Mitigating biofouling on reverse osmosis membranes via greener preservatives

by

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Biology (BSc), Le Moyne College, 2017

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF APPLIED SCIENCE

in the Department of Civil Engineering, University of Victoria

Supervisory Committee

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Abstract

Water scarcity is an issue faced across the globe that is only expected to worsen in the coming years. We are therefore in need of methods for treating non-traditional sources of water. One promising method is desalination of brackish and seawater via reverse osmosis (RO). RO, however, is limited by biofouling, which is the buildup of organisms at the water-membrane interface. Biofouling causes the RO membrane to clog over time, which increases the energy requirement of the system. Eventually, the RO membrane must be treated, which tends to damage the membrane, reducing its lifespan. Additionally, antifoulant chemicals have the potential to create antimicrobial resistance, especially if they remain undegraded in the concentrate water. Finally, the hazard of chemicals used to treat biofouling must be acknowledged because although unlikely, smaller molecules run the risk of passing through the membrane and negatively impacting humans and the environment. It is, therefore, integral to investigate techniques for prevention of biofouling and removal of mature biofilms that are effective, less damaging to the membrane, and safe for humans and the environment.

A common experimental setup is biofilm antimicrobial microdilution susceptibility tests. To acquire meaningful data from these tests, however, appropriate organisms must be tested. Manuscripts 1 investigates, via semi-systematic reviews, the question of what organisms are appropriate to represent the complexity of a biofilm in antimicrobial tests. Ultimately, we recommend utilizing the model biofilm-forming, pioneer organism, *Pseudomonas aeruginosa* for these studies.

Biofouling studies also must present data in a useful manner to the many disciplines that are interested in preventing or removing biofouling. Our goal is to investigate both via antimicrobial microdilution susceptibility tests. In Manuscript 2 we investigate the metrics of each discipline

with an interest in anti-biofouling studies. Ultimately we recommend utilizing both crystal violet stain to assess total biomass removal and the LIVE/DEAD *Bac*Light stain to assess cell vitality (including log reduction and MIC, BPC, MBIC, MBC, BBC, and MBEC), to satisfy the metrics of all interested disciplines. Finally, in Manuscript 3 we implement the recommendations from Manuscripts 1-2 for biofilm prevention and biofilm removal antimicrobial microdilution susceptibility tests. In this manuscript, we work with a subset of safer preservatives including, methylisothiazolinone, phenoxyethanol, and sodium benzoate. We found that methylisothiazolinone was the most effective antimicrobial, however, it was not the safest. Additionally, we investigated the relationship between MBIC and BPC, which was found to vary between the preservatives.

Ultimately, we have provided recommendations for biofilm antimicrobial susceptibility tests that produce widely applicable and useful metrics, as well as utilized these recommendations to investigate the efficacy of safer antimicrobials. All of this work provides a framework for which even safer and effective novel antimicrobials can be investigated.

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Appendix B. (Section S1)

Appendix B. (Section S2)

List of Abbreviations

RO	Reverse Osmosis
SWRO	Seawater Reverse Osmosis
MCDI	Membrane Capacitive Deionization
EPS	Extracellular Polymeric Substances
MBIC	Minimum Biofilm Inhibitory Concentration
BPC	Biofilm Prevention Concentration
MIT	Methylisothiazolinone
PE	Phenoxyethanol
SB	Sodium benzoate
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analysis
PCR	Polymerase Chain Reactions
qPCR	Quantitative PCR
CAMHB	Cation-adjusted Mueller Hinton Broth
CMIT	Chloromethylisothiazolinone
LB	Lysogeny broth
MIC	Minimum Inhibitory Concentration
CFU	Colony Forming Units
MBC	Minimum Bactericidal Concentration
BBC	Biofilm Bactericidal Concentration
MBEC	Minimal Biofilm-Eradication Concentration
CV	Crystal Violet Stain

Acknowledgements

I would like to acknowledge with respect the Lekwungen-speaking peoples on whose traditional territory the University of Victoria stands and the Songhees, Esquimalt and WSÁNEĆ peoples whose historical relationships with the land continue to this day.

I am grateful for the funding I received throughout this experience from my advisor, Heather Buckley, the Faculty of Graduate Studies, and the Mitacs Research Training Award.

I would like to acknowledge how lucky I was to have had Heather Buckley as my supervisor. Between her expertise; drive to push through the obstacles of interdisciplinary work; and unwavering support, whether it was for classes, research, or taking a break to get outside to enjoy this amazing province (which sometimes I did not need more encouragement to do), I cannot sufficiently express how truly grateful I am.

Thank you to my lovely co-op students, Audrey Piper Arroliga and Matthew Thibodeau. Thank you for being there through the many trials and tribulations of developing a protocol from scratch.

Thank you to Rebecca Hof, for your troubleshooting and emotional support, and for providing a great lab space to work in everyday (and always striving to make it better).

Thank you to the Civil Engineering Tech Support, especially Arielle, for supporting many aspects of this project.

Thank you to my fellow graduate students and co-ops in the Green Safe Water Lab, as well as my officemates from other lab groups, for providing research and sanity support.

Thank you to Patrick Lucey for taking me out into the field to experience the other side of civil engineering and for always reminding me to look around and be aware of what's going on out there.

And finally, thank you to my friends and family for always supporting me, whether in research or life! A special shout out to my mom for letting me move 4,863 km away to learn how to do my part to make the world a better place.

Dedication

To my second mom, Cate Tully, who was one of my biggest supporters to start this adventure.

You are a true inspiration and will be missed immensely.

Introduction

The UN World Water Development Report estimates that over 2 billion people, or one-quarter of the population, live in countries that already experience high water stress, and that amount is only expected to rise in the coming years [1]. Already, we can see impacts of freshwater scarcity across the globe, including Mexico [2], [3], the United States [4], [5], Jordan [6], [7], and China [8], [9], to name a few. Along with implementing better water management practices, we need to investigate and optimize cost-effective and energy-efficient processes for utilizing non-traditional water sources.

One promising process is desalination of water via reverse osmosis (RO). RO systems require the application of a force to push water containing solutes through a semipermeable membrane, trapping solutes on the feed side of the membrane and producing freshwater on the permeate side of the membrane. Feed water sources range from brackish water, which contains 1,000 to 10,000 ppm of dissolved salts, to seawater, which contains 10,000 to 35,000 ppm of dissolved salts [10]. Seawater RO (SWRO) is the most commonly utilized process for desalination [11] because the energy consumption of RO remains relatively constant, regardless of salt concentration, as compared to other desalination processes such as membrane capacitive deionization (MCDI) for which the energy consumption increases based on salt concentration [12]. Brackish water desalination via RO is also competitive with other desalination practices. For example, at salt concentrations of about 1,750 ppm, the energy requirement of MCDI desalination surpasses that of RO [12].

RO, however, has a major drawback, which is fouling of the membrane. Fouling of the membrane is the build up of inorganic and organic particulates and colloids, and microorganisms at the

membrane-water interface [13]. These foulants are problematic because they can clog the membrane, resulting in an increased energy input to maintain flux, as well as decreas2ed salt rejection [13]. We focus our work on biofouling, which is fouling of the membrane by microorganisms incorporated in biofilms. Biofouling occurs in four steps: 1) conditioning of the membrane by pioneer organisms or components of the bulk water, 2) attachment of pioneer organisms, 3 microcolony formation, and 4) formation of a mature biofilm in a matrix of extracellular polymeric substances (EPS). The biofilm is made up of up to 90% EPS [13]. Before step 2, biofouling is considered reversible because the biofilms can be removed by increasing shear force across the membrane, however, once the biofilm becomes a mature biofilm, it can no longer be removed by increasing shear force, and is considered irreversible fouling [14]. Irreversibly fouled membranes must eventually be removed from the system and treated to remove the biofilms; however, this results in paused production of permeate water and increases the complexity and cost of running a RO system. Additionally, common treatment methods, such as chlorine, can damage the membrane, decreasing the lifespan of the membrane [15]. Ultimately, biofouling is of considerable concern because even if the majority of organisms are removed from the membrane, the remaining organisms can regrow a biofilm [13].

Biofouling, therefore, must be mitigated to maintain cost effectiveness and low energy requirements of RO systems. One method for mitigating biofouling is treatment via antimicrobials, sometimes referred to as antifoulants or anti-biofouling agents. The efficacy of these chemicals can be determined by microdilution antimicrobial susceptibility tests [16]. For these studies, biofilms are grown, treated with a range of concentrations of the antimicrobial of interest, and the minimum biofilm inhibition concentration (MBIC) is determined. Additionally, biofouling prevention can be studied via antimicrobial susceptibility tests that treat suspension cultures before

biofilms have formed, leading to determination of biofilm prevention concentrations (BPC). However, from the perspective of treating biofouling on RO membranes, it is insufficient to only determine cell death or inhibition of growth; it is also important to determine the removal of EPS; therefore, the total biomass must also be detected. Aside from studying the efficacy of antimicrobials, antimicrobial treatments should also not damage the membrane, cause antimicrobial resistance, and have low hazard if they persist in the feed water concentrate.

In this thesis, we focused on applying safer preservatives currently used in home and personal care products, identified in [17], to treat biofouling on RO membranes. Before beginning bench-top experiments, we determined in Manuscript 1 which organisms are commonly used to test antimicrobial efficacy against biofilms in relation to biofouling on RO membranes via a semi-systematic review. We additionally identified which organisms are commonly found on RO membranes via genetic analysis via a separate semi-systematic review. Next, utilizing the data obtained via the two semi-systematic reviews, we determined which of the organisms were pioneer organisms, that could be targeted to prevent biofouling. We utilized the results from these analyses to recommend a representative pioneer, biofilm-forming organism that is found on RO membranes and has been used to study antimicrobial efficacy against biofilms before, and thus could lead to comparisons between studies. In Manuscript 2, we share our thought-process behind selecting two biofilm detection methods that will make our results applicable to relevant fields, due to this topic having inter-, multi-, and transdisciplinary implications. The conclusions from this manuscript can be applied to any high-throughput antimicrobial susceptibility test for biofilms. Finally, in Manuscript 3, we perform high-throughout microdilution antimicrobial susceptibility tests for biofilm removal (MBIC) and biofilm prevention (BPC). We tested the efficacy of three preservatives identified in Buckley *et al.* [17] including methylisothiazolinone (MIT),

phenoxyethanol (PE), and sodium benzoate (SB). The three chemicals range from high hazard to low hazard to human health and the environment respectively. We specifically screened these chemicals with a focus on biofilm prevention and removal efficacy for RO membranes, however, the high-throughput nature of the experimental setup allows the results to be extended to other membranes and biofilm scenarios.

Manuscript 1

Manuscript Title

The Best-Practice Organism for Single-Species Studies of Antimicrobial Efficacy Against Biofilms Is *Pseudomonas aeruginosa*

Authors

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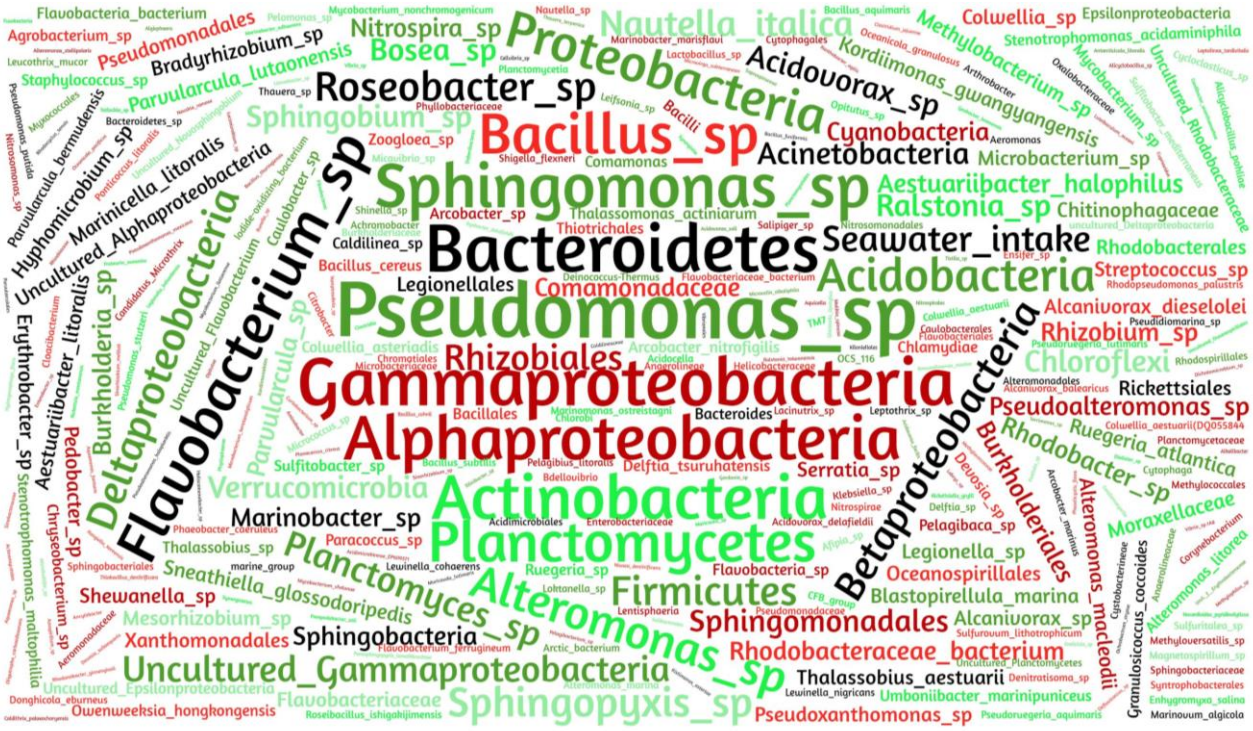
State of Publication

This manuscript was published August 27, 2020 as a Review in *Membranes* special issue "Membranes:10th Anniversary".

Author Contributions

A.M. Curtin, M.C. Thibodeau, and H.L. Buckley planned the semi-systematic review. A.M. Curtin and M.C. Thibodeau analyzed the literature for the semi-systematic review. A.M. Curtin performed analysis of the information derived from analyzing the literature. H.L. Buckley contributed to the interpretation of the results. A.M. Curtin lead manuscript preparation. H.L. Buckley provided feedback and revised the manuscript. H.L. Buckley secured funding for this project.

Graphical Abstract



Abstract

As potable water scarcity increases across the globe; it is imperative to identify energy and cost-effective processes for producing drinking-water from non-traditional sources. One established method is desalination of brackish and seawater via reverse osmosis (RO). However, the buildup of microorganisms at the water-membrane interface, known as biofouling, clogs RO membranes over time, increasing energy requirements and cost. To investigate biofouling mitigation methods, studies tend to focus on single-species biofilms; choice of organism is crucial to producing useful results. To determine a best-practice organism for studying antimicrobial treatment of biofilms, with specific interest in biofouling of RO membranes, we answered the following two questions, each via its own semi-systematic review: 1. Which organisms are commonly used to test antimicrobial efficacy against biofilms on RO membranes? 2. Which organisms are commonly identified via genetic analysis in biofilms on RO membranes? We then critically review the results of two semi-systematic reviews to identify pioneer organisms from the listed species. We focus on pioneer organisms because they initiate biofilm formation, therefore, inhibiting these organisms specifically may limit biofilm formation in the first place. Based on the analysis of the results, we recommend utilizing *Pseudomonas aeruginosa* for future single-species studies focused on biofilm treatment including, but not limited to, biofouling of RO membranes.

Key words: reverse osmosis; biofouling; single-species, prevention; semi-systematic review

1. Introduction

Across the globe, there are increasingly inadequate amounts of clean water to meet human and environmental needs. For example, as of 2017, 20% of the global population lacked clean drinking water [1]. This perilous situation is largely due to increasing global populations and increasing demands for water, as well as changing climate patterns. Fortunately, we have not yet effectively utilized non-traditional water sources, such as natural and human-caused brackish water sources (i.e., saltwater intrusion in overused groundwater aquifers) and seawater, which if treated could provide water for many. One way to treat this water is via reverse osmosis (RO). RO systems use an external force to push water across a semipermeable membrane from the feed side containing solutes to the permeate side containing potable water [2].

RO water treatment is a membrane-based technology that can enable the use of typically non-potable water sources, ranging from brackish water (1000–10,000 ppm) to seawater (10,000–35,000 ppm) [3]. Desalination via RO, however, is limited significantly by fouling of the RO membranes [4–8]. The most significant form of fouling is biofouling, which is involved in more than 45% of RO membrane fouling [4, 8–10] Biofouling occurs when a biofilm forms on membrane material at the water-membrane interface. A biofilm is a complex of microorganisms, including bacteria, fungi, and algae, and extracellular polymeric substances (EPS) [11–13]. The process of biofilm formation proceeds as follows: (1) conditioning of the membrane by EPS secreted by microorganisms or from the bulk water, (2) attachment of pioneer microorganisms, which are the organisms that condition the membrane and are the first to attach to it; (3) diversification, growth, and metabolism of attached microorganisms, and (4) development into a mature biofilm [12–14]. Mature biofilms consist of up to 90% EPS by mass [8, 13].

The steps of biofouling can be broken up into two categories: reversible and irreversible fouling. Reversible biofouling consists of loosely deposited or bound foulants on the membrane, which can be removed by backwashing the system or increased shear force [15]. Reversible fouling occurs before microcolony formation (Figure 1.1). Reversible fouling is still detrimental because it can result in temporarily stopping the production of potable permeate in order to backwash the system. The other type is irreversible biofouling, which includes biofilms from microcolony formation to mature biofilms in a matrix of EPS [16] (Figure 1.1). It cannot be removed by backwashing or increasing flux; this means the membrane needs to be removed and chemically treated or completely replaced. Often, chemical treatments, such as chlorine, damage the membrane, decreasing the membrane lifespan [17].

The low permeability of the biofilm causes membrane flux to decrease, which then requires increased energy input to maintain flux [9]. The accepted fouling model suggests that fouling effects flux in two phases [16, 17]. During the first phase, flux decreases rapidly due to the compaction of the membrane and the development of irreversible fouling. During the second phase, the rate of decrease in flux is smaller due to fouling, reaching a state of homeostasis between biofilm formation and sloughing off of the mature biofilm. The biofilm can also lead to the biodegradation or corrosion of the membrane due to acidic byproducts [9]. In addition, the torturous path of the heterogeneous structure of the biofilm inhibits back diffusion, which can lead to an increased degree of concentration polarization, leading to the increased passage of salt across the membrane [18]. This phenomenon is known as biofilm enhanced osmotic pressure (BEOP). Finally, anti-fouling treatment methods can wear down membranes, which shortens their lifespan [12]. Ultimately, biofouling decreases the efficiency of RO membranes, consequently increasing

operational and maintenance costs for water treatment plants [18–23]. These drawbacks cause RO to be disadvantageous for promoting cost-effective, sustainable communities and cities.

Common treatment methods are often reactive, meaning they attempt to treat biofouling after mature biofilms have formed [9]. Treating mature biofilms is difficult for many reasons. Firstly, physical cleaning methods are ineffectual or near impossible on spiral-wound membranes [24], which are the most common type of membrane used in RO systems [9]. The structure of mature biofilms also protects microorganisms from chemical and physical treatment methods. Additionally, microorganisms in biofilms experience enhanced gene transfer, which allows microorganisms to share beneficial traits, such as antimicrobial resistance, which increases the resistance of mature biofilms to treatment [14]. The protective mechanisms facilitated by the microenvironment of a mature biofilm, especially increased antibiotic resistance, make biofouling one of the most difficult forms of fouling to treat [14]. Biofouling is also considered the "Achilles heel" of RO because even if 99.9% of the microorganisms are removed from the membrane, the remaining microorganisms can re-form a biofilm [9]. Moreover, treating biofilms is a complex problem because the components of biofilms vary depending on the type of microorganisms in the water and on the environmental and operational conditions [25].

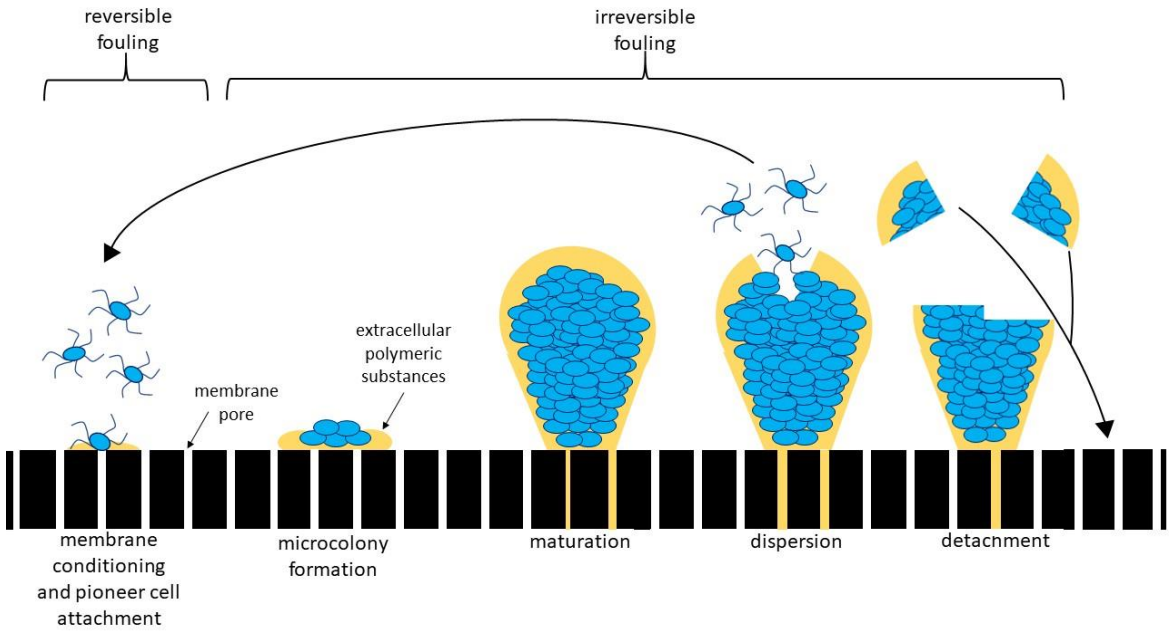


Figure 1.1. Biofouling occurs via a four-step process: (1) conditioning of the membrane by pioneer organisms or material in the bulk water, (2) attachment of pioneer organisms to the conditioned surface, (3) formation of microcolonies, and (4) formation of a mature biofilm consisting of a community of organisms in a matrix of extracellular polymeric substances (EPS) [26]. Up to 90% of the biofilm consists of the EPS matrix, most produced by the bacteria with in it, with the remaining 10% consisting of the bacterial organisms [9]. Before microcolony formation, biofouling is considered reversible fouling because it can be removed by shear force. However, after microcolony formation, biofilms are too strongly attached to the membrane to detach with increased shear force, therefore, requiring other treatment methods (i.e., chemical treatment).

Various prevention and treatment methods are being studied to attempt to mitigate biofouling, including chemical treatments (i.e., linoleic acid (plant fatty acid) [27]; nitric oxide [28]; urea [29]), membrane modifications (i.e., silver nanoparticles [30–33]; triclosan [34]; carbon nanotubes [35]; Arabic gum [36, 37]; hydrophilic membranes [38]; capsaicin [39]), and biological treatments (i.e., bacteriophages [40]; quorum-quenching [41, 42]). Before these methods can be employed, the treatment methods must be rigorously tested to determine anti-biofouling efficacy, impacts on the membranes, and safety for humans and the environment. Selecting appropriate organisms to test efficacy is integral, especially since studies are often performed on single-species biofilms, due to the complexities of multi-species biofilms [43]. Emphasis should be put on organisms that are integral to biofilm formation, namely, pioneer organisms. If treatment methods focus on

preventing pioneer organisms from surviving or producing EPS, a biofilm could potentially be avoided.

One way to identify common biofilm organisms is via genetic analysis [43]. If samples are collected early on in biofilm formation, pioneer organisms can be identified. The genetic analysis includes genotypic methods for identifying bacteria, which are more accurate than other common phenotypic methods of identification, i.e., morphological characteristics [43]. The most common method of genetic analysis of bacteria is 16S ribosomal RNA (rRNA) gene sequencing. First, researchers extract DNA from samples. Extraction methods range from total nucleic acids [43–45] via kits, such as the Soil Master™ DNA extraction kit [46], to the isolation of bacteria (i.e., via agar spread plates) and purification based on macro and micro-morphology [47, 48]. The former method samples all organisms, and the latter samples only organisms that are culturable; therefore, it leaves out viable but not culturable organisms. Next, researchers use primers homologous to portions of the 16S rRNA gene to amplify the DNA via polymerase chain reactions (PCR) [47–52]. The amplified DNA is then sequenced, and the sequences are processed, after which organisms can be identified using databases, such as EzTaxon [21] or NCBI Blast [53]. This identification method can lead to operational taxonomic unit (OTU) classifications when strains have less than 97–98% similarity; however, above 97% similarity, organisms must be differentiated via alternative approaches, such as DNA-DNA hybridization [51–53].

Researchers sequence fragments of the 16S rRNA gene because the gene codes for the RNA component of the 30S subunit of the bacterial ribosome, which is present in all bacterial species [54]. Additionally, the gene has multiple highly conserved regions with nine variable regions dispersed throughout. Researchers can design primers homologous to the conserved regions, which will lead to the amplification of portions of the gene that include the variable regions that are used

to distinguish species [55, 56]. Another benefit of 16S rRNA gene sequencing is the abundance of 16S sequence data available for bacterial organisms. As stated earlier, the major limitation of 16S rRNA gene sequencing is the inability to resolve species classification of strains with too high similarities [54]. For example, Janda and Abbott [57] found that 16S rRNA was able to determine the genus classification of 90% of isolates; however, it was only able to resolve species classification of 65–83% of strains.

This study attempted to answer the question of which organism should be the focus of anti-biofouling studies with an emphasis on biofouling prevention, rather than a mature biofilm removal perspective. We answered this question by performing two semi-systematic reviews to investigate the following related questions:

1. Which organisms are commonly used to test antimicrobial efficacy against biofilms on RO membranes?
2. Which organisms are commonly identified via genetic analysis in biofilms on RO membranes?
3. Based on the results of questions 1 and 2, we additionally answered the following question: Which of the identified organisms are pioneer organisms?

The first question will inform about organisms that are accepted model organisms for biofilm treatment. The answers to question 1 will allow researchers to choose organisms for their studies in such a way that they can compare their results to relevant existing literature. Utilizing an organism identified via question 2 will ensure that the organisms are relevant to biofilms on RO membranes. Finally, utilizing an organism identified in question 3 will be useful for identifying proactive treatments for biofouling. Ultimately, these analyses allow us to recommend a best-

practice organism for studying the treatment of biofilms with an emphasis on biofouling of RO membranes, especially biofilm prevention.

2. Semi-Systematic Review Methods

The semi-systematic reviews report pertinent information according to the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) and are guided by the reporting style in Cassivi et al. [58]. All authors agreed to the semi-systematic review protocol. Study selection was completed by (AC and MT). The Web of Science© database was used to identify peer-reviewed literature that satisfies the semi-systematic review criteria discussed below. We used the term semi-systematic review because only one database was utilized to collect articles [59].

2.1. Semi-Systematic Review: Which Organisms are Used for Anti-Biofouling Studies?

2.1.1. Eligibility Criteria

Studies that utilized bacterial organisms to test antimicrobial efficacy against biofilms on RO water treatment membranes.

2.1.2. Search Strategy

The following Boolean search terms were used for the semi-systematic review: '(reverse osmosis OR RO) AND (bio-fouling OR biofouling OR biofilm) AND (anti-microbial OR antimicrobial OR anti-bacterial OR antibacterial OR anti-fouling OR antifouling OR anti-foulant OR antifoulant OR bacteriostat * OR bactericid *)' AND water'.

2.1.3. Study Selection

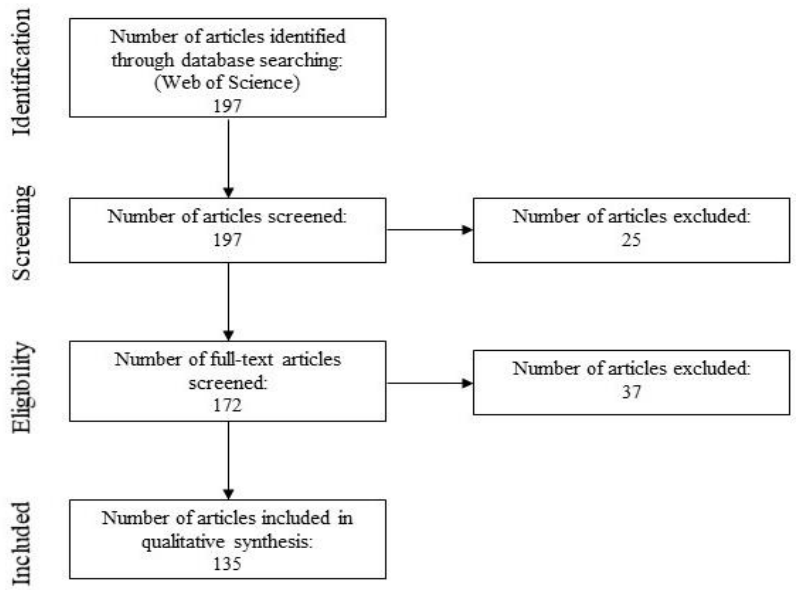
Studies were selected following the PRISMA flow chart reported in Moher et al. [60]. The final Boolean search terms resulted in 197 articles, which were exported to Mendeley (Figure 1.2a). The abstracts of these articles were analyzed to determine which were related to the purpose of the

semi-systematic review, which resulted in the removal of 25 articles. For the remaining 172 articles, the entire article was analyzed to determine whether it contained primary studies related to the purpose of the semi-systematic review. During this step, 37 articles were removed, including 13 review articles, 12 articles that used real water samples that were not analyzed to identify bacterial organisms, and 12 articles that did not include necessary content (i.e., not about RO).

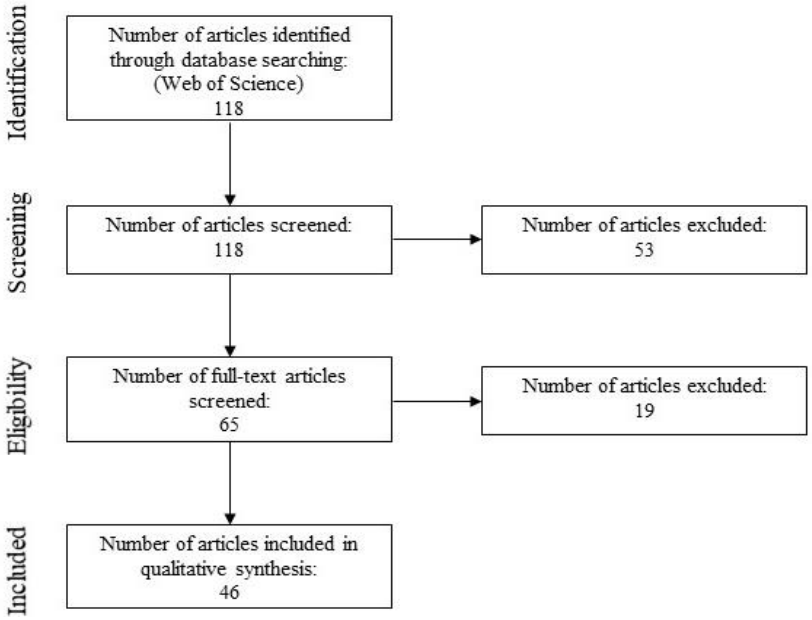
The final analysis, therefore, included 135 of the articles identified via the semi-systematic review (Appendix A, Table S1). The articles included studies that tested antimicrobial chemicals and membrane modifications in experiments ranging from biofilms in 96-well plates to bench-scale RO systems. Some articles included antimicrobial tests against suspension cultures (planktonic phase cells) prior to biofilm tests. We included all organisms used in those studies, even if some organisms were only used for the suspension culture aspect of the paper. We chose to include these because even though the organisms were not grown as biofilms, the data were still used to inform subsequent tests against biofilms.

2.1.4. Data Collection, Extraction, and Analysis

The 135 articles were analyzed to extract pertinent information, including general information (title, publication date, journal name), antimicrobial type (chemical, heavy metal, membrane modification, other), organism(s) tested, the organism phase (suspension culture, agar plate, or biofilm), biofilm detection method (i.e., LIVE/DEAD BacLight Stain, SEM, colony counting, measuring membrane flux, etc.), and whether or not the paper mentioned that the test bacteria was chosen because it is a model organism.



(**a**)



(**b**)

Figure 1.2. Flow chart of the selection process for articles via the semi-systematic review for Section 2.1 (**a**) and Section 2.2 (**b**), similar to Moher et al. [60].

2.2. Semi-Systematic Review: Which Organisms are Found in Biofilms on RO Membranes?

2.2.1. Eligibility Criteria

Studies that identified organisms in biofilms on RO membranes via 16S rRNA gene sequencing.

2.2.2. Search Strategy

The following Boolean search terms were used for the semi-systematic review: '(common OR pioneer OR divers * OR ((early AND coloniz *) OR (first AND coloniz *)) OR microb * analysis OR DNA analysis OR genetic analysis) AND (bacteria * OR organism * OR species OR microorganism OR microb *) AND (biofilm OR bio-fouling OR biofouling) AND (RO OR reverse osmosis) AND water'.

2.2.3. Study Selection

Studies were selected following the PRISMA flow chart reported in Moher et al. [60]. The final Boolean search terms resulted in 118 articles, which were exported to Mendeley (Figure 1.2b). The abstracts of these articles were analyzed to determine which were related to the purpose of the semi-systematic review, which resulted in the removal of 53 articles. For each remaining study, the entire article was analyzed to determine whether it related to the purpose of the semi-systematic review. During this step, 19 articles were removed, including 4 review articles, 5 articles that did not directly relate to RO or water treatment, and 5 articles that did not include a genetic analysis component.

The final analysis, therefore, included 46 of the articles identified via the semi-systematic review (Appendix A, Table S2). The articles included in the study performed 16S rRNA gene sequencing on RO biofouling samples retrieved from full-scale desalination plants, pilot-scale systems, and bench-scale systems. The feedwater sources included seawater, wastewater treatment plant influent and effluent, industry wastewater, membrane bioreactor effluent, tap water, drinking

water, and well water. We recorded the most specific taxonomical classification provided for each organism ranging from phyla to species level.

2.2.4. Data Collection, Extraction, and Analysis

The 46 articles were analyzed to extract pertinent information, including general information (title, publication date, journal name), feed water source(s) (i.e., seawater, wastewater treatment plant, etc.), level of taxonomical identification, and bacteria identified. We also analyzed the articles for mentions of pioneer organisms.

2.3. Phylogenetic Tree-Like Structure

A phylogenetic tree-like structure was constructed using the NCBI Taxonomy Database and Common Tree, a tool created to show a "hierarchical view of the relationships among the taxa and their lineages" [61–63]. The tree consisted of identifications from semi-systematic review 1. Due to the quantity of identified organisms, the tree was limited to class level identifications. The tree was viewed and manipulated using ggtree [64, 65].

3. Results

3.1. Semi-Systematic Review: Which Organisms are Used for Anti-Biofouling Studies?

One-hundred-and-thirty-five articles contributed to a consensus from the literature of common microorganisms used to test antimicrobial efficacy against biofilms on RO membranes. Upon analyzing these articles, thirteen genera were identified in at least one article, including *Acinetobacter* (1 occurrence), *Bacillus* (15), *Comamonas* (1), *Escherichia* (99), *Enterococcus* (1), *Klebsiella* (4), *Micrococcus* (1), *Methylobacterium* (1), *Pseudomonas* (51), *Serratia* (1), *Staphylococcus* (32), *Stenotrophomonas* (1), and *Sphingomonas* (4), resulting in a total of 212 organisms used in the 135 studies (Figure 1.3). Of the analyzed literature, approximately 93% of the identified organisms came from four different genera, including *Escherichia*, which was used

in 73% of the articles, *Pseudomonas*, which was used in 38% of the articles, *Staphylococcus*, which was used in 24% of the articles, and *Bacillus*, which was used in 11% of the articles. The remainder of the genera (5% of all of the identified organisms) occurred in less than 3% of the articles each.

The most commonly used strain was *Escherichia coli* (*E. coli*) K12 MG1655, which was used in 11 articles, followed by *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC PAO1, which was used in 10 articles. We also found that 73% of the articles included in the final analysis stated that the organism(s) they used was model or typical organisms or simulated biofouling. Five genera were not described in any of the articles as model organisms, including *Acinetobacter*, *Methylobacterium*, *Stenotrophomonas*, *Serratia*, and *Sphingomonas.*

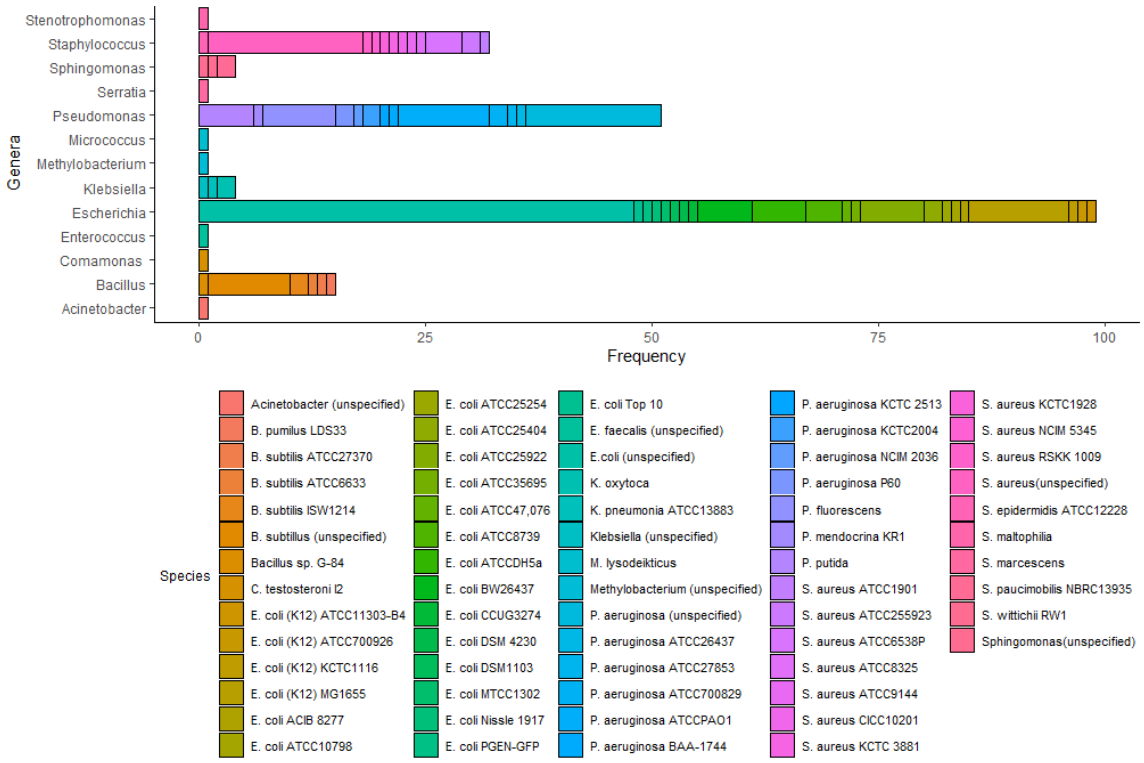


Figure 1.3. Frequency, in terms of the number of articles where the organism occurs, of use of microorganisms to study antimicrobial efficacy against biofilms on RO membranes cited in the literature. Data were acquired from an analysis of 135 articles related to biofouling in water treatment systems.

3.2. Semi-Systematic Review: Which Organisms are Found in Biofilms on RO Membranes?

Figure 1.4 shows the constructed phylogenetic tree, displaying the class and phyla diversity of the organisms that were identified in the analyzed literature. Forty-four classes were identified that corresponded to thirteen phyla, and an additional twenty phyla were identified but not resolved down to class level. Figure 1.5a shows the frequency with which organisms were identified in the thirty-three phyla, and Figure 1.5b shows the frequency organisms were identified in the forty-four classes.

Of the phyla, organisms in *Proteobacteria* (1090 identifications) accounted for 66% of all the identified organisms, corresponding to six times more organisms than the next highest phylum, *Bacteroidetes* (171 identifications). Following *Bacteroidetes*, were *Actinobacteria* (110

identifications), *Firmicutes* (90 identifications), and *Planctomycetes* (55 identifications). The remaining phyla combined accounted for only 7% of the identified organisms. At the class level, 32% of the identified organisms were part of the class *Alphaproteobacteria*, 24% in *Gammaproteobacteria*, 10% in *Betaproteobacteria*, 6% in *Actinobacteria*, 5% in *Flavobacteriia*, and 4% in *Bacilli*. The remaining 38 classes of organisms made up less than 20% of the total occurrences.

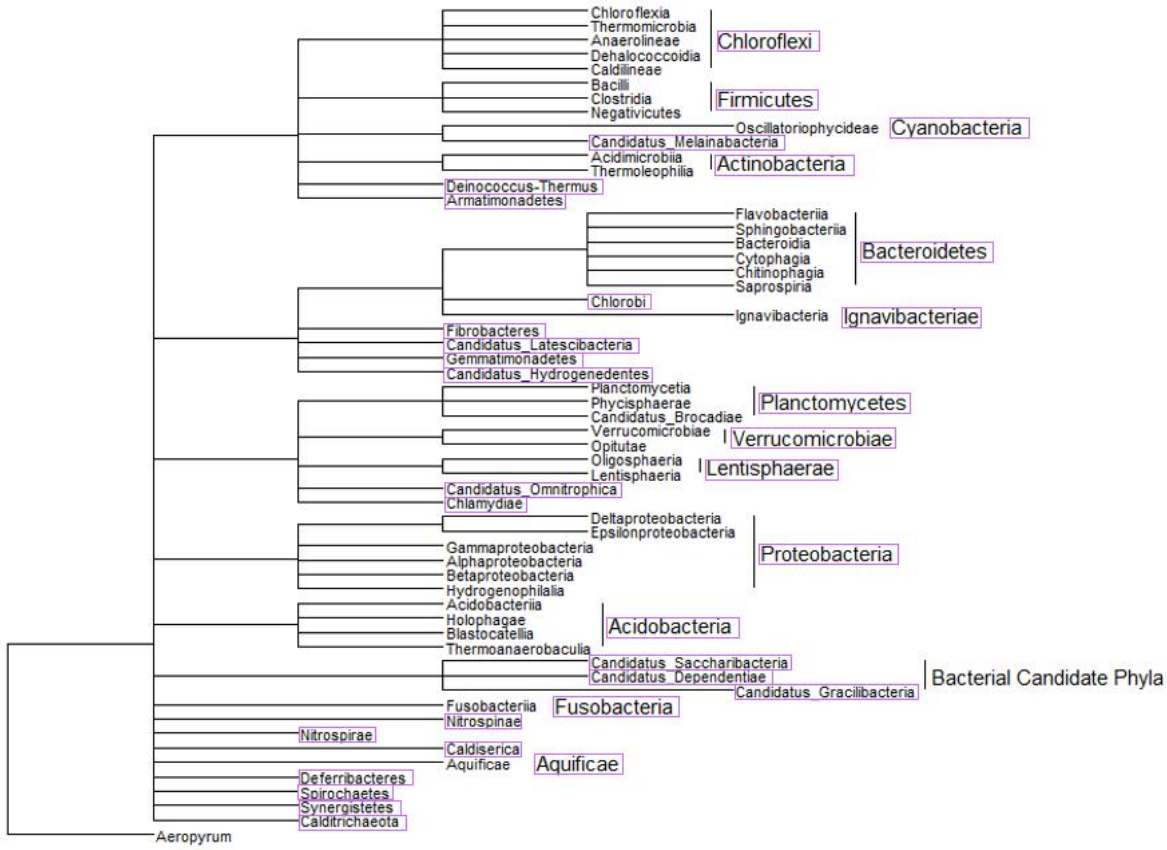


Figure 1.4. A phylogenetic tree-like structure was constructed of the organisms identified in semi-systematic review 1 down to class level (outlined in pink) to show the diversity of organisms that are found in biofilms on RO membranes. The tree-like structure shows hierarchal clusters between organisms based on data in the NCBI Taxonomy Database and Common Tree [61–65].

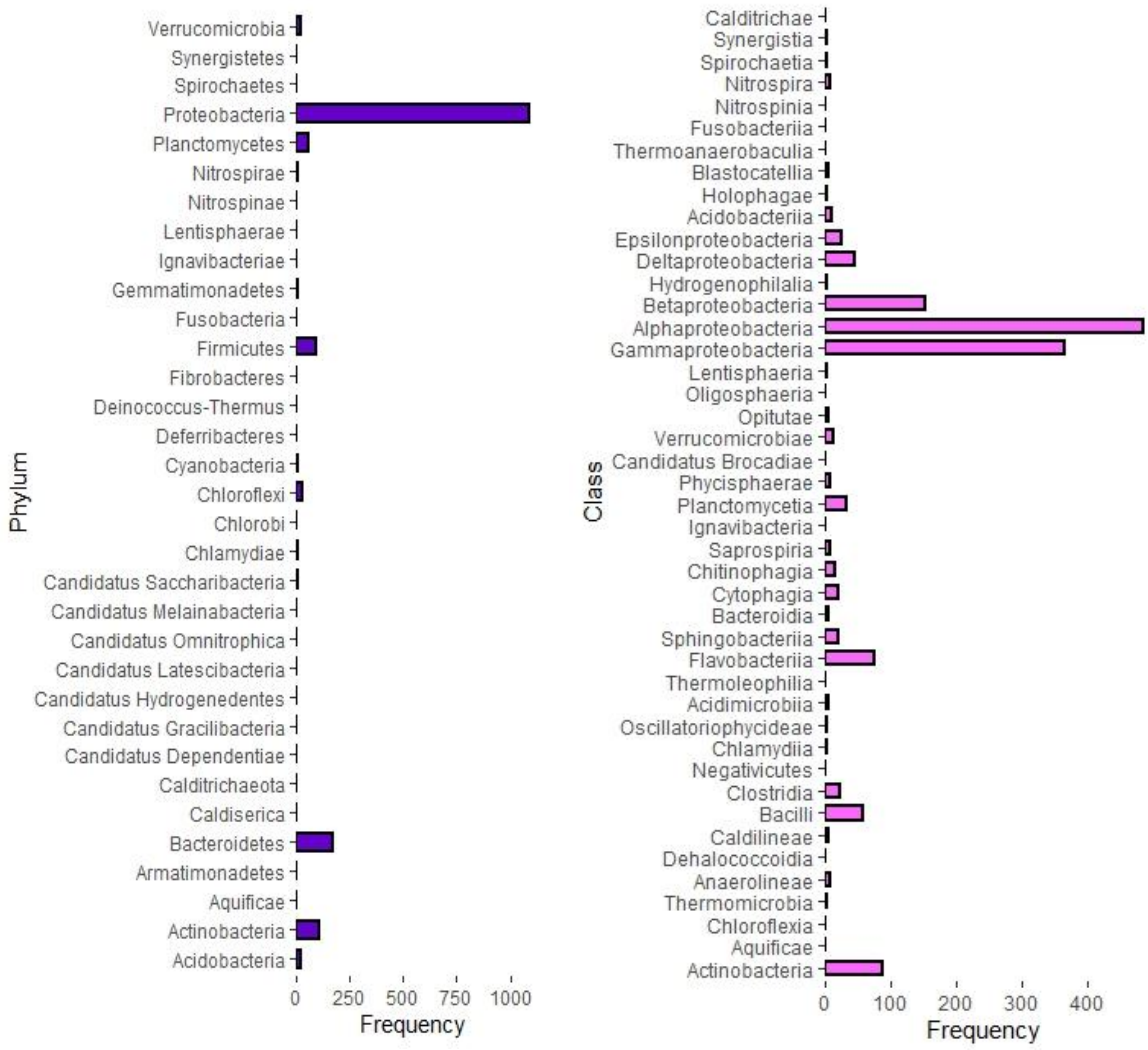


Figure 1.5. Phylum-level frequency of organisms identified through semi-systematic review 1 (**a**) Class-level frequency of organisms identified through semi-systematic-review. (**b**) The frequency is based on the number of times an organism from a specific phylum or class was identified in the analyzed literature.

4. Discussion

4.1. Which Organisms are Used for Anti-Biofouling Studies?

To choose successful antifoulants, studies against single-species biofilms should be performed against model biofilm-forming organisms. If tests are performed against model organisms, the results will be more representative of actual efficacy.

In this analysis, we found that many of the organisms used in the studies were chosen because they are considered model organisms. For example, *P. fluorescens* was used in [66] because it was considered convenient and a relevant model organism for biofilm formation. Similarly, [67] describes that *Pseudomonas* spp. are useful model organisms for studying biofouling based on the secretion of EPS. Additionally, Zhu et al. [68] stated that *E. coli* and *P. aeruginosa* were used in their study because they are commonly used as model bacteria in antibacterial studies. Only five of the genera, including *Acinetobacter*, *Methylobacterium*, *Stenotrophomonas*, *Serratia*, and *Sphingomonas*, have not been specifically described as model organisms in any of the analyzed literature; however, this does not mean that they are not model organisms. For example, *Sphingomonas* spp. are recommended as a model organism for biofouling, especially for studying initial attachment and growth of biofilms [69].

This analysis identified genera that contain model organisms that are commonly used to test antimicrobial efficacy against biofilms on RO membranes, which would provide relevant organisms for antimicrobial efficacy studies. However, there are some limitations to its application. Firstly, our analysis included organisms that were only tested in the suspension culture phase in a biofilm study. We included these organisms because we wanted to include all organisms that were used to inform about biofilms, not only the ones specifically grown as biofilms. The most common organism in our study, where this was the case, was *E. coli*. For example, Flemming and Wingender [70] noted that they did not use their *E. coli* strain for some of the biofilm tests they performed because it formed a mature biofilm very slowly and did not allow for clear comparisons between adhered cells and non-adhered cells. Therefore, although the researchers were not able to gather results for *E. coli* biofilms, they intended to use *E. coli* as a model biofilm organism. However, due to this phenomenon, we investigated concerns with *E. coli* biofilm

formation. In Narisawa et al. [71], the researchers found that there were 10-fold less *E. coli* W3110 cells incorporated in the biofilm compared to *E. coli* IAM1264, corresponding to 1.62×10^6 and 1.74×10^7 cells/well, respectively, highlighting the importance of strain selection. According to Spoering and Lewis [72], *P. aeruginosa* PAO1 biofilms contained about 10^8 cells/well in the biofilms, suggesting *P. aeruginosa* could lead to denser biofilms than the *E. coli* strains used in Narisawa et al. [71], which may explain why *E. coli* can lead to unsatisfactory biofilms (i.e., [70]). Cell density is especially important because biofilm detection methods, such as Crystal Violet stain, require sufficient biomass for accurate measurements [73].

Another possible limitation of this analysis is the inherent focus on opportunistic pathogens in biofilm research. For example, Davies [74] stated that many studies related to biofouling in water treatment-related systems focus on opportunistic pathogens, including *Pseudomonas*, *Staphylococcus*, and *Escherichia*. An opportunistic pathogen is an organism that normally has a commensal relationship with a host but can infect hosts under certain circumstances, such as individuals that have compromised immune systems. The organisms, therefore, are a public health concern, which warrants increased attention in the literature; however, these organisms may not be the most relevant from the perspective of biofouling on RO membranes. This could account for the high incidence of use of *E. coli* in 68% of the articles and *P. aeruginosa* in 35% of the articles. However, this is likely not a concern because one quality of opportunistic pathogens is the ability to form biofilms [74–77]. Additionally, *Pseudomonas* and *Staphylococcus* are specifically identified as opportunistic pathogens that are also biofilm formers in López et al. [78].

4.2. Which Organisms are Found in Biofilms on RO Membranes?

This semi-systematic review for question 2 determined thirty-three phyla of organisms corresponding to forty-four classes of organisms that were identified in various studies via 16S

rRNA analysis in biofouling samples from RO systems. Similar to results from question 1, the most common organisms identified were in the phylum *Proteobacteria* [21, 44, 79]. The top three most frequent classes of organisms were in *Proteobacteria*, including, *Alphaproteobacteria*, followed by *Gammaproteobacteria* and *Betaproteobacteria*. These results were supported by results found in [21, 44, 77, 79, 80]. For example, in Hörsch et al. [81], researchers found *Alphaproteobacteria* to be the most abundant class of organisms on RO membranes, followed by *Gammaproteobacteria*. Ivnitsky et al. [80] found *Gammaproteobacteria* to be the most abundant organisms; however, the author stated that after more than thirty days of activity, the biofilm became dominated by *Alphaproteobacteria* and *Betaproteobacteria*, which could explain why our study determined more *Alphaproteobacteria* than *Gammaproteobacteria* when analyzing the data from all of the studies that include data collection at multiple time points [80].

The next most common phylum was *Bacteroidetes*. Ferrera et al. [82] stated that *Bacteroidetes* was found in biofilms in samples taken at one and three months; thus, organisms in *Bacteroides* are considered regular members of biofilms on RO membranes, but not necessarily pioneer organisms [28, 83–85]. *Bacteroidetes* contain the second most common class of organisms, *Flavobacteriia*, and the eighth and ninth most common classes, including *Cytophagia* and *Sphingobacteriia*, respectively. All three of these classes are commonly found in mature biofilms on RO membranes [69, 86, 87].

The phylum *Actinobacteria* is the next most common, which includes the most common class, *Actinobacteria*. Studies have found that although early on the biofilms tend to include *Actinobacteria*, their abundance decreases over time [88, 89]. The phylum *Firmicutes* closely follows *Actinobacteria* in frequency. It contains the third most common class, *Bacilli*, and the seventh most common class, *Clostridia*. One study found that the amount of *Firmicutes* increased

over time as the mature biofilm formed [89]. *Planctomycetes* is the last phylum of bacteria with a relevantly high frequency. It contains the fifth most common class, *Planctomycetia*. One study found that organisms in *Planctomycetes* were of consistently high abundance throughout biofilm formation [85].

A major reason some of the previously mentioned phyla and classes are commonly found in biofilms on RO membranes is due to the production of EPS. EPS is integral for conditioning of the membrane for initial attachment and for the development of mature biofilms. According to Shang et al. [79], organisms in *Proteobacteria* produced more EPS than other bacterial phyla. For example, Ivnitsky et al. [81] identified that *Gammaproteobacteria* had superior attachment ability compared to other organisms due to the production of EPS, making it a common pioneer organism. Since EPS is so important for biofilms, it likely explains why organisms in *Proteobacteria* were most commonly identified in this study. More specifically, organisms in *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* produce amyloid adhesins, which constitute a large fraction of EPS in microcolonies [90]. Amyloids are insoluble and highly tolerant of denaturants, making removal of this EPS difficult [91]. Albertsen et al. [92] analyzed the genes of *Bacteroidetes* and characterized a gene for alginate production, which is another type of EPS. *Firmicutes* produce hydrophobic EPS, which clumps cells and contributes to biofilm formation [91]. Uniquely, the major reason organisms in *Planctomycetes* are believed to be effective biofilm formers is because they are budding bacteria, some of which are filamentous, which leads to aggregation of cells [85]. Organisms in this phylum tend to be found at the base of biofilms, suggesting they participate in early biofilm formation [85].

4.3. Which of the Identified Organisms are Pioneer Organisms?

Of the thirteen genera identified for question 1, eight of the genera, including *Acinetobacter*, *Bacillus*, *Escherichia*, *Methylobacterium*, *Pseudomonas*, *Staphylococcus*, *Stenotrophomonas*, and *Sphingomonas*, all contain species that are considered to be pioneer organisms of biofilms [14, 69, 89, 93–96]. The first step of biofilm formation on a membrane is conditioning by EPS, which is excreted directly onto the membrane by pioneer organisms or sourced from the bulk water [17]. EPS helps facilitate the attachment of organisms to the membrane in combination with flagella, type I pili, and outer membrane proteins [97]. For example, *P. aeruginosa* produces Pel and Psl, which are both important EPS for attachment to abiotic and biotic surfaces and for initial biofilm formation [70]. If treatment methods are focused on preventing the excretion of EPS and attachment of pioneer organisms, mature biofilms may not be able to form, negating the need for the complex treatment methods that are required for mature biofilms. It should be noted that an extensive EPS matrix, containing pores for water and nutrient flow, is an integral component of a mature biofilm; therefore, EPS is also important in mature biofilms. For example, *P. aeruginosa* produces alginate, which is a type of EPS that is not integral for initial biofilm formation but is important for producing the three-dimensional structure of a biofilm. Without alginate, *P. aeruginosa* biofilms are flat and thin [70].

For question 2, we found that the most common organisms in biofilms on RO membranes were from the phylum *Proteobacteria*. Shang et al. [79] concluded that the phyla *Proteobacteria* contained the main pioneer organism of marine biofilms on RO membranes, which was also supported by results from Ma et al. [98] and Hu et al. [99]. As stated earlier, Hörsch et al. [81] found *Gammaproteobacteria* to be the first colonizers of the membrane. Because *Gammaproteobacteria* are recognized as major pioneer organisms on RO membranes, testing

treatment methods on these organisms could provide representative models for biofouling prevention efficacy.

4.4. Comparison

Comparing the organisms identified in Section 3.1 to the organisms identified in Section 3.2 suggests that laboratory studies are being performed on organisms that are commonly found on RO membranes, ranging from pioneer organisms forming the biofilm to organisms involved in mature biofilms. The following classes contained pioneer organisms that were commonly used to study the anti-biofouling efficacy of antimicrobials: *Acinetobacter*, *Bacilli*, *Gammaproteobacteria*, and *Alphaproteobacteria*. The previous review found that the most commonly tested organisms were in the class *Gammaproteobacteria* in the genus *Escherichia*. *Escherichia* was likely tested most often because it is a model bacterial organism and because it is a common fecal contamination indicator used in water treatment [100, 101]. *Pseudomonas* was identified as the next most commonly used organism in the previous semi-systematic review. It is also in the class *Gammaproteobacteria*. In the case of *Pseudomonas*, we suggest that the use of *Pseudomonas* spp. in anti-biofouling efficacy studies relates to *Pseudomonas* spp. being model biofilm formers that produce EPS, which is an important characteristic of pioneer organisms. *Pseudomonas* spp. also have clinical health relevance due to their pathogenicity, which may contribute to its use in anti-biofouling studies (i.e., [102–104]).

We recommend utilizing the *Pseudomonas* spp. instead of *Escherichia* for anti-biofouling studies. One reason is due to concerns with the density of *Escherichia* biofilms, which will affect detectability. Additionally, some *Pseudomonas* species are pioneer organisms, which are useful from a biofilm prevention perspective. Finally, *P. aeruginosa* is an opportunistic pathogen, and therefore results from anti-biofouling studies in regard to RO may have applications in other fields.

5. Conclusions

Ultimately, antifouling efficacy tests should be performed on model biofilm-forming pioneer organisms that are commonly found in biofilms on RO membranes. A focus on pioneer organisms could cause results to be more relevant from a prevention perspective. The organisms that most closely fit that criteria are in the class *Gammaproteobacteria*. A common genus in this class that is already used for tests against biofilms is *Pseudomonas*. This genus contains organisms that are considered model biofilm formers as well as pioneer organisms. We recommend utilizing *P. aeruginosa*, which is commonly used for biofilm studies and identified via genetic analysis in biofilms on RO membranes. It should be acknowledged, however, that biofilms are very complex; therefore, single-species studies are only so useful, no matter which organisms are tested. It is integral that tests move beyond focusing on pioneer organisms and focus on the complex communities that make up biofilms to obtain the most realistic results for an anti-biofouling treatment method.

Supplementary Materials: Appendix A.: Table S1. Semi-systematic review raw data answering: Which organisms are used for anti-biofouling studies?, Table S2. Semi-systematic review raw data answering: Which organisms are found in biofilms on RO membranes?

Author Contributions: A.M.C., M.C.T. and H.L.B. planned the semi-systematic review. A.M.C. and M.C.T. analyzed the literature for the semi-systematic review. A.M.C. performed an analysis of the information derived from the analyzed literature. H.L.B. contributed to the interpretation of the results. A.M.C. led manuscript preparation. H.L.B. provided feedback and revised the manuscript. H.L.B. secured funding for this project.

Funding: This research was funded by Mitacs RTA, the W.E. Cowie Faculty Innovation Award, the IESVic Clean Energy Systems Accelerator Program, EcoCanada SWILP, NSERC USRA (M.C. Thibodeau), the UN Association of Canada Green Spaces Program, NSERC Discovery, CFI JELF, and BCKDF.

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Manuscript 2

Manuscript Title

Biofouling detection methods that are widely applicable and useful across disciplines: A critical review

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State of Publication

This manuscript is intended to be submitted as a Critical Review to *Membranes* special issue "Membranes: 10th Anniversary".

Author Contributions

A.M. Curtin performed this critical review with guidance by H.L. Buckley. A.M. Curtin lead manuscript preparation. H.L. Buckley provided feedback and revised the manuscript. H.L. Buckley secured funding for this project.

Abstract

Biofouling, or the buildup of microorganisms in a biofilm at the solid-water or water-air interface, is an interdisciplinary problem. Biofouling causes various issues including clogging systems, contaminating devices, and creating infections that are extremely difficult to treat, to name a few. Therefore, engineers, pharmacologists, microbiologists, wastewater treatment operators, chemists, food preservative formulators, home and personal care product formulators, and toxicologists all play a role in studying and have an interest in solving biofouling. High-throughput studies about biofilm prevention and removal can take the form of biofilm antimicrobial microdilution susceptibility tests. Due to vested interests of many disciplines, the results from biofilm antimicrobial microdilution susceptibility tests should be applicable and useful to each discipline. Via a critical review, we analyse the focuses, biological implications, and metrics required by each discipline. We then summarize the possible detection methods that could satisfy each desired metric. From the results of this analysis, we recommend two methods of biofilm detection, Crystal Violet stain and the LIVE/DEAD *Bac*Light stain, which correspond with three metrics, total biomass, log reduction, and MIC, BPC, MBIC, MBC, BBC, and/or MBEC. Determining these three metrics for each biofilm antimicrobial microdilution susceptibility test will causes this research to be widely applicable and useful across many disciplines.

Key words: biofouling, interdisciplinary, antimicrobial susceptibility tests, biofilm detection

1. Introduction

Research and practices that transcend inter-, multi-, and transdisciplinary boundaries are crucial to solving challenges that exist and emerge in the 21st century. Specifically, inconsistent communication styles, terminology, and reporting standards need to be standardized or made explicit. For example, Fruchter *et al.* [1] highlight the difficulties associated with investigating and communicating building designs in a multidisciplinary team consisting of structural engineers and architects. The authors suggest that due to varying models for design, personal idioms from respective professions, and different media platforms for presenting designs, multidisciplinary building design teams often have to interpret, extract, and re-enter design information in the idioms of their own profession. This costs time and often decreases the quality of the final product [1]. Like building design and construction, the challenge of addressing biofouling is a multisectoral problem, engaging researchers and practitioners from engineering, pharmacology, microbiology, wastewater treatment, chemistry, food preservative formulation, home and personal care product formulation, and toxicology. Thus, similar to Fruchter *et al.* [1], this field relies on successful inter-, multi-, and transdisciplinary communication. In this paper we highlight the focuses and metrics required by relevant disciplines in regard to biofouling studies and highlight two biofilm detection methods that, if used in conjunction, can satisfy the needs of each discipline simultaneously.

1.1 Biofouling

Biofouling is the build up of microorganisms in the form of a biofilm at the water-solid, or water-air interface. Biofouling occurs via a four step process: 1) conditioning of the membrane by pioneer organisms or non-biological material in the bulk water, 2) attachment of pioneer organisms to the conditioned surface, 3) formation of microcolonies, and 4) formation of a mature biofilm

consisting of a community of organisms in a matrix of extracellular polymeric substances (EPS) [2] (Figure 2.1). Up to 90% of the dry mass of a biofilm consists of the EPS, with the remaining 10% consisting of the bacterial organisms [3][4].

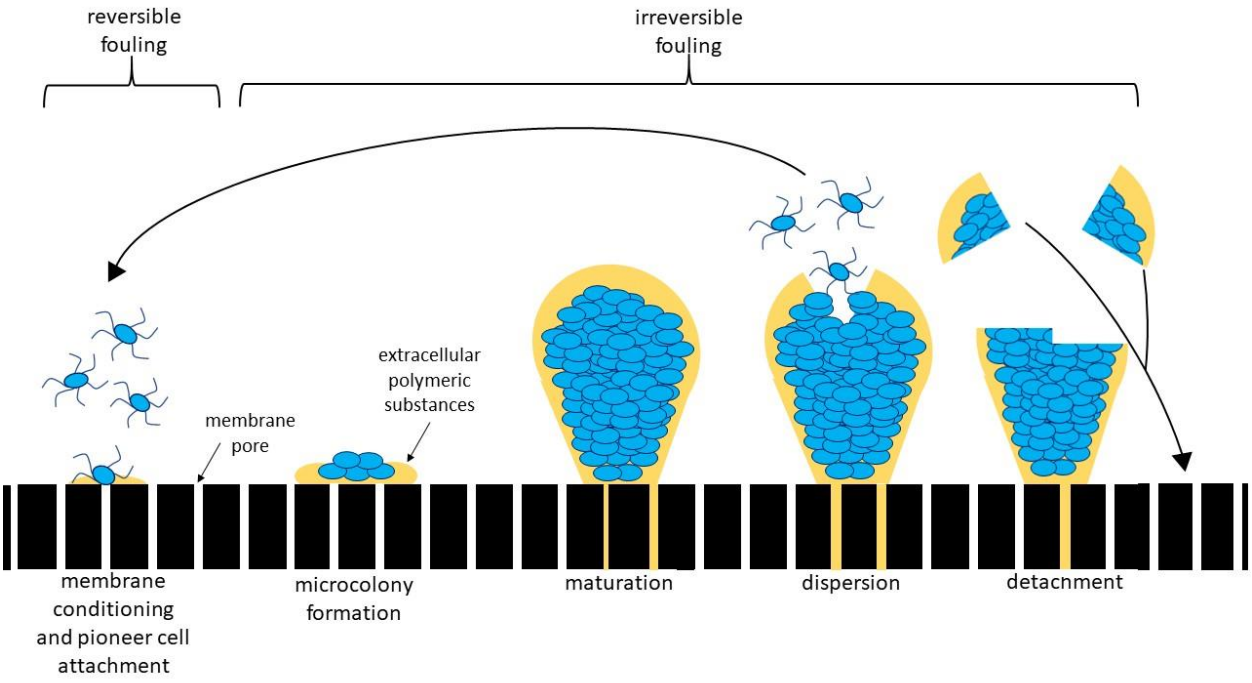

Figure 2.1. Biofouling begins by conditioning of the membrane by pioneer organisms or material in the bulk water followed by attachment of pioneer organisms to the conditioned surface, Next. A microcolony is formed by excretion of EPS. Finally, a mature biofilm forms due to growth and diversification of the microcolony. The mature biofilm, therefore, consists of a community of organisms in a matrix of extracellular polymeric substances. Organisms may disperse or detach from the biofilm based on environmental conditions (Reproduced from Figure 1.1, Manuscript 1) [2], [5]. Before microcolony formation, biofouling is considered reversible fouling because it can be removed by shear force. However, after microcolony formation, biofilms are too strongly attached to the membrane to detach with increased shear force, therefore, requiring other treatment methods (i.e. chemical treatment).

One technology that is impacted by biofouling is reverse osmosis (RO). Biofouling tends to buildup on the membrane-water interfaces. It can clog RO membranes, resulting in an increased energy requirement to maintain flux across the membrane. Eventually, biofouling of RO membranes requires the membrane to be removed from the system and treated or replaced. Unfortunately, although common treatment methods, such as chlorine, are effective, they can damage the membrane [6]. Increased energy requirements and decreased lifespan of RO

membranes caused by biofouling cause RO to be a less efficient and cost-effective process. As such, methods for effectively removing or managing biofilms while in place in a RO system have potential for significant economic and humanitarian benefit.

1.2 Biofouling Efficacy Tests

A simple and rapid method used for studying the efficacy of biofilm treatment by chemical intervention is antimicrobial susceptibility tests. Standard protocols for suspension culture antimicrobial susceptibility tests can be found in Zimmer *et al.* [5]. Suspension culture antimicrobial susceptibility tests generally entail treating suspension cultures with a range of concentrations of a chemical treatment either in centrifuge/culture tubes (macrodilution) or in multi-well plates (microdilution). Data is collected in the form of minimum inhibitory concentration (MIC), which is the lowest concentration of antibiotic that inhibits the visible growth of bacteria after overnight incubation [7].

In contrast to suspension cultures, protocols for biofilm antimicrobial susceptibility tests have not been standardized yet [8]. In general, biofilm antimicrobial susceptibility tests can either focus on biofilm prevention or biofilm removal. The detection method for biofilms, however, varies and the selection of detection method determines the metric (i.e. living cells, EPS removal, etc.). It is therefore integral to select biofilm detection method(s) based on the deliverables desired from the experiment and the application of the results.

1.3 Inherent Difficulties Detecting Biofilms

Compounding difficulty selecting a detection method for anti-biofouling efficacy, there are underlying challenges in simply detecting and quantifying biofilms. In general, biofilms are complex communities of various organisms, both living and dead, integrated in varying types of

EPS, therefore, developing a detection method that quantitatively determines all of these components is difficult [9]. Both Stiefel *et al.* [9] and Wilson *et al.* [10] investigate various quantitative and qualitative detection methods for biofilms and they conclude that a combination of methods is ideal for accurately detecting biofilms. Additionally, about 90% of a biofilm is made up of a matrix of EPS [4]. Any detection methods that depend on interacting with bacteria or the entire EPS must have the time and ability to diffuse throughout the matrix. Lastly, some methods require homogenizing the biofilm before analysis via methods such as sonication, vortexing, and scraping the biofilms off of the material on which they have formed. Completely removing and homogenizing the biofilm can be difficult, due to strongly adhered bacteria and EPS.

1.4 Limit of Scope

The following critical review addresses metrics that pertain specifically to the growth and prevention of biofilms themselves. It does not address other metrics of RO membrane performance that are impacted by biofouling, including membrane flux, or energy requirement. Studies that utilize bench-scale or pilot-scale RO systems benefit from maintaining the previously stated metrics because they are performance indicators and more readily tracked in a functioning RO system. However, our studies are intended to screen and provide recommendations of optimal antifouling chemistries to apply to RO systems, and as such are performed as high-throughput antimicrobial susceptibility tests (i.e. biofilms grown in 96-well plates). High-throughput antimicrobial tests do not have the components of a bench-scale or pilot-scale RO system; therefore, our focus is on biofilm detection methods, not RO system monitoring.

2. Goals, Metrics, and Challenges of Measuring Biofilms in Various Disciplines

2.1 Defining Goals and Metrics of Various Fields Studying Biofouling

Due to the negative impacts of biofouling on RO systems, hospital devices, and other systems and settings, researchers in various disciplines are investigating ways to mitigate biofouling. Biofouling treatment in these fields range from solubilized chemical treatments (i.e. linoleic acid (plant fatty acid) [11]; home and personal care product preservatives (Manuscript 3)), surface modifications (i.e. silver nanoparticle-impregnation [12], [13]), and biological treatments (i.e. bacteriophages [14], [15]). Due to the wide range of applications of biofouling treatment results, the results from biofouling treatment studies should be presented in a manner that is useful and accessible to all relevant disciplines.

Table 2.1 summarizes fields that are interested in biofouling treatment research deliverables including: RO engineers, pharmacologists and microbiologists, wastewater treatment plant operators, chemists, toxicologists, and preservative formulators (i.e. for food, home and personal care products). Each field has specific focuses for biofouling treatment research, which translate into microbiological implications and desired metrics for detection of efficacy.

Table 2.1. Disciplines interested in anti-biofouling research, their main focus in relation to biofouling, the biological implications they are interested in achieving through treatment methods, and what metric(s) the biological implications require.

Discipline	Focus	Biological Implications	Metric(s)
Engineer (Energy Requirement of RO)	Maintaining flux through the RO process; minimizing energy requirement of the system	Prevent bacteria from forming biofilms; Remove both the bacteria on a membrane and the EPS on a membrane	Total biomass removal
Engineer (Permeate Water Quality Produced by RO)	Permeate water quality	Decreasing bacterial concentration to a recommended level	Log reduction
Pharmacologist/Microbiologist	Dose of antimicrobial required for successful treatment	The minimum amount of antimicrobial required to inhibit bacterial growth (planktonic and biofilm)	MIC, BPC, MBIC, MBC, BBC, and/or MBEC (Table 2.2)
Wastewater Treatment Plant Operator	Remove odor and discoloration from water; Remove pathogens	Decreasing bacterial concentration to a recommended level	Log reduction
Chemist	Mode of action	Generally depends on interests of collaborators	Varies; commonly accompanied by or complementary to metric of collaborator
Toxicologist	Safety	Generally depends on interests of the collaborators	Varies; commonly accompanied by or complementary to metric of collaborator
Food/Home and Personal Care Products Preservative Formulator	Preventing color and odor changes in shampoo, etc.	Minimizing growth of planktonic bacteria	MIC (Table 2.2)

For example, RO engineers are interested in minimizing the impacts of biofouling on the energy requirement of RO systems and creating high quality permeate water that meets regulatory standards for drinking or discharge. For the former interest, engineers need to know the total biomass remaining after biofouling treatment because the EPS matrix that bacteria reside in must be prevented or removed from the membrane in addition to inhibition or killing of microorganisms, to maintain or resume normal RO processing [16], [17]. For the latter interest, engineers need to know organisms per liter (often calculated in laboratory studies as colony forming units per millilitre, or CFU) and log reduction of bacterial organisms. Log reduction is a way to express the relative number of microorganisms that were killed via disinfection. For example, a log reduction of 1 is equivalent to a 10-fold reduction of organisms or a 90% reduction of organisms. The World Health Organization Guidelines for Drinking-Water Quality present both maximum organisms per liter and log reduction recommendations for various organisms with associated health risks in drinking water [18]. Thus, the figures of merit for RO engineers are total biomass and log reduction. In contrast, pharmacologists and microbiologists are generally interested in determining viability of cells/cell count from biofilm susceptibility tests that either focus on biofilm prevention or biofilm removal. Biofilm prevention tests are similar to suspension culture antimicrobial susceptibility tests, in that a suspension culture is treated with the chemical of interest and the biofilm prevention is monitored. The suspension culture in biofilm prevention tests is at a higher concentration than that used for antimicrobial susceptibility tests in order to encourage biofilm growth. For biofilm prevention, data can be collected in the form of biofilm prevention concentration (BPC) (Table 2.2). For biofilm removal, a biofilm is grown and then treated. Data can be collected as minimum biofilm inhibitory concentration (MBIC), minimum bactericidal concentration (MBC), biofilm

bactericidal concentration (BBC), and minimal biofilm-eradication concentrations (MBEC), depending on experimental setup [7]. The benefit of utilizing biofilm susceptibility protocols that lead to BPC, MBIC, MBC, BBC, and/or MBEC is that the data is generalized to biofilms in centrifuge/culture tubes or multi-well plates (similar to [5]) or on pegs [19] and can, therefore, be applied to multiple situations. For example, the MBIC of a chemical against a certain type of bacteria can be determined via a biofilm susceptibility test in order to inform about biofilm removal from medical devices [20], however, that data can also be used to inform about biofilm removal from RO membranes (Manuscript 3).

Table 2.2. Different parameters available for antimicrobial susceptibility tests (adapted from Macià *et al.* [7]).

Parameter		Definition
MIC	Minimum inhibitory concentration	The lowest concentration of an antibiotic that inhibits the visible growth of a planktonic culture after overnight incubation
MBIC	Minimum biofilm inhibitory concentration	The lowest concentration of an antibiotic that resulted in an OD650 nm difference of ≤10% (1 log difference in growth after 6 h of incubation) of the mean of two positive control well readings
MBC	Minimum bactericidal concentration	The lowest concentration of an antibiotic producing a 99.9% CFU reduction of the initial inoculum of a planktonic culture
BBC	Biofilm bactericidal concentration	The lowest concentration of an antibiotic producing a 99.9% reduction of the CFUs recovered from a biofilm culture as compared to the growth control The
MBEC	Minimal biofilm-eradication concentration	The lowest concentration of an antibiotic that prevents visible growth in the recovery medium used to collect biofilm cells
BPC	Biofilm bactericidal concentration	Same as the MBIC, but bacterial inoculation and antibiotic exposure occur simultaneously

Unfortunately, metrics including BPC, MBIC, MBC, BBC, and/or MBEC do not inform directly about EPS removal, nor does total biomass directly indicate cell viability/cell count. Based on Table 2.1, we believe that studies that focus on antimicrobial efficacy against biofouling should collect data and report data in three forms: total biomass reduction, log reduction, and one or more of BPC, MBIC, MBC, BBC, and/or MBEC to satisfy the metrics of all of the relevant disciplines.

3. Available Detection Methods

In the following section, we investigate the biofilm detection methods that can satisfy the desired metrics including, total biomass, log reduction, and MIC/BPC/MBIC/MBC/BBC/MBEC.

3.1 Total Biomass

Total biomass is generally measured using dyes that bind to negatively charged molecules. These dyes can, stain cells and EPS, however, they do not allow for differentiation between the two or determination of live and dead cell counts. After staining samples with the dyes, the absorbance is read. Crystal Violet, Safranin Red, and Congo Red are three types of stains that can be used for total biomass biofilm detection [9] (Table 2.3). Each protocol is briefly discussed in Table 2.3, and further details are discussed in Section 5.3.

Table 2.3. Summary of biofilm detection methods for total biomass [9].

Total Biomass Detection			
Detection Method Name	Brief Protocol Description	Benefit(s)	Drawback(s)
Crystal Violet	Stain contents of wells (including cells and EPS); incubate for 30 minutes; rinse wells to remove excess stain; dissolve stain from well contents (i.e. via ethanol); read absorbance of well at 595 nm, compare to negative control	Inexpensive; highest absolute absorbance of the three	Sufficient biomass must be present to distinguish absorbance from the background noise
Safranin Red	Similar to Crystal Violet, but absorbance read at 535 nm	Inexpensive	Sufficient biomass must be present to distinguish absorbance from the background noise
Congo Red	Similar to Crystal Violet, but absorbance read at 500 nm, compare to negative control	Inexpensive	Sufficient biomass must be present to distinguish absorbance from the background noise

3.2 Log Reduction (Living Cells)

A simple, common way to determine log reduction is to spread plate the sample on an agar plate before and after treatment. The colonies that grow on the agar plates can be counted which leads

to the colony forming units per milliliter (CFU/mL) [21]. The log reduction can be determined by comparing the CFU/mL before and after treatment. This method, however, is time consuming and labor intensive, therefore alternative methods are often utilized.

There are various other methods for determining log reduction. The BacTiter-Glo assay is used to quantify the amount of ATP in a sample, which is only produced and retained by live cells [9]. After the addition of the BacTiter-Glo reagent (Promega), the luminescence is measured and compared to a standard curve to determine live cell count. The turbidity threshold method entails the addition of growth medium to treated samples, incubation, and monitoring of optical density (OD). A standard curve can then be used to determine the number of live cells based on OD readings. For the tetrazolium salt assay, tetrazolium salt is added to a biofilm sample and incubated for two hours. In that time, the salt is crystalized by microorganisms in the form of formazan, which can be dissolved in DMSO and the absorbance can be read. Since the absorbance reading is based on formazan production, which is converted by live microorganisms, it can lead to cell counts based on standard curves [9]. The LIVE/DEAD *Bac*Light Stain consists of fluorescent stains that intercalate with DNA. The stains label live cells green and dead cells red. The fluorescence can be read and compared to a standard curve or images of the biofilm can be taken and live cell counts can be determined by software such as ImageJ [9][10]. Each protocol is briefly discussed in Table 2.4, and further details are discussed in Section 5.5.

Each of these methods lead to live cell counts, however, they must be converted to log reduction. For biofilm prevention protocols, the live cell count is converted to log-reduction by comparing the final live cell count to the number of live cells inoculated at the beginning of the experiment.

For biofilm removal protocols, the live cell count in treated wells, is compared to the live cell count in positive control wells [7].

Table 2.4. Summary of biofilm detection methods for living cells, which can be translated to log reduction and minimum inhibitory concentration (MIC), biofilm prevention concentration (BPC),minimum biofilm inhibitory concentration (MBIC), minimum bactericidal concentration (MBC), biofilm bactericidal concentration (BBC), and minimal biofilm-eradication concentrations (MBEC) [9].

Living Cells:

Log Reduction/

MIC/BPC/MBIC/MBC/BBC/MBEC

Detection Method Name	Brief Protocol Description	Benefit(s)	Drawback(s)
Log reduction via spread plates	Prevention: aliquots of the stock culture are spread plated to determine starting concentration of cells; after treatment of cells aliquots are plated to determine final concentrations of cells; log reduction is calculated between starting concentration and final concentration Removal: biofilms are grown; after treatment of cells aliquots are plated of both the experimental wells and the positive control; these cell counts are compared to calculate log reduction	Accurate	Time consuming; labor intensive
BacTiter Glo assay	Detach bacteria from multi-well plate; add BacTiter-Glo reagent, incubate for 5 minutes, read the luminescence with a plate reader with gain of 135, 1 second per well	Accurate	Destructive; expensive
Turbidity Threshold assay	After treating biofilms, add broth; incubate plates on shaker incubator and measure OD at 600 nm every 30 minutes for 24 hours; compare OD to cell count		Time consuming; labor intensive
Tetrazolium Salt assay	Add 2-(4-iodophenyl)-3-(4-nitrophe- nyl)-5-phenyl-2H-tetrazolium chloride (INT) to each well; incubate the plate for 2 hours; remove medium; add dimethyl sulfoxide to dissolve the dye from the biofilms; read absorbance at 470 nm	Metabolic activity detection	Concerns with consistency and false-positives and false-negatives
LIVE/DEAD *Bac*Light Stain	Rinse wells; add stains solutions dissolved in 0.9% saline solution; incubate plates for 15 minutes while vortexing; read fluorescence with an excitation of 485 and emission of 528 and 640	Leads to live and dead cell count	Destructive; Issues with gram-positive bacteria

3.3 MIC/BPC/MBIC/MBC/BBC/MBEC (Living Cells)

As stated earlier, MIC for cells in planktonic phase is commonly determined by macro- or microdilution protocols [5]. Both protocols entail growing organisms, treating the organisms with a range of concentration of the antimicrobial, and visually determining the MIC. However, BPC/MBIC/MBC/BBC/MBEC rely on determination of live cells [7]. Detection methods that allow for the determination of BPC/MBIC/MBC/BBC/MBEC are similar to those for log-reduction. After determining live cells, the BPC/MBIC/MBC/BBC/MBEC are determined as values that are 90% lower than the positive controls [7]. Each protocol is briefly discussed in Table 2.4 and further discussed in Section 5.5.

4. Research Goals

Our aim is to select specific anti-biofouling efficacy detection methods to maximize inter-, multi-, and transdisciplinary applications of the results of biofouling treatment studies (Table 2.1). We therefore want to measure total biomass, log reduction, and BPC/MBIC/MBC/BBC/MBEC. Additionally, detection methods should be applicable to high-throughput antimicrobial susceptibility tests (similar to [5]), therefore, the selected detection methods need to be completed in a timely manner and be cost effective.

5. The Chosen Detection Methods

After reviewing the literature, we recommend two methods for determining anti-biofouling efficacy including the Crystal Violet stain (CV) and the LIVE/DEAD *Bac*Light Stain. Using both of these methods will allow for the determination of both total biomass, via CV, and viability of cells (log reduction and BPC/MBIC/MBC/BBC/MBEC), via the LIVE/DEAD *Bac*Light Stain.

5.1 Crystal Violet Stain

CV binds to negative charges, including bacteria and EPS. The protocol begins with adding CV to each well of the 96-well plate. The plate is then incubated for 30 minutes. Afterward, the contents of each well are aspirated out and each well is washed three times with sterile saline. Lastly, ethanol is added to each well to dissolve the biofilm-bound CV and the absorbance is read at 595 nm [9]. Total biomass removal is determined as a well with an absorbance reading that is not statistically different than the negative controls.

5.2 Why Crystal Violet Stain?

We recommend CV for detecting total biomass instead of Safranin Red and Congo Red (Table 2.3). We chose CV largely because it is a relatively simple and quick method for detecting biofilms [9], [10]. Additionally, researchers found that CV could detect up to 98.7% reduction in *Pseudomonas aeruginosa* biofilms, which is higher than the detectability for Safranin Red and Congo Red, two other total biomass stains [9]. Stiefel *et al.* [9] do identify one drawback common to total biomass detection methods, including CV; a high amount of biomass must be present to allow for distinction from the background noise. However, Stiefel *et al.* [9] found that CV was the best at detecting these changes because it has a higher absolute absorbance than other stains like Safranin Red and Congo Red, which makes it easier to differentiate from the background noise.

5.3 LIVE/DEAD *Bac*Light Stain Protocol

The LIVE/DEAD *Bac*Light Stain consists of two fluorescent nucleic acid dyes, SYTO9 and propidium iodide (PI). SYTO9 can permeate through the cell membrane and intercalate with the DNA of live and dead cells. SYTO9 fluoresces green. PI cannot permeate an intact cell membrane; therefore, it only intercalates with DNA of dead cells. PI fluoresces red. It has a higher affinity to DNA than SYTO9 therefore it replaces any DNA of dead cells intercalated with SYTO9 [22].

The protocol for the LIVE/DEAD *Bac*Light Stain starts by aspirating out the contents of the 96-well plate. Next, the wells are rinsed with sterile saline to remove any remaining planktonic cells. The stain is then added to the wells and incubated for 15 minutes while vortexing. Common practice is to read the fluorescence with a plate reader twice, both with an excitation of 585 nm and an emission of 528 nm for SYTO9 and 645 nm for PI [9]. Fluorescence readings can be compared to a standard curve for SYTO9 and PI fluorescence versus cell count to determine cell viability/cell count in experimental samples. The cell viability/cell count determined by the standard curve can be compared to the original inoculum amount or to the positive controls for prevention studies and removal studies, respectively. Other sources suggest using a confocal laser scanning microscope (CLSM) and Image J software to count fluorescent cells in photos take by the (CLSM), which does not require disturbing the biofilm, or a flow cytometer to count cells (i.e. [23], [24]).

5.4 Why LIVE/DEAD *Bac*Light Stain?

We recommend the LIVE/DEAD *Bac*Light Stain for determining log reduction and cell viability (Table 2.4). The LIVE/DEAD *Bac*Light stain protocol is advantageous because it can determine cell viability. It can also be detected with various devices including a plate reader, a confocal laser scanning microscope, and a flow cytometer. We will focus on utilizing a plate reader for fluorescence measurements. A limitation of this method is utilizing a vortex to homogenize the biofilms during stain incubation. We found that vortexing may not sufficiently disturb biofilms for a single-point fluorescence read, therefore an area scan was required which is quite time consuming (about 40 minutes per 96-well plate). To continue to make this an effective high-throughput detection method, a more homogenous solution needs to be made, for example by sonicating the biofilms (i.e. [25]).

An additional concerning limitation of this protocol is issues staining gram-negative cells. For example, Stiefel *et al.* [26] found that samples that contained 100% dead cells had 2.7 fold higher SYTO9 fluorescence than samples with 0% dead cells (100% living cells). The amplification of the SYTO9 stain by dead cells make it appear that samples of 100% dead cells contain more living cells than samples containing 100% live cells. In contrast, for *Staphylococcus aureus,* a gram-positive bacteria, there was a 9-fold weaker SYTO9 signal for 100% dead cells compared to 0% dead cells (100% living cells), more in line with expected results. Researchers suggest this may be related to difficulties of SYTO9 crossing the two cell membranes of gram-negative bacteria, therefore, when the cell is dead and has damaged cell membranes, it can more easily reach the DNA and is not replaced by PI quickly enough, leading to higher SYTO9 fluorescence readings for dead cells [26]. We remain wary of this concern, however, we believe that drawbacks of other viability assays outweigh this concern. Additionally, results in Manuscript 3 were not affected by this relationship as strongly as in Stiefel *et al.* [26].

We do not recommend the BacTiter-Glo assay because, although it is very accurate, it is prohibitively expensive for a high-throughput assay [26]. We did not choose the turbidity threshold assay because it is considered time consuming [26]. Unlike the LIVE/DEAD *Bac*Light Stain, which can be improved by implementing sonicating, the turbidity threshold assay cannot be shortened [7]. Lastly, we did not choose the tetrazolium salt assay because there are concerns with its consistency and false-negatives and false-positives due to background interference [27].

6. Conclusion

Biofouling is a concern in various disciplines; therefore, biofouling treatment results should be widely applicable so that they are useful across disciplines. In this critical review, we determined that assays aimed at measuring the effect of chemical interventions (antifoulants) on biofouling

will best serve a range of researchers and practitioners if they detect both total biomass removal, and cell viability/cell count, which can be translated to log reduction and BPC/MBIC/MBC/BBC/MBEC (as applicable). After analyzing possible methods for biofilm detection of total biomass and cell viability/cell count, guided by Stiefel *et al.* [9], we recommend a combination of two detection methods in studies: LIVE/DEAD *Bac*Light Stain and CV stain. Both methods are well aligned with a high-throughput protocol and the combination will lead to both total biomass and viability counts, which can lead to log reduction and BPC/MBIC/MBC/BBC/MBEC measurements. Other biofilm detection protocols discussed had drawbacks, such as consistency, cost, and duration that we believe make them less useful for biofilm detection via high-throughput susceptibility assays. By detecting and reporting both cell viability/cell count and total biomass, results from biofouling treatment research can be useful across disciplines.

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Manuscript 3

Manuscript Title

Anti-biofouling efficacy of three home and personal care product preservatives in regard to reverse osmosis: MBIC and BPC determinations

Authors

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State of Publication

This manuscript is intended to be submitted as a Research Article to *Membranes* special issue *Enhancing the Efficiency of Membrane Processes for Water Treatment.* We do not intend to include the appendix sections about protocol development.

Author Contributions

A.M. Curtin developed the protocol with guidance from H.L. Buckley. A.M. Curtin and M.C. Thibodeau performed laboratory research. A.M. Curtin performed analysis of the results with assistance from M.T. Thibodeau. H.L. Buckley contributed to the interpretation of the results. A.M. Curtin lead manuscript preparation. H.L. Buckley provided feedback and revised the manuscript. H.L. Buckley secured funding for this project.

Abstract

As the globe faces increasing water scarcity, it is essential to determine cost-effective and efficient methods of producing potable water, especially ones that focus on non-traditional water sources. Although reverse osmosis (RO) shows promise as a key-player in mitigating water scarcity, it is limited by biofouling. We therefore need to identify effective antifoulants, but they must also not damage the membrane, cause resistance, or negatively impact human health and the environment. One source for potential antifoulants is preservatives used in home and personal care products. We hypothesize that we can apply these safer preservatives to RO systems to remove or prevent biofouling. We tested three preservatives, methylisothiazolinone (MIT), phenoxyethanol (PE), and sodium benzoate (SB), via antimicrobial susceptibility tests against *P. aeruginosa* biofilms grown in 96-well plates to investigate both biofilm removal and biofilm prevention. Data collection was in the form of minimum biofilm inhibitory concentration (MBIC) and biofilm prevention concentration (BPC), respectively. The results showed that MIT was the most effective of the three preservatives, but unfortunately, it also is the highest hazard compound of the three. Due to efficacy and safety concerns, MIT, PE, and SB are not the final solution in safer antifoulants, however, this work demonstrates a process for determining the efficacy of novel, safer antifoulants and efficacy metrics for comparison. Additionally, we investigated the relationship between MBIC and BPC. We found that the MBIC and BPC for each preservative were consistently higher than the minimum inhibitory concentration (MIC) for each preservative reported in the literature, but the relationship between MBIC and BPC for each preservative was not consistent. Ultimately, further investigations into safer antifoulants, paired with a greater understanding of biofilm removal and prevention doses will help make RO a better solution for water scarcity.

Key words: reverse osmosis, antifoulant, antimicrobial susceptibility testing, safer preservatives

1. Introduction

Desalination via reverse osmosis (RO) is a promising technology for helping to mitigate water scarcity, however, it is limited by energy requirements to pump water across the semipermeable membrane. For example, Wayne and Mark [1] found that the high pressure pump at an RO facility accounted for 87% of the total energy requirement of the plant. Although the energy demand of the RO process has been decreased by improvements such as switching to hybrid RO systems that utilize low flow, high rejection membranes followed by high flow, low rejection membranes, future design changes are projected to only provide about 0.5 kWh/m^3 of improvements due to impacts to flux and pressure drop [2]. A complementary and critical improvement that remains for increasing RO energy efficiently, which will make the technology available for widespread application, lies in preventing fouling.

Fouling is the build-up of organic and inorganic constituents as well as microorganisms from the feed water at the water-membrane interface. Biofouling, which is the build-up of microorganisms, is a contributing factor in more than 45% of membrane fouling cases [3]. Biofouling occurs via a four step process: 1) conditioning of the membrane by pioneer organisms or material in the bulk water, 2) attachment of pioneer organisms to the conditioned surface, 3) formation of microcolonies, and 4) formation of a mature biofilm consisting of a community of organisms in a matrix of extracellular polymeric substances (EPS) (Figure 3.1) [4]. Up to 90% of the biofilm consists of the EPS matrix, most produced by the bacteria within it, with the remaining 10% consisting of the bacterial organisms [3]. The EPS matrix itself comprises ~90% water and 10% EPS by mass, including proteins, lipids, humic substances, and polysaccharides [5]. At the early stages of biofilm formation, the fouling is considered reversible because it can be removed by increasing shear across the membrane, however, once a mature biofilm has formed it cannot be

removed by increasing flux and is considered irreversible fouling. Irreversible fouling must by removed by chemical or physical means [6].

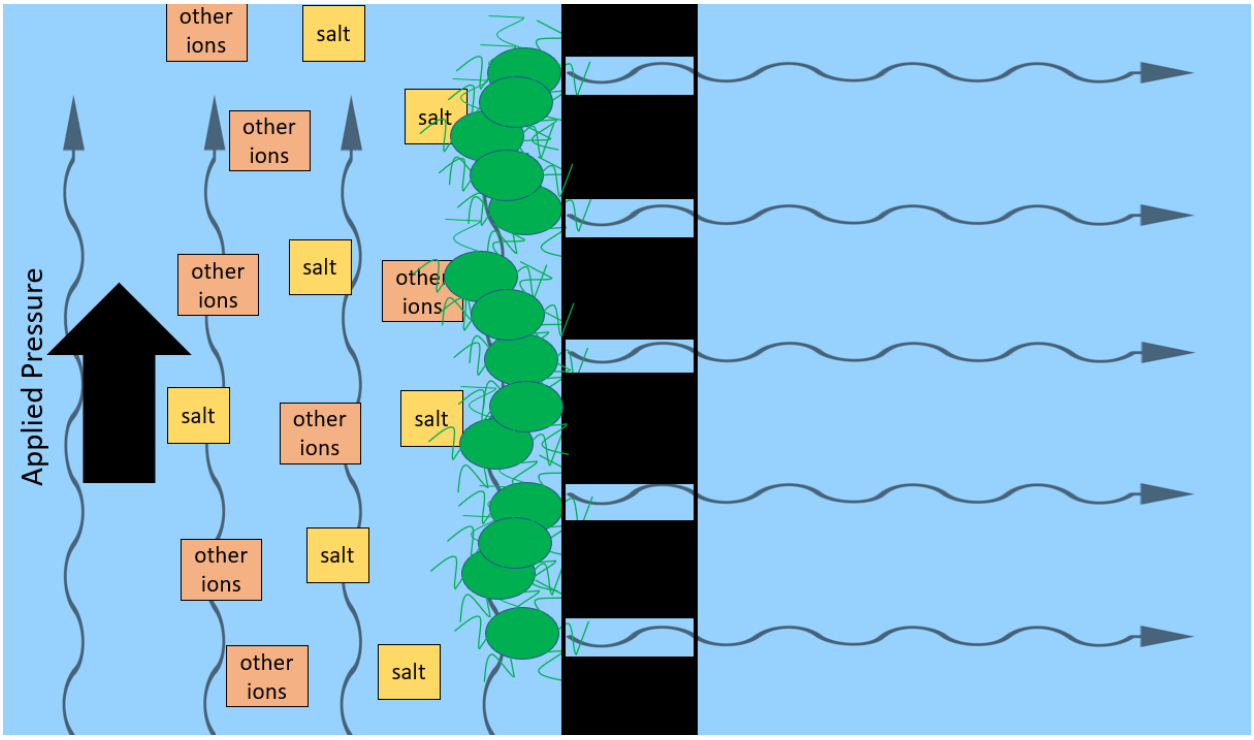

Figure 3.1. Biofouling begins by conditioning of the membrane by pioneer organisms or material in the bulk water followed by attachment of pioneer organisms to the conditioned surface, Next. A microcolony is formed by excretion of EPS. Finally, a mature biofilm forms due to growth and diversification of the microcolony. The mature biofilm, therefore, consists of a community of organisms in a matrix of extracellular polymeric substances. Organisms may disperse or detach from the biofilm based on environmental conditions [4], [7]. Before microcolony formation, biofouling is considered reversible fouling because it can be removed by shear force. However, after microcolony formation, biofilms are too strongly attached to the membrane to detach with increased shear force, therefore, requiring other treatment methods (i.e. chemical treatment).

Although solubilized chemical treatment methods, which are often biocidal, can be effective at removing biofilms, they also have associated concerns. Biocides, such as chlorine, tend to damage the membrane, decreasing its lifespan, which adds further costs to RO systems in order to regularly replace the membranes [8]. Organisms in biofilms can also develop resistance to biocides, making selecting the correct type(s) and combinations of biocides complex. Additionally, if 100% of the biofilm is not removed by the biocide, a typical situation due to the protection afforded by the dense EPS matrix, a biofilm will regrow [3], [5], [9]. It is also important to analyze the hazards

associated with biocides because they may remain in the concentrated waste or, although unlikely, small quantities of certain biocides may also pass through the membrane and enter the permeate water. For example, in Zodrow *et al.* [10] researchers measure the silver content in the filtrate which leads to the quantification of the amount of silver that remains on the membrane and can be used to determine risk of human health and environmental risk associated with this antimicrobial.

Due to health concerns related to biocides, such as endocrine disruption by triclosan [11], a method has been developed to analyze the antimicrobial efficacy and the safety of preservatives added to home and personal care products to prevent spoilage [12]. This method may have relevance in the field of RO water treatment. We hypothesize that the preservatives determined to be safer and effective in this previous work show merit as candidates for treating biofouling of RO membranes, and that they may have lower associated hazards than other common chemical anti-biofouling methods. Furthermore, the low hazard of these molecules, coupled with being of sufficient size to be largely rejected by a RO membrane, suggests it could be appropriate to add them to the feed stream of a RO system without having to take the system offline for cleaning. What trace quantities might pass through the membrane are likely to pose minimal hazard for human health and the environment.

However, antimicrobial testing for these safer chemicals has largely been performed on suspension cultures which can require 100-1000 times less chemical than biofilm treatment [13]–[15]; therefore, many results from previous studies cannot be directly applied to anti-biofouling efficacy for RO membranes. Tests that are representative of anti-biofouling efficacy must focus on biofilms.

We have developed a modified high-throughput protocol for testing anti-biofouling efficacy of three preservatives used in home and personal care products that range from high to low hazard level including 2-methyl-4-isothiazolin-3-one (MIT), 2-phenoxyethanol (PE), and sodium benzoate (SB) (Figure 3.2). We tested two anti-biofouling methods including biofilm prevention and biofilm removal. Data is presented in the form of biofilm prevention concentrations (BPC) and minimum biofilm inhibitory concentrations (MBIC) [15].

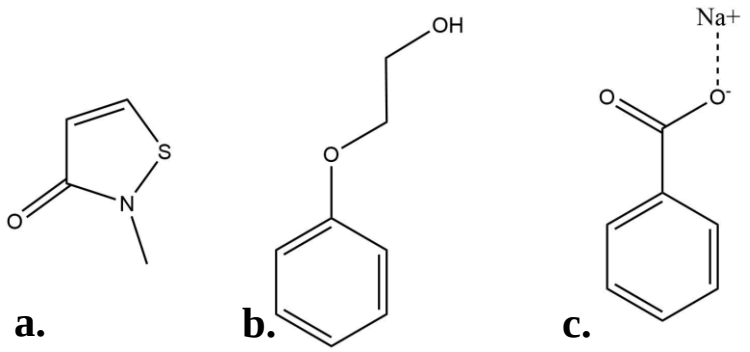

Figure 3.2. Two-dimensional chemical structures of methylisothiazolinone (a.), phenoxyethanol (b.), and sodium benzoate (c.).

2. Methods

We developed and used two different experimental set-ups to determine MBIC and BPC. The first investigated preservative efficacy against biofilms, resulting in MBIC data, and is referred to in the following sections as the Biofilm Removal Protocol. The second investigated preservative efficacy against suspension cultures at concentrations that generally lead to biofilms, resulting in BPC data, and is referred to as the Biofilm Prevention Protocol in the following sections. Figure 3.3 shows a flow diagram of all of the protocol components. Methods development information can be found in Appendix B.S2.

2.1 Materials and Chemicals

De-ionized (DI) water was obtained from a Millipore Milli-Q Advantage A10 Ultrapure Water Purification System. A 50% w/v aqueous solution of 2-methyl-4-isothiazolin-3-one (MIT) (C_4H_5NOS) was obtained from ACROS Organics (CAS: 2682-20-4). 2-phenoxyethanol (PE) ($C_8H_{10}O_2$) 99% purity was obtained from ACROS Organics (CAS: 122-99-6). Sodium benzoate (SB) ($C_7H_5NaO_2$) 98.0+% purity was obtained from TCI America™ (CAS: 532-32-1). Remell™ Mueller Hinton broth with cations (CAMHB) was obtained from ThermoFisher Scientific. Sodium chloride was obtained from Fisher Chemical (CAS: 7647-14-5). A 9g/L sterile saline solution was made by dissolving the sodium chloride in de-ionized water and autoclaving before use. Live/Dead *Bac*Light Bacterial Viability Kit, for microscopy and quantitative assessment (L7012) was obtained from Invitrogen. *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 10145™) (*P.* aeruginosa) was obtained from the University of Victoria Microbiology Department. Trypsin-EDTA (0.5%), No Phenol Red (10X) was obtained from Gibco. Corning™. Costar® Assay Plates (polystyrene 96-well plates, black with clear flat bottom, tissue culture treated) were obtained from FisherScientific. Costar® Assay Plates (polystyrene 96-well plates, clear with round bottom, tissue culture treated) were obtained from FisherScientific. We used a Cytation 5 Cell Imaging Multi-Mode Reader for fluorescence readings and imaging (University of Victoria).

2.2 Overnight Cultures (both protocols)

CAMHB powder was dissolved in DI water according the manufacturer's directions and autoclaved before use. Overnight cultures were made by inoculating 5 mL of CAMHB solution with an isolated colony of *Pseudomonas aeruginosa* (from an existing streak plate) via a disposable inoculating loop. The inoculated culture was vortexed then incubated for 18 hours in a 37°C shaker incubator set at 200 rpm.

Overnight cultures were used for both the biofilm prevention protocol and the biofilm removal protocol. We processed the overnight cultures for use in each protocol by centrifuging the overnight culture at 2095 xg for 8 minutes at approximately 21°C, to pellet *P. aeruginosa* . Longer centrifugation times tend to cause the pellet to be too difficult to break up in a later step. We then decanted the supernatant into a liquid waste container and resuspended the pellet in 5 mL of CAMHB via vortexing. We recommend holding the sample in the light to visually confirm that there are no remaining clumps of the *P. aeruginosa* pellet.

2.3 Preservative Stock Solution (both protocols)

The preservative stock solutions were prepared in CAMHB. The stock concentrations were 0.02 mM MIT (20X MIC [16]), 1,390 mM PE (20X MIC [17]), and 1,735 mM (50X MIC [18]) for SB. The PE was difficult to dissolve, therefore, we thoroughly vortexed the sample each time before an aliquot was taken.

2.4 Preservative Microdilution Plate (both protocols)

Preservative microdilution plates containing serial dilutions of the preservative can be made day-of or beforehand and stored at an appropriate temperature. Slightly different preparation methods were used for each protocol.

2.4.1 Preservative Microdilution Plate/Serial Dilution (Biofilm Removal Protocol)

The preservative microdilution plate for the biofilm removal protocol was prepared in a round-bottom 96-well plate because aliquots from this plate were added to a different plate. To perform serial dilutions for the biofilm removal protocol, first, we added 125 µL of CAMHB to each well in the 96-well plate except for wells in column 1. The CAMHB acted as both the diluent for the antimicrobial and the broth required for organisms to grow successfully. We then added 250 µL

of 1X CAMHB to column 1 in row A (negative control/no preservative) and C (positive controls). In column 1 in rows B (negative controls/contains preservative) and D-H (experimental) we added 250 µL of the preservative stock. We then performed a serial dilution across the 96-well plate. To do this, we mixed contents in column 1 by pipetting and then removed 125 µL from the well and expelled the contents into the corresponding wells in column 2. We mixed the contents in column 2 thoroughly and repeated the process until column 12, where the removed contents were discarded. This leads to a plate with 125 µL of contents per well with 2-fold dilutions across the plate. The plate contains a highest concentration that is equal to the preservative stock concentration (MIT= 600µg/mL, PE=960 mg/mL, and SB= 250 mg/mL).

2.4.2 Preservative Microdilution Plate/Serial Dilution (Biofilm Prevention Protocol)

The preservative microdilution plate for the biofilm prevention protocol was prepared in a flat-bottom 96-well plate because the dilution plate was used to grow biofilms. To perform serial dilutions for the biofilm prevention protocol, we added 80 µL of CAMHB to each well in the plate, except for wells in column 1. Next we added 160 µL of CAMHB to wells A (negative controls/no preservative) and C (positive controls) in column 1. The following steps varied for each preservative in order to lead to the desired final concentrations of preservative. To create a dilution plate with a highest preservative concentration that is three-quarters the preservative stock solution concentration, we added 40 µL of CAMHB and 120 µL of the preservative stock to wells B (negative control/contains preservative) and D-H (experimental) in column 1. This led to plates with the highest concentration (column 1 B and D-H) at 15X the MIC for MIT and PE (450 µg/mL MIT and 720 mg/mL PE). To create a dilution plate with a highest preservative concentration that is half the preservative stock solution concentration, we added 80 µL of CAMHB and 80 µL of the preservative stock to wells B and D-H in column 1.

Next, we performed a serial dilution in the same way as part 2.4. To do this, we mixed contents in column 1 by pipetting and then removed 80 μL from the well and expelled the contents into the corresponding wells in column 2. We mixed the contents in column 2 thoroughly and repeated the process until column 12, where the removed contents were discarded. This leads to a plate with 80 μL of contents per well with 2-fold dilutions across the plate.

2.5 Biofilm Removal Protocol (Figure of Merit: MBIC)

The biofilm removal protocol follows a modified version of [7]. Biofilm growth was performed in flat-bottom 96-well plates in order to encourage biofilm formation. First, we measured the optical density of the processed overnight culture of *P. aeruginosa* and determined the approximate cell number via a premade standard curve. We then diluted the processed overnight culture of *P. aeruginosa* to approximately 1 x 10^6 CFU/mL (determined via OD standard curve) [19]. We added 100 μL of the diluted bacterial suspension into each of the wells in rows C-H. We added 100 μL of CAMHB to wells in rows A and B. We secured the lid on top of the 96-well plate with tape and incubated it for 24 hours in a 37°C shaker incubator set at 200 rpm.

After incubation we aspirated the contents out of each well of the 96-well plate, ensuring not to hit the sides of each well, which is where *P. aeruginosa* biofilms are most often found [20]. Next, we rinsed each well once with 125 μL sterile saline. After thawing the pre-prepared preservative microdilution plate, we mixed each well thoroughly with a pipette and withdrew 100 μL per well. We then carefully expelled the 100 μL in the corresponding wells in the plate containing *P. aeruginosa* biofilms, avoiding the biofilms formed on the side of the well. We then secured the lid on top of the 96-well plate with tape and incubated the plate for 24 hours in a 37°C shaker incubator set at 200 rpm.

2.6 Biofilm Prevention Protocol (Figure of Merit: BPC)

The biofilm prevention protocol follows a modified version of [7] (Appendix B.S1, Figure S1). To begin, we measured the optical density of the processed overnight culture and determined the approximate cell number via a standard curve. We then diluted the overnight culture of *P. aeruginosa* to $5x10^6$ CFU/mL (determined via OD standard curve). After thawing the pre-prepared preservative microdilution plate and thoroughly mixing the contents of each well via pipette, we added 20 µL of the diluted bacteria to each well in the preservative microdilution plate using new tips for each row, leading to a final cell concentration of $1x10^6$ CFU/mL (the same concentration of cells used in the biofilm removal protocol) [19]. Each well in the 96-well plate should contain 100 µL total volume. We secured the lid on top of the 96-well plate with tape and incubated the plate for 24 hours in a 37°C shaker incubator set at 200 rpm.

2.7 Staining Protocol (both protocols)

The following steps of the protocol apply to both the biofilm prevention and removal protocols. First, we aspirated out all of the contents in each well of the 96-well plate. Next, we rinsed the plate once with 125 µL of sterile saline, in order to remove planktonic cells. Then we added 60 µL of 1X trypsin to each well of the 96-well plate, to attempt to remove the biofilms from the sides of the wells. We incubated the plate in the shaker incubator at 37°C at 200 rpm for four minutes. Meanwhile, in the dark we prepared a saline solution containing the LIVE/DEAD *Bac*Light Stain at final concentrations of 15 µM propidium iodide (PI) and 2.5 µM SYTO9. This stock was vortexed thoroughly. We then added 40 µL of the stain stock solution to each well in the 96-well plate. We wrapped the plate in foil and vortexed it for 15 minutes to further attempt to remove biofilms from the sides of the wells and create a homogenous solution. We then read the fluorescence intensity using Gen5 3.08 software and the Cytation 5 plate reader, performing a 5x5

well area scan (25 measurements per well) from the bottom of the plate, and ignoring well and carrier limitations. Green fluorescence was read at 485 nm excitation and 528 nm emission and red fluorescence was read at 485 nm excitation, 645 nm emission.

2.8 Data Analysis (both protocols)

2.8.1 Determining MBIC/BPC and Standard Error from Raw Data

The following analysis was completed for both the green and the red fluorescence data obtained from the plate reader for each experiment. For the broth negative control wells (A1-12) we calculated the mean and standard deviation for all readings for each well (25 measurements per well, therefore, 300 measurements total), which provided one value for the mean and standard deviation for broth negative controls. We calculated one mean fluorescence value with population standard deviation because each broth negative control well contained the same exact conditions. For the preservative control wells (B1-12), we calculated a mean and population standard deviation for each well. We calculated separate mean fluorescence values and standard deviations because each well contained a different concentration of the preservative, which could absorb light and affect emission values.

Next, we calculated one mean fluorescence and population standard deviation for the positive control. To do this, we first calculated the mean and population standard deviation of each positive control well (C1-12) from the 25 measurements per well, resulting in one mean fluorescence value and population standard deviation for each positive control well. Next, we subtracted the mean of the broth negative control wells (calculated above from wells A1-12) from each mean of the positive control wells (C1-12) and corrected the standard deviation. We subtracted the negative control well mean in order to remove the background fluorescence. Then we calculated the combined mean of the positive control wells and the combined variance, which was converted to

standard deviation. This led to one mean fluorescence value and standard deviation for the positive controls (minus background).

For the experimental wells (D-H 1-12), we calculated the mean fluorescence of each well containing the same concentration of preservative (25 measurements per well, 125 measurements for each concentration). This led to one mean fluorescence value and population standard deviation for each preservative concentration. Next, we subtracted the mean of the corresponding negative control well (i.e. (mean(D-H1)) minus mean B1) and corrected the standard deviation. This led to one mean fluorescence value and population standard deviation for each concentration of the preservative.

We combined replicates for each preservative by calculating the combined mean and calculating combined variance, which was converted to standard deviation first, and then standard error. Each resulting data set consisted of a mean fluorescence of the positive controls with standard error and mean fluorescence values of each concentration of the preservative with standard error.

2.8.2 Determining BPC and MBIC

Finally, both BPC and MBIC were determined by calculating the concentration at which the mean green fluorescence was 90% lower than the mean of the positive control wells [15].

2.8.3 ANOVA to Investigate the Variation of the Green Fluorescence

The first ANOVA was between the green fluorescence of the positive control wells and the green fluorescence of each experimental well separately for each preservative for both the removal and prevention protocol. One purpose of comparing the green fluorescence of the positive controls to the green fluorescence of the experimental wells of each experiment was to ensure that each MBIC and BPC value was statistically different than the positive control. We also analyzed these results

to investigate the variation in green fluorescence due to concentration of preservative for each experiment.

2.8.4 ANOVA to Investigate the Variation of the Red Fluorescence

Another ANOVA was between the red fluorescence of the positive controls and the red fluorescence of each experimental well separately for each preservative for both the removal and prevention protocol. One purpose of this ANOVA was to investigate whether there were red fluorescence values that were significantly different than the positive control. The other purpose was to investigate the variation in red fluorescence due to concentration of preservative for each experiment.

2.8.5 ANOVA to Investigate Consistency of the Positive Controls Across Experiments

We performed two other ANOVAs. One ANOVA was between the green fluorescence of each set of positive controls of each preservative for both the removal and prevention protocol. The other was between the means of the red fluorescence of each set of positive controls of each preservative for both the removal and prevention protocol. The purpose of these ANOVAs was to determine if the protocol lead to consistent positive controls across experiments.

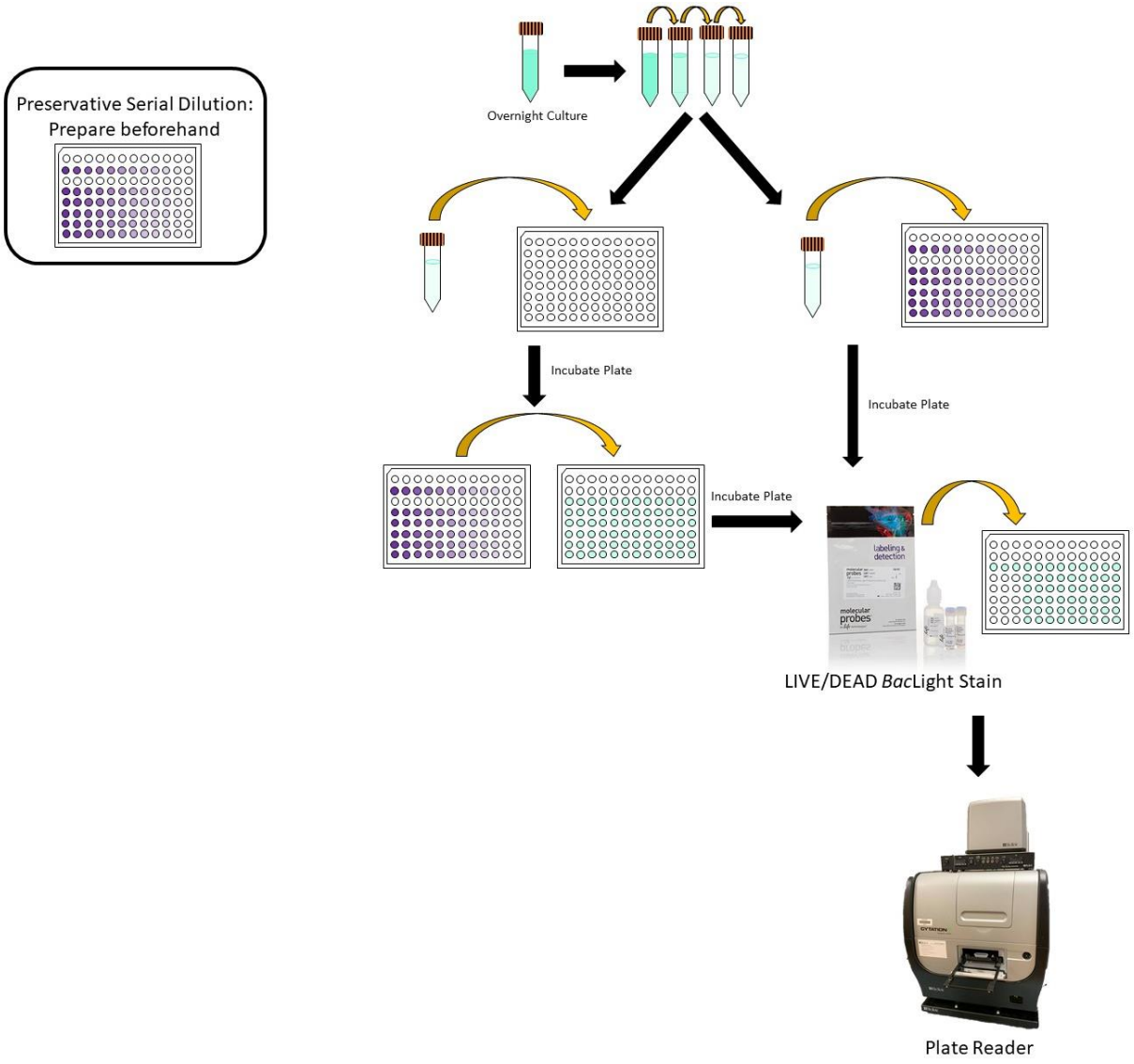

Figure 3.3. Flow diagram showing the experimental protocol. First we prepared an overnight culture. After 18 hours we serially diluted the overnight culture to the appropriate CFU/mL depending on whether or not we were following the Biofilm Removal Protocol (Section 2.5) or the Biofilm Prevention Protocol (Section 2.6). For the Biofilm Removal Protocol we inoculated a 96-well plate with the diluted overnight culture. We incubated the plate for 24 hours and then added the serially diluted preservative. For the Biofilm Prevention Protocol we directly inoculated the serially diluted preservative with the diluted overnight culture. We incubated the plate for 24 hours. For both of the protocols we next rinsed the plates and then added trypsin and the LIVE/DEAD *Bac*Light Stain. After 15 minutes incubation, we read the fluorescence with the plate reader.

3. Results

In general, for both the MBIC and the BPC, we found that the green fluorescence was higher at lower concentrations of the preservative and dropped dramatically, most often at the MBIC or BPC

(Figure 3.4 and 3.5). Additionally, we found that the red fluorescence tended to be higher at lower concentrations of the preservative and decreased at lower concentrations of preservative.

3.1 Biofilm Removal Results (MBIC)

MBIC values are provided in Table 4.1. Of the three antifoulants tested, the lowest MBIC value for biofilm removal was for MIT. The MBIC for biofilm removal by MIT was 75 µg/mL (Table 4.1; Figure 3.4a.). This corresponds with a concentration of MIT that resulted in a fluorescence value that was significantly lower than the positive control values according to the ANOVA ($p<0.001$) (Appendix B.S3, Table S1), indicative of a significant decrease in the extent of biofilm formation in the treated well as compared to the controls. The MBIC value for biofilm removal by PE was 120,000 µg/mL (Table 4.1; Figure 3.4b.). This corresponds with a fluorescence value that was significantly different than the control ($p<0.001$), however, 60,000 µg/mL and 30,000 µg/mL also were significantly different than the control ($p<0.001$), but the mean fluorescence for those concentrations was not 90% lower than the mean for the positive control (Appendix B.S3, Table S1). The MBIC value for biofilm removal by SB was 31,200 µg/mL (Table 4.1; Figure 3.4c.). This corresponds with a fluorescence value that was significantly different than the control ($p<0.001$), however, 15,600 µg/mL also was significantly different than the control, but the mean fluorescence was not 90% lower than the mean for the positive control ($p<0.001$) (Appendix B.S3, Table S1).

3.2 Biofilm Prevention Results (BPC)

BPC values are provided in Table 3.1. In the case of MIT and PE, the BPC values were lower than the MBIC, however, the MBIC and BPC were the same for SB. The BPC for MIT was lower than the BPC for PE. The BPC for MIT was 22.5 µg/mL (Table 3.1; Figure 3.4d.). The BPC for MIT corresponds with a fluorescence value that was significantly different than the control, however ,

11.3 and 5.63 µg/mL also were significantly different than the control ($p<0.001$), but the mean fluorescence was not 90% lower than the mean for the positive control (Appendix B.S3, Table S1). The BPC value for PE was 36,000 µg/mL (Table 3.1; Figure 3.4e.). This corresponds with a fluorescence value that was significantly different than the control, however, fluorescence at 18,000 µg/mL also was significantly different than the control ($p<0.001$), but the mean fluorescence was not 90% lower than the mean for the positive control (Appendix B.S3, Table S1). The BPC for SB was 25,000 µg/mL (Table 3.1; Figure 3.4f.). The BPC for SB corresponds with a fluorescence value that was significantly different than the control, however, the mean fluorescence at 12,500 µg/mL also was significantly different than the control ($p<0.001$), but the mean fluorescence was not 90% lower than the mean for the positive control (Appendix B.S3, Table S1).

Table 3.1. Minimum biofilm inhibitory concentration and biofilm prevention concentration values for each preservative with corresponding relative fluorescence units and standard error determined via Section 2.8.1.

Preservative	Minimum Biofilm Inhibitory Concentration (µg /mL)	Fluorescence (Relative Fluorescence Units)	Biofilm Prevention Concentration (µg /mL)	Fluorescence (Relative Fluorescence Units)
MIT	75	$2.3 \pm 0.7 * 10^3$	22.5	$1.8 \pm 0.3 * 10^3$
PE	120,000	$3.5 \pm 2.1 * 10^3$	36,000	$1.1 \pm 0.3 * 10^3$
SB	31,200	$1.1 \pm 0.2 * 10^4$	25,000	$9.7 \pm 0.9 * 10^2$

3.3 ANOVA Between Green Fluorescence of Positive Controls and Experimental Wells

The results of the ANOVA between the green fluorescence of the positive control wells and the green fluorescence of each experimental well separately for each preservative for both the removal and prevention protocol can be found in Appendix B.S3, Table S1.

3.3.1 Statistical Significance of MBIC and BPC

Green fluorescence values for all MBIC and BPC values were significantly different than the green fluorescence of the positive control ($p<0.001$) (Figure 3.4, Table S1)

3.3.2 Green Fluorescence of the Positive Control Compared to Experimental Wells

Higher concentrations of each preservative led to significantly lower green fluorescence values than the positive control. Some lower concentrations led to green fluorescence values that were not statistically different than the control, however, unexpectedly, at some lower concentrations of each preservative, green fluorescence values were higher than the positive control.

3.4 ANOVA Between Red Fluorescence of Positive Controls and Experimental Wells

Figure 3.5 shows box and whisker plots of the red fluorescence values for each of the preservatives for each protocol. The results of the ANOVA between the red fluorescence of the positive control wells and the red fluorescence of each experimental well for each preservative for both the removal and prevention protocol can be found in Appendix B.S3, Tables S2 and S3.

3.4.1 Red Fluorescence of Positive Controls Compared to Experimental Wells

First, we investigated the red fluorescence values for preservative concentrations that correspond to green fluorescence values that are significantly lower than the positive control (higher concentration of preservative). For the MIT biofilm removal protocol and of the MIT, PE and SB prevention protocol these red fluorescence values were statistically different than the corresponding positive controls ($p<0.01$) (Figure 3.5; Appendix B.S3, Table S2). The relevant red fluorescence of SB and PE for the biofilm removal protocol were not statistically different than the control.

Next, we investigated the red fluorescence values for preservative concentrations that correspond to green fluorescence values that are equal to or significantly higher ($p<0.05$) than the positive control (lower concentrations of the preservative) (Appendix B.S3, Table S2). The red fluorescence values at these concentrations appeared to be statistically similar or statistically higher than the positive control.

3.4.2 Comparing Red Fluorescence Between Experimental Wells

To compare between experimental wells, we focused on red fluorescence values for preservative concentrations that correspond to green fluorescence values that are significantly lower than the positive control (higher concentrations of the preservative) because there have been issues identified with fluorescence accuracy at lower dead cell concentrations [21] (Appendix B.S3, Table S3). We found that the red fluorescence at these concentrations were not statistically different from each other.

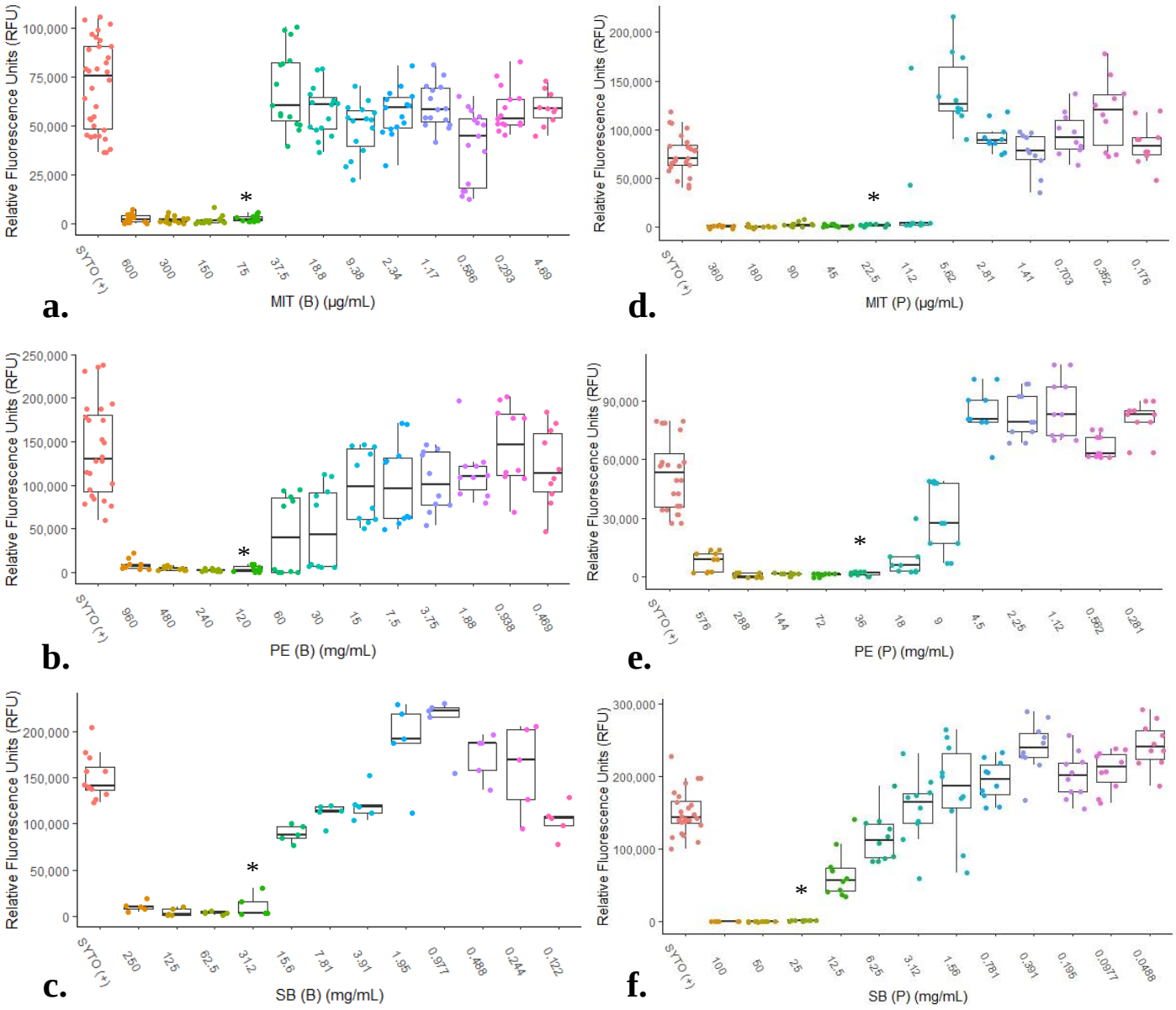

Figure 3.4. Box and whisker plots show the green fluorescence values for each biological replicate (5 samples/96-well plate) for the Biofilm Removal Protocol (a.-c.) and Biofilm Prevention Protocol (d.-f.) for each preservative (a. n=3 plates, b. n=2, c. n=2, d. n=2, e. n=2, f. n=2). The asterisk indicates the MBIC location (a.-c.) or the BPC location (d.-f.), both of which were determined as the lowest concentration at which the mean green fluorescence was 90% lower than the mean green fluorescence of the positive control (SYTO (+)). The MBIC and BPC locations were determined to be significantly different than the positive control ($p<0.001$) via an ANOVA. Adjusted p-values can be found in Appendix B.S3, Table S1.

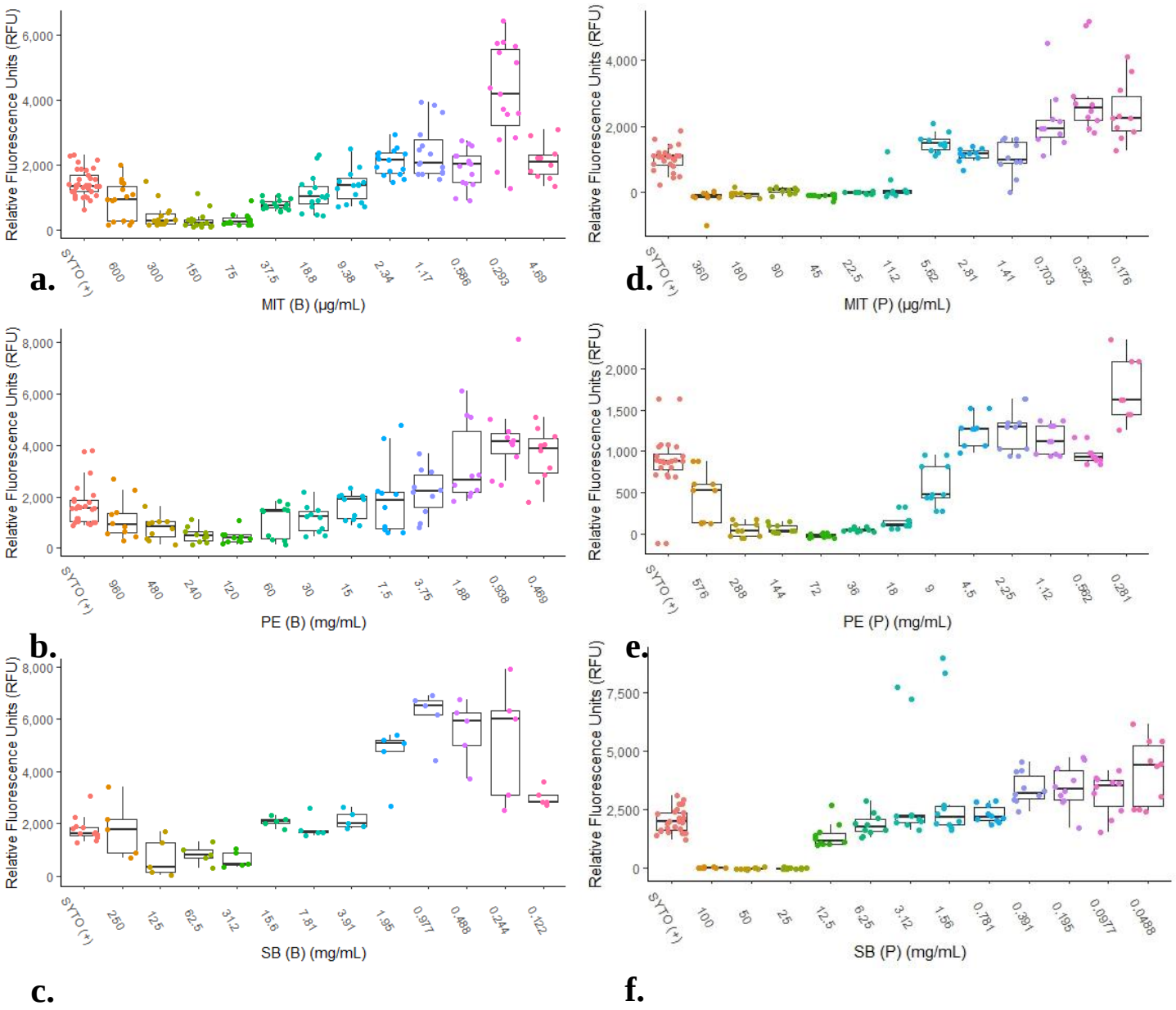

Figure 3.5. Box and whisker plots show the red fluorescence values for each biological replicate (5 samples/96-well plate) for the Biofilm Removal Protocol (a.-c.) and Biofilm Prevention Protocol (d.-f.) for each preservative (a. n=3 plates, b. n=2, c. n=2, d. n=2, e. n=2, f. n=2). This data was used for multiple ANOVAs and the adjusted p-values can be found in Appendix B.S3, Table S2.

3.5 ANOVA Investigating Positive Control Consistency

The ANOVA between green fluorescence values for the positive control for each experiment suggested that the MIT biofilm removal protocol and the MIT and PE prevention protocol were statistically different from the PE and SB biofilm removal protocol and the SB prevention protocol (Figure 3.6a.; Appendix B.S3, Table S4). The ANOVA between red fluorescence values for the positive control for each experiment suggested that the red fluorescence of the MIT and PE prevention protocol were statistically different from the other experiments ($p<0.01$ for MIT prevention protocol—MIT removal protocol, $p<0.001$ for the remainder) (Figure 3.6b., Appendix B.S3, Table S4). The SB prevention protocol was statistically different from the PE prevention protocol and MIT prevention protocol ($p<0.001$) and the MIT biofilm removal protocol ($p<0.001$) (Figure 3.6b.; Appendix B.S3, Table S4). The remainder of the experiments were not statistically different.

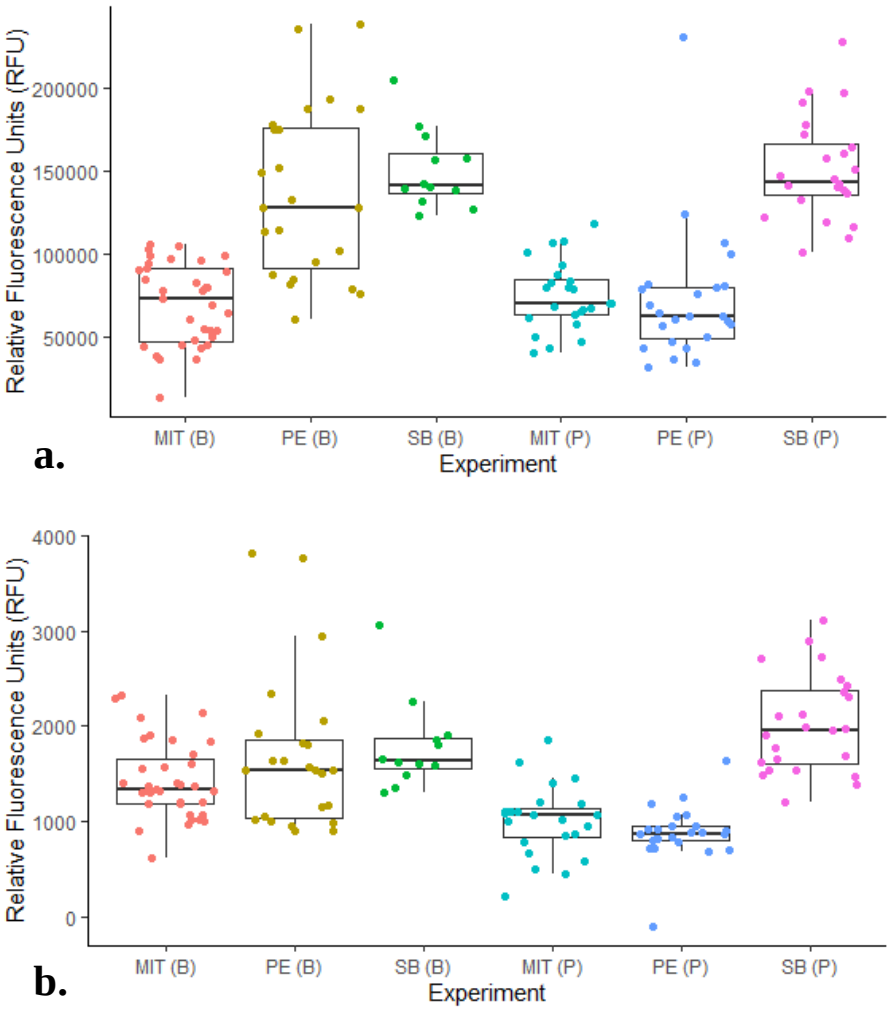

Figure 3.6. Comparison of means of green (a.) and red (b.) fluorescence of the positive controls for each experiment to investigate consistency of positive controls across experiments. Corresponding ANOVA results can be found in the Appendix B.S3 (Table S4).

4. Discussion

4.1 Minimum Biofilm Inhibitory Concentration and Biofilm Prevention Concentration

MBIC and BPC are determined as the lowest concentrations at which the resulting fluorescence value was at least 90% lower than the positive control. Our data showed that the MBIC for biofilm removal was almost three times higher than the BPC for biofilm prevention for MIT and PE (Table 4.1 and 2). More simply put: with MIT and PE, preventing biofilm formation requires less antifoulant that slowing growth of a biofilm once it is established. This relationship is supported by Güven and Onurdağ [22], which found the MIC to be smaller than the MBIC for multiple organisms. Although Güven and Onurdağ [22] studied MIC values, rather than BPC values, Fernández-Olmos *et al.* Fernández-Olmos *et al.* [23] suggests this may be an acceptable replacement because they found that the BPC for *P.* aeruginosa was equal to or only slightly higher than the MIC value, therefore a comparison between the two may be acceptable.

There are various mechanisms at play that cause BPCs to be lower than MBICs. One way that bacteria in biofilms have lower susceptibility to disinfectants is diffusion/reaction limitations [24]. Bacteria in mature biofilms are in a matrix of EPS that impedes the diffusion of disinfectants into the biofilm and thus, decreases disinfection reactions. For example, De Beer *et al.* [25] found that biofilms containing both *P. aeruginosa* and *Klebsiella pneumoniae* had 20% or less chlorine inside the biofilm than in the bulk water and the concentration of chlorine inside the biofilm did not reach equilibrium with the bulk water after 2 hours of treatment. Additionally, cells incorporated in biofilms express different phenotypes than planktonic cells in suspension cultures, which can result in increased resistance to disinfectants [26]–[29]. For example, researchers found that cells in biofilms have altered growth and activity rates that promote defense mechanisms and lead to cell membrane modifications that can impact resistance to disinfectants [30]–[34]. Bacterial cells

incorporated in biofilms also can undergo lateral gene transfer, which can include transferring genes for antimicrobial resistance [24].

The BPC for SB was found to be less than one time greater than the MBIC for SB. We hesitate to form a conclusion about this relationship until we perform more replicates. Additionally, the MIC for sodium benzoate is recorded as 5,000 μg/mL in the literature Stanojevic *et al.* [18], which if MIC is similar to BPC as suggested by [23], then the BPC should be lower. There are, however, cases where the MBIC and BPC are the same, such as for ciprofloxacin against *P. aeruginosa* [23], suggesting that the relationship may be valid. We plan to perform more replicates to investigate this further.

In our study, MIT was the most effective anti-biofouling chemical preservative with an MBIC of 75 μg/mL and a BPC of 22.5 μg/mL (Table 4.1). In the literature, reported values for the MIC of MIT are reported at 12.125 μg/mL - 30 μg/mL against *P. aeruginosa* [16], [35]. This range overlaps the BPC we found for MIT, owing to the fact that the prevention protocol closely mimics a suspension culture susceptibility test. The difference between our protocol and suspension culture susceptibility tests that lead to MIC values is that we added a higher concentration of cells to our 96-well plates, compared to the amount commonly used in suspension culture susceptibility tests (i.e. [12]) in hopes of promoting biofilm formation [36]. In the literature the MIC for PE ranges from about 3,200 μg/mL to 4,800 μg/mL [12], [37]. Reported MICs for PE are lower than the BPC we determined through our experiment, which was 36,000 μg/mL, and the MBIC, which was 120,000 μg/mL (Table 4.1). We were unable to find similar molecules in the literature that resulted in MBIC and BPC values greater than MIC, however, a similar relationship was found for *P. aeruginosa* treated with ceftazidime, tobramycin, and imipenem [23]. The recorded MIC of sodium benzoate is 5,000 μg/mL [18]. Our experiments with sodium benzoate suggest that the

MBIC, which was 31,200 μg/mL, is close to the BPC, which was 25,000 μg/mL (Table 4.1). This was the only MBIC from our study that could be corroborated by a reported MBIC, which was 32,768 μg/mL [22]. As stated earlier, a similar relationship between MBIC/BPC and MIC is supported in the literature [23].

We hypothesize that MIT had the lowest MBIC and BPC due to mode of action. The mechanism of action of MIT proceeds by MIT diffusing across the bacterial cell membrane and then the electron-deficient sulfur bond reacting with nucleophilic groups in the cell (Figure 3.7). Susceptible nucleophilic groups include thiol-containing compounds such as cysteine-containing proteins and glutathione enzymes, which prevent damage to the cell by reactive oxygen species [38]. MIT blocks the active site in these enzymes, preventing enzymatic activity from occurring. For example, Collier *et al.* [39] found MIT to readily react with thiol-containing compounds, inhibiting cellular growth within a few minutes of addition and causing cell death within a few hours. Since MIT reacts so readily, we believe it is able to inhibit biofilm formation and remove biofilms more effectively than the other preservatives tested.

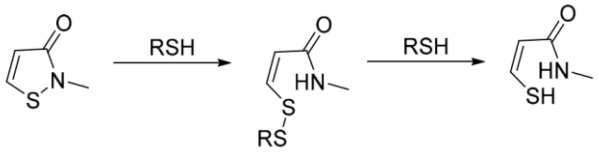

Figure 3.7. A representation of the mode of antimicrobial action of methylisothiazolinone during which it reacts with cysteine-containing proteins/gluthathione enzymes, denoted as RSH [38].

PE and SB had higher MBIC and BPC values compared to MIT, likely due to different modes of action. In the case of PE, the mechanism of action has been investigated but never fully elucidated [40]. Research suggests that multiple modes of action may cause PE to have antimicrobial activity, including membrane damage that allows cytoplasmic contents to leak out of the cell, however

researchers suggest this is only a contributing factor to cell death [40]. Other experiments tested PE at sub-lethal doses and found that PE also inhibits cell growth by inhibiting the synthesis of DNA and RNA [41], inhibiting energy metabolism [42], and disrupting the proton gradient [43]. In the case of SB, researchers suggest it is first converted to benzoic acid in acidic conditions [44], [45]. Once in the cell, the cell undergoes the energetically expensive process of pumping out the protons, which results in less energy available for growth [44], [46]. More specifically, Pongsavee [45] states that the benzoic acid inside the cell disrupts anaerobic fermentation of glucose via phosphofructokinase decreases which inhibits growth of cells and survival. The previous mechanisms of action are supported by Sagoo *et al.* [47] in which researchers found yeast cells were able to maintain viable numbers for about 20 minutes of exposure to SB while they pumped out protons, however, after the cells depleted their internal energy, the cells could no longer survive without an external energy source (i.e. glucose).

4.2 Variation in Green Fluorescence

We found that the green fluorescence at lower concentrations of the preservative was sometimes statistically higher than the green fluorescence of the positive control ($P<0.05$) (Figure 3.4, Appendix B.S3, Table S1). We believe this may be due to a phenomenon identified in Stiefel *et al.* [21]. Stiefel *et al.* [21] found that the green fluorescence of a sample of 100% dead *P. aeruginosa* cells was 2.7 fold higher than the green fluorescence of a sample of 0% dead *P. aeruginosa* cells. Stiefel *et al.* [21] identifies this phenomenon with gram-negative bacteria, but not gram-positive bacteria and, therefore, suggests that SYTO9 may not be able to pass through the two cell membranes of live gram-negative bacteria quickly enough to intercalate with DNA, which turns on the fluorescence. By contrast, SYTO9 can intercalate rapidly with the DNA of dead gram-negative bacteria, leading to artificially high green fluorescence with the presence of dead

cells. We believe this phenomenon may play a role in the green fluorescence values that result in higher green fluorescence values than the green fluorescence of the positive control that correspond to red fluorescence values that were higher than the red fluorescence of the positive control, however, the trend is not consistent across preservatives and concentrations.

4.3 Variation in Red Fluorescence

In general, the red fluorescence increased with a decrease in preservative concentration, indicating an increase in dead cells with a decreasing amount of preservative. This was somewhat unexpected. However, we believe that more cells could grow in the samples with less preservative, which meant more cells to die, whereas the higher concentration of preservative may have killed cells before more cells could grow and then die.

We also analysed the variation of the red fluorescence for each preservative for both the removal and prevention protocol (Figure 3.5; Appendix B.S3, Table S2). The mean red fluorescence of the MIT biofilm removal protocol and of the MIT, PE and SB prevention protocol were statistically different from the mean of the red fluorescence of each experiment's positive controls ($p<0.01$) (Figure 3.5; Appendix B.S3, Table S2). The red fluorescence of the SB and PE biofilm removal protocol were not statistically different than the control. Ultimately, we found some variation between the experimental wells and the positive controls ($p<0.05$), suggesting the number of dead cells varied with concentration. However, when we compared red fluorescence values between the higher concentrations of preservatives (that lead to significantly lower green fluorescence values), the values did not differ significantly for the PE biofilm prevention data (Figure 3.5; Appendix B.S3, Table S2). Stiefel *et al.* [21] suggests that relatively high amounts of dead cells are needed for precise determination of dead cells due to high background noise caused by unbound propidium iodide (red fluorescent molecule). We have some concerns with the red fluorescence due to the

limited statistical difference between the experimental and control data and between the experimental data. Due to this concern, in the future, we hope to corroborate dead cell amount determined by the LIVE/DEAD *Bac*Light Stain via alternative methods (i.e. [48]).

4.4 Consistency of Controls

Additionally, we investigated the consistency between the positive controls (Figure 3.6; Appendix B.S3, Table S4). The conditions of the positive controls across all experiments were exactly the same, therefore we would expect consistency between experiments. There was some consistency between experiments, however, multiple experiments were statistically different ($p<0.001$). We attribute this to insufficient homogenization of the biofilm via vortexing (Appendix B.S3, Figure S4). Although we read fluorescence using a 25-point area well scan, we still believe a non-homogenous solution impacts accuracy of readings. We suggest that future experiments sonicate samples to improve consistency across experiments.

4.5 A Brief Analysis of Hazard

Although MIT was found to be the most effective of the three preservatives tested, it has a slew of health concerns that have caused it to be a model of efficacy, but not safety in consumer products. MIT ranks as very high hazard level for acute mammalian toxicity, skin irritation/corrosivity, eye irritation/corrosivity, and acute aquatic toxicity, as well as high to moderate hazard level for endocrine activity, skin sensitization, and terrestrial ecotoxicity [49]. A highly noted hazard of MIT is contact dermatitis from direct contact or airborne exposure to the preservative in consumer items ranging from baby wipes and toilet paper to paint [50]–[52]. There were even two case of children who experienced toxic lung injury believed to be in-part caused by chloromethylisothiazolinone (MCIT – a halogenated variant) and MIT-containing humidifier disinfectants [53]. The other two preservatives have fewer identified hazards and are recognized

in the home and personal care products industry as potential safer alternatives to MIT. PE is ranked as a high hazard for eye irritation/corrosivity and moderately for reproductive toxicity, developmental toxicity, acute mammalian toxicity, and terrestrial ecotoxicity, while SB ranks at a moderate hazard level for terrestrial ecotoxicity [49].

5. Conclusion

In this study, we developed and implemented protocols to compare the relative efficacy of small molecule antifoulants, previously recognized as effective preservatives for preventing planktonic growth, against the formation (BPC) and continued growth (MBIC) of biofilms. Our study found MIT to be the most effective preservative for both preventing and removing biofilms of the preservatives we tested. This conclusion is supported by existing literature. Additionally, we found that the MBIC and BPC for the preservatives we tested were always higher than the reported MIC values, consistent with both literature precedent and biological defense mechanisms understood to be at play in biofilms. However, the relationship between BPC and MBIC varied between preservatives.

Although effective, MIT has many associated hazards, which likely makes it a good model for efficacy, but not for safety. Both PE and SB are safer preservatives, however, their efficacy is not as high as that of MIT. It is therefore integral to continue to search out and test the efficacy of other safer antimicrobials, such as octyl gallate [12]. Additionally, in recognition of the complex, multispecies nature of biofilms, future work will include analogous experiments on other biofouling organisms, such as *Staphylococcus aureus*, to corroborate our MBIC and BPC results for *P. aeruginosa*. By identifying efficacious and safe preservatives that can be applied to RO systems, we can help make RO a more energy-efficient and cost-effective process, making it a better candidate for mitigating water scarcity.

Acknowledgements:

Thank you: Ellen Busby and Stephen Redpath for supplying bacteria; Rebecca Hof for providing guidance and lab space; Kurtis Manke for help with coding.

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Discussion

This thesis consists of four manuscripts focused on biofilm antimicrobial susceptibility tests in regard to biofouling, with the goal being to mitigate biofouling on reverse osmosis membranes. The first three manuscripts consist of critical reviews that recommend protocol and metrics for anti-biofouling efficacy studies. The fourth manuscript utilizes the recommendations presented in the first three manuscripts to perform biofilm antimicrobial susceptibility tests.

Specifically, in Manuscript 1 we analyzed literature via a semi-systematic review to identify the common organisms used in studies that investigate antimicrobial efficacy against biofilms on RO membranes. We focused on pioneer organisms, the first organisms to attach to the membrane that lead to the formation of a mature biofilms, because we were interested in looking at prevention of biofilms, along with biofilm removal. We found that *E. coli* was the most common organism used for biofilm antimicrobial susceptibility tests, followed by *P. aeruginosa* and *S. aureus*. All three of these organisms were also identified as model, biofilm-forming organisms that are also pioneer organisms [18]–[24]. These qualities suggest all three would be effective challenge organisms for biofilm antimicrobial susceptibility testing [25].

In Manuscript 1 we also analyzed literature via a separate semi-systematic review to investigate the common organisms identified via genetic analysis on RO membranes. Again, we focused our analysis on pioneer organisms. Due to inconsistent taxonomical levels of identification, we analyzed data at phylum and class levels. The top three most frequent classes of organisms were in the phylum Proteobacteria including, Alphaproteobacteria, followed by Gammaproteobacteria and Betaproteobacteria. We theorize that the common occurrence of these organisms is due to relatively high production of EPS [26]. We then compare the results of the two semi-systematic reviews to identify the best organism to recommend for antimicrobial susceptibility tests based on

commonly occurring in biofilms on RO membranes, being a model-biofilm forming organism, being a pioneer organism, and being utilized in previous studies investigating anti-biofouling efficacy on RO membranes to allow for future comparisons. This analysis led to our recommendation of using *P. aeruginosa* (Gammaproteobacteria) for biofilm antimicrobial susceptibility tests because it meets all four requirements. Additionally, *P. aeruginosa* is a clinically relevant organism that has public health implications, which are relevant in RO systems that produce drinking-water quality permeate [27]–[29].

Manuscript 2 delved further into biofilm antimicrobial susceptibility tests by investigating the best-practice detection methods and corresponding metrics. Since biofouling is multidisciplinary concern, impacting disciplines including engineering, pharmacology, microbiology, wastewater treatment, chemistry, food preservative formulation, home and personal care product formulation, and toxicology, the results of biofilm antimicrobial susceptibility tests should be widely applicable. We analyzed the focuses and microbial implications (prevention of organisms, removal, etc.) relevant to each discipline, to gain an understanding of the metrics that each discipline requires. We found that collecting and presenting data in the form of total biomass reduction and cell viability/cell count, which can lead to log reduction, and MIC, BPC, MBIC, MBC, BBC, and/or MBEC, could satisfy the metrics of each discipline. We then investigated which detection methods were required to satisfy each metric. Ultimately, we recommended using a Crystal Violet stain to detect total biomass and the LIVE/DEAD *Bac*Light stain for cell vitality/cell count. The alternative detection methods for each metric were either not as effective or were too time consuming or labor intensive for a high-throughput biofilm antimicrobial susceptibility test.

Finally, Manuscript 3 utilized the recommendations from each of the previous manuscripts as part of prevention and removal antimicrobial susceptibility tests. We utilized three preservatives found

in home and personal care products including, methylisothiazolinone (MIT), phenoxyethanol (PE), and sodium benzoate (SB), in hopes that these chemicals were less damaging to the RO membrane than common anti-biofouling treatments in RO systems and they are relatively large, thus less likely to be pass through the membrane pores and be released into the permeate water and pose harm to humans and the environment. We performed tests against *P. aeruginosa* both in suspension culture, but at higher concentrations than for MIC data to encourage biofilm formation, and in mature biofilms. We detected biofilms with the LIVE/DEAD *Bac*Light stain, which has led to biofilm prevention concentrations (BPC) and minimum biofilm inhibition concentrations (MBIC) of the selected preservatives. Ultimately, MIT was the most effective preservative for both the BPC and the MBIC, however, it is the most hazardous of the three, therefore not a good solution for biofouling on RO membranes at large scale. It does, however, provide a metric of efficacy. We also found that in the case of MIT and PE, the MBIC is greater than the BPC. That was not the case for SB, for which the BPC was equal to the MBIC. Both of these relationships are supported in the literature. The nature of the high-throughput protocol utilized in Manuscript 3 allows the results to be applied to biofilm formation scenarios other than RO as well.

The laboratory work that led to Manuscript 3 involved iterative work (discussed in detail in Manuscript 3, Appendix S2) that ultimately led to the improved protocol we used. Additionally, there are further opportunities for protocol improvement in subsequent work. This includes sonicating biofilm samples to create a more homogenous solution, which leads to more accurate fluorescence readings of the LIVE/DEAD *Bac*Light stain. Additionally, we have concerns about the validity of the red fluorescence values at lower concentrations, therefore, we believe developing a standard curve comparing fluorescence and cell count would be valuable.

Conclusion

In conclusion, the first three manuscripts lead to recommendations for prevention and removal biofilm susceptibility tests, including organism choice and biofilm detection method and metrics. The final manuscript utilizes these recommendations in prevention and removal biofilm antimicrobial susceptibility tests via the preservatives MIT, PE, and SB. The conclusions and protocols in Manuscripts 1-3 can be applied to all high-throughput antimicrobial susceptibility tests, not just those related to biofouling of RO membranes. The tests appear to have successfully identified the BPC and MBIC for each preservative. These specific preservatives do not have sufficient combinations of safety and efficacy to be the final solution for biofouling prevention or removal from reverse osmosis membranes. However, the results from these studies can provide metrics of safety and efficacy for future studies on novel antimicrobials that can help make reverse osmosis a better solution for water scarcity.

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Appendix

A. Manuscript 1

Table S1. Semi-systematic review raw data answering: Which organisms are used for anti-biofouling studies?

Antifoulant Method	Organism(s)	Model Bacteria (Y if mentioned)	Type of Biofilm Detection Method	Source
composite membranes	E. coli ATCC25922 S. aureus ATCC255923	Y	LIVE/DEAD baclight stain	[1]
composite membranes	E. coli ATCC25922 S. aureus RSKK 1009	Y	colony counting	[2]
graphene oxide	Saccharomycetes		colony counting	[3]
methyl p-hydroxybenzoate potassium sorbate	L. monocytogenes P. putida Y. enterocolitica A. hydrophila			[4]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type) K. pneumonia ATCC13883 P. aeruginosa BAA-1744	Y	FESEM	[5]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)	Y	SEM	[6]
graphene oxide	E. coli ATCC25922 S. aureus ATCC9144 P. aeruginosa ATCCPAO1	Y	colony counting	[7]
composite membranes	E. coli (unspecified/unique sample type)	Y	measuring flux	[8]
graphene oxide	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)	Y	colony counting SEM LIVE/DEAD baclight stain	[9]
modified membrane	P. aeruginosa P60 Bacillus sp. G-84	Y	DAPI LIVE/DEAD baclight stain	[10]
bacteriophages	E. coli (K12) ATCC11303-B4	Y	measuring flux	[11]
quorum quenching	P. aeruginosa KCTC 2513		LIVE/DEAD baclight stain	[12]
modified membrane	E. coli (unspecified/unique sample type)		colony counting colony counting measuring flux	[13]

	S. aureus (unspecified/unique sample type)			
modified membrane	E. coli BW26437	Y	measuring flux	[14]
graphene oxide	Klebsiella (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type)		colony counting	[15]
graphene oxide	P. aeruginosa (unspecified/unique sample type)		measuring flux	[16]
composite membranes modified membrane	E. coli (K12) MG1655		DAPI PI	[17]
composite membranes	E. coli (unspecified/unique sample type)		counting fluorescent cells	[18]
modified membrane	E. coli (K12) MG1655	Y	PI DAPI colony counting	[19]
modified membrane	B. subtilis ISW1214 M. lysODeikticus	Y	colony counting LIVE/DEAD baclight stain	[20]
modified membrane	P. aeruginosa ATCCPAO1	Y	colony counting LIVE/DEAD baclight stain counting fluorescent cells	[21]
graphene oxide	E. coli BW26437		colony counting SEM	[22]
modified membrane	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)		colony counting colony counting measuring flux	[23]
modified membrane	E. coli (K12) MG1655	Y	counting fluorescent cells colony counting	[24]
modified membrane	P. aeruginosa ATCC700829	Y	colony counting SEM PI DAPI	[25]
modified membrane	E. coli ATCCDH5a	Y	ATP analysis ATP analysis TOC	[26]
composite membranes	S. aureus ATCC6538P E. coli ATCC8739		colony counting LIVE/DEAD baclight stain	[27]
graphene oxide	E. coli (unspecified/unique sample type) B. subtilis (unspecified/unique sample type)	Y	SEM measuring flux measuring flux	[28]
composite membranes	E. coli DSM 4230	Y	colony counting	[29]

composite membranes	E. coli ATCC25922			[30]
graphene oxide	E. coli ATCC35695	Y	colony counting LIVE/DEAD baclight stain	[31]
graphene oxide	E. coli (unspecified/unique sample type)	Y	colony counting	[32]
graphene oxide	E. coli BW26437		colony counting	[33]
composite membranes	P. aeruginosa (unspecified/unique sample type) E. coli (unspecified/unique sample type)	Y	measuring flux LIVE/DEAD baclight stain	[34]
composite membranes	B. subtilis (unspecified/unique sample type)		colony counting SEM	[35]
composite membranes	P. aeruginosa ATCCPAO1		colony counting LIVE/DEAD baclight stain	[36]
graphene oxide graphene oxide	E. coli (K12) MG1655 E. coli ATCC25922	Y	colony counting colony counting LIVE/DEAD baclight stain	[37]
composite membranes	E. coli (K12) MG1655 A. parasiticus JFS B. pumilus LDS33	Y	colony counting	[38]
composite membranes	E. coli CCUG3274		crystal violet	[39]
composite membranes	E. coli (unspecified/unique sample type)	Y	FESEM syto 9	[40]
composite membranes	S. aureus (unspecified/unique sample type) E. coli (unspecified/unique sample type)		measuring flux	[41]
composite membranes	E. coli DSM1103		colony counting	[42]
composite membranes	S. aureus (unspecified/unique sample type) E. coli (unspecified/unique sample type)	Y	colony counting	[43]
composite membranes	E. coli (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type)		FESEM	[44]
composite membranes	P. fluorescens	Y	LIVE/DEAD baclight stain	[45]
composite membranes	S. aureus (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type)	Y	LIVE/DEAD baclight stain	[46]

	E. coli (unspecified/unique sample type)			
composite membranes	P. aeruginosa ATCCPAO1		LIVE/DEAD baclight stain	[47]
composite membranes	E. coli ATCC25404 P. mendocina KR1		colony counting DAPI colony counting	[48]
composite membranes	E. coli (unspecified/unique sample type)		counting fluorescent cells	[49]
composite membranes	B. subtilis (unspecified/unique sample type) B. subtilis (unspecified/unique sample type) E. coli (unspecified/unique sample type) E. coli (unspecified/unique sample type)		colony counting	[50]
	P. aeruginosa KCTC2004		measuring flux	[51]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type) E. coli (unspecified/unique sample type)	`	LIVE/DEAD baclight stain measuring flux	[52]
composite membranes	E. coli (unspecified/unique sample type)	Y	colony counting	[53]
composite membranes	P. aeruginosa (unspecified/unique sample type)		LIVE/DEAD baclight stain	[54]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)		colony counting	[55]
composite membranes	P. aeruginosa (unspecified/unique sample type)		colony counting LIVE/DEAD baclight stain	[56]
composite membranes	E. coli (unspecified/unique sample type)		LIVE/DEAD baclight stain	[57]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)	Y	colony counting LIVE/DEAD baclight stain	[58]

	P. aeruginosa (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type)			
composite membranes	E. coli (unspecified/unique sample type) B. subtilis (unspecified/unique sample type)		measuring flux measuring flux	[59]
composite membranes	E. coli (unspecified/unique sample type) S. maltophilia		LIVE/DEAD baclight stain	[60]
composite membranes	E. coli ATCC25254		colony counting LIVE/DEAD baclight stain	[61]
	E. coli (unspecified/unique sample type) P. fluorescens		colony counting	[62]
composite membranes	P. aeruginosa (unspecified/unique sample type)		colony counting SEM LIVE/DEAD baclight stain	[63]
composite membranes	S. paucimobilis		LIVE/DEAD baclight stain LIVE/DEAD baclight stain	[64]
composite membranes	E. coli (unspecified/unique sample type)	Y	colony counting measuring flux	[65]
composite membranes	S. aureus (unspecified/unique sample type)	Y	colony counting	[66]
composite membranes	E. coli (K12) MG1655		colony counting SEM	[67]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)	Y	colony counting LIVE/DEAD baclight stain	[68]
composite membranes	E. coli (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type)		SEM	[69]
composite membranes	E. coli (unspecified/unique sample type) P. putida		colony counting LIVE/DEAD baclight stain	[70]

	P. putida			
composite membranes	P. aeruginosa ATCCPAO1		colony counting LIVE/DEAD baclight stain	[71]
composite membranes	P. putida B. subtilis (unspecified/unique sample type)	Y	LIVE/DEAD baclight stain	[72]
composite membranes	E. coli (unspecified/unique sample type)		colony counting	[73]
composite membranes	P. putida	Y	colony counting measuring flux	[74]
composite membranes	S. paucimobilis		LIVE/DEAD baclight stain measuring flux	[75]
composite membranes	E. coli (unspecified/unique sample type) Saccharomycetes		FESEM	[76]
	P. aeruginosa ATCCPAO1	Y	colony counting LIVE/DEAD baclight stain	[77]
composite membranes	P. fluorescens			[78]
	K. oxytoca		colony counting LIVE/DEAD baclight stain	[79]
composite membranes	P. fluorescens S. wittichii RW1		counting fluorescent cells LIVE/DEAD baclight stain counting fluorescent cells	[80]
composite membranes	E. coli ATCC25922 S. aureus ATCC6538P S. aureus ATCC6538P		OD LIVE/DEAD baclight stain	[81]
composite membranes	S. aureus (unspecified/unique sample type) E. coli (unspecified/unique sample type)		colony counting FESEM	[82]
composite membranes	P. fluorescens		counting fluorescent cells LIVE/DEAD baclight stain measuring flux	[83]
composite membranes	E. coli (unspecified/unique sample type)		colony counting	[84]
composite membranes	P. fluorescens P. putida		counting fluorescent cells	[85]
composite membranes	E. coli (unspecified/unique sample type) B. subtilis (unspecified/unique sample type)			[86]
composite membranes	P. putida P. fluorescens			[87]
composite membranes	E. coli MTCC1302	Y	SEM	[88]

			LIVE/DEAD baclight stain	
graphene oxide	S. aureus ATCC1901 E. coli ATCC8739 E. coli ATCC8739		colony counting colony counting colony counting	[89]
composite membranes	E. coli BW26437		LIVE/DEAD baclight stain	[90]
composite membranes	E. coli (K12) MG1655		counting fluorescent cells	[91]
graphene oxide	E. coli (unspecified/unique sample type)		colony counting	[92]
composite membranes	E. coli (unspecified/unique sample type)		LIVE/DEAD baclight stain	[93]
composite membranes	E. coli ATCC47,076 P. aeruginosa KCTC2004		colony counting LIVE/DEAD baclight stain	[94]
composite membranes	S. aureus KCTC 3881 P. fluorescens S. epidermidis ATCC12228		LIVE/DEAD baclight stain SEM	[95]
graphene oxide	E. coli (K12) ATCC700926		colony counting OD	[96]
composite membranes	E. coli (unspecified/unique sample type)		colony counting	[97]
composite membranes	S. marcescens		measuring flux OD	[98]
composite membranes	E. coli (unspecified/unique sample type)		colony counting SEM	[99]
composite membranes	B. subtilis ATCC6633 E. coli ATCC8739 C. testosteroni I2	 Y	colony counting LIVE/DEAD baclight stain SEM	[100]
composite membranes	E. coli ACIB 8277 S. aureus ATCC6538P	Y	colony counting SEM	[101]
composite membranes	E. coli (K12) MG1655		colony counting	[102]
composite membranes	E. coli (unspecified/unique sample type)		OD	[103]
composite membranes	S. aureus (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type)			[104]
composite membranes	E. coli DH5a S. aureus CICC10201	Y	colony counting	[105]
composite membranes	E. coli (unspecified/unique sample type)	Y	SEM	[106]
composite membranes	P. aeruginosa (unspecified/unique sample type)	Y	measuring flux	[107]
composite membranes	E. coli BW26437	Y	colony counting	[108]

	P. aeruginosa ATCC26437 S. aureus ATCC8325 P. aeruginosa ATCC26437		LIVE/DEAD baclight stain	
composite membranes	E. coli (K12) KCTC1116 S. aureus KCTC1928	Y	SEM	[109]
composite membranes	P. aeruginosa ATCC27853	Y	SEM	[110]
composite membranes	E. coli DH5a		colony counting SEM LIVE/DEAD baclight stain measuring flux	[111]
composite membranes	P. aeruginosa ATCC700829	Y	Dapi	[112]
composite membranes	E. coli (K12) MG1655		LIVE/DEAD baclight stain	[113]
composite membranes	E. coli (unspecified/unique sample type) B. subtilis (unspecified/unique sample type)		colony counting	[114]
composite membranes	E. coli (unspecified/unique sample type) B. subtilis (unspecified/unique sample type)		colony counting measuring flux	[115]
composite membranes	B. subtilis ATCC27370 E. coli ATCC10798	Y	colony counting	[116]
	E. coli BW26437	Y	colony counting	[117]
composite membranes	P. aeruginosa NCIM 2036 S. aureus NCIM 5345	Y	colony counting SEM measuring flux	[118]
composite membranes	P. aeruginosa ATCCPAO1	Y	LIVE/DEAD baclight stain	[119]
composite membranes	E. coli (unspecified/unique sample type) P. aeruginosa (unspecified/unique sample type) S. aureus (unspecified/unique sample type) Enterococcus faecalis (unspecified/unique sample type)	Y	OD LIVE/DEAD baclight stain	[120]
composite membranes	E. coli (unspecified/unique sample type)		measuring flux	[121]

graphene oxide	E. coli (unspecified/unique sample type)	Y	LIVE/DEAD baclight stain	[122]
composite membranes	E. coli (unspecified/unique sample type)	Y	SEM colony counting	[123]
composite membranes	E. coli (unspecified/unique sample type) S. aureus (unspecified/unique sample type)		colony counting LIVE/DEAD baclight stain SEM	[124]
composite membranes	E. coli ATCC8739 S. aureus ATCC6538P	Y	colony counting	[125]
composite membranes	P. aeruginosa ATCCPAO1	Y	colony counting SEM LIVE/DEAD baclight stain	[126]
composite membranes	E. coli (unspecified/unique sample type)	Y	colony counting SEM counting fluorescent cells	[127]
composite membranes	E. coli DH5a		SEM	
composite membranes	E. coli ATCC25922 S. aureus ATCC255923	Y	colony counting SEM counting fluorescent cells	
composite membranes	E. coli ATCCDH5a		colony counting	
composite membranes	E. coli (unspecified/unique sample type) B. subtilis (unspecified/unique sample type)		colony counting	
composite membranes	E. coli (unspecified/unique sample type)		colony counting	
composite membranes	E. coli Nissle 1917	Y	measuring flux	
	P. aeruginosa P60	Y	LIVE/DEAD baclight stain colony counting	
	P. aeruginosa ATCCPAO1	Y	colony counting LIVE/DEAD baclight stain OD measuring flux	

Table S2. Semi-systematic review raw data answering: Which organisms are found in biofilms on RO membranes?

Membrane Setup	**Water Type**	**Identification Method**	**Bacterial Identity**	**Source**
Moving Bed Biofilm Reactor inoculated with activated sludge	WWTP influent	16S rRNA	Acinetobacter Methyloversatilis Denitratisoma Lactobacillus	[128]

			Pseudomonas unidentified_Spirochaetaceae Methylotenera Sulfuritalea Sutterella Streptococcus	
RO membrane from pilot plant	/	16S rRNA	Deltaproteobacteria Bacilli Betaproteobacteria Sphingobacteria Flavobacteria Alphaproteobacteria Gammaproteobacteria	[129]
benchscale RO	Seawater sample	16S rRNA	Blastocatella fastidiosa (JQ309130) Nocardia cyriacigeorgica (BAFY01000107) Lysinimonas soli (JN378395) Propionibacterium acnes (AB042288) Luteibaculum oceani (KC169812) Spongiibacterium flavum (FJ348473) Blastopirellula marina (AANZ01000021) Planctomyces maris (ABCE01000043) Sphaeronema italicum (AY428765) Kordiimonas aquimaris (GU289640) Methlyoligella solikamskensis (JQ773444) Ochrobactrum oryzae (AM041247) Rhodoplanes elegans (D25311) Tepidamorphus gemmatus (GU187912) Labrenzia aggregate (AUUW01000037) Magnetospira thiphila (EU861390) Nisaea denitrificans (DQ665838) Tistlia consotensis (EU728658) Caedibacter acanthamoebae (AF132138) Seathiella chungangensis (KF482756) Limnobacter thiooxidans (AJ289885) Acidovorax temperans (AF078766) Acidovorax caeni (AM084006) Marinobacter algicola (ABCP01000031) Maricoccus atlantica (KC997601) Porticoccus hydrocarbonoclasticus (JN088732) Endoriftia Persephone (AFOC01000137) Thiohalomonas nitratireducens (DQ836238) Deulfuromonas svalbardensis (AY835388) Pedosphaera parvula (ABOX01000003) Juniperus virginiana (AF131092)	[130]

			Pinus thunbergii (D17510) Thermoanaerobaculum aquaticum (JX420244) Flagellimonas eckloniae (DQ191180) Fabibacter pacificus (KC005305) Nitrospina gracilis (L35504) Phycisphaera mikurensis (AP012338) Scalindua sorokinii (AY257181) Maritalea porphyrae (AB583774) Methyloceanibacter caenitepidi (AB794104) Labrenzia alexandrii (ACCU01000015) Hyphomonas oceanitis (AF082797) Roseovaruis mucosus (AJ534215) Phaeobacter caeruleus (AM943630) Micavibrio aeruginosavorus (CP002382) Pelagibius litoralis (DQ401091) Magnetospira thiophila (EU861390) Microvirga subterranean (FR733708) Roseovarius lutimaris (JF714703) Marinicauda pacifica (JQ045549) Polyangium brachysporum (AM410613) Legionella dresdenensis (AM747393) Theoalkalibirbrio thiocyanodenitrificans (AY360060) Alcanivorax balearicus (AY686709) Microbulbifer gwangyangensis (JF751045) Oceanibaculum pacificum (FJ463255) Methylophaga marina (X95459) Pelobacter carbinolicus (CP001734) Deulfohalobium retbaense (CP001734) Roseibacillus ishigakijimensis (AB331888) Roseibacillus ponti (AB331889)	
RO plant samples	/	16S rRNA	Acidovorax Flavobacterium Mycobacterium Tatlockia Aminobacter Hyphomicrobium Pedobacter Bacillus Leptothrix Rhodobacter Devosia Methylobacterium Sphingopyxis	[131]
Benchscale NF, RO, GDM	Shale gas wastewater	16S rRNA	Nitrosomonas Denitromonas Azoarcus Uncl._o__Oligosphaerales Psedoxanthomonas	[132]

			Methylobacterium Hyphomicrobium Dialister Alkalibacter Pseudomonas Pelagibacterium Uncl._d__Bacteria Planktosalinus Uncl._o__Sphingomonadales Uncl._f__Erythrobacteraeae Uncl._o__Bradymonadales Sphingopyxis C1-B045_f__Porticoccaceae Legionella Acetobacterium Muricauda Sphingosinicella Pusillimonas Labrenzia Citeitalea Paracoccus Glycocaulis PAUC26f_o__Solibacterales Chromatocurvus Iodidimonas Rubrimonas Thalassobaculum Lacimicrobium Uncl._f__Phycissphaeraceae Oceanibacterium Dichotomicrobium Tistlia Uncl._f__Cryomorphaceae Uncl._c__Deltaproteobacteria Arenibacter Magnetospira Methyloceanibacter Uncl._p__BRC1 Porphyrobacter Uncl._p__Hydrogenedentes Uncl._f__Rhodospirillaceae Alcanivorax Uncl._o__Rickettsiales Uncl._c__Gemmatimonadetes Rhodovulum Uncl._f__Halieaceae Methylophaga Roseavarius Pseudohongeilla Bryobacter Filomicrobium Uncl._p__Proteobacteria Marinobacter Uncl._c__Gammaproteobacteria Unc._f__Rhodobactaceae Uncl._c__Alphaproteobacteria Uncl._Rhizobiales Uncl._p__Bacteroidetes Rehaibacterium SM1A02_f__Phycisphaeraceae	
lab & pilotscale GDM	Seawater sample next to desalination plant	16S rRNA	Hydrogenedentes Firmicutes	[133]

			Candidatus Saccharibacteria Verrucomicrobia Proteobacteria Planctomycetes Chloroflexi Chlamydiae Bacteroidetes Actinobacteria	
benchscale RO	MBR effluent from WWTP	16S rRNA	env.OPS 17 Xanthomonadaceae Sphingomonadaceae Rickettsiales Incertae Sedis Rhizobiaceae P3OB-42 Moraxellaceae Methylophilaceae Hyaloperonospora arabidopsidis Helicobacteraceae Halothiobacillaceae Flavobacteriaceae Cytophagaceae Comamonadaceae Chitinophagaceae Burkholderiaceae Bradyrhizobiaceae Blastocatellaceae Anaerolineaceae	[134]
RO samples from PSDP	Seawater sample etc.	16S rRNA, phenotypes	Ochrobactrum Agrobacterium Sphingobium Sphingomonas Sphingopyxis Achromobacter Burkholderia Pandoraea Ralstonia Delftia Shewanella Acinetobacter Pseudomonas Cytophaga Flavobacterium Pedobacter Sphingobacterium Rhodococcus Microbacterium Cellulomonas Alicyclobacillus Bacillus Streptococcus	[135]
RO membrane from SWRO plant	Seawater sample	MAG analysis	Candidatus Omnitrophica Chlamydiae Acidobacteria Nitrospirae Gemmatimonadetes Verrucomicrobia Planctomycetes Cyanobacteria Chloroflexi Firmicutes Actinobacteria	[136]

			Bacteriodetes Proteobacteria	
Pilot scale RO	MBR effluent	16S rRNA	Pedobacter composti Pseudomonas pseudoalcaligenes Pseudomonas anguilliseptica Herbaspirillum huttiense Limnobacter thiooxidans Pseudomonas veronii Azoarcus tolulyticus Reyranella massiliensis Hydrogenophaga pseudoflava Hyphomicrobium vulgare Gemmobacter lanyuensis Pseudomonas stutzeri Pseudomonas caeni Brevundimonas denitrificans Pseudomonas mendocina Falvobacterium cucumis Gemmobacter megaterium Brevundimonas bullata Subtercola frigoramans Sphingobium fontiphilum Sphingpbium xenophagum	[137]
Sample membranes	Sea, brackish, well water	16S rRNA, ITS(fungi)	Acidobacteria Acidimicrobiales Actinomycetales Solirubrobacterales Cytophagales Flavobacteriales Saprospirales Chlamydiales Chlorobi Cyanobacteria Bacillales Nitrospirales Acetothermales Planctomycetes Phycisphaerales Planctomycetales Alphaproteobacteria Caulobacterales Kiloniellales Kordiimonadales Rhizobiales Rhodobacterales Rhodospirillales Sphingomonadales Burkholderiales Rhodocyclales Myxococcales Deltaproteobacteria Syntrophobacterales Gammaproteobacteria Legionellales Oceanospirllales Thiotrichales Xanthomonadales	[138]
RO, MF	Influent and effluent of MF-RO system	16S rRNA	Comamonadaceae Sphingomonadaceae Oxalobacteraceae Planktophila_f Flavobacteriaceae	[139]

			SAR11-4_f Chitinophagaceae Mycobacteriaceae Sphingobacteriaceae Moraxellaceae Lautropia_f Rhodobacteraceae Alcaligenaceae	
RO, UF	Influent and effluent of UF-RO system	16S rRNA	Starkeya Acidovorax Luteimonas Pelomonas Xanthobacter Hydrogenophaga Sphingopyxis Pseudoxanthomonas Bdellovibrio Bosea Afipia Sediminibacterium Gemmobacter Roseomonas Bacteriovorax Devosia Thermomonas Leifsonia Comamonas Flacovacterium Methyloversatilis Hyalangium Saccharibacteria Shinella Brevundimonas Chitinophaga Pseudomonas Citrobacter Legionella Herminiimonas Variovorax Parachlamydia Fluviicola Ohtaekwangia Pedobacter Ferruginibacter Nereida Sphingomonas Emticicia Roseateles Salinibacterium Hyphomicrobium Clostridium sensu stricto Mesorhizobium Adhaeribacter Cytophaga Cloacibacterium Phaselicystis	[140]
RO pilot system	Wastewater from oil refinery	16S rRNA, culture	Leifsonia Microbacterium Acidocella Ancylobacter Bosea Bradyrhizobium	[141]

			Breundimonas Devosia Ensifer Ferrovibrio Hirschia Kaistia Labrys Magnetospirillum Mesorhizobium Novosphingobium Parvularcula Pedomicrobium Rhizobium Rhizomicrobium Rhodobacter Shinella Sphingobium, Sphingopyxis Woodsholea Acidovorax Burkholderia Cupreavidus Limnobacter Methylibium Sulfuritale Zoogloea Blastocatella Runella Bdellovibrio Vampirovibrio Flavobacterium Alkanindiges Aquicella Escherichia Shigella Pseudomonas Rheinheimera Thioprofundum Thiothrix Nitrospira Coraliomargarita Opitutus Sediminibacterium Brevifollis	
RO plant	Wastewater from WWTP	16S rRNA, flow cytometry, HPC, ATP measurements	Rhodobacter Caldilinea Phyllobacteriaceae Stenotrophomonas Longilinea Phycisphaera Massilia Opitutus Nitrosopumilus Haliangiumcaldilineaceae Sinobacteraceae Nannocystineae Hymenobacter Rhodovulum Anaerolineaceae Owenweeksia Spirochaeta Barnesiella	[142]

			Legionella Hgci_clade Cystbacterineaeop3 (candidate division) Saprospiraceae Planctomycetaceae Candidatus_chloracidobacterium Psuedomonas Rhodospirillaceae	
Labscale NF	Wastewater effluent	High throughput illumina pyrosequencing	Betaprotobacteria Gammaproteobacteria Unclassified Proteobacteria Alphaporteobacteria Actinobacteria Verrucomicrobia Chloroflexi Minor phylum Deltaproteobacteria Nitrospira Bacteroidetes Acidobacteria Epsilonproteobacteria Unclassified Phylum Cyanobacteria Planctomycetes Firmicutes TM7 Deinococcus-Thermus Chlamydiae Synergistetes Gemmatimonadetes	[143]
AWPF (advanced water purification facility)	WWTP influent	Shotgun (WGS) sequencing	Acidovorax Acinetobacter Aeromonas Afipia Arcobacter Bacteroides Bifidobacterium Caulobacter Chryseobacterium Comamonas Elizabethkingia Flavobacterium Hydrogenophaga Klebsiella Limnohabitans Mycobacterium Pseudomonas Rhodocyclaceae Sediminibacterium Sphingobium Sphingopyxis Thauera Thiobacillus	[144]
/	WWTP influent	16S rRNA	Thauera Denitratisoma Propionivibrio Sphingobium Terrimonas Nitrosomonas Pseudomonas Ornatilinea	[145]

			Anaerolinae Planctomyces Filimonas Streptococcus Clostridium Ignavibacterium Comamonas Arcobactor Nitrospira Sphingomonas Microvirgula Bacteroides Undibacterium Mycobacterium Cloacibacterium Chryseobacterium	
DMF, (PA)RO with BER	Raw seawater	ATP analysis, 16S rRNA 454 pyrosequencing	Dehalococcoides Trichodesmium Nitrospira Sphingomonas Hyphomonas Sneathiella Erythrobacter Maricaulis Roseobacter Thiohalobacter Phaeobacter Oceanicola Thalassospira Alteromonas Marinobacter Algisphaera Micavibrio Cyanobacterium	[146]
RO sample from WWTP	Synthetic water inoculated with activated sludge	16S rRNA PCR	Strenotrophomonas maltophilia strain MHF ENV20 Bacillus cereus strain ZL-1 Bacillus sp. Sd-16 Bacillus cereus strain 2 Delftia tsuruhatensis strain BN-HKY6 Pseudomonas sp. SJT25	[147]
UF, NF, RO spiral wound membranes from dairy processing plants	Pastuerized milk, condensed water, UF whey permeate, Bleached cheese whey	16S rRNA (enzymatic pretreatment and a phenol-chloroform extraction)	Other Bacilli Other Firmicutes Alphaproteobacteria Methylobacterium Other Alphaproteobacteria Betaproteobacteria Burkholderia Petrobacter Other Betaproteobacteria Gammaproteobacteria Acinetobacter Citrobacter Cronobacter Klebsiella Pseudoalteromonas Psychrobacter Other Gammaproteobacteria Other Proteobacteria	[148]

Labscale RO	Suplemented TWW solution	16S rRNA (PCR amplification and Illumina sequencing)	Aeromonadaceae Alphaproteobacteria Bacteroidetes Bradyrhizobiaceae Burkholderiaceae Caulobacteaceae Chitinophagaceae Chromatiaceae Comamonadaceae Cystobacteraceae Cytophagaceae	[149]
Pilotscale SWRO	Treated feed water (Chlorine and sand filtration)	16S rRNA	Marinobacter adhaerens Vibrio atlanticus Ruegeria atlantica Muricauda lutimaris Parasphingopyxis lamellibrachiae Erythrobacter longus Roseovarius albus Rhodanobacter ginsengisoli Pelagibius litoralis Maribacter polysiphoniae Alcanivorax jadensis Hyphomonas chukchienses Parvularcula lutaonensis Sphingopyxis litoris Methylophaga thiooxydans Bacillus subtiliis	[150]
/	Feed water and permeate water from RO drinking plant	16S rRNA, MALDI-TOF MS	Ensifer adhaerens Stenotrophomonas Serratia Rhizobium Pseudomonas Enterobacter Acinetobacter Acidovorax Achromobacter Stenotrophomonas Maltophilia Ilyobacter Delafieldii Pseudomonas veronii Pseudomonas libanensis Pseudomonas jessenii Pseudomonas frederiksbergensis Pseudomonas koreensis Pseudomonas putida Pseudomonas nitroreducens Pseudomonas proteolytica Pseudomonas mandelii	[151]
MF membrane samples from wastewater treatment plant		16S rRNA, culturing	Enterobacteriaceae Moraxellaceae Pseudomonadaceae Comamonadaceae Xanthomonadaceae Phyllobacteriaceae Bacillaceae Paenibacillaceae Microbacteriaceae	[152]
Labscale forward osmosis system	Supplemented annamox effluent	16S rRNA Illumina sequencing	Prosthecobacter Dokdonella Dok59 Kucncnia Candidatus Jettenia Candidatus Brocadia	[153]

			Sediminibacterium	
KAUST desalination plant	Raw seawater Brackish water RO permeate Drinking water (chlorinated) RO influent RO permeate Spruce media permeate	16S rRNA pyrosequencing, PCR	Sphingomonadales Parvularculales Rhodobacterales Rhodospirillales Caulobacterales Rhizobiales Rickettsiales Rhodocyclales Nitrosomonadales Methylophilales Burkholderiales Hydrogenophilales Alteromonadales Thiotrichales Pseudomonadales Methylococcales Oceanospirillales Enterobacteriales Chromatiales Legionellales Aeromonadales Desulfobacterales Desulfarculales Syntrophobacterales Bdellovibrionales Myxococcales Desulfuromonadales Bacilli Ws3 Synergistia Oscillatoriophycideae Verrucomicrobiae Lentisphaeria Nitrospira Planctomycea Caldilineae Bacteroidia Phycisphaerae Opitutae Candidatus_Thiobios Anaerolineae Cytophagia Clostridia Planctomycetacia Sphingobacteria Synechococcophycideae Flavobacteria	[154]
ADOM desalination facility	Raw seawater Pre RSF water RSF anthracite layer (biofilm) RSF sand layer (biofilm) Post RSF water CF membrane Post CF water RO membrane modules (biofilm) Brine Product	16S rRNA pyrosequencing	Actinobacteria Alphaproteobacteria Bacteroidetes Betaproteobacteria Chloroflexi Deltaproteobacteria Acidobacteria Epsilonproteobacteria Planctomycetes Gammaproteobacteria Rhizobiales Rhodobacterales SAR11 cluster Nitrosomonadales	[155]

			Deltaproteobacteria Chromatiales Legionellales Oceanospirillales Xanthomonadales	
SWRO module	Raw seawater DMF inlet DMF outlet SWRO inlet SWRO permeate SWRO brine	16S rRNA pyrosequencing	Cyanobacteria Proteobacteria Gammaproteobacteria Alphaproteobacteria Bacillales Rhodobacteraceae Rhodospirillaceae Hyphomonadaceae Pseudomonas Janthinobacterium Kordiimonas Legionella Flavobacterium Methylophilus Ralstonia Corynebacterium Burkholderia Streptococcus Prevotella Methylobacterium Silicibacter Staphylococcus Acidocella Pedobacter	[156]
Labscale RO	Water inoculated with bacteria from industrial MBR and RO	454 pyrosequencing	Proteobacter Bacteroidetes Betaproteobacter Gammaproteobacteria Alphaproteobacteria Sphingobacteriia Chitinophagaceae Oxalobacteraceae Enterobacteriaceae Comamonadaceae Sphingobacteriaceae Aeromonadaceae Flavobacteriaceae	[157]
RO plant	Secondary WWTP effluent RO influent RO effluent Brackish water RO permeate Drinking water (chlorinated) RO influent RO permeate Spruce media permeate	16S rRNA pyrosequencing	Acidobacteria Sphingobacteria Bacteroidetes Firmicutes Fusobacteria Alphaproteobacteria Betaproteobacteria Deltaproteobacteria Epsilonproteobacteria Gammaproteobacteria	[158]
Labscale RO	UF permeate from HG-MBR	16S rRNA	Proteobacteria Acidobacteria Actinobacteria Bacteroidetes Sphingomonadales Rhizobiales Burkholderiales Xanthomonadales	[159]

			Pseudomonadales Sphingobacteriales	
Labscale RO	Raw seawater	16S rRNA pyrosequencing	Alphaproteobacteria Rhodobacteraceae bacterium Roseobacter Sulfitobacter Phaeobacter Antarcticicola litoralis Betaproteobacteria Gammaproteobacteria Cycloclasticus Colwellia Spongiibacter Pseudoalteromonas Marinobacter Pseudomonas Deltaproteobacteria Firmicutes Bacteroidetes Actinobacteria Loktanella Rhodovulum Pseudoruegeria Thalassobilus Sagittula, Sphingopyxis Erythrobacter Jannaschia Rhizobium Sphingobium Neptunomonas Moraxellaceae Glaciecola Shewanella Oceanospirillaceae bacterium Psychrobacter Alcanivorax Thiohalomonas Serratia Microbulbifer Cellvibrio Cycloclasticus sp. Phe42 (GQ345342) Colwellia sp. BSw20968 (GU166136) Spongiibacter sp. JAMGA14 (AB526337) Pseudoalteromonas sp. B149 (FN295744) Marinobacter sp. YKS2 (AB504895) Pseudomonas stutzeri BBSPN3 (GU594474) Pseudidiomarina sp. KYW314 (FJ768737)	[160]
Labscale RO	Raw seawater	16S rRNA pyrosequencing ATP analysis	Actinobacteria Cyanobacteria Firmicutes Lentisphaerae Planctomycetes Betaproteobacteria Epsilonproteobacteria	[161]

			Bacteroidetes Deinococcus-Thermus Fibrobacteres OD1 Verrucomicrobia Alphaproteobacteria Deltaproteobacteria Gammaproteobacteria Antarctobacter Citreicella Pelagibaca Rhodobacter Rhodobacteraceae Roseobacter Roseovarius nubinhibens Sulfitobacter Methylophaga Pseudidiomarina homiensis	
RO pilot plant	WWTP secondary effluent Activated sludge	16S rRNA	Afipia felis Bradyrhizobium Bradyrhizobium yuanmingense Ensifer Ensifer sinorhizobium Saheli Rhizobium etli Roseomonas Sinorhizobium Sphingomonadales Sphingomonas Sphingopyxis panaciterrae Aquamonas fontana Aquamonas Methylibium aquaticum Nitrosomonadaceae Pelomonas Zoogloea Helicobacteraeae Legionellaceae Methylococcaceae Methylococcales Methylothermus Pseudomonadaceae Pseudomonas entomophila Stentrophomonas acidaminiphila Gammaproteobacteria Kineosporiaceae Kouleothrix Microbacteriaceae Mycobacterium cosmeticum Mycobacterium nonchromogenicum Tetrasphaera Flavobacteriaceae Caldilinea Lactococcus lactis Anabaena Tm7	[162]
Fouled SWRO membranes	Raw seawater Pretreated seawater	16S rRNA ATP analysis	Proteobacteria Bacteroidetes	[163]
CF and RO samples from commercial desalination	/	Total Genomic DNA analysis	Aciditerrimonas ferrireducens IC-180	[164]

	16S rDNA	Winogradskyella sp. poriferorum UST030701-295 Bacillus algicola strain LS7 Bacillus zhanjiangensis JSM 099021 Bacillus cohnii DSM 6307 Bacillus vietnamensis 15-1 Bacillus decolorationis LMG 19507 Blastopirellula marina DSM 3645 Zavarzinella formosa A10 Mesorhizobium albiziae CCBAU 61158 Parvularcula lutaonensis CC-MMS-1 Leisingera aquimarina CCUG Pseudoruegeria lutimaris HD-43 Ruegeria lacuscaerulensis ITI-1157 Nautella italica CCUG 55857 Thalassobius aestuarii JC2049 Rhodobacter veldkampii ATCC35703 Parvularcula bermudensis HTCC2503 Rickettsia montanensis ATCC VR-611 Maribius salinus CL-SP27 Paracoccus fistulariae KCTC 22803 Phaeobacter daeponensis TF-218 Phaeobacter caeruleus CCUG 55859 Loktanella pyoseonensis JJM85T Pseudidiomarina taiwanensis strain PIT1 Kangiella koreensis DSM 16069 Kangiella aquimarina SW-154 Kangiella spongicola A79 Shigella flexneri ATCC 29903 Legionella brunensis 441-1 Legionella beliardensis strain Montbeliard A1 Thioalkalivibrio denitrificans ALJD Thioalkalivibrio thiocyanodenitrificans ARhD1 Vibrio diabolicus HE800 Photobacterium rosenbergii LMG 22223 Desulfuromusa ferrireducens 102 Bdellovibrio bacteriovorus HD100 Geobacter bemidjiensis Bem Geobacter bremensis Dfr1 Roseibacillus ishigakijimensis MN1-741 Candidatus Solibacter usitatus Ellin6076 Caldithrix palaeochoryensis MC Thermonema lapsum ATCC 43542 Adhaeribacter aquaticus MBRG1.5 Ekhidna lutea BiosLi/39 Wandonia haliotis Haldis-1 Meridianimaribacter flavus NH57N Bizionia echini KMM 6177

			Owenweeksia hongkongensis UST20020801 Flavobacterium haoranii LQY-7 Candidatus Aquirestis calciphila MS-Falk1-L Solitalea koreensis R2A36-4 Leptolinea tardivitalis YMTK-2 Bellilinea caldifistulae GOMI-1 Caldilinea aerophila STL-6-O1 Fusibacter paucivorans SEBR 4211 Thermaerobacter composti Ni80 Sporacetigenium mesophilum ZLJ115 Geosporobacter subterraneus VNs68 Caldicoprobacter oshimai JW/HY-331 Clostridium sp. pascui DSM 10365 Nitrospira marina Nb-295 Blastopirellula marina DSM 3645 Gemmata obscuriglobus UQM 2246 Blastopirellula marina DSM 3645 Zavarzinella formosa A10 Phycisphaera mikurensis FYK2301M01 Parvularcula bermudensis HTCC2503 Jhaorihella thermophila CC-MHSW-1 Ruegeria marina ZH17 Pseudoruegeria lutimaris HD-43 Afifella pfennigii AR2102 Erythrobacter flavus SW-46 Limnobacter thiooxidans CS-K2 Thauera terpenica 58Eu Malikia spinosa ATCC 14606 Azoarcus indigens VB32 Curvibacter delicatus LMG 4328 Balneatrix alpaca 4-87 Shigella sonnei GTC 781 Shigella flexneri ATCC 29903 Cellvibrio mixtus subsp. Mixtus ACM 2603 Marinicella litoralis KMM 3900 Thermomonas haemolytica A50-7-3 Thiohalocapsa marina JA142 Legionella gresilensis ATCC 700509 Natronocella acetinitrilica strain ANL 6-2 Haliangium tepidum SMP-10 Geobacter metallireducens GS-15	
Elsa purification plant RO membrane sample	River water	16S rRNA	Hippea maritima MH2 Cryobacterium psychrotolerans, DQ515963 Leifsonia kafniensis, AM889135 Clone ROM_78, HE575376 Clone ROM_17, HE575377 Leifsonia rubra, AJ459101 Salinibacterium amurskyense, AF539697 Clone ROM_5, HE575378	[165]

Clone ROM_96, HE575379
Frigoribacterium mesophilum, EF466126
Frigoribacterium faeni, AM410686
Frondihabitans australicus, DQ525859
Clone ROM_93, HE575382
Clone ROM_2, HE575380
Microcella alkaliphile, AJ717385
Microcella putealis, AJ717388
Yonghaparkia alkaliphile, DQ256087
Clavibacter michiganensis subsp. M, U09762
Clone ROM_92, HE575381
Propionicicella superfundia, DQ176646
Propionicimonas paludicola, AB078858
Micropruina glycogenica, AB012607
Clone ROM_77, HE575383
Nocardioides fonticola, EF626689
Nocardioides pyridinolyticus, U61298
Nocardioides terrigena, EF363712
Nocadrioides halotolerans, EF466122
Actinomadura chokoriensis, AB3311730
Clone ROM_18, HE575385
Actinomadura bangladeshensis, AB331652
Actinomadura livida, AF163116
Actinomadura yumaensis, AF163122
Actinomadura meyerae, AY273787
Clone ROM_1, HE575384
Actinomadura cremea subsp. Cremea, AF134067
Actinomadura glauciflava, AB184612
Actinomadura formosensis, AJ293703
Actinomadura napierensis, AY568292
Clone ROM_13, HE575386
Uncultured bacterium, FJ671519
Sphaerobacter thermophilus, AJ420142
Thermomicrobium roseum, M34115
Clone ROM_28, HE575388
Canthobacter flavus, X94199
Xanthobacter aminoxidans, AF399969
Clone ROM_3, HE575387
Xanthobacter agilis, X94198
Clone ROM_25, HE575389
Phenylobacterium conjuctum, AJ227767
Clone ROM_23, HE575390
Sphingomonas terrae, D13727

			Sphingomonas adhaesiva, D13727 Sphingopyxis ginsengisoli, AB245343 Sphingomonas macrogoltabidus, D13723 Sphingopyxis witflariensis, AJ416410 Clone ROM_20, HE575391 Thiobacillus sajanensis, DQ390445 Thiobacillus denitrificans, AJ243144 Clostridium estertheticum subsp. 1, AJ506115 Clostridium frigoris, AJ506117 Clostridium bowmanii, AJ506119 Clostridium tagluense, DQ296031 Clone ROM_87, HE575394 Clone ROM_15, HE575393 Clostridium peptidivorans, AF156796 Clostridium tetanomorphum, DQ241819 Clostridium aminovalericum, X73436 Clostridium jejuense, AY494606 Clone ROM_10, HE575392 Clostridium populeti, X71853 Clostridium phytofermentans, CP000885 Clone ROM_59, HE575395	
Biofouled SWRO membrane Cartridge Filter	Raw seawater	16S rRNA	Donghicola eburneus strain SW-277 (DQ667965) Uncultured bacterium clone S25_436 (EF574092) Loktanella sp.K4B-4 (FJ889559) Uncultured Rhodobacteraceae bacterium clone NdSurf79 (FJ753141) Thalassobius sp. (FJ889559) Marine sponge bacterium FILTER4C220m (EU34644) Roseobacter sp. (AY258102) Nautella sp. (FJ161344) Ruegeria sp. (FJ357642) Rhodobacteraceae bacterium (FM163068) Arctic bacterium NP26 (EU196330) Robiginitomaculum sp. G5 (FJ230838) Novosphingomonas sp. (AB070237) Sphingomonadaceae bacterium ACEMC 2-1 (FM07237) Marine gamma proteobacterium Fun-110 (DQ107393) Leucothrix muco (X87277) Isolate B1 Shewanella sp. (EF105395) Isolate B4 Isolate B6 Vibrio sp.1A8 (EU854873) Isolate B3	[166]

			Alteromonas sp. (FJ652055) Isolate B2 Uncultured planctomycete clone Hal 25 (AM422930) Uncultured Chloroflexi bacterium clone GoM IDB-09(EU735030) Lewinella cohaerens (AB301614) Uncultured bacterium clone SGUS1259 (FJ202110) Cellulaphaga sp. (AB180390) Isolate B5 Flavobacteria bacterium SOMBO 59 (AJ936938) Lacinutrix sp. (DQ530481) Uncultured Flavobacteria bacterium (AM 279207) Winogradskyella sp. (EU727254) Flavobacteriaceae bacterium ALC1 (EF527870)	
pilot scale SWRO plant	Raw seawater Permeates	16S rRNA	Aestuariibacter halophilus Aestuariibacter litoralis Alteromonas hispanica Alcanivorax dieselolei Alcanivorax balearicus Alcanivorax hongdengensis Alteromonas addita Alteromonas stellipolaris Alteromonas macleodii Alteromonas marina Glaciecola mesophila Colwellia aestuarii Colwellia polaris Colwellia piezophila Glaciecola chathamensis Glaciecola agarilytica Glaciecola polaris Glaciecola pallidula Umboniibacter marinipuniceus Saccharophagus degradans Microbulbifer salipaludis Arcobacter marinus Arcobacter nitrofigilis Arcobacter halophilus Sulfurovum lithotrophicum Nitratifractor salsuginis Sulfuricurvum kujiense Owenweeksia hongkongensis Wandonia haliotis Kordia periserrulae Marinovum algicola Thalassobius aestuarii Oceanicola pacificus Nautella italica Oceanibulbus indolifex Shimia marina Parvularcula bermudensis Parvularcula lutaonensis Parvibaculum indicum Ponticoccus litoralis Sulfitobacter dubius Pseudoruegeria aquimaris Dinoroseobacter shibae	[167]

			Glaciecola lipolytica Colwellia asteriadis Glaciecola punicea Aestuariibacter salexigens Marinobacterium marisflavi Marinobacterium lutimaris Neptuniibacter caesariensis Enhygromyxa salina Plesiocystis pacifica Nannocystis exedens Anaeromyxobacter dehalogenans Geobacter uraniireducens Phaselicystis flava Candidatus Arcobacter sulfidicus Kordiimonas lacus Kordiimonas gwangyangensis Devosia geojensis Altererythrobacter marinus Roseovarius mucosus Roseovarius tolerans Roseovarius halotolerans Oceanibaculum pacificum Nisaea nitritireducens Oceanibaculum indicum Sneathiella glossodoripedis Sneathiella chinensis Devosia subaequoris Marinicella litoralis Kistimonas asteriae Endozoicomonas montiporae Alteromonas litorea Alteromonas genovensis Thalassomonas actiniarum Thalassomonas viridans Thalassomonas haliotis Granulosicoccus coccoides Granulosicoccus antarcticus Marinobacter lutaoensis	
	Seawater Brackish water Wastewater Freshwater Activated sludge-treated waste effluent	16S rRNA Culturing	Simonsiella sp. Bosea sp. Rhizobium sp. Proteobacteria Cytophaga–Flexibacter–Bacteroides group Firmicutes Sphingomonas sp. Bacteroides Actinobacteria Rhizobiales Dermacoccus sp. Microbacterium sp. Rhodopsedumonas sp. Bradyrhizobium Bosea Planctomycetes Acidobacter Pseudomonas spp. Corynebacterium Pseudomonas Bacillus Arthrobacter Flavobacterium	[168]

			Aeromonas Penicillium Trichoderma Mucor Acinetobacter Lactobacillus Alcaligenes Moraxella Micrococcus Serratia	
full scale desalination plant	open intake water; RO feed water after conventional pre-treatment	PCR= 16S rRNA tree	Alphaproteobacteria Defluvicoccus AACY020273010 Hyphomicrobiaceae DQ431901 Parvularcula FJ516787 Parvularcula EU236361 Phyllobacteriaceae EU236398 Rhizobiales GQ348477 Rhodobacteraceae FJ716871 Roseovarius EF471647 marine group AEGEAN-169 EF471704 marine group AEGEAN-169 AACY023897748 Rhodospirillaceae GQ264068 SAR116 EU799440 SAR116 AY664095 SAR11clade DQ009166 SAR11clade EU805335 Rhodospirillales EU237396 SAR11clade EP879548 OCS 116 clade AB106120 OCS 116 clade EU236400 Micavibrio FJ202882 Rhodobium_2 FJ745192 Rickettsiales EF 516885 Rickettsiales EU804393 Betaproteobacteria Ideonella AB240317 Deltaproteobacteria Nannocystineae EU283371 SAR324clade EF574189 Gammaproteobacteria Rheinheimera EF076757 Ectothiorhodospiraceae GQ246404 SAR86 clade AACY020555669 SAR86 clade FJ745006 SAR86 clade EF574960 SAR86 clade FJ745145 SAR86 clade EF575172 Actinobacteria Acidimicrobineae EP609371 Bacteroidetes Bruminmicrobium AF507867 Cryomorphaceae EU183317 Flavobacteriaceae EU799420 Flavobacterium EF573073 Flavobacteriaceae EF572094 Polaribacter AY794064 Bizionia EU143366	[169]

			Flavobacteriaceae FJ545454 Flavobacterium GQ988780 Flexibacteraceae AF406540 Haliscomenobacter EF644787 BD1-5 BD1-5 FJ203485 Chloroflexi Caldilineacea AB250571 Anaerolineae EU050928 Deferribacteres SAR406clade AACY023373955 Planctomycetes Planctomyces DQ811897 Planctomyces DQ395893 Planctomycetaceae AY 592313 Planctomyces FJ664808 Planctomyces DQ811897 Planctomycetaceae FJ 202841 Verrucomicrobia Verrucomicrobiales DQ300578 Incertae_sedis FJ478940	
spiral-wound RO mems from RO unit	seawater	PCR via recombinant cells= 16S rRNA gene	Salipiger sp. PTG4-12 Pelagibaca sp. F6 Thalassobius aestuarii Tf-212 Rhodobacteraceae bacterium F9 Uncultured a-proteobacterium (AF473929) Leisingera aquamarina LMG 24366 Ruegeria atlantica SS-05 Nautella italica LMG 24365 Nautella italica R-28753 Oceanicola granulosus HTCC2516 Roseobacter sp. SPO804 Uncultured Sulfitobacter sp. (AY697912) Sulfitobacter mediterraneus (Y17387) Sphinogomonas sp. JQ1-2 Uncultured Novosphingobium sp. Parvularcula sp. CC-MMS-1 Uncultured Pseudorhodobacter sp. (DQ917873) Alcanivorax sp. Mho1 Pseudoxanthomonas sp. P2-3 Pseudomonas borepolis Alicyclobacillus pohliae CIP 109385 Bacillus sp. Eur1 9.5 Uncultured actinobacterium clone (DQ070787) Uncultured high G+C gram-positive bacterium (AJ241005) Candidatus Microthrix Calida TND2-4 (DQ147284) Uncultured Planctomyces sp. Clone (AB189347) Planctomyces sp. Schlesner 664 Lewinella nigricans Uncultured CFB group bacterium clone (AF406541)	[170]

			Uncultured Cytophagales bacterium (AF355051) Uncultured Flavobacterium sp. (AM259763) Fouled RO membrane 39 alphaproteobacteria sewater intake 31 alphaproteobacteria seewater intake 68 alphaproteobacteria sewater intake 12 alphaproteobacteria sewater intake 9 alphaproteobacteria Fouled RO membrane L9 alphaproteobacteria Fouled RO membrane 30 alphaproteobacteria Fouled RO membrane 20 alphaproteobacteria Fouled RO membrane 14 alphaproteobacteria Fouled RO membrane L45 alphaproteobacteria Fouled RO membrane L6 gammaproteobacteria Sewater intake 43 ammaproteobacteria Sewater intake 58 ammaproteobacteria Fouled RO membrane 48 Firmicute Fouled RO membrane 3 Firmicute Sewater intake 2 Actinobacter Fouled RO membrane L14 Actinobacter Fouled RO membrane L40 Planctomycetes Fouled RO membrane L34 Bacteriodetes Fouled RO membrane 01 Bacteriodetes Seawater intake 55 Bacteriodetes Seawater intake 52 Bacteriodetes	
NF-polyamide tubular mem; 200Da MWCO	tertiary quality wastewater effluents; synthetic media mimicking intermediate quality effluents	DGGE analysis; PCR	uncultured rape rhizosphere bacterium uncultured bacterium AY053480 uncultured eubacterium AY038612 uncultured Bacteroidetes AJ583191 CFB group bacterium metal-contaminated soil bacterium uncultured bacterium AY212682 uncultured actinobacterium UBA534677 blackwater bioreactor bacterium BW AF394172 uncultured alphabacterium AB193878	[171]

uncultured deltabacterium AY218599
uncultured deltabacterium AF414588
uncultured gammabacterium AF418944
uncultured Ralstonia sp. Beta bacterium
uncultured betabacterium AY444992
gammaproteobacterium A40-1

Chitinophaga pinensis
Flavobacterium ferrugineum
Dipareforma spartinacae
Flavobacterium columnare
Curtobacterium sp.
Microbacterium keratanolyticum
Microbacterium laevaniformans
Bacillus silvestris
Brevibacillus sp. PLC-3
Bacillus sp. CPB 6
Bacillus sphaericus
Bacillus sp. ARI 3
Bacillus fusiformis
Bacillus aquamarinus
Sphingomonas sp. SKJH -30
Sphingomonas paucimobilis
Sphingomonas subarctica
Sphingomonas sp. C28242
Sphingomonas capsulata
Cystobacter fuscus
Myxococcus xanthus
Pseudomonas putida
Pseudomonas sp. NZ 024
Pseudomonas marginalis
Pseudomonas veronii
Pseudomonas sp. AEBL 3
Pseudomonas sp.
Pseudomonas sp. AY66343
Pseudomonas pavonaceae
Pseudomonas anguilliseptica
Legionella pneumophila
Rolstonia sp. AY177368
Rolstonia sp. AY 177364
Rolstonia sp. FRA 01
Rolstonia sp.
Rickettsiella grylli
Ralstonia oxalatica
Ralstonia paucula
Wautersia sp.
Ralstonia taiwanensis
Ralstonia eutropha
Burkholderia anthina
Delftia sp.
Burkholderia cepacia
Burholderia sp.
Ralstonia pickettii
Delftia acidovorans
Delftia tsuruhalensis
Acidovorax avenae

			Acidovorax delafieldii Acidovorax sp. Hydrogenophaga palleronii Hydrogenophaga pseudoflava Hydrogenophaga flava Rhodanobacter lindanoclasticus Frateuria aurantia Dyella japonica	
MF, Spiral-Wound; full scale membrane process for water purification	secondary effluent (domestic WWTP or waterworks)	DNA extraction=16S rDNA clone library; FISH	Paracoccus/Rhodobacter sp. Hyphomicrobium sp. Azospirillum sp. Xanthobacter sp. Bosea sp. Environmental isolate Environmental clones Thermomonas haemolvtica Mycobacterium nonchromogenicum Ctophagales sp. Bacteroidetes sp. Environmental clones Flavobacterium sp. Planctomycetes sp. Nostocoida limicola Bradyrhizobium sp. Zoogloea sp. Rhizobium sp. Caulobacter sp. Mesorhizobium sp. Agrobacterium sp. Bordetella hinzii Stenotrophomonas acidaminiphila Nevski ramose Brevibacterium sp. Gordonia sp. Aureobacterium sp. Bacillus sp. Staphylococcus sp. Flavobacterium ferrugineum Afipia genosp Rhodopseudomonas palustris Magnetospirillum sp. Methylocystis parvus Rhodocyclus tenuis Dechlormonas agitaus Tiobacillus Q Legionella sainthelensi Holophaga foetida Geothrix fermentans Sphingomonas sp. Rhodospeudomonas sp. Dermacoccus sp. Microbacterium sp. Bacillus sp.	[172]
MBR-RO (GE Osmotics), SE-MF (full-scale hollow fiber MF mem- domestic WW), PW-RO (full-scale, spiral wound RO mem- potable water)	lab-scale MBR for wastewater + RO concentrate	T-RFLP (nitrate reductase); Bacteria-specific primers= 16S rRNA gene clone library	Oligotropha carboxidovorans S28	[173]

RO clone RO229
RO clone RO160
Rhodopseudomonas sp. TUT3631
Rhodospeudomonas palustris TUT3620
Bosea sp. BMA-4
PW-RO isolate RO3
RO clone RO161b
SE-MF isolate MF18
Bosea thiooxidans BI-42
RO clone RO215
Methylocella silvestris BL2
RO clone RO154
Alphaproteobacterium CRIB-02
Uncultured bacterium clone 661238
RO clone RO53
Alphaproteobacterium Shinshu-th1
RO isolate ROi16
Xanthobacter tagetidis TagT2C
Se-MF isolate MF22
Uncultured bacterium clone aab54f12
Mesorhizobium genosp. AA isolate Cs6145
RO isolate ROi51
Shinella zoogloeoides ATCC 19623
Gram-negative bacterium isolate DM1
Sinorhizobium meliloti Rm1021
Ochrobactrum sp. CGL-X
Brucella sp. YBJA-1
RO clone RO233
RO isolate ROi52
RO clone RO238
Uncultured Ochrobactrum sp. Clone p3
Ochrobactrum anthropi CCUG 44770
RO isolate ROi15
Ochrobactrum anthropi CCUG 44770
Ochrobactrum sp. B2
RO isolate ROi43
Sphingomonas sp. JQ1-3
Uncultured bacterium clone KRA30+14
Hydrogenophagaatypica BSB 41.8
RO clone RO219
Uncultured betaproteobacterium clone ccslm2112
RO clone RO118
Uncultured bacterium clone TSAI28
Hydrogenophaga intermedia S1
RO isolate ROi28
Denitrobacter sp. BBTR53
Castellaniella defragrans TJ4
RO isolate ROi27
Thermomonas brevis LMG 21746T
RO isolate ROi19
Thermomonas haemolytica A50-7-3
RO isolate ROi7

Stenotrophomonas maltophilia
isolate FLX
RO isolate ROi55
RO clone RO156
Stenotrophomonas acidaminiphila
AMX19
RO isolate ROi44
Pseudoxanthomonas kaohsiungensis
J36
RO clone RO127
RO isolate ROi22
Uncultured bacterium clone SX3-79
Chimaereicella alkaliphila AC74
RO clone RO224
RO clone RO74
cf. Bergeyella CCUG 46293
Uncultured bacterium clone SS-54
RO clone RO157
Uncultured soil clone M26_Pitesti
Uncultured candidate division TM7
bacterium clone
RO clone RO230
Uncultured bacterium clone 54
Uncultured division TM6 bacterium
clone NOS7.2WL
RO isolate ROi31
Microbacterium aurum DSM 8600
RO clone RO 28
Isosphaera-like str. CJugI1
Plactomycete str. 563
Aquifex pyrophilus Kol5a

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B. Manuscript 3

S1. Experimental Setup

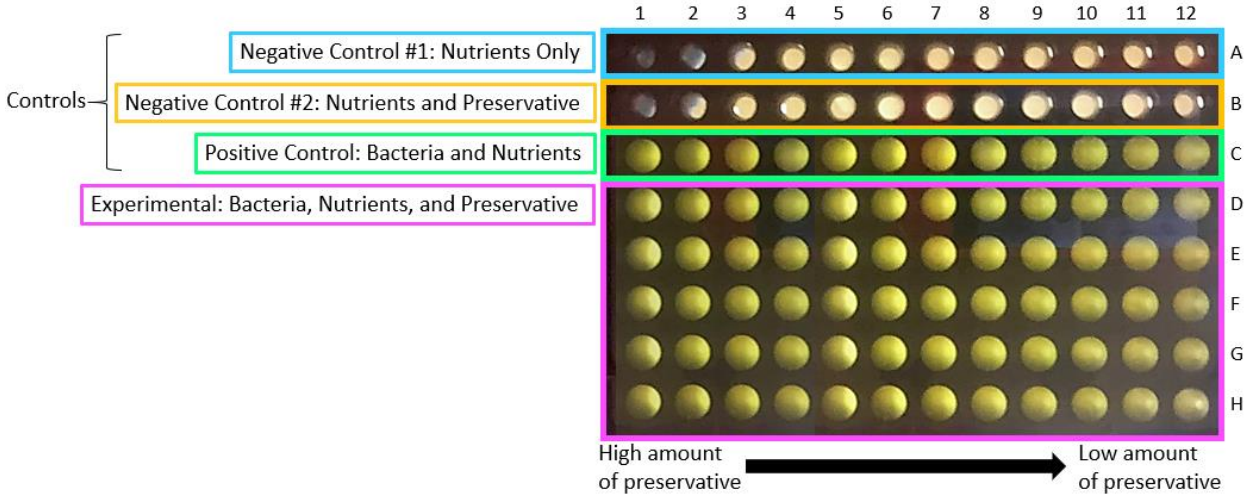

Figure S1. 96-well plate experimental set-up for both protocols (Section 2.5 and Section 2.6).

S2. Protocol Development

A summary of the trials and tribulations associated with developing this protocol. Additionally, the summary includes improvements for future related research.

S.2.1 *Escherichia coli* K12 W3110 Biofilms

The first organism we chose to use for this study was *Escherichia coli* K12 W3110 (*E. coli*). We chose to test this organism because it is used as a common fecal contamination indicator in water treatment (i.e. [1]) , and based on a literature source that indicated it would be a reasonable biofilm former (Manuscript 1 and 2). We attempted to grow biofilms with this *E. coli* strain by incubating it for 24 hours in a 37°C shaker incubator set at 200 rpm. After incubation we fluorescently stained the biofilm and saw that many cells appeared alive (green fluorescence) and clumped together in what may have been a biofilm (Figure 2). We chose to move forward with tests, however, after

rinsing and staining the plate, there were no longer cells in the plate. This led us to believe that a biofilm had not formed. We investigated whether this strain of *E. coli* can form biofilms and we found that [2] did find that was the case, however they found 10.7-fold less cells incorporate in the biofilm compared to another strain of *E. coli*. That corresponded to 0.56% of cells added to the sample incorporating into the biofilm [2]. Research suggests that the reason that *E. coli* K12 W3110 forms smaller biofilms is because it is F^-, meaning it is missing the F episome [2], [3]. The F episome is a type of genetic material that can replicate independently of the host and allows bacteria containing the factor to pass on genes to bacteria that do not contain the factor. Having the F episome has been shown to be important for biofilm formation [3]. This led us to believe that the clump of cells visualized by the stain was likely not a biofilm (Figure S2). We attempted to grow the biofilm for 48-72 hours and after no further success we decided to cease attempting to grow biofilms with *E. coli* K12 W3110.

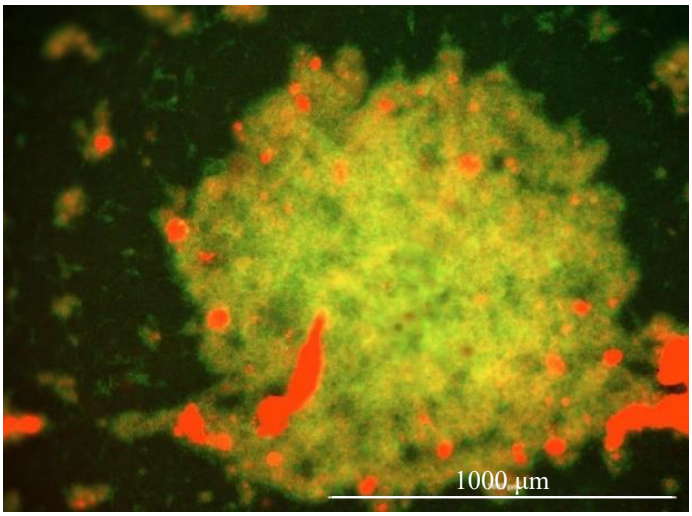

Figure S2. An image taken by the Cytation5 plate reader of a fluorescently stained clump of cells at the bottom of a well of a 96-well plate. This well was inoculated with *E. coli* K12 W3110 in hopes of growing a biofilm. We were never successful creating a biofilm with this strain of bacteria. The intense red areas may be clumps of stain, rather than clumps of dead cells, as [4] states that unbound PI possess strong background signal, however, we did not investigate further whether it was clumps of stain or clumps of dead cells.

S.2.2 Lysogeny Broth (LB) versus Cation-adjusted Mueller Hinton Broth (CAMHB)

Originally, we performed our tests using LB (Lysogeny Broth) because it is an acceptable media for the organisms we hoped to test. However, upon closer review, specifically in [5], we realized that we needed to perform tests with cation-adjusted Mueller Hinton broth (CAMHB.) CAMHB is recommended for susceptibility tests because it produces reproducible results, it is low in inhibitors that can effect some antibiotics, it is an acceptable nutrient broth for many pathogens, and there are many experiments that use CAMHB as a nutrient source for which acquired results can be compared [5]. We switched broth as soon as we identified this preference in the literature.

S.2.3 Rinsing the Wells via the "Dump" Method

Once we were having success growing *P. aeruginosa* biofilms we moved forward with the protocol. Before adding the antifoulant and before adding the stain we needed to rinse out the plates to replenish nutrients and remove planktonic cells so that we were only measuring the fluorescence of cells incorporated in the biofilm, respectively. To rinse out the wells we followed the rinse protocol in [6]. We dumped the liquid out of the 96-well plate into a bucket, refilled the wells with saline, and dumped out the contents again. We repeated this two more times. After following this protocol, we had contaminated control wells (Figure S3a). We tried multiple variations to this protocol i.e. shaking the plate differently, changing the angle of the bucket, waiting longer to flip the plate over after dumping etc., however, we continuously had contamination in the control wells. We eventually decided to discontinue using the dump method from O'Toole [6] and switch to rinsing plates via a multichannel pipette which was successful, although time-consuming (Figure S3b.) [7]–[9].

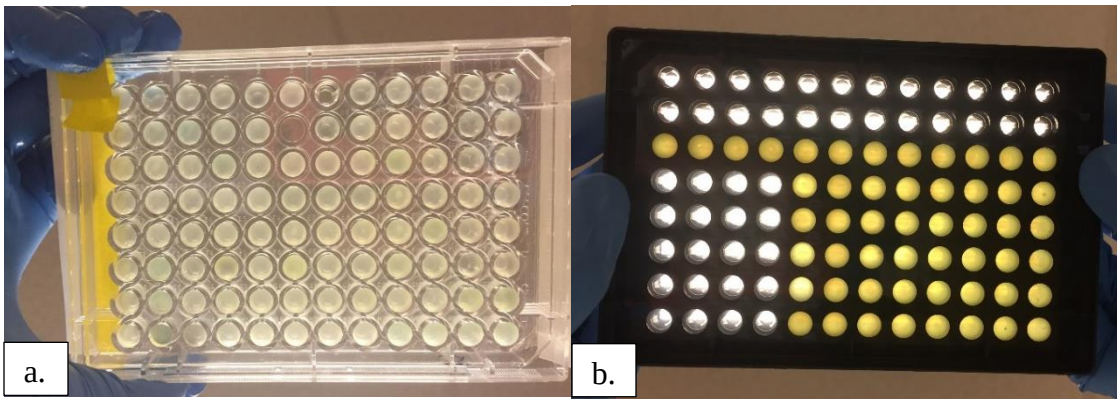

Figure S3. A 96-well plate after using the dump method of rinsing the wells (a.). The top two rows are control wells that should not be tinted green, indicating *P. aeruginosa* growth. A 96-well plate after rinsing the wells via a multichannel pipette (b.). Noteworthy are the two top wells that are clear indicating no contamination.

S.2.4 Stain Issues

The remaining hurdle for this protocol was related to the fluorescent stain. First, we realized vortexing was not sufficiently removing the biofilms from the plate (Figure S4a. and b). To attempt to improve removal of bacteria from the sides of the well, we added a trypsin incubation step. We incubated 60 µL of 1X trypsin per well for four minutes before vortexing the plate, which seemed to help break up the biofilms a bit more. We also extended the amount of time we vortexed the plate from 10 minutes to 15 minutes. Changes to the protocol helped break up the biofilm more, however, we suggest that future studies sonicate their sample to more effectively remove bacteria from the wells and create a more homogenous solution, which would be better for analyzing with the LIVE/DEAD *Bac*Light Stain (i.e. [10]–[13]).

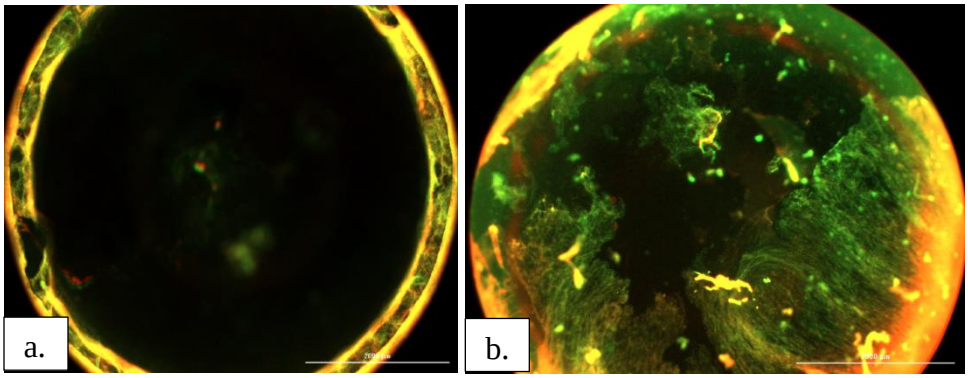

Figure S4. Both images are of wells after vortexing the plates with the fluorescent stain. In a. the biofilm is concentrated along the sides of the well, whereas in in b. the biofilm is on both the sides and extends into the center. Both wells do not contain homogenous solutions, which we need for more accurate fluorescent readings via the plate reader.

The other issue we experienced with the stain relates to reading the fluorescence. Originally, we were using the plate reader to read fluorescence values at one point in the well. Since the wells clearly do not contain homogenous solutions even after adding the trypsin and extending vortex time, we switched to an area well scan. We set the plate reader to a 5x5 area scan, which takes 25 measurements per well, at high sensitivity. This was chosen as a compromise between time and accuracy. Figure S5 shows a comparison of different scan types of green fluorescence. The 7x7 scan was likely slightly more accurate due to more data points collected, however it took forty minutes to collect data for just the green stain, compared to twenty minutes for the 5x5 scan. Ultimately, we decided the 7x7 time scan was too time-consuming and could impact the red stain which needed to be read after the green fluorescence. We were concerned that the red stain could be impacted and possibly damaged due to increased number of excitation at 485 nm for the 7x7 scan compared to the 5x5 scan, therefore by limiting the number of fluorescent reads by using the 5x5 scan, we may have decreased that risk. For example, there was a decrease in RFU values from the first 5x5 scan to the second and third, suggesting repeated reads decrease the quality of the fluorescent molecule. Stiefel *et al.* [4] found photo-bleaching was a concern for PI, but was a greater concern for SYTO9. Since there was a minute difference in duration between high versus low intensity scans, we chose high intensity.

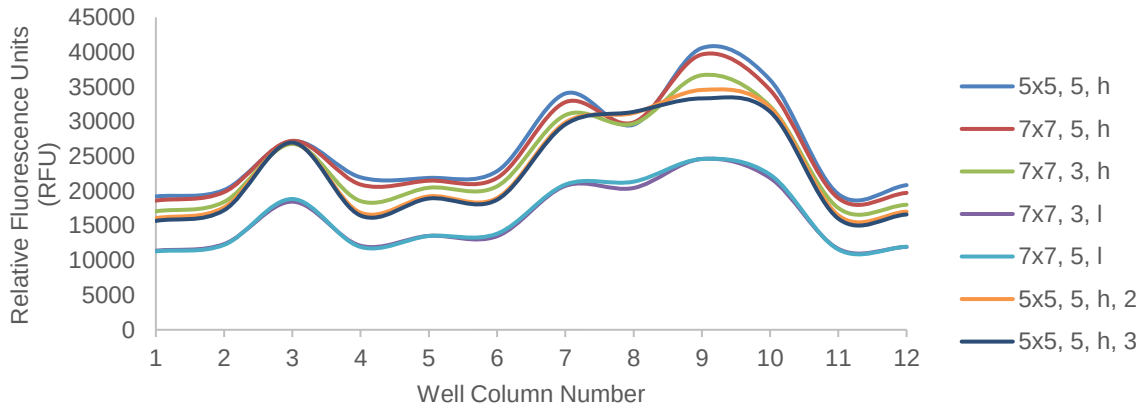

Figure S5. We read the green fluorescence (SYTO9) of row C of a 96-well plate with biofilms via an area well scan to determine the effect of different options on the plate reader and multiple reads per well. Scan options includ number of points read per well (5x5 or 7x7), seconds per read (5, 3), and sensitivity (high (h) or low (l). Scans had lower fluorescence per well than usual, however, we could still ascertain a relationship between fluorescence and scan options/number of repeated reads.

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S3. ANOVA Results

Table S1. Results from the ANOVA and Tukey Test between the mean green fluorescence of the positive control wells and the mean green fluorescence of each experimental well for each combination of preservative and method combination. (B) indicates the biofilm removal protocol and (P) indicates the biofilm prevention protocol.

***p<0.001 , **p<0.01, *p<0.05

Comparison	Concentration (µg/mL)	Adjusted p-value (Tukey Test)	Significance
MIT (B)—SYTO (+)	600	0.0000000	***
	300	0.0000000	***
	150	0.0000000	***
	75	0.0000000	***
	37.5	0.9999992	
	18.8	0.1398199	
	9.38	0.0001082	***
	4.69	0.5086176	
	2.34	0.0879071	
	1.17	0.4090118	
	0.586	0.0000000	***
	0.293	0.1806649	

Comparison	Concentration (µg/mL)	Adjusted p-value (Tukey Test)	Significance
MIT (P)—SYTO (+)	360	0.0000000	***
	180	0.0000000	***
	90	0.0000000	***
	45	0.0000000	***
	22.5	0.0000000	***
	11.3	0.0000039	***
	5.63	0.0000000	***
	2.81	0.7306539	
	1.41	1.0000000	
	0.70	0.4575712	
	0.352	0.0002401	***
	0.176	0.9918320	

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
PE (B)—SYTO (+)	960	0.0000000	***
	480	0.0000000	***
	340	0.0000000	***
	120	0.0000000	***
	60	0.0000001	***

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
PE (P)—SYTO (+)	576	0.0000000	***
	288	0.0000000	***
	144	0.0000000	***
	72	0.0000000	***
	36	0.0000000	***

	30	0.0000011	***
	15	0.2250530	
	7.5	0.3066775	
	3.75	0.3912137	
	1.88	0.8790954	
	0.938	1.0000000	
	0.469	0.9801082	
	18	0.0000000	***
	9	0.0000702	***
	4.5	0.0000000	***
	2.25	0.0000000	***
	1.13	0.0000000	***
	0.563	0.0794945	
	0.281	0.0000003	***

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
SB (B)—SYTO (+)	250	0.0000000	***
	125	0.0000000	***
	62.5	0.0000000	***
	31.2	0.0000000	***
	15.6	0.0008770	***
	7.81	0.1419539	
	3.91	0.5510000	
	1.95	0.1989383	
	0.977	0.0017026	**
	0.488	0.8721060	
	0.244	0.9999663	
	0.122	0.0316408	*

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
SB (P)—SYTO (+)	100	0.0000000	***
	50	0.0000000	***
	25	0.0000000	***
	12.5	0.0000000	***
	6.25	0.1973339	
	3.13	1.0000000	
	1.56	0.3983745	
	0.781	0.0342607	*
	0.391	0.0000000	***
	0.195	0.0057141	**
	0.098	0.0006573	***
	0.049	0.0000000	***

Table S2. Results from the ANOVA and Tukey Test between the mean red fluorescence of the positive control wells and the mean red fluorescence of each experimental well for each combination of preservative and method combination. (B) indicates the biofilm removal protocol and (P) indicates the biofilm prevention protocol

***p<0.001 , **p<0.01, *p<0.05

Comparison	Concentration (µg/mL)	Adjusted p-value (Tukey Test)	Significance
MIT (B)—SYTO (+)	600	0.2561957	
	300	0.0000283	***
	150	0.0000012	***
	75	0.0000014	***
	37.5	0.0423537	*
	18.8	0.9298454	
	9.38	0.9999999	***
	4.69	0.1057175	
	2.34	0.0444946	
	1.17	0.0000513	***
	0.586	0.3943843	
	0.293	0.0000000	***

Comparison	Concentration (µg/mL)	Adjusted p-value (Tukey Test)	Significance
MIT (P)—SYTO (+)	360	0.0000031	***
	180	0.0000432	***
	90	0.0004952	***
	45	0.0000136	***
	22.5	0.0001364	***
	11.3	0.0027494	**
	5.63	0.4356524	
	2.81	0.9999905	
	1.41	1.0000000	
	0.70	0.0000066	***
	0.352	0.0000000	***
	0.176	0.0000000	***

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
PE (B)—SYTO (+)	960	0.9406196	
	480	0.3284298	
	340	0.0574960	
	120	0.0282743	*
	60	0.8603528	
	30	0.9339102	
	15	1.0000000	

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
PE (P)—SYTO (+)	576	0.0019459	**
	288	0.0000000	***
	144	0.0000000	***
	72	0.0000000	***
	36	0.0000000	***
	18	0.0000000	***
	9	0.1858845	

	7.5	0.9997204	
	3.75	0.9753475	
	1.88	0.0009959	***
	0.938	0.0000000	***
	0.469	0.0000149	***
	4.5	0.0094620	**
	2.25	0.0039739	**
	1.13	0.1338841	
	0.563	0.9966274	
	0.281	0.0000000	***

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
SB (B)—SYTO (+)	250	1.0000000	
	125	0.5399469	
	62.5	0.7153192	
	31.2	0.4380918	
	15.6	0.9999937	
	7.81	1.0000000	
	3.91	0.9999194	
	1.95	0.0000141	***
	0.977	0.0000000	***
	0.488	0.0000000	***
	0.244	0.0000002	***
	0.122	0.3572968	

Comparison	Concentration (mg/mL)	Adjusted p-value (Tukey Test)	Significance
SB (P)—SYTO (+)	100	0.0004031	***
	50	0.0002536	***
	25	0.0002681	***
	12.5	0.9521734	
	6.25	0.9999999	
	3.13	0.3465565	
	1.56	0.0758505	
	0.781	0.9999893	
	0.391	0.0716341	
	0.195	0.0481503	*
	0.098	0.2579004	
	0.049	0.0002440	***

Table S3. Results from the ANOVA and Tukey Test between the mean red fluorescence of each experimental well for each combination of preservative and method combination. The purpose of this ANOVA was to investigate whether the red fluorescence was sensitive enough to detect quantities of red cells, especially at the lower values of dead cells. (B) indicates the biofilm removal protocol and (P) indicates the biofilm prevention protocol. The yellow highlighted p values are below 0.05.

MIT (B) Concentration (µg/mL)	Adjusted p-value (Tukey Test)
300-600	0.6174540
150-600	0.2585248
75-600	0.2666217
37.5-600	0.9999944
18.8-600	0.9992228
9.38-600	0.7548937
2.34-600	0.0000411
1.17-600	0.0000000
0.586-600	0.0012178
0.293-600	0.0000000
4.69-600	0.0002542
150-300	0.9999982
75-300	0.9999987
37.5-300	0.9368611
18.8-300	0.0989340
9.38-300	0.0035806
2.34-300	0.0000000
1.17-300	0.0000000
0.586-300	0.0000000
0.293-300	0.0000000
4.69-300	0.0000000
75-150	1.0000000
37.5-150	0.6574540
18.8-150	0.0197349
9.38-150	0.0004051
2.34-150	0.0000000
1.17-150	0.0000000
0.586-150	0.0000000
0.293-150	0.0000000
4.69-150	0.0000000
37.5-75	0.6680324
18.8-75	0.0207437
9.38-75	0.0004321
2.34-75	0.0000000
1.17-75	0.0000000
0.586-75	0.0000000
0.293-75	0.0000000
4.69-75	0.0000000
18.8-37.5	0.9447784
9.38-37.5	0.3427746
2.34-37.5	0.0000023
1.17-37.5	0.0000000
0.586-37.5	0.0000945
0.293-37.5	0.0000000
4.69-37.5	0.0000225
9.38-18.8	0.9984059
2.34-18.8	0.0023929
1.17-18.8	0.0000032
0.586-18.8	0.0365538
0.293-18.8	0.0000000
4.69-18.8	0.0074938
2.34-9.38	0.0744321
1.17-9.38	0.0003655
0.586-9.38	0.4208578
0.293-9.38	0.0000000
4.69-9.38	0.1254556
1.17-2.34	0.9597557
0.586-2.34	0.9998987
0.293-2.34	0.0000000
4.69-2.34	1.0000000
0.586-1.17	0.5545806
0.293-1.17	0.0000000
4.69-1.17	0.9938107
0.293-0.586	0.0000000
4.69-0.586	0.9997757
4.69-0.293	0.0000000

PE (B) Concentration (mg/mL)	Adjusted p-value (Tukey Test)
480-960	0.9996457
240-960	0.9553972
120-960	0.8938958
60-960	1.0000000
30-960	1.0000000
15-960	0.9897020
7.5-960	0.7157870
3.75-960	0.4036094
1.88-960	0.0000852
0.938-960	0.0000000
0.469-960	0.0000019
240-480	0.9999904
120-480	0.9998040
60-480	0.9999708
30-480	0.9997213
15-480	0.6542293
7.5-480	0.1745140
3.75-480	0.0552328
1.88-480	0.0000016
0.938-480	0.0000000
0.469-480	0.0000000
120-240	1.0000000
60-240	0.9840221
30-240	0.9596738
15-240	0.2465187
7.5-240	0.0328924
3.75-240	0.0076152
1.88-240	0.0000001
0.938-240	0.0000000
0.469-240	0.0000000
60-120	0.9514218
30-120	0.9017132
15-120	0.1584140
7.5-120	0.0174428
3.75-120	0.0037081
1.88-120	0.0000000
0.938-120	0.0000000
0.469-120	0.0000000
30-60	1.0000000
15-60	0.9683436
7.5-60	0.5883150
3.75-60	0.2906308
1.88-60	0.0000381
0.938-60	0.0000000
0.469-60	0.0000008
15-30	0.9881843
7.5-30	0.7023881
3.75-30	0.3903077
1.88-30	0.0000781
0.938-30	0.0000000
0.469-30	0.0000018
7.5-15	0.9998865
3.75-15	0.9907983
1.88-15	0.0108837
0.938-15	0.0000004
0.469-15	0.0004869
3.75-7.5	0.9999998
1.88-7.5	0.1121506
0.938-7.5	0.0000144
0.469-7.5	0.0092422
1.88-3.75	0.3026314
0.938-3.75	0.0001043
0.469-3.75	0.0389366
0.938-1.88	0.4351890
0.469-1.88	0.9997887
0.469-0.938	0.9287236

SB (B) Concentration (mg/mL)	Adjusted p-value (Tukey Test)
125-250	0.7769850
62.5-250	0.8858184
31.2-250	0.6983440
15.6-250	0.9999991
7.81-250	1.0000000
3.91-250	0.9999877
1.95-250	0.0004303
0.977-250	0.0000000
0.488-250	0.0000011
0.244-250	0.0000126
0.122-250	0.6276988
62.5-125	1.0000000
31.2-125	1.0000000
15.6-125	0.4498894
7.81-125	0.7359785
3.91-125	0.3669039
1.95-125	0.0000003
0.977-125	0.0000000
0.488-125	0.0000000
0.244-125	0.0000000
0.122-125	0.0082685
31.2-62.5	1.0000000
15.6-62.5	0.5987707
7.81-62.5	0.8560842
3.91-62.5	0.5087085
1.95-62.5	0.0000008
0.977-62.5	0.0000000
0.488-62.5	0.0000000
0.244-62.5	0.0000000
0.122-62.5	0.0160460
15.6-31.2	0.3691721
7.81-31.2	0.6532194
3.91-31.2	0.2943456
1.95-31.2	0.0000002
0.977-31.2	0.0000000
0.488-31.2	0.0000000
0.244-31.2	0.0000000
0.122-31.2	0.0055462
7.81-15.6	0.9999998
3.91-15.6	1.0000000
1.95-15.6	0.0023263
0.977-15.6	0.0000001
0.488-15.6	0.0000074
0.244-15.6	0.0000785
0.122-15.6	0.9022163
3.91-7.81	0.9999966
1.95-7.81	0.0005462
0.977-7.81	0.0000000
0.488-7.81	0.0000015
0.244-7.81	0.0000162
0.122-7.81	0.6735957
1.95-3.91	0.0035748
0.977-3.91	0.0000002
0.488-3.91	0.0000121
0.244-3.91	0.0001264
0.122-3.91	0.9431998
0.977-1.95	0.2973702
0.488-1.95	0.9229752
0.244-1.95	0.9988320
0.122-1.95	0.2269414
0.488-0.977	0.9970580
0.244-0.977	0.8871674
0.122-0.977	0.0000681
0.244-0.488	0.9999852
0.122-0.488	0.0028397
0.122-0.244	0.0204420

MIT (P) Concentration (µg/mL)	Adjusted p-value (Tukey Test)
180-360	0.9999994
90-360	0.9989849
45-360	1.0000000
22.5-360	0.9999604
11.2-360	0.9810289
5.62-360	0.0000000
2.81-360	0.0000198
1.41-360	0.0001110
0.703-360	0.0000000
0.352-360	0.0000000
0.176-360	0.0000000
90-180	0.9999994
45-180	1.0000000
22.5-180	1.0000000
11.2-180	0.9997168
5.62-180	0.0000001
2.81-180	0.0001657
1.41-180	0.0008264
0.703-180	0.0000000
0.352-180	0.0000000
0.176-180	0.0000000
45-90	0.9999649
22.5-90	1.0000000
11.2-90	1.0000000
5.62-90	0.0000015
2.81-90	0.0011872
1.41-90	0.0051478
0.703-90	0.0000000
0.352-90	0.0000000
0.176-90	0.0000000
22.5-45	0.9999998
11.2-45	0.9973542
5.62-45	0.0000000
2.81-45	0.0000652
1.41-45	0.0003435
0.703-45	0.0000000
0.352-45	0.0000000
0.176-45	0.0000000
11.2-22.5	0.9999889
5.62-22.5	0.0000004
2.81-22.5	0.0004190
1.41-22.5	0.0019648
0.703-22.5	0.0000000
0.352-22.5	0.0000000
0.176-22.5	0.0000000
5.62-11.2	0.0000087
2.81-11.2	0.0047663
1.41-11.2	0.0182362
0.703-11.2	0.0000000
0.352-11.2	0.0000000
0.176-11.2	0.0000000
2.81-5.62	0.9500000
1.41-5.62	0.7884679
0.703-5.62	0.2480776
0.352-5.62	0.0000030
0.176-5.62	0.0106691
1.41-2.81	1.0000000
0.703-2.81	0.0027019
0.352-2.81	0.0000000
0.176-2.81	0.0000248
0.703-1.41	0.0005906
0.352-1.41	0.0000000
0.176-1.41	0.0000041
0.352-0.703	0.1051258
0.176-0.703	0.9954972
0.176-0.352	0.7494056

PE (P) Concentration (mg/mL)	Adjusted p-value (Tukey Test)
288-576	0.0212199
144-576	0.0339285
72-576	0.0022393
36-576	0.0234207
18-576	0.2429392
9-576	0.9901648
4.5-576	0.0000000
2.25-576	0.0000000
1.12-576	0.0000006
0.562-576	0.0007734
0.281-576	0.0000000
144-288	1.0000000
72-288	0.9999862
36-288	1.0000000
18-288	0.9994250
9-288	0.0002080
4.5-288	0.0000000
2.25-288	0.0000000
1.12-288	0.0000000
0.562-288	0.0000000
0.281-288	0.0000000
72-144	0.9998668
36-144	1.0000000
18-144	0.9999104
9-144	0.0003887
4.5-144	0.0000000
2.25-144	0.0000000
1.12-144	0.0000000
0.562-144	0.0000000
0.281-144	0.0000000
36-72	0.9999768
18-72	0.9398370
9-72	0.0000123
4.5-72	0.0000000
2.25-72	0.0000000
1.12-72	0.0000000
0.562-72	0.0000000
0.281-72	0.0000000
18-36	0.9995943
9-36	0.0002369
4.5-36	0.0000000
2.25-36	0.0000000
1.12-36	0.0000000
0.562-36	0.0000000
0.281-36	0.0000000
9-18	0.0071858
4.5-18	0.0000000
2.25-18	0.0000000
1.12-18	0.0000000
0.562-18	0.0000000
0.281-18	0.0000000
4.5-9	0.0000059
2.25-9	0.0000022
1.12-9	0.0001680
0.562-9	0.0559847
0.281-9	0.0000000
2.25-4.5	1.0000000
1.12-4.5	0.9999242
0.562-4.5	0.4797079
0.281-4.5	0.0003629
1.12-2.25	0.9990853
0.562-2.25	0.3427060
0.281-2.25	0.0008347
0.562-1.12	0.9247059
0.281-1.12	0.0000138
0.281-0.562	0.0000000

SB (P) Concentration (mg/mL)	Adjusted p-value (Tukey Test)
50-100	1.0000000
25-100	1.0000000
12.5-100	0.2568400
6.25-100	0.0216705
3.12-100	0.0000007
1.56-100	0.0000000
0.781-100	0.0009829
0.391-100	0.0000000
0.195-100	0.0000000
0.0977-100	0.0000004
0.0488-100	0.0000000
25-50	1.0000000
12.5-50	0.2108032
6.25-50	0.0159680
3.12-50	0.0000005
1.56-50	0.0000000
0.781-50	0.0006757
0.391-50	0.0000000
0.195-50	0.0000000
0.0977-50	0.0000002
0.0488-50	0.0000000
12.5-25	0.2159583
6.25-25	0.0165661
3.12-25	0.0000005
1.56-25	0.0000000
0.781-25	0.0007067
0.391-25	0.0000000
0.195-25	0.0000000
0.0977-25	0.0000003
0.0488-25	0.0000000
6.25-12.5	0.9992813
3.12-12.5	0.0436943
1.56-12.5	0.0070539
0.781-12.5	0.8481554
0.391-12.5	0.0066296
0.195-12.5	0.0043414
0.0977-12.5	0.0294680
0.0488-12.5	0.0000280
3.12-6.25	0.3920401
1.56-6.25	0.1203982
0.781-6.25	0.9996817
0.391-6.25	0.1151967
0.195-6.25	0.0847741
0.0977-6.25	0.3109126
0.0488-6.25	0.0014089
1.56-3.12	0.9999968
0.781-3.12	0.9188862
0.391-3.12	0.9999955
0.195-3.12	0.9999654
0.0977-3.12	1.0000000
0.0488-3.12	0.7733439
0.781-1.56	0.6019530
0.391-1.56	1.0000000
0.195-1.56	1.0000000
0.0977-1.56	0.9999998
0.0488-1.56	0.9769254
0.391-0.781	0.5893360
0.195-0.781	0.5045607
0.0977-0.781	0.8674374
0.0488-0.781	0.0289512
0.195-0.391	1.0000000
0.0977-0.391	0.9999998
0.0488-0.391	0.9792013
0.0977-0.195	0.9999965
0.0488-0.195	0.9903174
0.0488-0.0977	0.8453271

Table S4. Results from the ANOVA and Tukey Test between the mean green and red fluorescence of the positive controls for each preservative and method combination. The purpose of this ANOVA was to investigate the consistency between experiments. (B) indicates the biofilm removal protocol and (P) indicates the biofilm prevention protocol. ***p<0.001 , **p<0.01, *p<0.05

Comparison (Green)	Adjusted p-value (Tukey Test)	Significance
PE (B)-MIT (B)	0.0000000	***
SB (B)-MIT (B)	0.0000000	***
MIT (P)-MIT (B)	0.9915160	
PE (P)-MIT (B)	0.9993362	
SB (P)-MIT (B)	0.0000000	***
SB (B)-PE (B)	0.8669511	
MIT (P)-PE (B)	0.0000000	***
PE (P)-PE (B)	0.0000000	***
SB (P)-PE (B)	0.7058052	
MIT (P)-SB (B)	0.0000000	***
PE (P)-SB (B)	0.0000000	***
SB (P)-SB (B)	1.0000000	
PE (P)-MIT (P)	0.9999197	
SB (P)-MIT (P)	0.0000000	***
SB (P)-PE (P)	0.0000000	***

Comparison (Red)	Adjusted p-value (Tukey Test)	Significance
PE (B)-MIT (B)	0.3819934	
SB (B)-MIT (B)	0.2690279	
MIT (P)-MIT (B)	0.0246129	**
PE (P)-MIT (B)	0.0008719	***
SB (P)-MIT (B)	0.0002944	***
SB (B)-PE (B)	0.9923254	
MIT (P)-PE (B)	0.0001039	***
PE (P)-PE (B)	0.0000019	***
SB (P)-PE (B)	0.2147220	
MIT (P)-SB (B)	0.0003216	***
PE (P)-SB (B)	0.0000143	***
SB (P)-SB (B)	0.7990158	
PE (P)-MIT (P)	0.9440156	
SB (P)-MIT (P)	0.0000000	***
SB (P)-PE (P)	0.0000000	***