Removal of antibiotic-resistant bacteria and antibiotic resistance genes affected by varying degrees of fouling on anaerobic microfiltration membranes

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New membrane

Increase in fouling severity

- Plasmid
- Bacterium
- Driving force
Removal of antibiotic-resistant bacteria and antibiotic resistance genes affected by varying degrees of fouling on anaerobic microfiltration membranes

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ABSTRACT

An anaerobic membrane bioreactor was retrofitted with polyvinylidene fluoride (PVDF) microfiltration membrane units, each of which was fouled to a different extent. The membranes with different degrees of fouling were evaluated for their efficiencies in removing three antibiotic-resistant bacteria (ARB), namely, *bla*<sub>NDM-1</sub>-positive *Escherichia coli* PI-7, *bla*<sub>CTX-M-15</sub>-positive *Klebsiella pneumoniae* L7 and *bla*<sub>OXA-48</sub>-positive *Escherichia coli* UPEC-RIY-4, as well as their associated plasmid-borne antibiotic resistance genes (ARGs). The results showed that the log removal values (LRVs) of ARGs correlated positively with the extent of membrane fouling and ranged from 1.9 to 3.9. New membranes with a minimal foulant layer could remove more than 5-log units of ARB. However, as the membranes progressed to subcritical fouling, the LRVs of ARB decreased at increasing operating transmembrane pressures (TMPs). The LRV recovered back to 5 when the membrane was critically fouled, and the achieved LRV remained stable at different operating TMPs. Furthermore, characterization of the surface attributed the removal of both the ARB and ARGs to adsorption, which was facilitated by an increasing hydrophobicity and a decreasing surface zeta potential as the membranes fouled. Our results indicate that both the TMP and the foulant layer synergistically affected ARB removal, but the foulant layer was the main factor that contributed to ARG removal.
1. INTRODUCTION

Municipal wastewater treatment plants have been identified as hotspots for the enrichment of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) and can potentially contribute to the dissemination of ARB and ARGs into the environment. In contrast to secondary biological activated sludge processes, which are unable to achieve good removal efficiencies for ARB and ARGs, aerobic membrane bioreactors (aeMBRs) were reported in prior studies to achieve >5.5-log removal of bacteria and >2.67-log removal of ARGs. However, operating an aeMBR would require high energy consumption rates, and that process produces waste sludge that is viewed as a hotbed for ARB and ARGs. A relatively more sustainable alternative to an aeMBR would be an anaerobic membrane bioreactor (anMBR). An anMBR couples a membrane-based filtration process with anaerobic fermentation, which would not only eliminate the need for aeration but also generate methane as an energy source. Furthermore, anaerobic fermentation has lower sludge production rates compared to activated sludge processes.

Despite the advantages of anMBRs compared to aeMBRs, the main drawback for both technologies is membrane biofouling. Biofouling causes a decrease in water flux and higher energy consumption rates and operational costs. Even though several approaches have been developed to alleviate the biofouling of membranes (e.g., backwash, chemical wash, and sonication), the total eradication of the foulant layer is not possible. Therefore, a conventional norm that subcritically fouled membranes remain in operation as long as their flux is not compromised exists. Its existence means that
membranes have an additional foulant layer that has been shown to improve the rejection
of bacterial cells and that cleaning the membrane to remove the foulant layer would lead
to a reduction of 1 in the log removal values (LRVs) for the bacterial cells in an aeMBR
12. The mechanism by which fouled membranes retain particles in wastewater results
from many factors (e.g., surface characteristics and pore blockage)13-15. To illustrate,
Cho et al. reported that steric exclusion and aromatic/hydrophobic and charge interactions
affect the removal efficiency of natural organic matter13. In addition, pore blockage by a
cake layer also served to increase the number of retained particles14, 15.

Earlier studies have reported an approximately 3 to 6-log removal of bacteria in aeMBRs
6, 7, 12. However, with increased fouling, the transmembrane pressure (TMP)
concomitantly rises during the operation, and the operating pressure to drive water
through the membrane must increase to maintain a constant flux. Therefore, an increase
in the TMP at constant flux may affect the membrane rejection rates. Specifically, this
study hypothesizes that an increase in the operating filtration pressure may compromise
the LRV for ARB achieved by fouled membranes. This hypothesis has not been
systematically assessed in most studies, particularly those that evaluate the performance
of anMBRs.

Furthermore, several differences exist between the aerobic and anaerobic foulant layers.
To illustrate, Xiong et al. studied the foulant layers attached to membranes connected to
aerobic and anaerobic membrane bioreactors of the same reactor configuration16. Their
findings suggest that when both membrane bioreactors were operated under similar
conditions, differences occurred in the characteristics of both the aerobic and anaerobic foulant layers, specifically in both the concentration and molecular weight of not only the extracellular polymeric substances (EPS) but also the soluble microbial products (SMP).

In addition, Yun et al. proved that the anoxic cake layer was more uniform compared to the aerobic one \(^{17}\). These differences may mean that the LRVs previously reported for aeMBR systems may not be representative of anMBR systems. As anMBRs are increasingly under consideration for municipal wastewater treatment, differences in their foulant layers compared with those of aeMBRs suggest the existence of a knowledge gap regarding their ability to remove ARB and ARGs.

In the present study, we evaluated the removal efficiency for ARBs and ARGs achieved by anaerobic microfiltration (MF) membranes that were fouled to varying degrees (i.e., N0: new membrane; F1: membrane harvested at \(\sim 20\) kPa; F2: membrane harvested at \(\sim 40\) kPa; and F3: membrane harvested at \(\sim 60\) kPa). Three types of pathogenic ARB (i.e., \textit{E. coli} PI-7, \textit{K. pneumoniae} L7 and \textit{E. coli} UPEC-RIY-4) and their associated plasmid-borne ARGs (i.e., \textit{bla}_{NDM-1}, \textit{bla}_{CTX-M-15} and \textit{bla}_{OXA-48}) were applied as model contaminants. The genes \textit{bla}_{NDM-1}, \textit{bla}_{CTX-M-15} and \textit{bla}_{OXA-48} code for carbapenemases and extended-spectrum beta-lactamases, which are enzymes that confer resistance to carbapenems and other beta-lactam antibiotics. Carbapenems compose a new class of beta-lactam antibiotics that are typically used as a last-resort treatment against gram-negative bacterial infections. The ARB that are resistant as well as the ARGs that confer resistance to such antibiotics are listed as global concerns by the World Health Organization (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-
needed/en/). These ARB and ARGs were therefore used to determine whether anaerobic membrane bioreactors have the capability of removing these contaminants from wastewaters. Specifically, the aims of this study are to elucidate whether the fouling of anaerobic membranes to varying degrees affects the removal efficiency of biological, emerging contaminants (i.e., ARGs and ARB) and whether the removal rates vary when a fouled membrane is subjected to different operating pressures.

2. MATERIAL AND METHODS

2.1. Reactor configuration and operating conditions. The anMBR operated in this study followed the same configuration (Figure S1A) as that operated in an earlier study [16]. Briefly, the reactor was fed with synthetic wastewater having a chemical oxygen demand (COD) of 750 mg/L and operated at 35 °C and a pH of 7. Two separate runs were conducted (i.e., Run 1 and Run 2), spaced approximately 4 months apart. For each run, three PVDF microfiltration (MF) membranes (GE Osmonics, Minnetonka, MN, USA) that were individually housed in cassette holders were connected in parallel to the anaerobic reactor. The membranes had a nominal pore size of 0.3 µm. The anMBR was operated at a 300 mL/min recirculation rate. Biogas was used to scour the membrane surface at a gas sparging rate of 100 mL/min. The flux was maintained at approximately 7 L/m²/h (LMH), while the changes in TMP were recorded by a pressure gauge connected to each membrane module (Figure S2). The COD of the effluent was quantified weekly.
2.2. Membrane characterization. An FEI Nova Nano scanning electron microscope (SEM) was used to characterize a cross section of the cake layer at 5 kV. In preparation for the SEM examination, membrane samples with dimensions of 1 by 2.5 cm were air-dried and then each affixed to an aluminum stub. Iridium was sputtered at a thickness of 3 nm onto the membrane surface with a K575X Emitech sputter coater (Quorum Technologies, UK). Atomic force microscopy (AFM) was used to characterize the membrane surface topography. The air-dried membranes were first attached to a support plate and then imaged by an Agilent 5500 AFM system (Agilent Technologies Inc., Palo Alto, CA, USA) in contact mode. Silicon cantilevers (Applied NanoStructures, Inc.; CA; USA) with a resonance frequency of 11–19 kHz and a force constant of 0.1-0.6 Nm\(^{-1}\) were used. The acquired AFM images were post-processed by Gwyddion software. For each membrane, five random 10 x 10 µm square pictures were scanned. The contact angle was measured by an EasyDrop shape analyzer (Krüss, Hamburg, Germany) in static mode at ambient temperature to evaluate whether the membranes were hydrophilic (< 90°) or hydrophobic (> 90°). Ultrapure water was used as the probing liquid, and the mean values were determined from five different independent specimens. The surface zeta potential was measured in a Nano Zetasizer surface cell cuvette containing trace particles (Nano ZS Zen3600, Malvern, UK) at pH 7 and 25 °C, as described elsewhere \(^{18}\). The membranes to be measured were placed inside the cell cuvette, and the surface zeta potential was evaluated by measuring the zeta potentials of the tracer particles at different distances from the membrane surface. A linear change with distance is observed, and the value at a distance equal to zero is extrapolated automatically by the instrument.
2.3. Determination of polysaccharides (PS) and proteins (PN) in soluble extracellular polymeric substances (EPS). The PS and PN concentrations in the soluble EPS fraction from fouled membranes were quantified based on the modified protocols specified by an earlier study. Briefly, the membranes were harvested, placed in 30 mL of sterile 1X M9 minimal salt medium and then vortexed at maximum speed for 2 min to detach the biofilm from the membrane. A 10 mL aliquot of suspension was centrifuged at 10,000 × g for 10 min, and the supernatant was filtered through a 0.22-µm syringe filter (VWR US, Radnor, PA, USA) before its PS and PN contents were determined. The PSs were determined in triplicate for each sample by the phenol-sulfuric acid method. The PNs were quantified in triplicate by a Total Protein Kit (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Filtration experiment for ARB. The MF membranes enclosed in their cassette modules were harvested for the filtration experiments at the TMPs of ~20 kPa, ~40 kPa and ~60 kPa (hereafter named F1, F2 and F3, respectively) (Figure S1B). The membranes harvested at ~20 kPa and ~40 kPa represent subcritically fouled membranes having an increase in the foulant layer corresponding to the TMP level. The membranes harvested at ~60 kPa correspond to critically fouled membranes. The definition for subcritically fouled and critically fouled membranes was made based on the slope of the relationship between the TMP profile and the duration (Figure S2). The subcritically fouled membranes were defined as those with a TMP that increased at an exponential rate to maintain a constant flux (ca. 7 LMH). The critically fouled membranes were defined as those with TMP that reached a plateau and were no longer capable of further increases to
maintain a constant flux. Three types of ARB, namely, \( \textit{bla}_{\text{NDM-1}} \)-positive \textit{E. coli} PI-7\textsuperscript{20} marked with green fluorescence protein, \( \textit{bla}_{\text{CTX-M-15}} \)-positive \textit{K. pneumoniae} L7\textsuperscript{21} and \( \textit{bla}_{\text{OXA-48}} \)-positive \textit{E. coli} UPEC-RIY-4 were spiked into sterile 1X M9 minimal salt medium (Sigma-Aldrich, St. Louis, MO, USA) to obtain a final \( \text{OD}_{600} \) of \(~0.3\). The growth conditions for each ARB are provided in Supplementary Material S1. The culture suspension with bacteria was independently filtered through the F1, F2 and F3 membranes. The same culture suspensions were also filtered through new membranes. All the filtration experiments were operated at a recirculation rate of 300 mL/min and a nitrogen sparging rate of 100 mL/min to approximate the same conditions experienced in the anMBR. During the filtration experiment, the filtration TMP was incrementally adjusted for F1, F2 and F3 to determine whether an increase in filtration pressure would affect the removal efficiencies achieved by each type of fouled membrane. The permeate was sampled every 4 h prior to increasing the filtration TMP. For example, the filtration TMP for F1 was incrementally increased from 5 kPa to 10 kPa, then to 15 kPa and finally to 20 kPa (the permeates collected at each TMP are hereafter referred to as F1-1, F1-2, F1-3 and F1-4, respectively). Similarly, for F2, the TMP was incrementally adjusted from 10 kPa to 20 kPa, 30 kPa and finally 40 kPa (the permeates collected at each TMP are hereafter referred as F2-1, F2-2, F2-3 and F2-4, respectively). For F3, the filtration TMP was increased from 10 kPa to 20 kPa, 40 kPa and 60 kPa sequentially (the permeates collected at each TMP are hereafter referred as F3-1, F3-2, F3-3 and F3-4, respectively) (Table S1). The filtration experiment with the new membrane was performed at fluxes matching those of the F1 membranes. In all instances, no filtration TMP was observed with the new
membrane since no foulant layer existed. The permeate was collected at the same
abovementioned intervals to provide corresponding controls, and these permeates are
hereafter referred as N0-1, N0-2, N0-3 and N0-4, respectively. Table S1 summarizes the
matrix of conditions used in this study. At the end of the filtration experiments, all the
membranes were harvested for characterization, as detailed in section 2.3. The biofilm
attached to the membranes and the permeate were processed and extracted for their total
DNA, as previously described 19.

2.5. Filtration experiment for ARGs. A 60 mL sample of each bacterial host was
incubated in a 200 rpm shaker incubator for 17 h at 37 °C and then extracted for its
plasmids by the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA).
Earlier work sequenced the blaNDM-1-positive plasmid 3, and the associated plasmid size
was known. In addition, the present study sequenced the blaCTX-M-15-positive plasmid
associated with K. pneumoniae L7 and the blaOXA-48-positive plasmid associated with E.
coli UPEC-RIY-4 via the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The
specific protocols detailing the sequencing and assembly are provided in Supplementary
Material S2. The plasmids were spiked into sterile 1X M9 minimal salt medium to a final
concentration of ~10^5 copies/mL. The filtration experiment for the ARGs was conducted
in a manner similar to that described in section 2.4 but without the increments in the
filtration TMP. After the filtration experiment, the membranes were harvested for
characterization, as detailed in section 2.3. Six milliliters of each biomass suspension and
permeate were frozen at -80 °C and lyophilized using a Christ Alpha 1-2 LDplus freeze
The lyophilized biomass suspension and permeate were subjected to DNA extraction, as previously described.  

2.6. Quantification of ARB and ARGs. The ARB were quantified by determining their associated ARG marker. The quantification of the associated ARGs (i.e., bla\text{NDM-1}, bla\text{CTX-M-15} and bla\text{OXA-48}) was conducted with an Applied Biosystems 7900HT Fast Real-Time PCR (qPCR) System (Thermo Fisher Scientific, Carlsbad, CA, USA). The TaqMan probes and corresponding primer sequences are listed in Table S2. The qPCR standards for the associated genes were prepared as described previously. The thermal cycle and detection limit are shown in Supplementary Material S3. In addition, to verify the qPCR result, flow cytometry by the BD FACSCanto II system ((BD Biosciences, San Jose, CA, USA) was used to determine the cell counts of the bla\text{NDM-1}-positive E. coli PI-7, based on the green fluorescence protein.  

2.7. ARB adsorption comparison. To compare adsorption between the ARB and the foulant layer, isothermal titration calorimetry (ITC) was applied (Malvern ITC200, Malvern, UK). ITC measures the heat change that occurs when two substances interact. E. coli PI-7, K. pneumoniae L7 and E. coli UPEC-RIY-4 were respectively incubated in a 200 rpm shaker incubator for 17 h at 37 °C and then centrifuged at 10,000 g for 10 min to obtain cell pellets. The cell pellets were resuspended and washed twice with M9 minimal salt medium and recentrifuged before the washed pellets were finally resuspended in M9 minimal salt medium and diluted to a final OD\text{600} of 0.3. Ten milliliters of the different foulant suspensions from section 2.3 was filtered through 40 µm Falcon™ Cell Strainers
(Fisher Scientific, USA). The filtrate was respectively aliquoted and then diluted 1:4 v/v in M9 minimal salt medium. Two microliters of the filtrate was injected into 200 µL of each ARB suspension at 25 °C using a 750 rpm stirring speed and 4 s duration. Each sample was injected into the ITC machine a total of 12 times, with a 120 s interval between each injection. The resulting values were analyzed and plotted using Origin (version 7).

2.8. Statistical analysis. Significance was analyzed either by a two-tailed t-test, available in Microsoft Excel 2013, or by one-way ANOVA, available in Minitab Express.

3. RESULTS

3.1. Differences in the thickness and the PS and PN concentrations of F1, F2 and F3 membranes. As fouling progressed from F1 to F3, the thickness of the foulant layer, as evidenced from the cross-sectional images of the membranes, revealed a corresponding increase (Figure S3). Its thickness for F1 was approximately 2.99 ± 0.17 µm in Run 1 and 3.96 ± 0.26 µm in Run 2 (Table 1). When the TMP increased to ~40 kPa (i.e., F2), the foulant layer thickness increased by > 2 times compared to that for F1 (8.80 ± 0.13 µm in Run 1 and 10.43 ± 0.48 µm in Run 2). F3, the critically fouled membrane, had a foulant layer thickness of 26.92 ± 3.24 µm in Run 1 and 20.33 ± 0.43 µm in Run 2. The estimated biovolume increased from F1 to F3 with the increasing thickness (Table 1). The polysaccharide (PS) concentration increased significantly as fouling progressed (t-test, p < 0.01), rising from 17.53 and 25.63 µg/cm² in Run 1 and Run 2, respectively, for
F1 to 73.89 and 45.18 µg/cm² in Run 1 and Run 2 for F2. For F3, the PS concentration further increased to 96.56 and 133.27 µg/cm² in Run 1 and Run 2, respectively (Figures S4A and S4C). Similarly, the protein (PN) concentration also increased significantly from F1 to F3 (t-test, p < 0.01). The PN concentration in the foulant layer was 4.36 and 5.00 µg/cm² in Run 1 and Run 2, respectively, for F1. These values increased for F3 to 19.36 and 25.50 µg/cm² in Run 1 and Run 2, respectively (Figures S4B and S4D).

3.2. Differences in the surface characteristics of F1, F2 and F3 membranes. Among all the tested membranes, the new membrane (i.e., N0) had the roughest surface area (Rₐ = 118.3 ± 27.0) and was significantly rougher than the three fouled membranes (i.e., F1, F2 and F3) (t-test, p < 0.01) (Table 1). As the membranes fouled, the hydrophobicity of the membrane surfaces increased (Table 1). The new PVDF membrane was hydrophilic (78.7 ± 3.8°), but the presence of a foulant layer caused the contact angle to increase to 90.2 ± 2.3° for F1, indicating that the membrane surface became hydrophobic. The contact angle further increased to 95.5 ± 2.3° and 107.4 ± 1.6° for F2 and F3, respectively. In addition, the new membrane exhibited the lowest surface zeta potential (i.e., -44.3 ± 4.4 mV) (Table 1). In contrast, the surface zeta potential values for the fouled membranes were -17.3 ± 0.3 mV, -19.2 ± 1.9 mV and -25.8 ± 2.3 mV; these membranes showed a significantly lower negative charge than that measured for the new membrane (t-test, p < 0.01).

3.3. ARGs showed smaller particulate sizes but a higher negative charge compared to ARB. The results obtained from the dynamic light scattering technique indicated that
the average particulate size of *E. coli* PI-7, *K. pneumoniae* L7 and *E. coli* UPEC-RIY-4 was 1899.4 ± 235.4 nm, 1956.2 ± 253.0 nm, and 2141 ± 33.9 nm, respectively. In contrast, all of the three plasmids were smaller than 565 nm (Figure S5A). The zeta potential assessments of plasmids showed that all three plasmids and the ARBs were negatively charged. The absolute zeta potential value of plasmids was greater than 22 mV; in contrast, the absolute charge of bacteria was always less than 15 mV (Figure S5B).

### 3.4. Increase in LRV for ARGs with fouling severity.

In Run 1, the new membrane achieved an LRV of 2.75 for the plasmid encoding the *bla*<sub>NDM-1</sub> gene. The LRVs achieved by F1, F2 and F3 were higher, reaching 3.48, 3.60 and 3.84, respectively (Figure 1A). A similar increment in the LRV was observed for the plasmid encoding the *bla*<sub>OXA-48</sub> gene. To illustrate, the LRV achieved by the new membrane was 1.90, but this value increased to 3.37 for F2. No *bla*<sub>OXA-48</sub>-encoding plasmid was detected in the F1 or F3 permeates. Likewise, no *bla*<sub>CTX-M-15</sub>-encoding plasmid was detected in any of the permeate samples, and no LRVs could be obtained.

To confirm that the LRVs of the ARG-encoding plasmids were higher in the presence of fouled membranes, a replicate run was conducted. In Run 2, the LRVs achieved by new membrane were 2.30, 2.13 and 2.42 for the plasmids encoding *bla*<sub>NDM-1</sub>, *bla*<sub>CTX-M-15</sub> and *bla*<sub>OXA-48</sub>, respectively (Figure 1C). In contrast, all the LRVs for the fouled membrane were higher than 2.76, and the LRV increased as fouling progressed. The LRV of the plasmid encoding *bla*<sub>NDM-1</sub> achieved by F1, F2 and F3 were 1.3-, 1.2- and 1.5-fold higher than that achieved by the new membrane. Similarly, the LRV for the plasmid encoding
blaCTX-M-15 increased to 3.35 and 3.45 on the F1 and F2 fouled membranes, and no detectable blaCTX-M-15 was present in the F3 permeate. In addition, the LRVs for the plasmid encoding blaOXA-48 were higher using the fouled membranes compared to the new membrane (Figure 1C).

3.5. Foulant layer enhanced the adsorption of ARGs. The abundance of plasmids encoding the blaNDM-1 gene and attached to the new membrane was approximately 1.90 x 10^3 and 5.51 x 10^2 copies/cm² in Run 1 and Run 2, respectively. In contrast, the respective abundance of these plasmids attached to the F1, F2 and F3 membranes increased to 2.61 x 10^5, 1.46 x 10^4 and 3.11 x 10^4 copies/cm² for Run 1; and these abundances were significantly higher than that obtained for this plasmid using the new membrane (t-test, p < 0.01) (Figure 1B). In Run 2, the abundances of the plasmid encoding blaNDM-1 and attached to the fouled membranes were up to 74 times higher than the abundance of this plasmid detected on the new membrane (Figure 1D). Similar trends were observed for the other two plasmids (Figures 1B and 1D).

3.6. New membrane and critically fouled F3 membrane displayed high LRVs for ARBs. The qPCR results indicated that new membrane can achieve a high LRV for all the tested ARB. To illustrate, the average LRVs were 6.50, 6.50 and 5.39 in Run 1 for E. coli PI-7, K. pneumoniae and E. coli UPEC-RIY-4, respectively. The same range of LRVs was achieved in Run 2 (Figures 2A to 2C). In contrast, the average LRV achieved by F1 for E. coli PI-7 decreased to 3.48 and 4.99. The average LRV further decreased to 1.28 and 2.18 in the two runs for E. coli PI-7 when a more heavily fouled F2 membrane
was tested. However, the average LRV for the F3 treatment increased in both runs to approximately that achieved by the new membranes (Figure 2A), and the LRVs obtained for F3 and for the new membranes were not significantly different (one-way ANOVA, p > 0.05). Specifically, the LRV for *E. coli* PI97 was 5.19 in Run 1 and 4.51 in Run 2. These LRVs achieved by F3 were, however, significantly higher than those achieved by F2 (one-way ANOVA, p < 0.01). The same trend was also observed for the average LRVs of *K. pneumoniae* and *E. coli* UPEC-RIY-4 as the severity of membrane fouling progressed (Figures 2B and 2C).

A further evaluation was undertaken to determine whether an increase in the filtration pressure would affect the LRV achieved by membranes fouled to different degrees. For the F1 and F2 subcritically fouled membranes, an increase in filtration pressure resulted in a decreased LRV in most instances. For example, with the exception of the LRVs achieved for all the ARB by the F1 membrane in Run 2, both runs showed that the subcritically fouled F2 membranes experienced up to 2-log declines in the LRVs for all three ARB groups when the filtration pressure was increased (Figures 2A to 2C). In both runs, the LRVs achieved by the F2 membranes at the higher filtration pressures (i.e., F2-3 and F2-4) were significantly less than the LRVs achieved at the lower filtration pressures (i.e., F2-1 and F2-2) (one-way ANOVA, p < 0.05).

In contrast to the subcritically fouled membranes, increasing the filtration pressure applied to the critically fouled F3 membrane did not significantly affect the average LRV of any tested ARB (one-way ANOVA, p > 0.59).
3.7. Foulant layer enhanced the attachment of ARB. The abundance for *E. coli* PI-7 attached per cm$^2$ of the new membrane was 4.92 x 10$^6$ in Run 1, which was significantly lower than that of the F1, F2 and F3 membranes (t-test, p < 0.05) (Figure 3A). In Run 2, the abundance of *E. coli* PI-7 attached per cm$^2$ of the F1, F2 and F3 membranes was 2.41 x 10$^7$, 2.40 x 10$^7$, and 4.25 x 10$^7$, respectively, and these abundances were significantly higher than that on the new membrane by as much as 1-log unit (t-test, p = 0.01) (Figure 3B). Similarly, the abundance of *K. pneumoniae* L7 and *E. coli* UPEC-RIY-94 attached to the new membrane was significantly lower than that on the F1, F2 and F3 membranes in both runs (t-test, p < 0.05) (Figures 3A and 3B).

The interaction between the ARB and the foulant layer was confirmed by ITC (Figure 4). Compared to the blank which did not contain any foulant, the presence of foulant obtained from F1, F2 and F3 membranes released significantly more heat upon interaction with the three ARB (t-test, p < 0.01). In addition, the heat release was highest when foulant layer obtained from F3 was present. To illustrate, the heat change for F1 was 0.035 µcal/s upon interaction with *E. coli* PI-7, and this value increased by 46.9% and 59.9% for F2 and F3, respectively, compared to that for F1.

3.8. Experiment to verify the LRV for *E. coli* PI-7. To verify the result obtained by qPCR, flow cytometry was applied as an alternative method to assess the LRV and the abundance of the *E. coli* PI-7 cells adhered to the membrane surfaces (Figure S6). To illustrate, the average, *E. coli* PI-7 LRV for the new membrane was 5.95 in Run 1 and 5.19 in Run 2. For F1, the LRV decreased to 2.89 in Run 1 and 3.35 in Run 2. However, the LRV recovered to 3.92 in Run 1 and 4.27 in Run 2 for F3. A further evaluation
regarding the abundance of *E. coli* PI-7 attached to the membrane surfaces revealed higher cell counts on the fouled than on the new membranes (Figure S7). The number/cm\(^2\) of *E. coli* PI-7 attached to the new membrane was 6.64 x 10\(^4\) in Run 1 and 1.40 x 10\(^5\) in Run 2. In contrast, the abundance of *E. coli* PI-7, measured as the number of bacteria attached per cm\(^2\) of fouled membrane (i.e., F1, F2 and F3), ranged from 2.37 x 10\(^6\) to 1.57 x 10\(^7\) in Run 1 and from 3.62 x 10\(^6\) to 3.20 x 10\(^7\) in Run 2.

4. DISCUSSION

The World Health Organization has warned in their 2014 Global Report on Surveillance that “antimicrobial resistance threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi”\(^24\).

Because of the widespread use of antibiotics, resistant organisms, including their mobile genetic elements, exist almost ubiquitously in humans, animals, food and the environment. In particular, wastewater has been identified as an important reservoir that can disseminate such resistant organisms and mobile genetic elements into the environment or to end users during reuse events\(^1,25\). These concerns may impede subsequent effort to reuse treated wastewaters.

As such, an effective treatment process is needed to remove ARB and ARGs from wastewater prior to its discharge or reuse. An earlier study assessed the LRVs for these emerging contaminants (i.e., ARB and ARGs) achieved by full-scale aerobic MBRs and determined that the concentrations of ARGs (e.g., tetW, tetO, and sulI) and ARB (e.g.,
tetacycline- and sulfonamide-resistant bacteria) in the post-MBR permeate stream were
1 to 3-log units less than those achieved by activated sludge processes\(^8\), suggesting that
membrane systems are more effective than the conventional activated sludge in removing
these emerging contaminants from wastewater. Studies have also been conducted to
assess the LRVs for contaminants (e.g., organic micropollutants, bacterial pathogens, and
viruses) achieved by anMBRs\(^{26-28}\), which in recent years, are proposed as a sustainable
alternative to aerobic MBRs for municipal wastewater treatment. For instance, Wei et al.
investigated the removal of 15 organic micropollutants by a laboratory-scale anMBR and
found 80–92% rejection rates for most organic micropollutants\(^{26}\). Harb et al. further
reported that a laboratory-scale anMBR can achieve > 1.7-log removal of opportunistic
pathogenic species from municipal wastewater\(^ {27}\). In addition, Wong et al. reported a 3.7
log removal of coliphages by an anMBR\(^ {28}\).

Nevertheless, most of these existing studies do not evaluate for the removal efficiencies
of ARBs and ARGs by anMBRs. In the present study, the LRVs of three plasmids
encoding for ARGs ranged from 2.76 to 3.84-log units when the anaerobic membrane
became increasingly fouled (Figures 1A and 1C). These reported LRVs approximately
match the LRV obtained by Wong and coworkers for viruses\(^ {28}\). This similarity occurred
despite differences between the viruses and plasmids evaluated in the two studies.
Viruses such as adenovirus, enterovirus and coliphage have genome sizes that range from
3600 nt to 48 kbp, depending on whether the virus is a single-stranded RNA virus or a
double-stranded DNA virus. These genome sizes would equate to an approximate viral
diameter of 30 to 100 nm. In comparison, these sizes are much smaller than those
of the plasmids that were evaluated in the present study. The plasmid sequencing did not result in a complete assembly of the plasmids that encode for \textit{bla}_{CTX-M-15} and \textit{bla}_{OXA-48}.

However, the assembled contigs of the partial plasmidic genomes have already revealed a size as large as 110 kbp and 55 kbp for these two plasmids (i.e., \textit{bla}_{CTX-M-15} and \textit{bla}_{OXA-48}). Similarly, the IncF plasmid encoding for \textit{bla}_{NDM-1} is reported to have a size as large as 110 kbp. Collectively, the plasmids assessed in the current study show comparatively larger genome sizes and are thus likely to have larger particulate sizes compared to viruses. To verify this supposition, the particulate size of all three plasmids used in the current study were further assessed by a dynamic light scattering technique and were found to be ca. 460 to 560 nm in diameter (Figure S5A).

Furthermore, viruses have capsid proteins that are hydrophobic, while extracellular plasmids are generally hydrophilic due to the exposed sugar-phosphate bond of DNA. In the present study, significantly more plasmids were found attached to the fouled compared to the new membranes. This pattern occurred despite no clear correlation between the attachment preference and the surface characteristics. For example, hydrophobicity increases as anaerobic membranes become progressively fouled, and both the membrane surface and the plasmids showed a negative charge (Table 1 and Figure S5B). These conditions would have led to hydrophobic-hydrophilic and charge repulsion between the membrane surfaces and plasmids. However, the abundance of the plasmids that attached to the membranes remained within the same range (Figures 1B and 1D). This effect may have been due to the presence of an electrolyte in the M9 minimal salt medium used during the filtration experiment, which led to a decrease in the repulsion.
force exhibited in hydrophobic-hydrophilic and charge interactions. In addition, new membrane exhibits a rougher surface area compared to fouled membranes, which is generally thought to facilitate adhesion. Instead, a significantly lower abundance of attached plasmids occurred on the new membranes compared to the fouled ones with relatively smooth surfaces (Figures 1B and 1D).

Despite a lack of understanding regarding the exact mechanisms governing the attachment of plasmids to anaerobic membranes, the presence of foulant improved the removal efficiency for all three of the plasmids. However, the LRVs achieved by membranes fouled to different degrees (i.e., F1, F2, and F3) were similar. This pattern occurred despite an increase in the thickness of the foulant layer (Table 1), suggesting that size exclusion and biovolume are less important factors than adsorption in removing plasmids (Figures 1B and 1D). A higher adsorption of plasmids on the fouled membranes compared to the new membrane was likely due to the higher protein and polysaccharide concentrations measured in the foulant layer of the fouled membranes, which facilitated interaction and the adsorption of the plasmids to the foulant layer. In addition, the presence of a cake layer on the fouled membranes would induce a more severe concentration polarization than that experienced on the new membrane. Previous studies have shown that concentration polarization improves the rejection of volatile organic compounds, perfluorooctane sulfonates, boron and arsenic. Similarly, concentration polarization may have also contributed to the high LRVs for the ARGs achieved by the fouled membranes evaluated in the current study.
Apart from ARG filtration, the current study also investigated the LRVs for three ARB. Prior studies have determined that both *E. coli* and *K. pneumoniae* possess a hydrophobic cell surface, which would facilitate bacterial attachment to the hydrophobic anaerobic membranes (Figure 3). In addition, the relatively lower negative charge of bacteria compared to plasmids would have resulted in less charge repulsion and thus adsorption onto the membranes (Figure S5B). Although the number of cells attached to the three fouled membranes was significantly higher compared to the new membrane, no significant difference existed among the number of cells attached to the three fouled membranes. This observation implies that other factors (e.g., extracellular polymeric substances, cake layer thickness) and not solely surface properties affect the adhesion of ARB to fouled membranes. To verify this supposition, ITC was used as an alternative method to detect and compare the interaction between the ARB and the foulant layers. Unlike the traditional atomic force microscope (AFM) method, which modifies the AFM tips to detect the interaction force, the ITC method measures the heat change that occurs when two molecules interact, which is directly proportional to the level of interaction. When compared to the heat change obtained from the interaction between the bacterial suspensions and the control (i.e., M9 minimal salt medium with no biofilm suspension), more heat was released when the biofilm suspensions obtained from the fouled membranes were injected into the bacterial suspensions (Figure 4). This observation indicates that the ARB can interact with the biofilm matrix, hence accounting for the higher attachment of cells onto the fouled membranes compared to the new one.
The present study further aims to determine whether different filtration pressures applied to these fouled membranes affect their ARB removal efficiencies. The results suggest that when anaerobic membranes are subcritically fouled and the filtration pressure is increased, the removal of ARB is compromised. However, a lower LRV was not observed when the membranes were critically fouled, despite a subsequent increase in filtration pressure.

The ARB used in this study were found to be of ca. 2000 nm in particulate size (Figure S5A and Figure S8). Antimicrobial molecules are known for inducing alterations in the bacterial envelope and in its mechanical properties, such as the cell wall elasticity. Prior study indicates that the cell wall is an important factor in triggering the passage of bacteria through membranes with a relatively small pore size. An earlier study found that antibiotics present in the suspension medium affect the mechanical properties of the bacterial cell wall by decreasing its rigidity, thereby resulting in a greater number of cells passing through a membrane and entering into the permeate stream. However, whether ARB have a higher deformability compared to antibiotic-susceptible bacteria is unknown.

Gram-negative bacteria were also demonstrated to cross through membrane barriers by deforming themselves under high filtration pressures of 10 to 950 kPa. Earlier study has also shown that the particle size in the cake layer from anaerobic membranes decreases from the top to the bottom layer, resulting in a corresponding decrease in porosity across the different layers. This gradual decrease in pore size formed funnel-like structures that would likely facilitate the passage of less rigid cells through membrane pores. Coupled with findings suggesting that an increase in transmembrane...
pressure could enlarge membrane pore size\textsuperscript{52}, these factors can possibly explain why subcritical fouling would lower the LRV for ARB when the filtration pressure increases.

However, the LRVs achieved by the critically fouled membranes returned to a lower level approximating that achieved by the new membranes (Figure 2). This result could be explained by the total blockage of membrane pores via an irremovable foulant layer at that fouling level; therefore, any further increase in the filtration pressure was unable to force deformed cells through the membrane barrier. A total pore blockage is facilitated by the high protein concentration measured in the biofilm matrix of the anaerobic membrane in this study (Figures S4B and S4D) and in an earlier study\textsuperscript{16}. These high protein contents may, in turn, contribute significantly to the blockage of anaerobic membrane pores\textsuperscript{53,54}.

In summary, the findings from the present study suggest that as anaerobic membranes fouled progressively, the total LRV for the ARGs increased while that for the ARB initially decreased before subsequently stabilizing at an LRV similar to that of new microfiltration membranes. In particular, for subcritically fouled membranes, a lower LRV for the ARB was attained with an increase in filtration pressure. However, the same compromise in the LRVs was not observed when the membranes were critically fouled. These results collectively suggest that an MBR shows promise in removing at least 2 to 3-log units of ARB and ARGs, especially when operated in the long term to favor high removal rates of both ARB and ARGs.
5. Acknowledgments

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Supplementary Information Available

Methods: Growth conditions for antibiotic-resistant bacteria; Plasmid sequencing and assembly; Real-time PCR and detection limit test; Analysis of particulate sizes; Zeta potential analysis.

Tables: Matrix of conditions used in this study; Primers and fluorogenic probes for the specific detection of resistance genes.

Figures: Schematic diagram of anaerobic membrane reactors and experimental setup for the ARB and ARG filtration experiment; Changes in transmembrane pressure for the different microfiltration membranes; Cross-sectional SEM images of membranes; Concentration of polysaccharides and proteins in soluble EPS; Particulate size and zeta potential of ARB and ARGs; LRV of *E. coli* PI-7 assessed by flow cytometry; Abundance of *E. coli* PI-7 attached to membranes assessed by flow cytometry; SEM images of ARB.
Table 1 Roughness, hydrophilicity and surface zeta potential of different membranes. N.A. denotes not applicable.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Thickness (µm)</th>
<th>Estimated dried biovolume (mm³)ᵃ</th>
<th>Roughness</th>
<th>Hydrophilicity</th>
<th>Surface zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 1</td>
<td>Run 2</td>
<td>Rₐ (nm)ᵇ</td>
</tr>
<tr>
<td>N0</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>118.3 ± 27.0</td>
</tr>
<tr>
<td>F1</td>
<td>2.99 ± 0.17</td>
<td>3.96 ± 0.26</td>
<td>15.0</td>
<td>19.8</td>
<td>79.2 ± 10.4</td>
</tr>
<tr>
<td>F2</td>
<td>8.80 ± 0.13</td>
<td>10.4 ± 0.48</td>
<td>44.0</td>
<td>52.2</td>
<td>61.7 ± 6.5</td>
</tr>
<tr>
<td>F3</td>
<td>26.9 ± 3.24</td>
<td>20.3 ± 0.43</td>
<td>134</td>
<td>102</td>
<td>51.1 ± 6.5</td>
</tr>
</tbody>
</table>

ᵃEstimated dried biovolume was determined by multiplying the average thickness by the membrane surface area.
ᵇRₐ is the arithmetic average of the absolute values of the surface height deviations measured from the mean plane.
ᶜRₗ is the root mean square average for height deviation taken from the mean image data plane.
FIGURE LEGENDS

**Figure 1.** Log removal value (LRV) and abundance of ARGs attached on membranes. (A) LRV reported in Run 1; (B) Copies of ARGs attached per surface area of membrane in Run 1; (C) LRV reported in Run 2; (D) Copies of ARGs attached per surface area of membrane in Run 2. ◊ and □ indicate that the LRV could not be determined because the \( bla_{\text{CTX-M-15}} \) plasmid and \( bla_{\text{OXA-48}} \) plasmid, respectively, were below the qPCR detection limit for that sample.

**Figure 2.** ARB log removal value (LRV) of different membranes evaluated by qPCR. (A) LRV for *E. coli* PI-7 with plasmid encoding \( bla_{\text{NDM-1}} \) in Run 1 and Run 2, (B) LRV for *Klebsiella pneumoniae* L7 with plasmid encoding \( bla_{\text{CTX-M-15}} \) in Run 1 and Run 2, (C) LRV for *E. coli* UPEC-RIY-4 with plasmid encoding \( bla_{\text{OXA-48}} \) in Run 1 and Run 2. (N0: new membrane; N0-1, N0-2, N0-3 and N0-4 denote samples collected at 0 kPa. F1 and F2: subcritically fouled membranes harvested at ~20 kPa and 40 kPa, respectively; F1-1, F1-2, F1-3 and F1-4 denote samples collected at 5 kPa, 10 kPa, 15 kPa and 20 kPa, respectively; F2-1, F2-2, F2-3 and F2-4 denote samples collected at 10 kPa, 20 kPa, 30 kPa and 40 kPa, respectively. F3: critically fouled membrane harvested at ~60 kPa; F3-1, F3-2, F3-3 and F3-4 denote samples collected at 10 kPa, 20 kPa, 40 kPa and 60 kPa, respectively. * indicates significant difference.)

**Figure 3.** Abundance of ARB attached per unit surface area of membrane in (A) Run 1, and (B) Run 2. Abundances were quantified by qPCR. Each type of ARB attached on F1, F2, and F3 was significantly higher than that attached on new membrane (p < 0.05).

**Figure 4.** Isothermal titration calorimeter (ITC) results for diluted suspension containing the foulant layer of F1, F2 and F3 membranes with each ARB. The heat change for the foulant layer sampled from F1, F2 and F3 and each ARB was significantly higher than that measured for the control (blank).
REFERENCES


27. Harb, M.; Hong, P.-Y., Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: a


Figure 1.
Figure 2.

E. coli PI-7 with $bla_{NDM-1}$

K. pneumoniae L7 with $bla_{CTX-M-15}$

E. coli UPEC-RIY-4 with $bla_{OXA-48}$
Figure 3.
Figure 4.