtained by fluorescence microscopy with fluorescence intensity from microplate analysis. Continuous cross-flow experiments were done employing similar conditions used in the soaking method to evaluate the reliability of the soaking method and the quantification through fluorescence microplate analysis in determining biofouling potential on RO membranes used in real filtration conditions.

**MATERIALS AND METHODS**

**Sample preparation**

For all experiments, deionized (MilliQ) autoclaved water was used as control sample (blank). Secondary effluent water from Higashi Hiroshima wastewater treatment plant was used as sample water. Average (± standard deviation (SD)) raw water quality for the secondary effluent water was 4.028 (0.752) mg L\(^{-1}\) dissolved organic carbon (DOC), 4.700 (1.273) mg L\(^{-1}\) total suspended solids, 1.104 (0.023) mS conductivity, and pH 6.22 (0.04). To accelerate biofilm formation on the membrane surface, secondary effluent water samples for soaking and continuous cross-flow experiments were added with glucose (Wako Pure Chemical Industries, Ltd, Japan) and/or *Bacillus subtilis* (JCM 2499, Riken, Japan) to enhance nutrient concentration and/or microorganism growth, respectively.

**Bacteria stock preparation**

*B. subtilis* has emerged as an alternative model organism for studying the molecular basis of biofilm formation (Vlamakis et al. 2015). It has also been found to be one of the many bacterial species that participate in biofouling on RO membranes (Ridgway et al. 1983; Matin et al. 2011), and thus has been used in studies that involve seawater RO membrane biofouling (Lee et al. 2010) and in the development of antibacterial polyamide (PA) RO membranes (Saeki et al. 2013), and thus the use of *B. subtilis* is acceptable for enhancing biofilm development on the membrane surface. The bacterial stock was prepared by growing overnight cultures in 3% tryptic soy broth (TSB, Merck, Germany) with shaking at 45 rpm at 37 °C for 24 hours. The bacterial cells were harvested by centrifugation at 2,000 rpm for 5 minutes and washed at least three times with 0.85% NaCl (Nacalai Tesque, Inc., Japan). The pellet was resuspended in 0.85% NaCl to achieve an optical density of 0.4–0.5 (OD 550 nm, UV-1800, Shimadzu), resulting in a bacterial concentration in the range of 3–6 × 10\(^8\) cfu mL\(^{-1}\) in the stock mixture, indicated by the McFarland turbidity standard (bioMérieux, Inc., USA). This stock of *B. subtilis* suspension was then added to the sample waters using the necessary dilutions required by the experiment.

**Optimum conditions for biofilm formation**

In order to confirm enhancement of biofilm formation by using secondary effluent water and addition of glucose and to determine the optimum time that is required for biofilm formation, unfiltered secondary effluent water without glucose, unfiltered secondary effluent water with 1 mM glucose and blank water were used as soaking solutions. The optimum soaking time was determined by varying the soaking time from 6 to 48 hours. Since all secondary effluent
feedwaters contain microorganisms, which can be regarded as colloidal particles and are removed during filtration pretreatment to limit biological growth (Pandey et al. 2012), the pretreatment, if needed, for the secondary effluent water is then evaluated. Secondary effluent water samples were filtered with GF 75 (0.3 μm pore size, Advantec), GF/F (0.7 μm pore size, Whatman), and GF/B glass filters to determine the effect of filtration as pretreatment for the removal of particulates in the sample water. To determine the extent of increase in biofilm growth, the amount of DOC is increased by adding 1 mM glucose to the secondary effluent water. To further achieve an enhanced biofouling behavior, a suspension of *B. subtilis* was added to the nutrient-enhanced samples (with 1.00 mM glucose) and the dilution necessary to produce such enhancement was determined.

**Soaking experimental procedure**

A commercial thin-film composite PA RO membrane (NTR 759HR, Nitto Denko, Japan) was used for the soaking experiments. The membranes were received as a flat sheet and were cut using sterile scissors into 2 cm × 2 cm pieces. Three membranes for each sample water were analyzed by placing the cut membranes in separate wells of a six well-plate container containing 10 mL of the test water. The well-plate containers were wrapped in aluminum foil and shaken at 45 rpm and at 37 °C. After filtering, the membranes were easily detached. The secondary effluent membrane containing 10 mL of the test water. The six well-plate containers were wrapped in aluminum foil and shaken at 45 rpm and at 37 °C to allow biofilm formation. The conditions used for biofilm formation (soaking time, pretreatment, and amount of *B. subtilis*) were based on optimization studies.

**Filtration experiments**

In order to simulate the conditions that happen during water treatment and to evaluate the reliability of the soaking method and subsequent fluorescence intensity quantification as a biofouling potential test, 24-hour filtration experiments were conducted. The secondary effluent water was first filtered through a GF/B glass filter (1 μm, Whatman). The secondary effluent water samples contained 3 × 10⁶ to 6 × 10⁶ cfu mL⁻¹ of *B. subtilis*. Three litres of the sample water was fed in a laboratory scale continuous cross-flow unit equipped with a pump, feedwater reservoir, and a conductivity meter (multi-function water quality meter, MM-60R, DKK-TOA Corporation, Japan) (Supplementary Figure 1, available with the online version of this paper). Permeate and retentate were recirculated to the feedwater reservoir and the filtration was run at a constant applied pressure of 1.5 MPa, feed temperature of 25 °C, initial flux of ~0.8 m³ m⁻² d⁻¹, and a cross-flow rate of 50 mL min⁻¹. NTR 759HR membranes used for these filtration experiments were received as circular sheets with 75 mm diameter size. After filtration, the membrane was retrieved and was very carefully cut with sterile scissors into six 2 cm × 2 cm pieces for the determination of biofilm amount.

**SYTO 9 staining procedure**

After soaking or filtration experiments, the membranes were retrieved, and then subjected to staining. The green dye SYTO 9, from the BacLight™ Bacterial Viability Kit L13152 (Invitrogen/Molecular Probes, USA), stains both live and dead cells with a fluorescent green color. The SYTO 9 solution was prepared according to manufacturer specifications and the solutions were kept in the dark and inside the refrigerator until analysis. A 2-cm piece of the membrane was stained with 100 μL of SYTO 9 and then allowed to stand for 30 minutes at the minimum. After 30 minutes, for the soaked membranes, the excess dye was washed off with 100 μL of deionized filtered (0.22 μm, Millipore) autoclaved water while cut filtered membranes were not washed. Repeated experiments showed that for the soaked membranes, this washing procedure did not remove the biofilm on the membrane surface whereas the foulants on the filtered membranes were easily detached. All measurements reported herein are within the precision errors described below. All stained membranes were then subjected to fluorescence microscopic analysis and fluorescence microplate analysis.

**Fluorescence analysis**

Biofilm formed on the dyed membranes is quantified by coverage (expressed as percentage) and fluorescence intensity. The green fluorescence from microorganisms present was viewed with a fluorescence microscope (Olympus CX-RFL-2). Sixteen microscopic field shots were obtained from each membrane using Canon EOS Kiss X-50. The images obtained were then analyzed for coverage using the free software ImageJ (Abramoff et al. 2004). Fluorescence intensity was analyzed using the Gemini EM microplate reader with SoftMax®Pro microplate data acquisition & analysis software with excitation scan set at 485 nm and emission scan set at 545 nm. SoftMax®Pro microplate data acquisition & analysis software gave fluorescence intensity values for 144 points per membrane. The percentage coverage and fluorescence intensity values are then reported as...
averages of three pieces of soaked membranes, and averages of six cut pieces from the membrane used in filtration. Consequently, precision is reported as standard deviations for \( n = 3 \) and \( n = 6 \) membranes, for soaking and filtration experiments, respectively.

**Biofouling potential test on three PA RO membranes**

The soaking method was used to evaluate the biofouling potential of three commercially available PA RO membranes: ES20 (Nitto Denko, Japan), NTR 759HR, and SU 700 (Toray Industries, Japan). Fluorescence intensity of biofilm formed on the membrane surface after 24 hours of soaking in secondary effluent water with 1 mM glucose and *B. subtilis* concentration range of \( 5 \times 10^7 \) to \( 6 \times 10^7 \) cfu mL\(^{-1}\) was determined. Contact angle, as a measure of the surface hydrophobicity of the virgin membranes, was determined to characterize the surface of the membranes using Drop Master DM-300 (Kyowa Co., Japan). Ten water contact angle measurements at different locations on one membrane sample were carried out to get average and standard deviation values.

**RESULTS AND DISCUSSION**

**Optimum conditions for biofilm formation**

Fluorescence intensities from the biofilm formed on the membrane were determined after 6, 24, and 48 hours and results are shown on Figure 1. These results indicate that optimum growth of microorganisms is expected to occur after around 24 hours of soaking the RO membranes in secondary effluent water for both with and without glucose, and thus 24 hours was employed for biofilm formation time. The decrease in intensity at 48 hours signifies that the amount of biofilm on the membrane has lessened and can be explained by possible detachment of microorganisms from the membrane surface and dispersal due to nutrient resource limitation (Vlamakis *et al.* 2013).

Secondary effluent waters can have variable loads of suspended solids, colloidal materials, organics, and bacteria, and to reduce cell deposition, and bacterial growth in actual RO operations, appropriate pre-treatment methods are employed (Pandey *et al.* 2012). After establishing the amount of time needed for biofilm formation (Figure 1), the effect of filtration in removing particles from the secondary effluent water on the fluorescence intensity of the biofilm formed was determined (Supplementary Figure 2, available with the online version of this paper). The extent of increase of biofilm growth by adding DOC in the form of glucose was also determined. To assess biofilm formation on all samples after 24 hours, difference of fluorescence intensity (ΔFluorescence intensity) values from fluorescence intensity of membrane samples soaked in test waters after 24 hours and the fluorescence intensity of membranes at zero hour (i.e. virgin membranes) were determined. Membranes soaked in blank water samples showed the lowest ΔF of 11, while filtered samples without added glucose using 0.3 μm, 0.7 μm, and 1.0 μm filters have lower intensity values, 20, 19, and 28, respectively. In contrast, membranes soaked in unfiltered samples without glucose have ΔF of 47. As expected, addition of the 1.0 mM glucose greatly enhanced the fluorescence intensity of the biofilm formed on the membrane surface, with ΔF of 99, 98, 85, and 355 for 0.3 μm-, 0.7 μm-, 1.0 μm-filtered, and unfiltered sample waters, respectively, signifying increased biofilm formation due to the added DOC source for the microorganisms. These results also indicate that the sensitivity of the soaking method and detection of fluorescence intensity is low without the added carbon, and thus conditions to increase sensitivity should be done. As expected also, filtration through 0.3 μm to 1 μm filters reduced the biofilm formation. Bacteria in the secondary effluent water may not exist in completely dispersed form but are in floc form. Since filtration would significantly reduce the bacterial number that could form biofilm in 24 hours, for the establishment of conditions necessary for the soaking method, secondary effluent water was not filtered. Moreover, since

![Figure 1](https://iwaponline.com/wst/article-pdf/73/12/2978/363298/wst073122978.pdf)
concentration of particulate matters in the secondary effluent water was very variable, addition of microorganisms in the soaking method was evaluated next in order to have a stable biofouling potential (Supplementary Figure 3, available with the online version of this paper).

Results of average intensity values (±SD) indicate that unfiltered secondary effluent water samples with 1.00 mM glucose with no *B. subtilis*, and samples with 3–6 × 10^4 cfu mL^{-1}, 3–6 × 10^5 cfu mL^{-1}, and 3–6 × 10^6 cfu mL^{-1} of *B. subtilis* suspension had relatively the same fluorescence intensity: 194 ± 43, 191 ± 26, 187 ± 30, and 220 ± 34, respectively. There was marked increased in fluorescence intensity in samples containing 3–6 × 10^7 cfu mL^{-1} of *B. subtilis* (451 ± 95) indicating more bacterial growth and thus this concentration was considered for all soaking experiments.

**Biofilm formation determination during soaking method**

In order to evaluate the new method, the relationship between amount of glucose concentration in the sample waters and the percentage coverage and fluorescence intensity from the biofilm formed on the membrane surface using the optimum soaking conditions were determined. An increase in percentage coverage (Figure 2(a)) and fluorescence intensity (Figure 2(b)) was not observed from 0 to 0.05 mM glucose concentration. A continuous increase in coverage and intensity was observed from 0.05 to 0.80 mM glucose concentration due to biofilm growth and development, and a linear correlation \( R^2 = 0.96 \) (Figure 3) exists between percentage coverage and fluorescence intensity in this range. From 0.80 mM glucose concentrations, the coverage and fluorescence intensity leveled off, suggesting that addition of 0.80 or 1.00 mM final concentration of glucose into the secondary effluent water is enough to accelerate biofilm formation for the evaluation of biofouling potential on the membrane.

The increase in percentage coverage and fluorescence intensity with glucose concentration between 0.05 and 0.80 mM, corresponding to 3.6 to 58 mg L^{-1} carbon, shows that this new method can also be applied to evaluate biofouling potential of water within this range. Glucose is a typical biodegradable organic matter and, therefore, this new method will be applicable to relatively organically polluted water with 3.6 to 58 mg L^{-1} of biodegradable organic carbon.

**Biofouling determination in real filtration conditions**

Previous studies show that biofilm formation occurs within 24 hours of contact between membrane surface and feedwater (Schneider et al. 2005; Bar-Zeev et al. 2012), although the extent of biofilm growth and accumulation varies depending on the conditions employed. To assess the reliability of the soaking method and fluorescence intensity quantification as a biofouling potential test, cross-flow filtration experiments were performed. Results showed that
percentage coverage ranged from ~75 to 95% (Figure 4(a)), suggesting an almost completely covered fouled membrane. Fluorescence intensity increased from 0 to 1.00 mM glucose concentration as shown in Figure 4(b), signifying a steady biofilm growth and accumulation on the membrane surface. The results indicate that in conditions of greater chance of biofouling, such as filtration during water treatment, the biofilm steadily covers the whole surface area of the membrane (thus an almost constant coverage observed), and when the membrane is fully covered, biofilm growth and accumulation in the vertical direction takes place.

Figure 5 shows that fluorescence intensity values from the biofilm formed during filtration conditions, even at no additional glucose, are at least eight times higher than fluorescence intensity values obtained from biofilms that were formed during the soaking method. This can be explained by the difference in the amount of liquid or amount of glucose in contact with the surface area as expressed in terms of liquid volume, or glucose amount, to membrane surface ratio, that is, 2.50 and 68.0 mL cm⁻², and 0.0225 and 0.611 mg cm⁻² in soaking and filtration experiments, respectively. As also shown in Figure 5, fluorescence intensity values from biofilm formed during filtration and soaking experiments have a slightly linear correlation ($R^2 = 0.87$), indicating the capability of the soaking method to depict biofouling potential in real filtration conditions. All conditions considered, except for the amount of glucose at the start of filtration and thus different biofouling potentials, continuous filtration would still lead to a rise in fluorescence intensity and reach a maximum because the feedwater continuously delivers nutrient, which the microorganisms in the feedwater can use for biofilm growth and development (Chen et al. 2013).

Biofouling potential test on three PA RO membranes

Membrane characteristics (surface hydrophobicity, surface charge, chemical composition, roughness, and surface morphology and microtopography) are known to influence biofouling, specifically on the initial adhesion of bacteria on the membrane surface (Habimana et al. 2014). It has been reported that membranes with neutral or highly negative surface charge, with smooth surfaces, and that are less hydrophobic tend to minimize membrane fouling (Norberg et al. 2007). Thus, the biofouling potential test developed in this study was applied to three commercially available PA RO membranes, and the fluorescence intensities obtained were compared to virgin membranes’ contact angles, as a measure of surface hydrophobicity. Figure 6 shows fluorescence intensity of ES 20 is lowest (120 ± 30), while NTR 759HR (300 ± 85) and SU 700 (317 ± 105) have comparable fluorescence intensity values. Contact angle measurements of virgin membranes revealed a similar trend with ES 20 having the lowest contact angle, 15.6 ± 5.3°, while NTR 759HR and SU 700 have similar values, 30.8 ± 5.0° and 31.5 ± 2.2°, respectively. Contact angle results indicate that ES 20 will have the least biofouling
tendency out of all the three membranes studied. This is supported by the fluorescence intensity values obtained where ES 20 showed the lowest value, suggesting least biofilm formation. A previous study showed that greater accumulation of cells was observed on RO and NF membranes with higher initial hydrophobicity, indicated by higher contact angle values (Khan et al. 2011), which could explain these results observed. These results further showed the applicability of the method developed in assessing biofouling potential on PA RO membranes.

Comparison of fluorescence microscopic analysis and fluorescence microplate analysis

Under soaking conditions, both methods of detection were able to portray the biofilm growth and accumulation on the surface of the membrane, indicated by the linear correlation in Figure 3. In most biofilm development, during the first 24 hours, the microorganisms tend to cover the surface of the membrane, rather than grow in thickness (Bar-Zeev et al. 2012). When the membrane surface is completely covered, microorganisms tend to grow and accumulate, thus increasing in thickness or volume. Although epifluorescence microscopy can provide two-dimensional distribution of microorganisms (Al-Juboori & Yusaf 2012) as well as bacterial activity and viability, examination of the depth of the biofilm is not possible (Wolf et al. 2002), as shown in Figure 4(a). Thus, fluorescence microplate analysis holds an advantage in determining the propensity for biofouling of highly polluted waters since it could depict growth and accumulation of a fully biofilm-covered membrane.

In addition, despite ImageJ software having macros to automate often-repeated tasks (Abramoff et al. 2004), in this study, repeated trials indicate more reliable results when individual pictures/ shots are assessed for coverage by manually adjusting the brightness slider based on the microorganisms stained with the fluorescent green color, while hue and saturation ranges are fixed. Therefore, increasing the number of membrane samples as well as the microscopic field shots corresponds to added amount of time allotted for data processing. In contrast, microplate assay boasts of its simple high-throughput method (Merritt et al. 2005). Combining these points, it is more advantageous to use the fluorescence microplate analysis for detecting biofouling potential on RO membranes.

Furthermore, the capability of the soaking method and fluorescence microplate analysis in simulating biofouling potential tendencies using a laboratory cross-flow filtration set-up has important implications in the ability of the method to analyze fouled spiral-wound RO commercial membranes and spacers. Fluorescence microscopy with fluorescence staining has been used in detecting and quantifying biofoulants (Khan et al. 2011). However, with the simplicity offered by just cutting portions of the fouled membrane, the quantitative analysis of biofouling given through fluorescence intensity measurements particularly for highly fouled membranes, and the quick output rendered by the microplate analysis, biofouling occurrence in fouled commercial membranes can now be easily analyzed using the method developed.

CONCLUSION

A new, capable, and rapid method was developed by analyzing fouled RO membranes for the determination of biofouling potential. The method involved soaking small pieces of the RO membranes in test waters added with microorganisms and glucose for accelerated biofilm formation, and the biofilm formed on the membrane surface was measured by fluorescence intensity through fluorescence microplate analysis. A correlation between fluorescence intensity values obtained from biofilms formed on the membrane during soaking (no pressure) and filtration (under constant pressure) conditions indicated the reliability of the membrane soaking method as a biofouling potential indicator. This method can be applied for determination of biofouling potential in water with more...
than 3.6 mg L\(^{-1}\) easily degradable organic carbon and showed capability of assessing biofouling potential of three commercially available PA RO membranes. On top of being a novel method that gives biofouling potential based on direct analysis of biofilm formed on the membrane, the capability of showing biofouling potential tendencies in real filtration conditions and the fast output of data analysis are clear benefits of using the soaking method and fluorescence microplate analysis for the determination of biofouling potential on RO membranes.

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