AQUA
Water Infrastructure, Ecosystems and Society

IWA PUBLISHING

 AQUA — Water Infrastructure, Ecosystems and Society Vol 72 No 3, 313 doi: 10.2166/aqua.2023.217

Efficacy testing of non-oxidizing biocides for polyamide membrane biofouling prevention using a modified CDC biofilm reactor

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ABSTRACT

Biofouling is one of the most challenging obstacles faced by reverse osmosis (RO) membrane systems to supply potable water. Currently, biofouling is imperfectly handled by RO feed water pre-chlorination, which is associated with the production of carcinogenic disinfection by-products. To propose a safer alternative to control biofouling in RO drinking water applications, this study investigates the efficacy of five biocides to prevent and remove *Pseudomonas aeruginosa* biofilms from RO membranes: 2-methyl-4-isothiazolin-3-one (MIT); 2,2-dibromo-3-nitrilopropionamide (DBNPA); sodium bisulfite (SBS); sodium benzoate (SB); and ethyl lauroyl arginate (LAE). Experiments were conducted on the Center for Disease Control (CDC) Biofilm Reactor (CBR) with biocidal dosing estimated on 96-well microtiter plates. Confocal Scanning Laser Microscopy (CLSM) and Scanning Electron Microscopy (SEM) were used to analyze the biocides' anti-biofilm efficacies under dynamic conditions relative to minimum biofilm inhibitory and eradication concentrations. The results in this study indicated that LAE presented the best anti-biofilm efficacies in treating *P. aeruginosa* biofilms when compared to all studied biocides; it not only prevented biofilm formation (>98%) but also it effectively removed pre-established biofilms (>99%) from RO membrane coupons. Therefore, due to safety and efficacy, LAE is an excellent candidate for controlling biofouling in drinking water RO membrane systems.

Key words: biocides, biofilm prevention, biofouling, drinking water, polyamide membranes, reverse osmosis

HIGHLIGHTS

- LAE is an excellent candidate to control biofouling in RO systems.
- MIT and DBNPA are excellent models for anti-biofilm efficacy in biofouling studies.
- SBS is not efficacious in treating pre-established biofilms.
- SB presents moderate anti-biofilm efficacies.

INTRODUCTION

Reliance on unconventional water resources is becoming increasingly necessary to bridge the water demand–supply gap. Of these sources, desalinated water is critical in relieving the global impacts of water stress, with the potential of providing a climate-independent and virtually unlimited supply of high-quality potable water (UN-Water 2020). Currently, reverse osmosis (RO) leads the desalination industry, boasting the highest energy efficiency of both thermal and membrane-based technologies and accounting for 69% of the installed desalination capacity (Zarzo & Prats 2018; Feria-Díaz *et al.* 2021). Despite the growing amount of research and resources expended on RO technology, optimization remains elusive due to fouling – the build-up of colloidal and particulate inorganic, organic, and biological matter on the membrane's surface (Eke *et al.* 2020). Biofouling, in particular, has been identified as the most difficult to control, as living cells are proliferative, and many of the stressors applied as treatment exacerbate natural defense mechanisms, such as planktonic cell attachment and secretion of extracellular polymeric substances (EPS), that support and strengthen biofilm formation (Nguyen *et al.* 2012; Kucera

2019). Almost any degree of surface colonization can lead to significant reductions in permeate flux and salt rejection, which can result in substantial economic losses as a result of reduced efficiency, a shorter membrane lifetime, and the need for physical and chemical mitigation strategies (Kucera 2015, 2019).

Presently, controlling the impacts of biofouling on RO performance is achieved through two primary means: (1) disinfection and (2) cleaning techniques (Nguyen *et al.* 2012; Kucera 2015, 2019). Chlorine is the most commonly applied disinfectant, killing a wide range of microorganisms via oxidation at low concentrations (Nguyen *et al.* 2012; Kim & Park 2015; Kucera 2015). However, its use in RO systems is limited by the polyamide membranes' susceptibility to oxidative degradation, the formation of harmful disinfection by-products, and its corrosivity and toxicity (Kim & Park 2015; Kucera 2015, 2019; Silva *et al.* 2020). Membrane incompatibility, in particular, is detrimental to biocidal efficacy, as oxidative biocides must be removed prior to membrane contact. This leaves the system downstream and most critically the membrane itself, vulnerable to bacterial regrowth. Since no pretreatment method is 100% efficacious, physical cleaning techniques that dislodge foulants using mechanical force are required as secondary treatment options (Nguyen *et al.* 2012; Kucera 2015, 2019). These techniques, such as membrane flushing, are efficacious at removing loosely bound layers but cannot remove mature biofilms and residual foulants (Kucera 2019; Matin *et al.* 2021). Furthermore, they are only applied in response to biofilm formation – the maturity of which is marked by a significant increase in mechanical and chemical resistance (Flemming *et al.* 1997). This is largely due to the secretion of the EPS, which serves to protect biofilm cells and anchor them to the RO membrane, resulting in enhanced biocidal tolerance (Bridier *et al.* 2011). Most notably, biocides have difficulty penetrating the 3D matrix of mature biofilms and those that do are delivered to cells in sub-lethal concentrations, creating the ideal environment for the propagation of antimicrobial resistance (Donlan 2002; López *et al.* 2010; Bridier *et al.* 2011; Kucera 2015; Curtin *et al.* 2021). Current methods are only able to reduce and delay biofilm formation; therefore, it is necessary to use preventative and membrane-compatibility treatments to promote sustainable RO desalination.

One approach that has demonstrated capacity for long-term biofouling mitigation in potable water treatment systems is the application of non-oxidizing biocides (Kucera 2015; Kim & Park 2016; Curtin *et al.* 2020). This class of antifouling compounds has the potential to meet the majority of the requirements for an ideal biocide listed by Bates (1998), including being low risk to human health and the environment, showing high efficacy in the prevention and removal of all types of microorganisms and providing increased compatibility with the polyamide membrane and all other system components. In this study, based on the above-mentioned characteristics, five non-oxidizing biocides – 2,2 dibromo-3-nitrilopropionamide (DBNPA), 2-methyl-4-isothiazolin-3-one (MIT), sodium bisulfite (SBS), sodium benzoate (SB), and ethyl lauroyl arginate (LAE) – were selected and investigated for their ability to prevent and control biofouling in RO systems. Our previous review (Da-Silva-Correa *et al.* 2022) provided an in-depth analysis of the selected biocides and summarized important research gaps such as the identification of inhibitory concentrations and laboratory-scale biofouling prevention studies that are explored in our current work.

A critical characteristic of an ideal biocide is high efficacy (the ability to produce an intended result) in the prevention and removal of microorganisms (Bates 1998). A biocide's efficacy is largely subject to its Mechanism of Action (MoA) (Kucera 2015, 2019). Biocides in this study can be divided into two categories accordingly: electrophiles and membrane-active biocides. MIT and DBNPA are moderate electrophiles that disrupt biofilm growth by entering the cell wall of the microorganism, by diffusion or active cell transport, resulting in permanent damage to intracellular proteins, carbohydrates, lipids, and nucleic acids (Williams 2007; Kucera 2015, 2019; Siddiqui *et al.* 2017). SBS, SB, and LAE are membrane-active biocides that disrupt the cell membrane function and structure (Penna *et al.* 2002; Kucera 2015; Kim & Park 2016; Curtin *et al.* 2021). Besides their primary MoAs, the biocides present multiple pathways that lead to cell inactivation or death (Penna *et al.* 2002; Hydranautics Nitto Group Company 2015; Kucera 2015, 2019; Kim & Park 2016; Curtin *et al.* 2021). The diverse nature of these MoAs makes it difficult for microorganisms to build biocidal resistance (Williams 2007; Hydranautics Nitto Group Company 2015; Kucera 2015; Kim & Park 2016), increasing the longevity of biofouling mitigation.

Of the biocides in this study, three are already applied in potable water applications: MIT, DBNPA, and SBS (Penna *et al.* 2002; Kucera 2015, 2019; Oh *et al.* 2017). MIT has been successfully implemented in both membrane cleaning and membrane storage, but the ability of MIT to prevent biofouling has not been studied (Kucera 2015). DBNPA, an effective non-oxidizing biocide has been applied in RO systems; however, DBNPA may not be used directly to treat the water due to its toxicity (Bertheas *et al.* 2012; Kucera 2015). SBS has been dosed as the reducing agent to remove free chlorine after chlorine disinfection and has strong antimicrobial properties. It also poses a low hazard to the environment, supporting its potential use as a preventative biocide (Penna *et al.* 2002; Kucera 2015, 2019). The remaining biocides in this study, SB and LAE, are of

very low risk to human and environmental health (Kim & Park 2016; Buckley *et al.* 2017; Da-Silva-Correa *et al.* 2022). SB has been used as a preservative in cosmetic, personal care products and as an antimicrobial in food such as meat and dairy products (Cosmetic Ingredient Review 2001; Code of Federal Regulations 2019), while LAE is a common food additive (Kim & Park 2016). SB is inexpensive and highly soluble in water and both have demonstrated strong antimicrobial properties, making them highly suitable for potable water RO systems (Kim & Park 2016). Despite these favorable qualities, further studies are required to prove their efficacy in inhibiting and eradicating biofilms.

In RO applications, it is essential to determine the optimal concentration of biocides. These concentrations may be referred to as the Minimum Biofilm Inhibiting Concentration (MBIC), the Minimum Biofilm Eradication Concentration (MBEC) and are typically determined under static conditions. The MBIC is the minimum concentration at which the biocide may prevent biofilm growth, while the MBEC is the minimum concentration at which the biocide may remove existing biofilms. Previous studies have reported the MBIC and MBEC values for MIT as 22.5 and 78 mg/L, respectively, and for SB as 25,000 and 32,000 mg/L, respectively (Curtin *et al.* 2021); however, there are no data reporting the MBIC and MBEC values for DBNPA, SBS, and LAE. Therefore, these values must be derived experimentally. The MBIC and MBEC of each biocide are critical to achieving biofilm prevention, removal, and attaining the lowest, most effective biocide dosage in the RO system. Once MBIC and MBEC values have been determined, they can be used to investigate biocidal efficacy under dynamic conditions.

In a dynamic system hydrodynamics at a surface greatly impact biofilm stability and development (Stoodley *et al.* 2002). To this end, several standardized laboratory systems, aptly named biofilm reactors (bioreactors) are available – each one defined by the unique range of hydrodynamic conditions they produce (Azeredo *et al.* 2017; Gomes *et al.* 2018). In this work, we use the Center for Disease Control (CDC) Biofilm Reactor (CBR). Originally developed by the CDC for the study of *Legionella pneumophila* disinfection in potable water systems (Donlan *et al.* 2001), the CBR is a continuously stirred tank reactor that has since demonstrated versatile and broad application in the study of biofilm growth and resistance (Kappachery *et al.* 2010; Lade & Paul 2015; Lee *et al.* 2017a; Ferrer-Espada *et al.* 2019; Werner *et al.* 2019; Johnson *et al.* 2021). It is well reported that high shear stress promotes adherence to surfaces and the development of a stronger EPS due to a diminishing hydrodynamic boundary layer; thereby creating biofilms that are more resistant to mechanical and chemical antifoulant strategies (Donlan *et al.* 2001; Stoodley *et al.* 2002). The ASTM International has approved two methods for the growth of reproducible *Pseudomonas aeruginosa* biofilms under high shear within the CBR: Standard Test Method E2562 (ASTM International 2017) and Standard Practice E3161 (ASTM International 2018). These methods have been widely applied in the investigation of biofilm prevention and removal in RO systems through various strategies, including the addition of quorum quenching compounds (Kappachery *et al.* 2010; Kim *et al.* 2021), mucolytic agents (Kappachery *et al.* 2012), copper-based disinfectants (Lee *et al.* 2017a, 2017b, 2020), and chlorinated biocides (Yu *et al.* 2013).

Kappachery *et al.* (2010), examined the application of vanillin (a natural quorum quenching compound) as a continuous feed or shock dosing treatment by exposing polyamide membranes to 1×10^6 CFU/mL of *Aeromonas hydrophila* for incubation periods of 1–7 days under standard CBR biofilm growth conditions (ASTM International 2018). Biofilm growth in the presence of vanillin was suppressed by over 93% as measured by surface coverage, average thickness, total biomass, and total protein, that demonstrates high viability as a continuous feed treatment. However, it had no effect once the biofilm was already formed, excluding it as a feasible shock treatment. Similar results were found in their 2012 study using N-acetylcysteine, where a preliminary determination of the Minimum Inhibitory Concentration (MIC) was performed in polystyrene microtiter plates prior to efficacy testing against a multi-species culture (*A. hydrophila, Pseudomonas putida, Stenotrophomonas sp.*, and *Serratia marcescens*) in the CBR (Kappachery *et al.* 2012). These investigations highlight the practicality of the CBR in temporally varied experiments and its replicability in both biofilm prevention and removal studies. To study the impacts of biofouling on RO performance metrics, permeate flux, and salt rejection, Lee *et al.* (2020) used a laboratory-scale cross-flow RO system in conjunction with a CBR. The researchers noticed that the efficacies of various biocide combinations were lower in the pressurized unit due to heightened compression and densification of the EPS; however, results regarding relative biofilm inactivation efficacy were consistent across the two methods (Lee *et al.* 2017b, 2020). These works provide evidence for the relevancy and scalability of the CBR as a primary testing method within this context and set a precedent for the validity of future work, such as our own.

Aim and novelty of the study

This study yields a preliminary assessment of green chemistries as alternative approaches for the prevention and removal of biofilms in drinking water RO membrane applications and provides a stronger understanding of potential biocidal

performance in RO modules using a smaller, safer, and simpler dynamic system. Specifically, this work (a) estimates the MBEC and MBIC of non-oxidizing biocides not currently reported in the literature, including DBNPA, LAE, and SBS, using microtiter plates; and (b) tests the relative efficacy of the selected biocides, including MIT, PE, SB, DBNPA, LAE, and SBS, in removing or preventing biofilms on RO membrane coupons in a CDC biofilm reactor (CBR 90, BioSurface Technologies Corporation, Bozeman, MT, USA). It is important to note that in this study each biocide is dosed, compared at their optimal concentrations (as determined by MBIC and MBEC static testing) and not at the same concentration, i.e. the relative efficacy under dynamic conditions as compared to static tests is determined. The results of this article are paramount to solving the issues of biofouling in potable water applications, such as the production of carcinogenic by-products due to chlorine-feed water pretreatment. The work presents a feasibility analysis of the application of greener strategies for biofouling control in RO systems.

MATERIALS AND METHODS

Bacterial strain and growth conditions

P. aeruginosa strain ATCC 10145™ was used in the MBIC/MBEC experiments as well as in the CDC biofilm reactor experiments because it is a well-known model biofilm organism commonly applied to study the anti-biofouling efficacy of antimicrobials applicable to RO systems (Kucera 2019; Curtin *et al.* 2020, 2021). This is because, in addition to *P. aeruginosa* being a known biofilm-forming pioneer organism commonly found in RO systems, *P. aeruginosa* forms a single-species biofilm that is extremely hard to treat due to its resilient nature and multi-drug resistance; making anti-biofilm efficacy studies with this microorganism relatively translatable to more complex biofilms (Heydorn *et al.* 2000; Poole 2011; Kucera 2015, 2019; Kim & Park 2016; Nagaraja *et al.* 2017; Suwarno *et al.* 2018; Curtin *et al.* 2020, 2021). The inoculum was prepared by streaking out one isolated colony of *P. aeruginosa* from a Tryptic Soy Agar (TSA) (Thermo Fisher Scientific, MA, USA) plate, which was stored at 4 °C. *P. aeruginosa* TSA plates were made from *P. aeruginosa* stocks stored at −80 °C in glycerol (Allkja *et al.* 2020; Curtin *et al.* 2021). A single colony of *P. aeruginosa* was transferred to a falcon tube with 5 mL of full-strength Tryptic Soy Broth (TSB) (Thermo Fisher Scientific, MA, USA) and was incubated in an incubator shaker (VWR 1575 Incubator Shaker, orbital diameter of 1.9 cm) for 18 h at 37 °C and 200 rpm (Allkja *et al.* 2020; Curtin *et al.* 2021). The bacterial cells were harvested at the exponential growth phase (OD = 1.0 [600; WPA CO 8,000 cell density meter]). In the MBIC and MBEC protocol, the overnight culture was centrifuged (3,000 rpm, 22 °C, 10 min, centrifuge model: Allegra X-12R), resuspended in 5 mL of full-strength TSB (MDL Number: MFCD00132536, Sigma-Aldrich Canada) and diluted to a concentration of approximately 10^6 CFU/mL. In the experiments performed in the CDC biofilm reactor, the overnight culture was resuspended in 5 mL of deionized (DI) water (Milli-Q IQ 700, Millipore Sigma, Darmstadt, Germany) and diluted to approximately 10^5 CFU/mL.

Determination of MBICs and MBECs

The two protocols applied to determine MBIC and MBEC values followed the same experimental procedure described in Curtin *et al.* (2021). In short, the total mean green fluorescence, after staining, associated with the total live biofilm biomass after 24 h was investigated for 12 different concentrations of each biocide. The experiments presented four experimental wells per biocide concentration, 12 wells for the positive control (MIT [CAS-Number: 2682-20-4, Sigma-Aldrich, Canada], bacteria and nutrients), 12 wells for the negative control (bacteria and nutrients), and 24 wells for the references (12 wells with nutrients only; 12 wells with nutrients and biocide) (Allkja *et al.* 2020; Curtin *et al.* 2021). A second 96-well plate was used to determine the background fluorescence of the positive control (96-wells with MIT and nutrients). The references were used to remove background fluorescence (Curtin *et al.* 2021). The mean fluorescence values were calculated by subtracting corresponding references from the experimental wells and controls' fluorescence values (Allkja *et al.* 2020; Curtin *et al.* 2021). Supplementary material, Figure S1 illustrates the 96-well plate arrangement for the determination of MBICs and MBECs. Supplementary material, Figure S2 illustrates the 96-well plate arrangement for the determination of the background fluorescence of MIT (nutrients and biocide). The experiments were performed in triplicate on independent weeks.

In the MBEC protocol, 100-μL aliquots of the bacterial cells, resuspended in TSB, were added to the experimental wells, controls, and references in a black-sided, clear-bottom polystyrene 96-well plate (Thermo Fisher Scientific, MA, USA) according to Supplementary material, Figure S1. The plate was incubated in the incubator shaker for 24 h at 200 rpm and 37 °C to allow biofilm growth (Curtin *et al.* 2021). After incubation, the planktonic bacterial suspension was gently removed by rinsing each well three times with a multichannel pipette (Biorad Multichannel Micropipette, eight-channel, 20–200 μL; 1660495)

fitted with 200 μL of sterile DI water (Allkja *et al.* 2020; Shatila *et al.* 2020; Curtin *et al.* 2021). The rinsing step was performed by inserting the 200 μL tip slowly at 45° avoiding touching the sides and bottom of the wells (Allkja *et al.* 2020). After planktonic cell removal, biocides were added to the plate and incubated in the incubator shaker for 24 h at 200 rpm and 37 °C (biocide treatment) (Allkja *et al.* 2020; Curtin *et al.* 2021). The biocides were prepared in a separate 96-well plate (Costar 96-well Cell Culture Plate, flat bottom with low evaporation lid, polystyrene, Corning Incorporated, NY, USA) and added to the experimental, reference, and control wells to yield the following final concentrations: SB: 40,000–20 mg/L [CAS-Number: 532-32-1; Botanic Planet, ON, Canada], LAE: 20,000–10 mg/L [CAS-Number: 60372-77-2; The US Agricultural Research Service, CA, USA]; DBNPA: 3,000–1 mg/L [CAS-Number: 10222-01-2; Sigma-Aldrich, Canada]; and MIT: 2,250–1 mg/L, according to the plate arrangement presented in Supplementary material, Figure S1. The plate inoculation, incubation, and biocide treatment in the MBIC protocol followed the same procedure described in the MBEC protocol; however, biocides were co-incubated with the bacterial cells, meaning that plate inoculation and biocide treatment occurred simultaneously (Macia *et al.* 2014; Curtin *et al.* 2021).

After plate incubation and biocide treatment, biofilms were stained with the LIVE stain (SYTO9; 5 μM) and DEAD stain (propidium iodide (PI), 30 μM) from the LIVE/DEAD Baclight viability kit (L 7012; Invitrogen, MA, USA). A 150-μL mixture of the stain solution diluted in DI water was added to each well slowly at 45° (avoiding the sides and bottom of the wells) using a multichannel pipette (Allkja *et al.* 2020; Curtin *et al.* 2021). The plate was then incubated at room temperature in the dark for 15 min. After staining, plates were rinsed one time with DI water to remove stain residues as prescribed by the manufacturer. Finally, green (excitation wavelength: 485 nm; emission wavelength: 528 nm) and red (excitation wavelength: 485 nm; emission wavelength: 645 nm) fluorescence were measured in a plate reader (Cytation 5, Gen5 3.08 software, Biotek, VT, USA) (Curtin *et al.* 2021). The MBIC was determined as the minimum concentration of a biocide that can reduce more than 90% of the mean green fluorescence values of experimental wells when compared to the negative control (Macia *et al.* 2014; Curtin *et al.* 2021). In the MBIC protocol, the efficacy of the biocides in preventing biofilm formation is assessed. Similarly, the MBEC was determined as the minimum concentration of a biocide that can reduce more than 90% of the mean green fluorescence values of experimental wells when compared to the negative control (Macia *et al.* 2014; Curtin *et al.* 2021). In the MBEC protocol, the efficacy of biocide in removing a pre-established biofilm is determined.

Synthetic feed water and RO polyamide membranes

The synthetic feed water for biofouling experiments in the biofilm reactor was designed based on standard pH and temperature for RO studies (pH 7 and 25 °C) and typical water quality of RO system influent after pretreatment, exacerbating parameters known to cause biofouling in RO systems: nutrient and microorganism concentration (*P. aeruginosa* at 10^5 CFU/mL) (Kucera 2015, 2019). Nutrient concentration (TSB) in the feed water was set to approximately 30 mg/L of equivalent Total Organic Carbon (TOC) in the CDC biofilm reactor (Suwarno *et al.* 2014, 2018). Background salinity was set to 500 mg/L (NaCl; 7647-14-5; Thermo Fisher Scientific, MA, USA) (Kucera 2015). To ensure anti-biofilm efficacies, the selected biocides (LAE, MIT, DBNPA, PE, SB, and SBS [CAS-Number: 7631-90-5; Sigma-Aldrich, Canada]) were dosed at 2× MBIC values for biofilm prevention tests and 2 × MBEC values for biofilm removal studies (Magnusson *et al.* 1995; Kucera 2015; Rivera Aguayo *et al.* 2020). Commercial RO polyamide membranes (TriSep, YMACM34205) were used for biofouling experiments. The RO membranes were cut into 12 mm diameter discs, sterilized with 70% ethanol, and affixed onto the coupons of the biofilm reactor with the feed side facing the center of the reactor (Kappachery *et al.* 2010; Suwarno *et al.* 2012, 2014; Werner *et al.* 2019).

Anti-biofouling efficacy testing in the CDC biofilm reactor

The two protocols applied for biofilm prevention and biofilm removal studies were adapted from the ASTM standard test method for quantification of 48 h *P. aeruginosa* biofilms using CDC biofilm reactors (CBR 90, BioSurface Technologies Corporation, Bozeman, MT, USA): ASTM E2562. The CBR contains eight rods with three coupons in each rod. Prior to the biofouling experiments, CBR, tubing, and carboys were sterilized as described in previous studies (Huang *et al.* 2016; Lee *et al.* 2017a). Furthermore, RO membranes and the high-pressure pump (Optos Series 2HM, Eldex, Napa, CA, USA) were sterilized as described in Suwarno *et al.* (2014). The CBR system was set up as depicted in Rautiola (2013). To evaluate the efficacy of the selected biocides in removing biofilms from RO membranes, 12 mm polyamide membrane coupons were affixed onto the coupons of the biofilm reactor with the feed side facing the center of the reactor using 3M double-sided

tape (Lee *et al.* 2017a). The bioreactor was inoculated with approximately 10^5 CFU/mL of *P. aeruginosa* to grow a 24 h *P. aeruginosa* biofilm. For the first 24 h, the CDC reactor was operated in batch mode at 100 rpm, 25 °C, pH 7 and an operation volume of 350 mL (TOC of 30 mg/L and NaCl of 500 mg/L). For the last 24 h, synthetic feed water solution was continuously fed to the CDC reactor at 11.7 mL/min under the same conditions to allow further biofilm development. After 48 h of growth, biofilms were treated for 24 h with a biocide concentration at 2× MBEC to test biofilm removal efficacy. Biofilm prevention experiments followed the same procedure as the biofilm removal studies with the 48 h of *P. aeruginosa* biofilm growth period done in the presence of biocides at 2× MBICs (Kappachery *et al.* 2010; Kucera 2015; Nagaraja *et al.* 2017; Curtin *et al.* 2020). In other words, to test biofilm prevention efficacy, *P. aeruginosa* biofilms were grown over 48 h in the presence of biocide (prevention protocol). To test biofilm removal efficacy, the bioreactor was inoculated to grow a biofilm unencumbered over 48 h (removal protocol). After treatment, Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM) were used to visualize and quantify biofilms and bacterial cells (Heydorn *et al.* 2000; Donlan 2002; Nagaraja *et al.* 2017). The biofouling experiments were performed in triplicate on independent weeks.

Confocal scanning laser microscopy

For a quantitative analysis of biofilm prevention and removal efficacies, the LIVE/DEAD Baclight viability kit (LN 7007) was used to stain nucleic acids, differentiating intact bacterial cells (LIVE) bound to the SYTO 9 stain from ruptured bacterial cells (DEAD) bound to PI (Nagaraja *et al.* 2017; Curtin *et al.* 2021). Furthermore, the Concanavalin A stain (C11252 Concanavalin A Alexa Fluor 488 conjugate kit) was used to quantify EPS (biofilm matrix), as it binds to polysaccharides, which are the main component of the biofilm matrix (Heydorn *et al.* 2000; Nagaraja *et al.* 2017). The membrane coupons containing biofilms were stained following the specifications of the kits discussed herein. In summary, after biocide treatment in the bioreactor, the RO membranes with biofilms were rinsed three times with DI water to remove planktonic bacteria (Donlan 2002; Stepanović *et al.* 2007; Suwarno *et al.* 2012; Nagaraja *et al.* 2017) and then stained for 15 min at room temperature using the following dye concentrations: (i) SYTO 9 at 5 μM, (ii) PI at 30 μM, and (iii) Concavalian A at 50 μM. After staining, the RO membranes were rinsed with DI water to remove residual dyes and wet mounted on clean glass slides (Nagaraja *et al.* 2017; Oh *et al.* 2017). Furthermore, the biofilm matrix, as well as live and dead bacterial cells were visualized in a Zeiss LSM 880 CLSM at 20× magnification with representative areas of 531.4 μm × 531.4 μm and a resolution of 1,784 × 1,784 pixels (16 bit) (Heydorn *et al.* 2000; Lade & Paul 2015; Nagaraja *et al.* 2017). Biofilm analysis was performed in the IMARIS software from CLSM Z-stacks images collected from at least three randomly selected areas on the RO membrane (version9.8.0, Bitplane) (Lade & Paul 2015; Nagaraja *et al.* 2017). In this software, the CLSM 3D images were processed and biofilm thickness, biofilm volume, biofilm appearance, and live-to-dead bacterial ratio (anti-biofilm efficacy) were quantified and analyzed as described in Heydorn *et al.* (2000), Donlan (2002), Lade & Paul (2015) and Nagaraja *et al.* (2017).

Scanning electron microscopy

For a qualitative analysis and further assessment of anti-biofilm efficacy, biofilms developed on RO membrane coupons were visualized in SEM (SEM Hitachi S-4800) (Hazrin-Chong & Manefield 2012; Shatila *et al.* 2020). For imaging preparation, after biocide treatment, the biofilm membrane coupons were rinsed three times with DI water to remove planktonic cells, then immersed in 2.5% glutaraldehyde (CAS-Number: 111-30-8, Thermo Fisher Scientific, MA, USA) and diluted in a phosphate-buffered saline (PBS; Cytiva, Marlborough, MA, USA) solution at 4 °C for 4 h for bacteria fixation. The membrane coupons were then rinsed two times with DI water. After rinsing, the biofilms were dehydrated in each of the following ascending ethanol concentrations for 5 min at room temperature: 30, 50, 70, 80, 96, and 100% (Donlan 2002; Stepanović *et al.* 2007; Hazrin-Chong & Manefield 2012; Shatila *et al.* 2020). After dehydration, the biofilms were dried with the following concentrations of hexamethyldisilazane (HMDS; CAS-number: 999-97-3, Thermo Fisher Scientific, MA, USA) for 30 min at room temperature: 50 and 100% (Hazrin-Chong & Manefield 2012). Finally, the biofilm membrane coupons were coated with gold by sputter deposition for 120 s (Anatech Hummer VI Sputter Coater). The SEM images were taken with magnifications of 1,000 × ; 5,000 × ; and 10,000× at a voltage of 1 kV (Hazrin-Chong & Manefield 2012; Gomes & Mergulhão 2017; Shatila *et al.* 2020; Schu *et al.* 2021).

Statistical analysis

Statistically significant differences were analyzed using a one-way ANOVA and Student's *t*-test using Microsoft Excel software. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

MBICs AND MBECs

To find the operational biocide dosage for biofouling experiments in the CBR, the MBIC and MBEC values of DBNPA, SBS, and LAE for *P. aeruginosa* were estimated. It is important to note that the MBICs and MBECs for *P. aeruginosa* for the two remaining biocides in this study were already found in the literature (MIT – MBIC: 22.5 mg/L and MBEC: 78 mg/L; SB – MBIC: 25,000 mg/L and MBEC: 32,200 mg/L) (Curtin *et al.* 2021). Therefore, Table 1 only presents the MBICs and MBECs of DBNPA, SBS, and LAE.

LAE and DBNPA presented the lowest MBICs among the studied biocides, with values of 63 mg/L (0.15 mM) and 125 mg/L (0.50 mM), respectively. Compared with SBS, which presented an MBIC value of 1,667 mg/L (16.05 mM), both chemicals demonstrate high efficacy in preventing *P. aeruginosa* biofilms under static conditions. This result is unsurprising, as DBNPA and LAE function under similar MoAs, mainly killing microorganisms by damaging their cell membrane (Kucera 2015; Kim & Park 2016; Farinelli *et al.* 2021). Although SBS is also a cell membrane-active biocide, its main mechanism of action is through oxygen scavenging, which often requires higher concentrations to be effective. For instance, the MIC of SBS for *P. aeruginosa* is 780 mg/L – more than 10 times the MICs of DBNPA and LAE (Penna *et al.* 2002; Kim & Park 2016; Kucera 2019). It should also be noted that the MBIC of LAE was approximately two times lower than that of DBNPA. This relationship is best supported by Kim & Park (2016), which found the same correlation between the MIC of LAE (31.3 mg/L) and the MIC of DBNPA (62.5 mg/L) for *P. aeruginosa*. These results suggest that LAE is more effective at inhibiting biofilm formation than DBNPA (Kim & Park 2016), a performance difference that can be primarily attributed to the cationic nature of LAE, which strengthens interactions with negatively charged surfaces of microorganisms and facilitates disturbance of cell membrane structure and potential (Kim & Park 2016).

In general, all the MBIC values were lower than the MBEC values for their respective biocides. DBNPA had the lowest MBEC value (375 mg/L or 1.55 mM) out of the tested biocides followed by SBS with MBEC of 2,500 mg/L (24.05 mM) and LAE with a MBEC of 5,000 mg/L (11.90 mM) (Table 1). The same trend was observed by Curtin *et al.* (2021) and Güven & Kaynak Onurdağ (2014). These results indicate that preventing biofilm formation requires less biocide than removing an established biofilm. A possible explanation for this trend is that in the MBIC protocol, the biofilms were exposed to the biocides in an earlier stage of development, while in the MBEC protocol, the biocides were applied to mature biofilms (strong biofilms). This is because mature biofilms present a higher resistance to antimicrobials for several reasons, such as the lower diffusion rate of antimicrobials into the biofilm due to a more developed biofilm matrix and the increased presence of persisters in the biofilm microbial community (Donlan 2002; López *et al.* 2010; Curtin *et al.* 2021).

ANTI-BIOFOULING EFFICACY TESTING IN THE CDC BIOFILM REACTOR

Confocal scanning laser microscopy

CLSM analyses were carried out in order to investigate the anti-biofilm efficacy of several non-oxidizing biocides in preventing and removing *P. aeruginosa* biofilms on commercial RO membranes. *P. aeruginosa* is a common biofilm pioneer in RO systems. For this experiment, 48 h *P. aeruginosa* biofilms were grown on polyamide RO membranes in a CBR under conditions designed to mimic conditions in RO systems for potable water applications. For the prevention protocol, the biofilms were developed from the beginning of the experiment in the presence of biocides at two times their corresponding MBIC values for 24 h. For the biofilm removal protocol, the biofilms were treated with biocides at two times their

Table 1 | MBICs and MBECs of DBNPA, SBS, and LAE for *Pseudomonas aeruginosa*

	DBNPA mg/L	SBS	LAE
MBIC	125	1,667	63
MBEC	375	2,500	5,000

Note: MBIC, minimum biofilm inhibitory concentration; and MBEC, minimum biofilm eradication concentration. The MBIC and MBEC values were determined to be significantly different from the growth control (Reference 2, Supplementary material, Figure S1) via Student's *t*-test ($p < 0.05$). Each MBEC and MBIC value in the triplicate experiments showed not to be significantly different from each other via ANOVA ($p > 0.05$) in the respective biocide-associated experiments. MBIC (Supplementary material, Figures S4–S6) and MBEC (Supplementary material, Figures S8–S10) graphs can be found in the supplementary material, available in the online version of this paper.

corresponding MBEC values after the 48-h *P. aeruginosa* biofilm was established. The CLSM analysis compared biofilm quantification parameters between untreated biofilms and treated biofilms. The biocides' anti-biofilm efficacy was quantified and analyzed in terms of biofilm appearance, biovolume, biofilm thickness, and live-to-dead bacterial ratio as described in Heydorn *et al.* (2000), Lee *et al.* (2017a), Nagaraja *et al.* (2017) and Werner *et al.* (2019). Figure 1 shows the reconstructed CLSM images of 48 h of *P. aeruginosa* biofilms grown in the CDC biofilm reactor (biofilm appearance) as a result of the treatment with each biocide. Table 2 summarizes the biocides' anti-biofilm efficacy metrics for all biocides.

From the results shown in Figure 1 and Table 2, it is clear that the 48 h untreated *P. aeruginosa* biofilms were dominated by green fluorescence, indicating the viability of microorganisms. This observation is confirmed by the high live-to-dead bacterial ratio of 54.18 ± 8.80 observed for the untreated biofilms, indicating a higher proportion of live biofilm cells in the sample. Furthermore, the untreated biofilms were characterized by very dense biofilms with biovolume and biofilm thickness of $6.88 \pm 0.78\ \mu m^3/\mu m^2$ and $13.4 \pm 1.9\ \mu m$, respectively, confirming that mature biofilms were successfully established in the CDC reactor.

Overall, the tested biocides presented high efficacy in preventing *P. aeruginosa* biofilms. Values of biovolume, biofilm thickness, and live-to-dead bacterial ratio for all biocides were significantly decreased when compared to the untreated biofilms

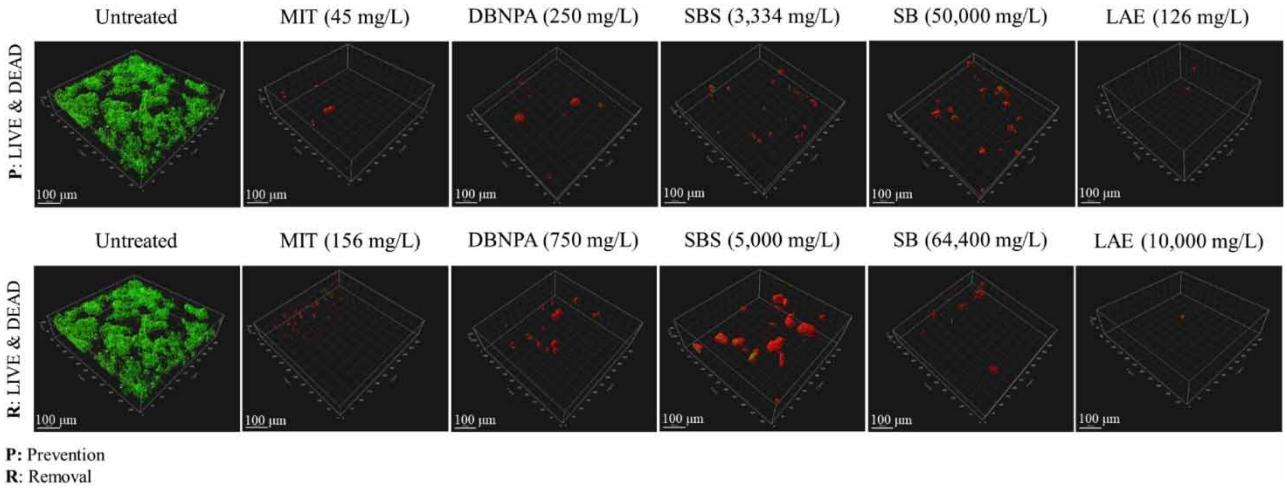

Figure 1 | Reconstructed CLSM images of 48-h *Pseudomonas aeruginosa* biofilms. *P*: Biofilm *P*revention (co-incubation of 48-h *P. aeruginosa* biofilms with biocides at 2× MBIC values). *R*: Biofilm *R*emoval (treatment of 48-h pre-established *P. aeruginosa* biofilms with biocides at 2× MBEC values). In live and dead, green refers to live biofilm biomass and red refers to-dead biofilm biomass. Images were captured at magnification 20×. Please refer to the online version of this paper to see the colored figure. http://dx.doi.org/10.2166/aqua.2023.217.

Table 2 | Anti-biofilm efficacy metrics

	Metrics	Untreated	MIT	DBNPA	SBS	SB	LAE
P	Biocide concentration (mg/L)	–	45	250	3,334	50,000	126
	Biocide concentration (mM)	–	0.4	1.0	32.1	347.2	0.3
	Biovolume ($\mu m^3/\mu m^2$)	6.88 ± 0.78	0.05 ± 0.01	0.70 ± 0.28	0.14 ± 0.03	0.60 ± 0.22	0.08 ± 0.02
	Biofilm thickness (μm)	13.4 ± 1.9	8.6 ± 2.3	7.4 ± 2.4	7.7 ± 1.5	7.8 ± 0.9	8.4 ± 2.7
	Live-to-dead bacterial ratio	54.18 ± 8.80	0.43 ± 0.29	0.13 ± 0.04	0.14 ± 0.03	0.22 ± 0.05	0.21 ± 0.09
	Biofilm prevention efficacy (%)	–	99.3 ± 0.1	89.9 ± 4.0	98.0 ± 0.4	91.2 ± 3.2	98.9 ± 0.3
R	Biocide concentration (mg/L)	–	156	750	5,000	64,400	10,000
	Biocide concentration (mM)	–	1.4	3.1	48.1	447.2	23.8
	Biovolume ($\mu m^3/\mu m^2$)	6.88 ± 0.78	0.16 ± 0.06	0.31 ± 0.03	1.26 ± 0.27	0.25 ± 0.10	0.03 ± 0.01
	Biofilm thickness (μm)	13.4 ± 1.9	7.7 ± 1.0	10.6 ± 0.4	13.7 ± 0.3	7.2 ± 0.4	9.1 ± 2.1
	Live-to-dead bacterial ratio	54.18 ± 8.80	0.26 ± 0.01	0.20 ± 0.02	0.51 ± 0.13	0.39 ± 0.22	0.45 ± 0.08
	Biofilm removal efficacy (%)	–	95.7 ± 0.9	95.5 ± 0.4	81.7 ± 4.0	96.4 ± 1.5	99.6 ± 0.2

Note: P, Prevention; and R, Removal. Calculated by IMARIS 9.8.0 from CLSM images. Values are shown as mean ± standard deviation, obtained from independent triplicate experiments. In the biofilm prevention protocol, the biocides were dosed at 2× MBIC values. In the biofilm removal protocol, the biocides were dosed at 2× MBEC values.

($p < 0.05$; Student's *t*-test) (Table 2). MIT and LAE had the highest biofilm prevention efficacies of 99.3 ± 0.1% and 98.9 ± 0.3%, respectively, followed by SBS (98.0 ± 0.4%), SB (91.2 ± 3.2%), and DBNPA (89.9 ± 4.0%) (Table 2). Ideal anti-biofouling candidates applicable to RO systems should be able to prevent biofilms on RO membranes as well as remove them. According to the results displayed in Figure 1 and Table 2, LAE was the most effective biocide in removing established *P. aeruginosa* biofilms from RO membranes with an efficacy of 99.6 ± 0.2%, followed by SB (96.4 ± 1.5%), MIT (95.7 ± 0.9%), DBNPA (95.5 ± 0.4%), and SBS (81.7 ± 4.0%). All the anti-biofilm efficacy metrics were significantly reduced when compared to the control samples ($p < 0.05$; Student's *t*-test) in the biofilm removal experiments in the bioreactor, except for biofilm thickness in the SBS treatment (Table 2). This indicates that SBS could not significantly reduce the thickness of biofilms when compared to untreated biofilms in the removal experiments. In general, all the treated biofilms were dominated by red fluorescence with very low live-to-dead bacterial ratio values (<1), indicating a low proportion of living biofilm cells on the membrane samples (Figure 1 and Table 2). Furthermore, all the treated biofilms were less dense than the untreated biofilms, with biovolume values varying from 1.26 ± 0.27 to 0.03 ± 0.01 $\mu m^3/\mu m^2$ in both protocols (prevention and removal) (Table 2).

MIT and DBNPA are biocides commonly used to control biofouling in offline RO water treatment applications. The MIT treatment in the prevention protocol was able to reduce the biofilm volume and biofilm thickness to 0.05 ± 0.01 $\mu m^3/\mu m^2$ and 8.6 ± 2.3 μm, respectively. In the removal protocol, MIT was able to reduce the biofilm volume and biofilm thickness to 0.16 ± 0.06 $\mu m^3/\mu m^2$ and 7.7 ± 1.0 μm, respectively. It is no surprise that MIT showed remarkable efficacy in preventing (99.3 ± 0.1%, Table 2) and removing (95.7 ± 0.9%, Table 2) *P. aeruginosa* biofilms from RO membranes with relatively low biocide concentrations (Figure 1). In part, this can be attributed to the ability of MIT to not only disrupt the biofilm matrix but also present a wide spectrum of inhibitory pathways that may lead to the inhibition of enzyme activity, cell membrane damage, and impairment of microbial respiration (Williams 2007; Kucera 2015; Silva *et al.* 2020; Curtin *et al.* 2021). Based on the CLSM images and the anti-biofilm efficacy metrics displayed in Table 2, DBNPA is less effective than MIT in treating *P. aeruginosa* biofilms on RO membranes. DBNPA-treated biofilms had the highest average biovolume value in the prevention experiments (0.70 ± 0.28 $\mu m^3/\mu m^2$) and the second-highest average biovolume value in the removal experiments (0.31 ± 0.03 $\mu m^3/\mu m^2$). Although MIT and DBNPA presented biofilm prevention and removal efficacies up to approximately 99%, human and environmental health hazards attributed to the use of these biocides limit their applications in inline potable water applications (Kucera 2015, 2019; Da-Silva-Correa *et al.* 2022). These characteristics suggest that MIT and DBNPA should only be used as models for anti-biofilm efficacy in biofouling studies.

SBS is also a commercial biocide commonly used in RO applications. Due to its low risk to humans and the environment, SBS is usually applied to limit microbial growth in long-term RO membrane storage and to remove chlorine after feed water pretreatment in RO membrane modules for drinking water applications (Kucera 2015, 2019). According to Figure 1 and Table 2, the SBS treatment resulted in a strong inhibition of biofilm formation with a biofilm prevention efficacy of 98.0 ± 0.4%. However, SBS was the least efficacious (81.7 ± 4.0%) of all the studied biocides in removing existing *P. aeruginosa* biofilms, only reducing biofilm volume to 1.26 ± 0.27 $\mu m^3/\mu m^2$. This result suggests that SBS was not able to disrupt the biofilm matrix, as supported by previous studies (Penna *et al.* 2002; Kucera 2015, 2019). Therefore, SBS is unlikely to be the most optimal biocide for RO drinking water applications.

LAE and SB are biocides that present low risk to humans and the environment but have yet to be investigated for their application in RO systems (Kim & Park 2016; Buckley *et al.* 2017; Curtin *et al.* 2021; Da-Silva-Correa *et al.* 2022). Both biocides are applied as food preservatives and have several features ideal for RO potable water applications, such as excellent antimicrobial properties, high solubility in water, low hazard, and high biodegradability (Kim & Park 2016; Curtin *et al.* 2021; Da-Silva-Correa *et al.* 2022). According to Table 2, SB presented moderate efficacies in inhibiting (91.2 ± 3.2%) and removing (96.4 ± 1.5%) *P. aeruginosa* biofilms compared to the other studied biocides. The moderate anti-biofilm efficacies observed for SB are likely correlated to pH, which was kept at 7 – a typical pH in RO water treatment applications but much higher than the effective pH for SB (pH 4) (Kucera 2015; Buckley *et al.* 2017; Curtin *et al.* 2021). Consequently, high doses were required to achieve even moderate anti-biofilm efficacies. This suggests that SB may not be suitable for addressing biofouling in RO systems, as its application could be quite expensive and its allowable concentration is a cause for concern.

On the other hand, LAE presented excellent anti-biofilm properties while requiring relatively low biocide doses. In general, it was noted that LAE was more effective than all commercial biocides (MIT, DBNPA, and SBS) in treating *P. aeruginosa* biofilms on RO membranes (Table 2 and Figure 1). This observation is best supported by Kim & Park (2016), who compared

the anti-biofilm effect of LAE with two commercial biocides (DBNPA and chlorine) at concentrations up to 62.5 mg/L against four different types of single-species biofilms (*Escherichia coli*, *P. aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*) in a complex growth medium through a static biofilm assay. The researchers concluded that LAE showed superior biofilm prevention and removal efficacies against all tested single biofilms when compared to DBNPA and chlorine (Kim & Park 2016). Furthermore, LAE was the most efficacious biocide in removing existing biofilms from RO membranes among all studied biocides (99.6 ± 0.2%) and the second-best biocide in inhibiting biofilm formation (98.9 ± 0.3%). Sun *et al.* (2022), studied the effects of LAE on the detachment of 24 h *P. aeruginosa* biofilms grown in an *in vitro* flow cell system. The researchers observed that exposure of biofilms to LAE at concentrations up to 42 mg/L for 1 h was responsible for a biofilm removal of approximately 68%. The results reported by Sun *et al.* (2022), support the high anti-biofilm efficacies observed for LAE in our study and highlight the importance of appropriate biocide concentrations in the treatment of biofilms. The superior anti-biofilm properties of LAE are possibly attributed to the fact that LAE can not only disrupt biofilm matrix, but also it inhibits biofilm formation by several mechanisms such as quorum sensing inhibition and cell membrane damage (Kim & Park 2016; Kim *et al.* 2018; Sun *et al.* 2022). Therefore, considering its high efficacy, not only for biofilm removal but also for biofilm inhibition and safety, LAE is an excellent candidate to address biofouling in RO drinking water applications.

Interestingly, biofilms grown in the CBR exhibited higher tolerance to biocide treatments when compared to the treated biofilms in the 96-well plate experiments. The biocides were dosed in the bioreactor at concentrations two times higher than their respective MBIC and MBEC values with the goal of guaranteeing treatment efficacies greater than 90%. However, not all biocides achieve this efficacy. For instance, SBS presented a biofilm removal efficacy of 81.7 ± 4.0% and DBNPA presented a biofilm prevention efficacy of 89.9 ± 4.0% (Table 2). This is most likely a consequence of harsher hydrodynamic conditions within the CBR that directly impact biofilm growth (Donlan 2002; López *et al.* 2010; Ferrer-Espada *et al.* 2019). Biofilms grown in the CBR develop under a heightened cross-flow velocity – a condition that increases cell interactions at the surface but limits nutrient transfer throughout the matrix – resulting in enhanced secretion of EPS and, therefore, microcolonies of greater thickness, density, and complexity (Gomes *et al.* 2014; Ferrer-Espada *et al.* 2019). These architectural characteristics influence microbial metabolism through the imposition of diffusion limits, controlling a biofilm's response to biocidal treatment (Bridier *et al.* 2011). Another possible explanation for the discrepancy in anti-biofilm efficacies between static and dynamic techniques is the difference in attachment layers. Donlan (2002) reported that rough surfaces increase microbial colonization and usually result in stronger biofilms (Donlan 2002). Since RO membranes present surfaces with higher roughness than the microtiter plates (Donlan 2002; López *et al.* 2010; Ferrer-Espada *et al.* 2019), it is unsurprising that biocidal resistance was elevated within the CBR. The results from the present study emphasize the importance of testing biocides in conditions akin to those found in RO system applications, as they have a major effect on the treatment of biofilms.

Scanning electron microscopy

SEM was used to analyze morphological changes in biofilm structures upon exposure to biocides in the CDC biofilm reactor experiments, as well as to validate CLSM results. Figure 2 shows the SEM micrographs of 48 h *P. aeruginosa* biofilms developed in the biofilm prevention protocol. Figure 3 displays the SEM images of 48 h *P. aeruginosa* biofilms developed in the biofilm removal protocol. As shown in Figures 2 and 3, untreated biofilms presented healthy, smooth, rod-shaped bacterial cells with uniform size, and distribution (Werner *et al.* 2019; Liu *et al.* 2020). Unsurprisingly, biofilms treated with biocides had a high number of damaged bacterial cells. In Figure 2, the results of the prevention protocol revealed a thin monolayer of EPS remaining on the RO membranes and no visible biofilm cells. This result was consistent across all biocides, with the exception of SB, which presented a thick layer of EPS. In line with CLSM results, the removal protocol was less effective at treating biofilms, exhibiting a significant number of damaged biofilm cells and a thicker biofilm matrix. Damage, characterized by an unstructured cell shape, appeared as elongated or shrunken cells with visible holes in the cell membranes – similar observations to those found by Gomes & Mergulhão (2017), which studied the effect of biocides on cell morphology.

According to Figure 3, biofilms treated with MIT and SB appeared to have a thin monolayer of EPS with a large number of damaged cells. In contrast, DBNPA presented few bacterial cells with a much thicker EPS layer. Previous biofilm studies indicated that DBNPA is not suitable for removing existing biofilms from RO applications because it cannot effectively disrupt the biofilm matrix (Kucera 2015, 2019; Siddiqui *et al.* 2017). However, our SEM images showed that when DBNPA is applied at a higher concentration than what is applied in full-scale RO installations (up to 30 mg/L), it has a significant effect on the treatment of existing biofilms (Kucera 2015, 2019). Furthermore, SEM results confirmed that SBS was not efficacious at removing

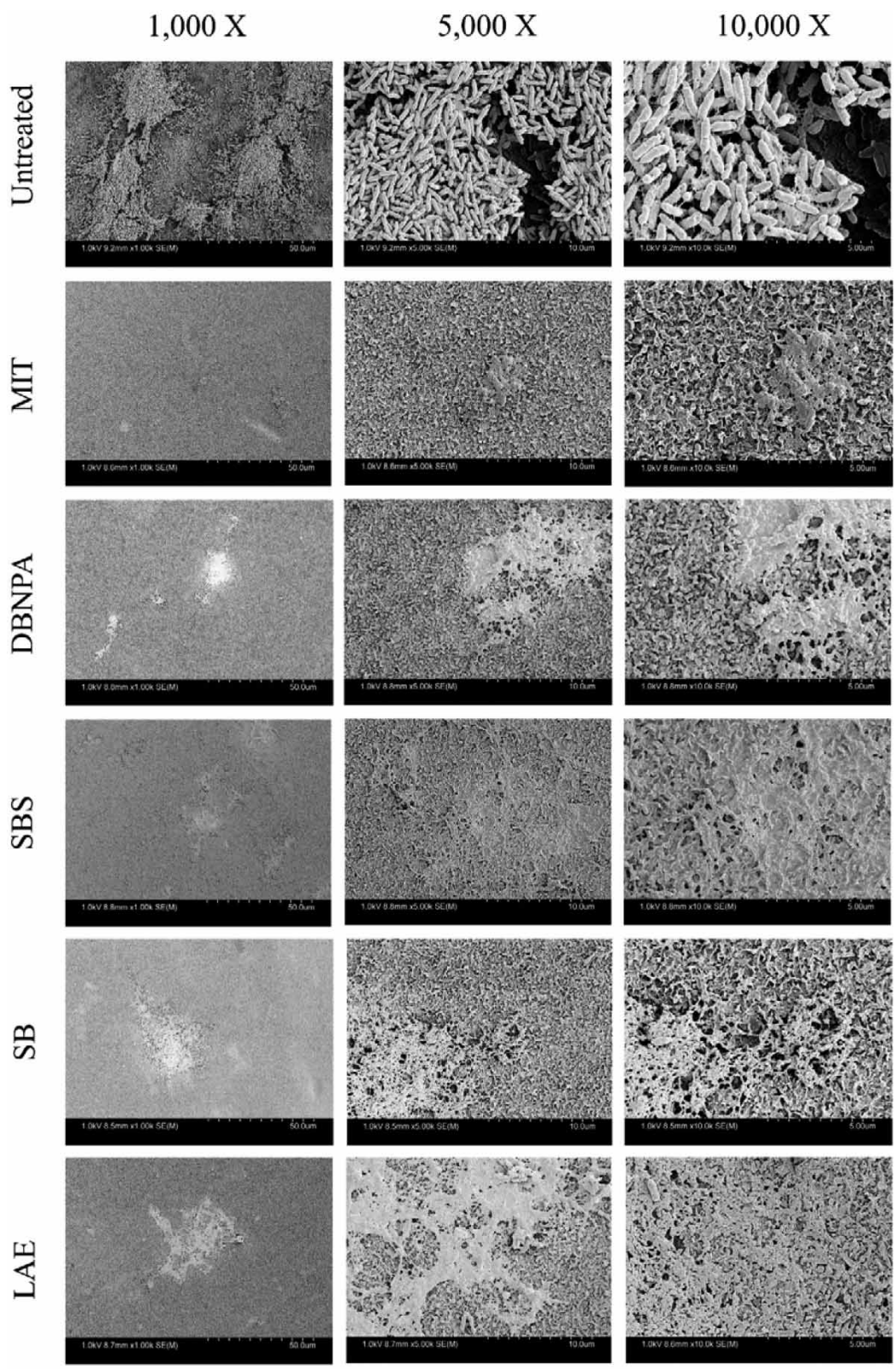

Figure 2 | Representative SEM images of 48-h *Pseudomonas aeruginosa* biofilms, co-incubated with biocides at 2× MBIC values, on polyamide RO membranes (biofilm prevention). Scales bars represent 50.0 μm (at 1,000×), 10.0 μm (at 5,000×), and 5.00 μm (at 10,000×).

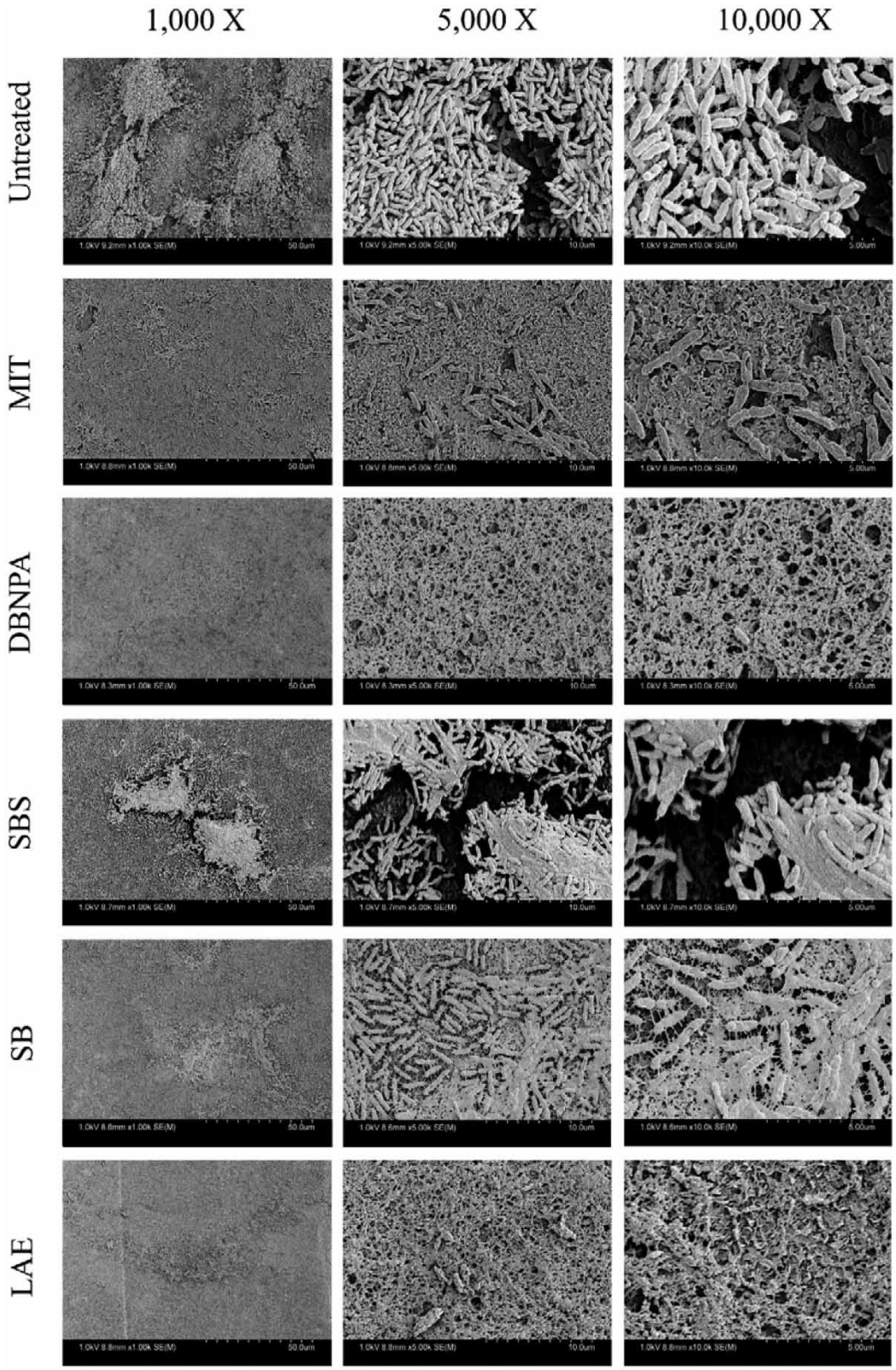

Figure 3 | Representative SEM images of 48-h pre-established *Pseudomonas aeruginosa* biofilms, treated with biocides at 2× MBEC values, on polyamide RO membranes (biofilm removal). Scales bars represent 50.0 μm (at 1,000×), 10.0 μm (at 5,000×), and 5.00 μm (at 10,000×).

biofilms, since it was unable to disrupt the typical 3D structure of biofilms or reduce the number of healthy biofilm cells (Figure 3). Finally, the LAE treated biofilms presented a thin monolayer of EPS and an insignificant number of biofilm cells remaining after treatment. Therefore, the results of the SEM images suggest that LAE and MIT are the most efficacious biocides, with their treatment resulting in the highest removal of EPS and the lowest number of biofilm cells. Additionally, the SEM results also confirmed that treatment aimed at preventing biofilm formation (Figure 2) is more effective than treatment intended to remove existing biofilms (Figure 3). All these observations are consistent with the CLSM results. Furthermore, the results of the SEM analysis provided additional information on the morphological properties of the biofilms. Together, the CLSM and SEM analyses prove to be powerful tools to promote a comprehensive understanding of biocidal efficacy.

CONCLUSIONS AND PROSPECTS

RO technology has the potential to end water shortages worldwide, as it can generate fresh water from different water sources. However, the efficiency and reliability of this membrane-based purification technique are threatened by biofouling since it (a) reduces permeate production; (b) increases energy demands; and (c) produces carcinogenic by-products consequent to feed water treatment with chlorine. In order to propose a safe solution for the reduction of detrimental effects of biofouling in RO systems, this study investigated the ability of five non-oxidizing biocides in preventing or removing biofilms from RO membranes. Biofouling tests were performed in a CDC biofilm reactor with biocidal concentrations estimated on 96-well microtiter plates. Generally, all biocides significantly impact biofilm health with removal and prevention efficacies ranging from 80 to 99%. Results also revealed that treatments aimed at preventing biofilms were more effective than those aimed at removing biofilms, which suggests that a continuous dose of biocidal is the best way to handle biofouling in the RO system. Specifically, the results from this study showed that:

- MIT and DBNPA (commercial biocides) were efficacious in removing and preventing biofilms from RO membranes, proving to be good models of anti-biofilm efficacy in biofouling studies. However, due to their toxicity to humans and the environment, they can only be applied in offline potable water applications.
- SBS was efficacious in preventing biofilm formation, but not in removing biofilms from RO membranes. Thus, SBS is not the optimal solution for biofouling control in RO systems.
- SB presented high–moderate efficacies in biofilm prevention and removal. However, the large concentration required to achieve those efficacies limits its application in RO drinking water applications.
- LAE effectively prevented and removed biofilms from RO membranes. Due to its high anti-biofilm efficacy and safety, LAE is an excellent candidate to control biofouling in RO drinking water applications.

Although LAE demonstrates great promise as an alternative biofilm treatment in RO systems, further research should be done before incorporating this chemical into biofouling control programs. Therefore, the following research studies are recommended: (a) determine LAE's anti-biofilm efficacy for different microorganisms common in RO units; (b) determine LAE's anti-biofilm efficacy against multi-species biofilms; (c) verify LAE's RO membrane-compatibility through short and long-term experiments; and (d) perform anti-biofouling tests on laboratory-, pilot-, and full-scale RO treatment systems.

ACKNOWLEDGEMENTS

This work was done at the Civil Engineering Department, Green Safe Water Lab, University of Victoria, Victoria, British Columbia, Canada. The authors acknowledge the provision and support of the SEM, CLSM, BioCore, and CAMTEC research facilities at the University of Victoria.

AUTHOR CONTRIBUTIONS

L.H.D.-S.-C. led the manuscript preparation; and performed the biofouling experiments in the CDC biofilm reactor with assistance from K.A., N.E.G., R.G., and N.R.. L.H.D.-S.-C and N.E.G. performed the 96-well plates experiments. L.H.D.-S.-C. performed the analysis and discussion of the data with assistance from K.A. and N.E.G. H.L.B. secured funding for this project and provided feedback and revised the manuscript.

FUNDING

This research was supported by NSERC Alliance Missions, Mitacs Globalink, OFI, CFI-JELF, BCKDF, and the University of Victoria.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 9 December 2022; accepted in revised form 6 February 2023. Available online 20 February 2023