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Nitric Oxide Treatment for the Control of Reverse Osmosis Membrane Biofouling

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Biofouling remains a key challenge for membrane-based water treatment systems. This study investigated the dispersal potential of the nitric oxide (NO) donor compound, PROLI NONOate, on single- and mixed-species biofilms formed by bacteria isolated from industrial membrane bioreactor and reverse osmosis (RO) membranes. The potential of PROLI NONOate to control RO membrane biofouling was also examined. Confocal microscopy revealed that PROLI NONOate exposure induced biofilm dispersal in all but two of the bacteria tested and successfully dispersed mixed-species biofilms. The addition of 40 μM PROLI NONOate at 24-h intervals to a laboratory-scale RO system led to a 92% reduction in the rate of biofouling (pressure rise over a given period) by a bacterial community cultured from an industrial RO membrane. Confocal microscopy and extracellular polymeric substances (EPS) extraction revealed that PROLI NONOate treatment led to a 48% reduction in polysaccharides, a 66% reduction in proteins, and a 29% reduction in microbial cells compared to the untreated control. A reduction in biofilm surface coverage (59% compared to 98%, treated compared to control) and average thickness (20 μm compared to 26 μm, treated compared to control) was also observed. The addition of PROLI NONOate led to a 22% increase in the time required for the RO module to reach its maximum transmembrane pressure (TMP), further indicating that NO treatment delayed fouling. Pyrosequencing analysis revealed that the NO treatment did not significantly alter the microbial community composition of the membrane biofilm. These results present strong evidence for the application of PROLI NONOate for prevention of RO biofouling.

Membrane technology, for the conversion of seawater and wastewater through desalination and reclamation processes into potable water, is vital for sustainable water management. However, membrane fouling by bacterial biofilms remains a key challenge for these technologies (1–3). Initially, the adsorption of organic species and suspended particles on the wetted membrane surface form a conditioning film. This enables attachment of planktonic cells to the membrane surface, followed by the formation of microcolonies and biofilm maturation, where bacterial cells are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (1, 4, 5). The EPS is typically composed of polysaccharides, proteins, and nucleic acids. The attachment of microorganisms to the membrane surface is affected by factors such as the membrane material, the roughness of the membrane surface, hydrophobicity, and membrane surface charge (6). Biofilm bacteria have several advantages over single planktonic cells, including optimization of growth and survival, improved acquisition of nutrients, and increased protection against environmental stresses, including shear forces (2, 5, 7).

Biofilm formation on membrane surfaces results in a severe decline in flux, or an increase in transmembrane pressure (TMP; defined as the pressure gradient of the membrane, or the average feed pressure minus the permeate pressure) to maintain flux, higher energy consumption, and a deterioration of system performance and product water production (3, 8, 9). As the adhesive and cohesive matrix of biofilms, EPS has been suggested to be the predominant culprit for biofouling of water treatment membranes (1, 10, 11). The EPS is composed mainly of polysaccharides and proteins, which form hydrogel matrices (12). Common techniques to reduce membrane fouling include membrane cleaning and pretreatment of the feed water. However, microorganisms may survive pretreatment processes such as coagulation, flocculation, sand filtration, ultrafiltration, and cartridge filtration to subsequently colonize and foul the system (3). Membrane cleaning by physical or chemical methods is used to regenerate the function of fouled membranes, and the methods used and the frequency of cleaning depend on the type of foulant as well as the resistance of the membrane to chemical cleaning agents (13). However, these cleaning methods frequently shorten membrane life, further increasing operational costs (11, 14). Despite the widespread use of such chemicals, they are ineffective in removing or killing the membrane biofilms (1), and regrowth quickly occurs, resulting in...
poor system performance (15). Therefore, new strategies of biofouling prevention are required to reduce such impacts.

Recent research has demonstrated that the gas molecule and important biological messenger nitric oxide (NO) is a signal for biofilm dispersal, inducing the transition from the biofilm mode of growth to the free swimming planktonic state (16, 17). NO induces biofilm dispersal by stimulating phosphodiesterase activity, resulting in the degradation of cyclic di-guanylate monophosphate (c-di-GMP), culminating in changes to gene expression that favor the planktonic mode of growth (17). NO, which has a short half-life in aqueous environments, can be delivered to biofilms by using chemical compounds that generate NO in solution. The NO donor compound, sodium nitroprusside (SNP), was shown to disperse biofilms of *Pseudomonas aeruginosa*, as well as other bacterial species, including mixed-species biofilms at low, nontoxic concentrations (16, 18). SNP is a well-characterized NO donor and is applied medically to control vasodilation. It has a half-life of approximately 2 min in aqueous solutions at neutral pH. Solutions of SNP are stable, that is, they do not release NO, at high pH, e.g., pH 10. A recent study investigated the biofilm dispersal potential of three NO donor compounds (MAHMA NONOate, SNP, and PROLI NONOate) using *P. aeruginosa* PAO1 (19). The results showed that MAHMA NONOate could reduce bacterial biofilms by up to 40% over a 2-h exposure period but that this reduction was partially due to growth inhibition. The addition of SNP reduced biofilm biovolume by 40% over a 24-h period but led to enhanced growth over shorter periods. Due to the short half-life of the PROLI NONOate (2 at pH 7.4 and 37°C) (20), this NO donor compound quickly dispersed PAO1 biofilms, reducing the biovolume by 30% after 1 h of exposure (19), with no inhibitory or growth effects observed.

It has also been shown that NO stimulates biofilm formation in *Nitrosomonas europaea* (21), *Azospirillum brasilense* (22), and *Shewanella oneidensis* (23) or that NO had no effect on biofilms (24, 25). Thus, different bacteria may show individual responses to NO, and hence it is uncertain what the overall effect of NO might be on a natural, mixed-species biofilm community. Therefore, the aim of this study was to determine the potential of PROLI NONOate to disperse individual- and mixed-species biofilms formed by bacteria isolated from fouled industrial reverse osmosis (RO) and membrane bioreactor (MBR) membranes. In addition, this study examined the application of PROLI NONOate as a novel strategy to control membrane biofouling in a laboratory-scale RO system.

**MATERIALS AND METHODS**

**Bacterial isolates.** Bacteria from industrial MBR and RO membranes were isolated and identified as previously described (19). In addition, biofilm scrapings were taken from six 9-cm² membrane segments from a 5-year-old spiral wound RO module (Kranji NeWater plant, Singapore). Membrane scrapings were mixed together, and bulk genomic DNA was extracted from two 0.1-g samples using sodium dodecyl sulfate hexadecyltrimethyl ammonium bromide (26). A portion of the biofilm scraping was cultured overnight in R2A broth at 30°C with shaking at 200 rpm to prepare glycerol stocks of a mixed RO bacterial community. DNA was extracted from the cultured cells as described above to determine the bacterial composition after culturing.

**Pyrosequencing.** DNA samples from both the raw membrane scraping and the R2A culture were sequenced using the 454 pyrosequencing platform (Research and Testing Laboratory, Texas, USA) using the eubacterial primers Gray2BF (5’-GAGTTTGTATCNGGCCTGCTG-3’) and Gray519R (5’-GTNTTACNGCGGCGGTGTC-3’) (27) for the initial PCR amplification.

**Sequence analyses.** The quality and adaptor trimming of the amplification sequencing reads was performed using QIME (version 1.8.0) (28). The trimmed reads were then clustered into representative sequences of operational taxonomic units (OTUs), based on 97% genetic similarity thresholds for the species level, with the Uclust algorithm (29). Chimera screening and deprecation was performed on the representative sequence using UCHIME (30). After chimera depletion, the sequences were aligned against the Greengenes OTU database (31) using PyNAST (32). The relative abundance for each OTU at the species level was then exported to PRIMER-E (Plymouth Routines in Multivariate Ecological Research) version 6 (33) to calculate the Bray-Curtis distances.

**NO dispersal of batch (petri dish-grown) biofilms.** The optimal concentration and time exposure of the PROLI NONOate for biofilm dispersal had been previously determined (19). A 10-ml liquid culture of each bacterial isolate was grown aerobically overnight in its isolation medium (R2A broth, nutrient broth, or tryptone soya broth) at 30°C with shaking at 200 rpm. The bacterial culture was diluted to an optical density at 600 nm (OD600) of 0.1 (approximately 1 × 10⁸ CFU ml⁻¹), and 1 ml was used for batch biofilm experiments. For mixed biofilms, 1 ml of each diluted bacterial culture was mixed together, and 1 ml of this suspension was used. The 12 RO and 10 MBR bacterial isolates used for mixed biofilms had been previously identified (19). Biofilms were cultivated for 24 h on a glass slide in a petri dish immersed in 19 ml M9 (28.2 mM NaH₂PO₄, 11.4 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl, 2 mM MgSO₄, 0.09 mM CaCl₂, and 22 mM glucose) with gentle shaking (50 rpm) at room temperature (24°C). After 24 h, the medium was replaced with 20% M9 medium and incubated for a further 1 h, at which time PROLI NONOate (Cayman Chemicals) solution in 10 mM NaOH was added to give a final concentration of 40 μM. For the untreated control, an equal amount of 10 mM NaOH was added. For each experiment, treated and untreated biofilms formed by a mixture of isolates were run in triplicate. Each experiment was repeated three times to give an overall average. After 1 h of exposure, slides were gently washed in 0.85% NaCl solution and stained using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen) as per the manufacturer’s instructions. The slides were then washed again in 0.85% NaCl solution to remove excess stain. Live, SYTO 9-stained cells and dead, propidium iodide (PI)-stained cells were visualized by confocal laser scanning microscopy (CLSM) (Nikon Eclipse 90i, part of the A1R hybrid confocal spectral imaging system) at a magnification of ×20, with an argon laser (488-nm wavelength for SYTO 9) and a diode laser (561-nm wavelength for PI). For each slide, 2 stack (three-dimensional [3D]) confocal images were obtained from 9 locations covering the glass surface, and the average biovolume (μm³) was calculated using IMARIS software (Bitplane; version 7.3.1). Biofilms on glass slides were stored in petri dishes containing water-soaked tissue paper to ensure humidity. In addition, slides were analyzed in a specific order so that any drying out of biofilm would not bias the results. No coverslips were used in order to protect biofilm integrity. For each experiment, the percentage of biofilm dispersal was calculated by comparing the average biovolume of NO-treated slides to the untreated control.

**Reverse osmosis system.** The RO pressure reactor was assembled as two independent cells in parallel, allowing one cell to be used as an untreated control. Each stainless steel RO cell had a flat plate geometry and flow channel sizes of 150 by 30 by 0.8 mm (length by width by height) with an effective area of 0.0045 m². The design (Fig. 1) and operation have been previously described (34) and represent conditions that simulate typical large-scale RO processes. Each 10-liter feed tank was equipped with a stirrer (IKA; model Eurostar) and was cooled using a chiller (PolyScience) to maintain the feed solution at 23 ± 1°C (Fig. 1). A high-pressure centrifugal pump (Hydra-Cell) was used to deliver the feed solution and maintain the cross-flow velocity (CFV) at 0.28 m s⁻¹, while system pressure was set to 362.6 lb/in² using a flow control valve (Swagelok; model SS-4R3A). The feed flow rate was monitored with a turbine flow meter.
Experiments were initiated by continuous injection of the bacterial suspension into the flow line at a dilution rate of 1:800, giving an input load of $10^{6}$ CFU ml$^{-1}$. The bacterial solution was replaced every 24 h. Experiments were conducted at a constant flux (30 LMH), and the difference in pressure between the feed and permeate stream (TMP) was monitored continuously. For PROLI NONOate injection, the bacterial suspension was replaced with sterile 0.034 M NaCl, and the bacterial injection tubing was flushed for 10 min at 2 ml min$^{-1}$ to remove excess bacterial cells. PROLI NONOate (10,000 µM) in 10 mM NaOH was then injected into the flow line for 30 min at a dilution rate of 1:250 (1.6 ml min$^{-1}$), giving a final concentration of 40 µM. For the untreated control, 10 mM NaOH, without PROLI NONOate, was used as a control. After dosing, the bacterial suspension was injected into the flow line as described before. PROLI NONOate was injected into the treated cell every 24 h. This dosing interval was selected, as multiple dosing over shorter time periods did not enhance biofilm dispersal in previous experimental work (19). Fouling was defined here as the increase in TMP over time during the filtration process at constant flux. Therefore, a higher TMP within the same time frame or the same TMP within a shorter time frame represents more severe fouling.

Membrane autopsy. The fouled membranes were removed from the RO modules for examination at the conclusion of the experiment, and 1.5 cm was removed from each end of the membrane while the remainder was aseptically cut into 8 segments. Six segments (1 by 3 cm) of membrane covering the inlet, middle, and outlet of the RO cell were analyzed for microbial communities and polysaccharide and protein extraction to determine the concentrations of nutrients remaining equal in each parallel unit.

NO treatment for prevention of membrane biofouling. The membrane fouling rate of an untreated RO cell was compared to the parallel RO cell dosed with 40 µM PROLI NONOate every 24 h. The RO cells were inoculated with either *P. aeruginosa* PAO1 or the mixed bacterial community cultured from the membrane scraping. The bacteria were grown overnight in either 750 ml nutrient broth (PAO1) or R2A broth (mixed community) at 30°C with shaking at 200 rpm. The bacterial cells were subsequently harvested by centrifugation at 18,514 g for 10 min. The pellet was washed and resuspended in 0.034 M NaCl to an OD$_{600}$ of 0.1. The bacterial suspension was injected into the system before the feed solution entered the RO cells by using an injection pump (ELDEX; model VWS) to ensure that the concentration of nutrients remained equal in each parallel unit.

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(Revised 30 March 2015. Accepted 9 April 2015.)

*EXPERIMENTAL PROCEDURE*

Air pressurized to 5 bars was used to maintain a constant flux of 30 LMH (liters/m$^2$/h) on the RO units while the feed solution was filtered through the system. Pressure transducers (Ashcroft) were used to monitor the TMP (transmembrane pressure) of the RO units to prevent excessive backpressure. An injection pump (ELDEX; model 5882) was connected to the feed stream to pump the bacterial suspension into the flow line at a dilution rate of 1:800, giving an input load of $10^{6}$ CFU ml$^{-1}$. The bacterial solution was replaced every 48 h. Experiments were conducted at a constant flux (30 LMH), and the difference in pressure between the feed and permeate stream (TMP) was monitored continuously. For PROLI NONOate injection, the bacterial suspension was replaced with sterile 0.034 M NaCl, and the bacterial injection tubing was flushed for 10 min at 2 ml min$^{-1}$ to remove excess bacterial cells. PROLI NONOate (10,000 µM) in 10 mM NaOH was then injected into the flow line for 30 min at a dilution rate of 1:250 (1.6 ml min$^{-1}$), giving a final concentration of 40 µM. For the untreated control, 10 mM NaOH, without PROLI NONOate, was used as a control. After dosing, the bacterial suspension was injected into the flow line as described before. PROLI NONOate was injected into the treated cell every 24 h. This dosing interval was selected, as multiple dosing over shorter time periods did not enhance biofilm dispersal in previous experimental work (19). Fouling was defined here as the increase in TMP over time during the filtration process at constant flux. Therefore, a higher TMP within the same time frame or the same TMP within a shorter time frame represents more severe fouling.

Membrane autopsy. The fouled membranes were removed from the RO modules for examination at the conclusion of the experiment, and 1.5 cm was removed from each end of the membrane while the remainder was aseptically cut into 8 segments. Six segments (1 by 3 cm) of membrane covering the inlet, middle, and outlet of the RO cell were analyzed by fluorescent staining and CLSM observation to quantify the live and dead cells and the polysaccharide volume (representative of EPS). For the remaining two segments (3 by 3 cm), viable bacterial counts were determined, as were the concentrations of polysaccharide and protein extracted from the membrane surface.

Fluorescent staining and CLSM. Segments of membrane were gently washed in 0.145 M NaCl solution and stained using SYTO 9 and PI (3 µg ml$^{-1}$ each) or 100 µg ml$^{-1}$ ConA-fluorescein isothiocyanate (FITC) (Sigma-Aldrich). The segments were then washed again in NaCl solution to remove excess stain, mounted onto glass slides, and viewed using CLSM as detailed above. For each section, z stack (3D) confocal images were obtained from 5 locations covering the membrane surface, and the average biovolume (µm$^3$) and surface coverage (%) were calculated using IMARIS (Bitplane; version 7.3.1).

Viable bacterial counts. Using a cell scraper, the biofilm was removed from each 3- by 3-cm segment of membrane and resuspended in individual tubes containing 3 ml sterile phosphate-buffered saline (PBS) and vortexed for 30 s. For PAO1, viable heterotrophic counts were determined using a KAREI (5 and 0.2 µm) microfilter (KAREI; 5 and 0.2 µm) installed downstream of the pressure cell to prevent excess bacteria from entering the feed tank. Microfilters (KAREI; 5 and 0.2 µm) were installed downstream of the pressure cell to prevent excess bacteria from entering the feed tank. Microfiltration was used to prevent excess bacteria from entering the feed tank.
using a modified method from Miles et al. (38). In brief, three 10-μl drops of 10⁻¹ to 10⁻⁶ dilutions were pipetted onto nutrient agar. For the mixed RO bacterial community, viable counts were determined by spread plating, in triplicate, 100 μl of 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions on R2A agar (Oxoid). Plates were incubated at room temperature for 24 h before counting. Viable cells were expressed as CFU/cm² of membrane.

EPS extraction and quantification. The remaining bacterial suspension was centrifuged at 18,514 g for 10 min, and the supernatant was transferred to a clean centrifuge tube for analysis of the soluble EPS fraction. The bound EPS was extracted by adding 2 ml 0.145 M NaCl and 12 μl of 37% formaldehyde (Sigma-Aldrich). The polysaccharide content of the EPS was measured by the phenol-H₂SO₄ method (39). Briefly, 0.5 ml of 5% phenol and 2.5 ml of concentrated H₂SO₄ (Merck) were added, and the solution was vortexed and incubated at 4°C for 1 h. After incubation, 2 ml of 1 M NaOH and 0.5 ml Milli-Q water were added, and the solution was vortexed and incubated at 4°C for 3.5 h. Subsequently, the bacterial suspension was centrifuged at 18,514 g at 4°C for 20 min, and the supernatant was transferred to a clean centrifuge tube. A blank control consisted solely of the EPS extraction reagents. The polysaccharide content of the EPS was measured by the phenol-H₂SO₄ method (39). Briefly, 0.5 ml of 5% phenol (Sigma-Aldrich) and 2.5 ml of concentrated H₂SO₄ (Merck) were added to 1 ml of sample and incubated at room temperature for 10 min. The absorbance of the solution was measured at 492 nm (Shimadzu; model UV1800) and compared against a standard curve generated using glucose (Sigma-Aldrich). The protein content of the EPS was analyzed using the Coomassie (Bradford) protein assay kit (Thermo Scientific). A 1-ml volume of the sample was mixed with 1 ml of working reagent and incubated at room temperature for 10 min, and the absorbance was measured at 595 nm (Shimadzu; model UV1800). Bovine serum albumin (Thermo Scientific) was used to generate a standard curve for quantification of proteins.

DNA extraction and pyrosequencing. For the mixed bacterial community, the biofilm was removed from three 3-cm² segments of membrane using a cell scraper for DNA extraction to compare the untreated and PROLI NONOate-treated membrane bacterial communities. DNA extraction, pyrosequencing, and sequence analysis were performed as described above.

RESULTS

NO induced dispersal of MBR and RO isolate batch (petri dish-grown) biofilms. Under batch conditions, the bacterial isolates differed in their response to NO, with the percentage of biofilm dispersal ranging from 10% to 46% for the RO and MBR isolates (Fig. 2). A mixed biofilm consisting of all 12 RO isolates (19) showed 17% dispersal, while a mixed biofilm consisting of all 10 MBR isolates (19) showed 23% dispersal (Fig. 2). An increase in biofilm formation in the presence of PROLI NONOate was observed for one RO isolate, Duganella sp. (8%), and one MBR isolate, Acinetobacter sp. (35%) (Fig. 2). In all batch experiments, the ratio of dead to live cells did not increase after exposure to PROLI NONOate (data not shown). Batch biofilms were established using a complex, mixed-species community recovered from fouled RO membranes to determine the effect of NO on biofilm dispersal. The NO-induced dispersal for the RO bacterial community biofilm was 25% (standard deviation [SD] = 7; n = 3 experiments) (data not shown), which was comparable to the artificially recreated biofilms based on RO and MBR isolates (Fig. 2). Overall, the results suggest that NO can induce dispersal in RO and MBR isolates as well as mixed-species biofilms. Therefore, experiments were designed to test the effect of NO on dispersing biofilms in an RO system.

PROLI NONOate treatment for the prevention of RO membrane biofouling by PAO1. Biofilm formation in the RO module differs from that in the batch systems described above, where the RO module includes continuous flow and flux (the passage of water through the membrane), which facilitate transport of bacteria and nutrients to the surface. Additionally, the cross-flow increases aeration, which may result in faster inactivation of NO through binding to oxygen. Therefore, before testing the effect of NO on a mixed-species biofilm, the effect of NO on dispersing biofilms of the well-characterized P. aeruginosa was first tested.

Results from multiple replicate experiments in the absence of PROLI NONOate treatment showed that the TMP rise in both modules was very similar when inoculated with PAO1, with only a 0.3% difference in the time required to achieve a maximum TMP.
increase of 120% (Fig. 3A). If the experiment was stopped after one module had reached the maximum TMP percentage increase, the maximum TMP percentage difference between the two modules was only 6%. Thus, differences of >6% between fouling rates were considered to be significant.

The RO membrane without PROLI NONOate treatment fouled in 140 h, a TMP increase of 120% (Fig. 3B). The TMP initially showed a slow increase, ~20% in the initial 100 h, followed by an abrupt rise in TMP over the next 40 h. Over the same 140-h time period, the TMP of the RO injected with PROLI NONOate increased by only 68%, a 52% difference between the treated and untreated membranes fouled with PAO1. The biofilm biovolume (total cells) of the treated membrane was 28% less than the untreated membrane (Fig. 3C). Additionally, the mean viable bacterial count of the treated membrane (3.89 × 10^6) was 18% lower than that of the untreated membrane (4.72 × 10^6 CFU cm⁻²), reflecting the lower number of bacterial cells present on the membrane. The ratio of dead to live cells was greater in the untreated membrane compared to the treated membrane (Fig. 3C). The ratio of dead to live cells was greater in the untreated membrane (0.34) than in the PROLI NONOate-treated biofilm (0.27), revealing that the PROLI NONOate treatment did not have a bactericidal effect. The untreated PAO1 biofilm was thinner than the untreated control (24 compared to 32 μm, treated compared to control) and had a lower surface coverage (83% compared to 99%, treated compared to control), with more areas of unfouled membrane (Fig. 3D). Staining with ConA-FITC revealed that the average polysaccharide biovolume was 20% less for the treated membrane than for the control (Fig. 3C). This difference was also illustrated by EPS extraction, revealing a 52% reduction in polysaccharides on the treated membrane. A 25% reduction in protein was also observed for the treated membrane (Fig. 3C). Collectively, the data clearly show that NO induced dispersal of the PAO1 biofilm, leading to reductions in CFU and biofilm matrix components, and improved performance of the RO module. These results demonstrated that NO could be used in the complex RO module to induce dispersal at concentrations derived from the batch biofilm experiments, and therefore the experiment was repeated using an RO bacterial community.

**PROLI NONOate treatment for the prevention of RO membrane biofouling by a mixed RO bacterial community.** As described above for PAO1, the variability of fouling between the two RO modules was determined in the absence of NO addition. The TMP rise in both modules, when fouled with the R2A-cultured mixed RO bacterial community, was similar to that observed for the PAO1, with a 0.4% difference in the time required for a maximum TMP increase of 120% (see Fig. S1 in the supplemental material). The TMP profile was similar to that of the PAO1, with a slow initial increase in TMP, ~20% in the initial 92 h, followed by a more rapid increase where the maximum TMP was reached in the next 25 h (see Fig. S1). If the experiment was stopped after one module had reached the maximum TMP percentage increase, a maximum TMP percentage difference between the two modules was 8%, and therefore differences of >8% were considered to be significant.

The untreated RO membrane fouled in 117 h, a TMP increase of 120% (Fig. 3A). If the experiment was stopped after one module had reached the maximum TMP percentage increase, the maximum TMP percentage difference between the two modules was only 6%. Thus, differences of >6% between fouling rates were considered to be significant.

![Image](https://aem.asm.org/)

**FIG 3** (A) TMP profiles of the RO system inoculated with PAO1, both cells untreated. TMP values were recorded every 1 min over the experimental period. (B) TMP profiles of the RO system inoculated with PAO1, untreated versus treated with 40 μM PROLI NONOate every 24 h. (C) RO membrane autopsy results for untreated versus treated: biofilm biovolume as determined by staining with SYTO 9 and PI; biofilm biovolume as determined by staining with ConA-FITC; polysaccharide concentration as determined by EPS extraction; protein concentration as determined by EPS extraction. Error bars are SD, n = 3 experiments. (D) PAO1 biofilm on untreated versus treated membranes. Representative confocal images showing biofilm structure via live and dead bacterial cells stained with SYTO 9 and PI. Magnification, ×20. Scale bar = 100 μm.
of 112% (Fig. 4A). Over the same 117-h time period, the TMP of the PROLI NONOate-treated RO module increased by only 20%, a 92% difference between the NO-treated and untreated membranes. The biofilm biovolume (total cells) of the treated membrane was 29% less than that of the untreated membrane (Fig. 4B). Additionally, the number of CFU of the treated (8.85 × 10^6 CFU cm^-2) membrane was 23% lower than that of the untreated membrane (1.15 × 10^7 CFU cm^-2). The ratio of live to dead cells was lower in the untreated biofilm (0.83) than in the PROLI NONOate-treated biofilm (0.92), indicating that the PROLI NONOate was not bactericidal. The biofilm surface coverage (59% compared to 98%, treated compared to control) and thickness (20 μm compared to 26 μm, treated compared to control) was also significantly lower on the NO-treated membrane, which also showed more areas of unfouled membrane (Fig. 4C). There was 48% less polysaccharide and 66% less protein in the EPS extract of the treated membrane biofilm relative to the control (Fig. 4B).

Due to the decreased biofouling rate in the PROLI NONOate-treated RO cell, the experiment was repeated and modified where the NO treatment was continued until the TMP reached its maximum. The RO membrane without PROLI NONOate fouled in 115 h, a total TMP increase of 130% (Fig. 5). Over the same 115-h time period, the TMP of the RO injected with PROLI NONOate increased by only 33%, a difference of 97% between the NO-treated and untreated membrane, a similar result to the previous experiment. However, biofouling could not be prevented indefinitely, and the treated RO membrane eventually became fully fouled after 147 h, a 22% difference in time required to achieve the maximum TMP.

**Bacterial community composition of the membrane biofilm communities.** To determine the effect of NO treatment on the mixed-species biofilm community on the RO membranes, the community present in the initial scraping, the R2A cultured community, as well as the community that ultimately formed biofilms on the membrane surface during fouling were first determined. The raw biofilm scraping taken from the industrial RO membrane was quite diverse, containing at least 9 bacterial phyla (data not shown). The community was represented mainly by Proteobacteria (91%), followed by Actinobacteria (5.3%) and Bacteroidetes (2.75%) at lower abundances (data not shown). The Proteobacteria classes were dominated by Alphaproteobacteria (77.35%), followed by Betaproteobacteria (12.1%) and Gammaproteobacteria (1.4%) (data not shown). The Bray-Curtis index at the species level revealed an 87% similarity between duplicate samples. The Proteobacteria (85.3%) remained as the dominant phylum in the cultured samples, followed by Bacteroidetes (11.9%) (data not shown). The most abundant families were Bradyrhizobiaceae (33% ± 1%), an unknown family of the order Rhizobiales (18% ± 2%), Spingomonadaceae (12% ± 2%), Nitrosomonadaceae (8%), Rhodospirillaceae (8% ± 1%), Hyphomicrobiaceae (5% ± 1%), and Mycobacteriaceae (3%). The Bray-Curtis index at the species level revealed an 87% similarity between duplicate samples. The Proteobacteria (85.3%) remained as the dominant phylum in the cultured samples, followed by Bacteroidetes (11.9%) (data not shown). The Gammaproteobacteria class dominated the scraping samples (62.9%), followed by Betaproteobacteria (22.1%) (data not shown). The dominant families after culturing were Aeromon-
adaceae (43% ± 8%), Oxalobacteraceae (17% ± 3%), Pseudomonadaceae (12% ± 2%), and Flavobacteriaceae (6% ± 2%). The communities of the duplicate planktonic samples were very similar, with an 85% similarity at the species level as determined by the Bray-Curtis index.

When the bacterial communities of two untreated membrane biofilms from the two experiments described above were examined at maximum TMP, the Bray-Curtis index at the species level revealed a 92% similarity between samples (see Fig. S3 in the supplemental material), showing good reproducibility of the biofilm community between the parallel cells of the RO system. The dominant phylum of the membrane biofilm was Proteobacteria (59.2%), followed closely by Bacteroidetes (40.6%). Of the Proteobacteria, the Betaproteobacteria class dominated the membrane biofilm samples (35.4%), followed by Gammaproteobacteria (22.6%) and Alphaproteobacteria (1.3%) (data not shown). The dominant class of the Bacteroidetes phylum was Sphingobacteria (34.3%). Despite bacteria of the family Aeromonadaceae being the most abundant in the R2A cultures (see Fig. S2), the dominant bacterial family of the membrane biofilms was Chitinophagaceae (23%) (see Fig. S3), suggesting that this organism is a strong biofilm former and thus plays a significant role in the biofouling of RO system membranes. Other dominant bacterial families within the membrane biofilms were Oxalobacteraceae (23%), Enterobacteriaceae (14%), Comamonadaceae (13%), Sphingobacteriaceae (11%), Aeromonadaceae (7%), and Flavobacteriaceae (6%) (see Fig. S3).

Bacterial community composition of the membrane biofilms and the effect of PROLI NONOate. The untreated and treated membrane biofilms were examined after 115 and 147 h, respectively, once they had reached maximum TMP. In comparison to the untreated control, dosing with PROLI NONOate led to a decrease in the biofilm percentages of Chitinophagaceae (decrease from 43% to 20%), Sphingobacteriaceae (decrease from 13% to 4%), and Comamonadaceae (decrease from 12% to 7%) but an increase in the percentages of Oxalobacteraceae (increase from 9% to 21%), Enterobacteriaceae (increase from 11% to 29%), and an unknown family of the order Rickettsiales (increase from 6% to 9%) (see Fig. S3). However, the same six families of bacteria dominated the biofilms of both the treated and untreated membranes. The Bray-Curtis index at the species taxonomic level revealed a 76% similarity between samples, indicating that the PROLI NONOate treatment did not significantly alter the microbial composition of the membrane biofilm (see Fig. S3). The results from the Bray-Curtis similarity index dendrogram indicated that the variation between the RO runs was higher than the variation between the untreated and PROLI NONOate-treated membrane biofilms, further suggesting that the NO treatment did not significantly alter the biofilm community.

DISCUSSION

The growing need to improve water production for developed and developing nations has focused attention on membrane-based purification methods. However, biofilm formation and biofouling remains a significant issue for the technology, and thus there is a real need for novel approaches to control biological fouling. One recent discovery has been that bacteria produce and respond to NO and use this as a natural dispersal signal (16–18, 40, 41). Here, we have investigated the potential of PROLI NONOate to disperse bacteria isolated from fouled industrial reverse osmosis (RO) and membrane bioreactor (MBR) membranes and explored the potential application of PROLI NONOate as a novel strategy to control membrane biofouling in a laboratory-scale RO system.

It has been demonstrated in this study that micromolar concentrations of PROLI NONOate induce biofilm dispersal in a range of bacteria isolated from industrial membrane bioreactor and RO membranes. Interestingly, results showed that different bacteria responded differently to NO exposure, with biofilm dis-
persal percentages ranging from 10% to 46%. These results correspond with the literature where NO induces biofilm dispersal in some bacteria (17) but may stimulate biofilm formation in others (21–23), where it was observed here that NO induced biofilm formation for Acinetobacter spp. and Duganella spp. The response of these bacteria was different from that observed in a former study, when the NO donor compound MAHMA NONOate was employed (19). This difference is likely a consequence of the growth inhibitory nature of the MAHMA NONOate, which was shown to not be the case for the PROLI NONOate (19). In addition, each NO donor compound has a different half-life/release rate of NO, which may have impacted the bacteria differently (20, 42, 43). The concentration and time of NO gas exposure may trigger different dispersal behavior in certain species of bacteria and would be worth investigating in future studies. Overall, the mixed biofilm effects are similar for both NO donor compounds tested, and hence the data reveal that it is not easy to predict effects on mixed communities under realistic conditions by using single species. Importantly, the results showed that PROLI NONOate is not bactericidal at the concentration tested in this study. The biofilm ratio of dead to live cells did not increase for any bacterial genera tested after exposure to this compound.

Previous studies have investigated only NO-induced biofilm dispersal of P. aeruginosa and other bacterial species in batch experiments (16, 18, 19). However, in this study, it has been shown that the regular addition of an NO donor compound to a continuously operating laboratory-scale RO system can significantly reduce the rate of biofouling by both a single species of P. aeruginosa and also, more importantly, a mixed bacterial consortium isolated from a fouled industrial RO membrane. In the mixed bacterial community, dosing with PROLI NONOate led to a decrease in the biofilm percentages of Chitinophagaceae, Sphingobacteriaceae, and Comamonadaceae but an increase in the percentages of Oxalobacteraceae, Enterobacteriaceae, and an unknown family of the order Rickettsiales. It therefore also seems apparent in this study that while the dispersal of some bacteria is induced during exposure to low levels of NO, biofilm formation may be encouraged in others. It is important to note that the pyrosequencing does not provide an absolute quantification of the number of each organism present, only a relative abundance. Therefore, it is possible that all of these organisms are generally dispersed, but their individual responses differ, as shown in Fig. 2, resulting in a change in their relative abundances. Despite these different responses, the same six families of bacteria dominated the biofilms of both the treated and untreated RO membranes, with a microbial composition similarity of 76%. Thus, the addition of PROLI NONOate did not significantly alter the microbial composition of the membrane biofilm. In addition, the increase in certain biofilm members did not affect the success of the PROLI NONOate treatment to dramatically reduce the rate of biofouling in the RO system.

Previous work has shown that while the aggregation of bacterial cells on the membrane surface may decrease salt rejection and permeate flux by a biofilm-enhanced osmotic pressure mechanism, the EPS biofouling layer adversely impacts permeate flux by increasing the hydraulic resistance to permeate flow (10, 11). A recent study showed that the biofouling rate is dependent on both the quantity of EPS components and the surface coverage of the membrane biofilm, rather than the number of bacterial cells present (34). A lack of biofilm polysaccharides combined with areas of unfouled membrane dramatically reduced any hydraulic resistance to permeate flow, thus delaying the rate of biofouling (34). This study showed similar results, where a reduction in EPS constituents (protein and polysaccharide) and a decreased biofilm surface coverage after NO treatment led to significantly lower biofouling rates. A reduction in biofilm cells was also observed after PROLI NONOate treatment. There is an increasing interest in developing alternative, nontoxic methods to control microbial fouling in membrane-based water purification, and in particular there has been a focus on modification of the biofilm development program. For example, Yeon et al. recently demonstrated that disruption of microbial cell-cell signaling could similarly delay the TMP rise associated with biofouling of a membrane bioreactor (44). Thus, biologically derived strategies may represent an additional approach for the control of fouling communities.

This study has contributed to existing knowledge on the structure and diversity of industrial RO membrane biofilm communities (3, 35, 45–49). The advancement of modern molecular methods, such as pyrosequencing, combined with ever-decreasing analysis costs enable current studies to portray a detailed characterization of sample microbial diversity and abundance. Identifying common bacteria that contribute to the biofouling of industrial RO membranes could enable the development of tailored solutions for its control. The industrial RO membrane biofilm supported a bacterial community dominated by Proteobacteria, with a relatively low abundance of other phyla. The dominance of Proteobacteria on a fouled industrial RO membrane has been observed in other studies (3, 15, 35, 45–48), covering RO plants in the Netherlands (3), Australia (48), Israel (47), Italy (49), and Singapore (35, 46). The dominant class within this phylum was observed to be Alphaproteobacteria, also similarly observed in other studies (3, 35, 46, 48), followed by Betaproteobacteria (3, 35, 48). Two studies by Bereschenko et al. (15, 45) revealed that Betaproteobacteria are dominant over Alphaproteobacteria within immature biofilms, and this changes to a dominance of Alphaproteobacteria over Betaproteobacteria as the biofilm matures (15, 45). This is consistent with the complex community used here to inoculate the RO system, which was dominated by Alphaproteobacteria, and that was collected from fouled membranes that had been in use for more than 5 years. However, after inoculation and fouling for approximately 5 days, the community was observed to be dominated by Betaproteobacteria, consistent with previous observations of early-stage biofouling communities.

A recent study has exposed the difficulty in determining the most predominant and problematic microbial species in a specific water recycling plant, because species composition varies significantly from one plant to another depending on site-specific conditions such as feed water quality and seasons (48). However, by comparing the bacterial community data from the raw Kranji NeWater membrane scraping in this study with that of other published work, some common bacteria can be observed to occur on fouled industrial RO membranes. These include bacteria of the orders Sphingomonadales (47, 48), Rhizobiales (46, 47), Pseudomonadales (47), Xanthomonadales (47), and Burkholderiales (45, 47), bacteria in the families Comamonadaceae (3, 15, 45, 47), Nitrosomonadales (48), Flavobacteriaceae (48), Microbacteriaceae (48), Pseudomonadaceae (48), Kineosporiaceae (48), and Planctomycetaceae (3), and bacteria of the genera Bacillus (19, 35), Bradyrhizobium (15, 35, 48), Rhizobium (48), Sphingomonas (3, 15, 35, 45, 46, 48, 49), Sphingopyxis (3, 15, 45, 48), Pseudomonas (3, 15, 19, 45, 48), Mycobacterium (3, 15, 48), Hyphomicrobium (3,
Membrane demonstrate strong evidence for the application of PROLIRO module to reach its maximum TMP. Importantly, community NONOate also led to a 22% increase in the time required for the film surface coverage and thickness. The addition of PROLI NONOate to a laboratory-scale RO system reduced the rate of biofilm formation by bacteria isolated from industrial membrane bioreactor systems. The addition of PROLI NONOate every 24 h to a laboratory-scale RO system, and Miles Rzechowicz and Stanislaus Suwarno for designing the lab.

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