I, Chloe JA Shea, hereby submit this original work as part of the requirements for the degree of Master of Science in Molecular Genetics, Biochemistry, & Microbiology.

It is entitled:
Effectiveness of Novel Compounds at Inhibiting and Killing P. aeruginosa and S. epidermidis Biofilms

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Effectiveness of Novel Compounds at Inhibiting and Killing *P. aeruginosa* and *S. epidermidis* Biofilms

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Abstract

*Pseudomonas aeruginosa* (Pa) and *Staphylococcus epidermidis* (Se) are opportunistic human pathogens whose ubiquitous nature and capacity to form antibiotic-resisting biofilms cause them to be a growing source of healthcare-associated infections. Increasing resistance to available medicine makes them important targets for new treatments that could be incorporated into surfaces upon which a biofilm could develop, such as indwelling catheters, tubing, and pacemakers.

Potential new compounds were discovered in a high-throughput screening of 40,000 compounds against Pa and Se biofilms. We assessed the effectiveness of 13 of these novel compounds against Pa biofilms and 20 compounds against Se biofilms. The compounds used in this study were divided into four groups. Group 1 (compounds 3, 7, 8, and 11): compounds effective against Pa, Se, and the additional opportunistic human pathogen *A. baumannii* (Ab) biofilms; Group 2 (compounds 1, 2, 9, 10, 12, 13): compounds only effective against Pa and Ab biofilms; Group 3 (compounds 14-16): compounds only effective against Pa biofilms; and Group 4 (Se1-Se16): compounds only effective against Se biofilms.

Confocal laser scanning microscopy was used to take images of Pa and Se biofilms under two conditions: inhibition of biofilm formation, in which the compound was co-cultured with the bacteria; and biofilm killing, in which the compound was added to an existing biofilm. Seven of the compounds screened against Pa biofilms (compounds 1, 2, 3, 7, 8, 13, and 14) were effective at inhibiting the biofilm at 10 μM, but only one compound was effective at killing the Pa biofilm at 300 μM (compound 3). Therefore, from all of the compounds used in this study, only compound 3 was effective at both inhibiting and killing the Pa biofilm. However, compounds 7,
8, 10, 11, 14, and 15 caused dispersion of Pa biofilm, and compounds 7, 8, 9, 11, and 14 caused a change in morphology from a rod to an elongated, filamentous-like shape.

Eleven of the compounds screened against Se biofilms (compounds 3, 7, 8, 11, Se3, Se 4, Se9, Se13, Se14, Se15, and Se16) were effective at inhibiting biofilm formation at 10 μM, while seven compounds (Se3, Se4, Se10, Se12, Se13, Se14, and Se15) were effective at killing the Se biofilm at 300 μM. Therefore, five of these compounds (Se3, Se4, Se13, Se14, and Se15) were effective at both inhibiting and killing the Se biofilm. None of the compounds screened against Se caused dispersion of the biofilm or morphological changes.

Additionally, Minimum Inhibition Concentration experiments were conducted on the four compounds of group 1; no large difference was observed between aerobic and anaerobic conditions. Compounds 7 and 8 were very effective, generally requiring less than 10 μM to inhibit both Pa and Se planktonic cultures, while compounds 3 and 11 required higher concentrations. The compounds of this group were also investigated for cytotoxicity on A549 and differentiated THP-1 human cell lines and were found to be nontoxic up to 260-570 μM, depending on the compound used.
Disclaimer

The views expressed in this article are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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**List of Abbreviations**

| Ab       | Acinetobacter baumannii          |
| CLSM     | Confocal Laser Scanning Microscopy |
| DMSO     | Dimethyl sulfoxide               |
| FBS      | Fetal Bovine Serum               |
| LB       | Lysogeny Broth                   |
| LDH      | Lactate Dehydrogenase            |
| MH       | Mueller Hinton                    |
| MIC      | Minimum Inhibitory Concentration |
| OD       | Optical Density                   |
| Pa       | Pseudomonas aeruginosa            |
| PBS      | Phosphate Buffered Saline        |
| PMA      | Phorbol 12-myristate 13-acetate   |
| PQ       | Paraquat dichloride               |
| RFU      | Relative Fluorescence Units       |
| RPM      | Rotations per minute              |
| Se       | Staphylococcus epidermidis        |
| TSB      | Tryptic Soy Broth                 |
1. Introduction

1.1 Bacteria

*Pseudomonas aeruginosa* (Pa), *Staphylococcus epidermidis* (Se), and *Acinetobacter baumannii* (Ab) are opportunistic human bacterial pathogens [1-4]. They are particularly dangerous due to their ubiquitous nature and capacity to form antibiotic-resisting biofilm infections [1, 2, 5], and are a growing source of healthcare-associated infections [6, 7].

Pa is a Gram-negative, rod-shaped bacterium that can grow under both aerobic and anaerobic conditions [2, 8]. It is one of the most common opportunistic pathogens that affect humans [3]. Infection is especially prevalent and dangerous in cystic fibrosis patients, where it inhabits the airways [8, 9], but is also a danger for cancer patients, burn victims, and individuals with compromised immune systems [3, 10].

Se is a Gram-positive [11] coccus capable of surviving in both aerobic and anaerobic conditions by switching from respiration to fermentation [12]. It is a normal component of human flora, and usually survives on the skin without harming its host [13]. However, Se biofilms can grow on catheters and other medical implants inserted into the body, and are a major source of biomaterial infections [14]. Se is another important opportunistic pathogen that can cause serious illness in immune-compromised patients [15].

Ab is a Gram-negative rod and is strictly aerobic [5]. It frequently emerges in outbreaks, particularly in intensive care units [5], and drug resistance is becoming a large problem worldwide [6, 7], especially in shrapnel related accidents in the wars in Iraq and Afghanistan. The sources of infection are similar to that of Pa and Ab; those persons undergoing invasive surgery, people with
catheters, and people exposed to high amounts of antibiotics are at the highest risk for infection [5].

Taken together, Pa, Se, and Ab are three extremely prevalent microorganisms whose pervasive presence and a decreasing supply of effective medicine make them important targets for new treatments. Although a variety of antimicrobials exist for combatting Pa, Se, and Ab infections, and common disinfectants are used for eliminating microorganisms from surfaces to which biofilms may attach, all three of these microorganisms are becoming increasingly resistant to both antibiotics and disinfectants [6, 7, 16]. Pa has a wide variety of mechanisms through which resistance is developed or possessed innately; it can prevent the access to the antibiotic through multidrug efflux pumps; inactivate the antibiotics through β-lactamases; and rapidly acquire mutations in vivo allows it to develop new defenses [16]. Ab can also express β-lactamases that offer resistance, and multidrug efflux pumps have been found present in resistant strains [16]. Moreover, Ab can rapidly transfer antimicrobial-resistance genes from resistant to susceptible strains [5]. Se has also developed mechanisms of resistance to certain antibiotic treatments. For example, it can escape neutrophil digestion and can detect antimicrobial peptides [13]. Finally, the biofilm structures themselves confer resistance, as their organized arrangement prevents antibiotics from accessing the majority of bacteria [2, 15, 17]. The antimicrobials to which these biofilms are resistant include kanamycin for Pa [9], methicillin and vancomycin for Se [11], and ciprofloxacin for Ab [4]; resistance is so extensive that some strains express some form of resistance to almost every available antibiotic [5, 16], and the implanted device must be surgically removed to eliminate the biofilm [17].
Due to increased resistance to these important pathogens, it is necessary to identify and develop new compounds. These could be incorporated into or on surfaces upon which a biofilm could develop, including indwelling catheters, tubing, pacemakers, bone, vital organs and skin.

### 1.2 Compounds

The compounds used in these experiments were selected from a 40,000 compound library, part of over 300,000 compounds initially supported by the Proctor and Gamble company in Cincinnati, Ohio. A high-throughput screening of these 40,000 compounds was previously conducted to determine compound candidates for further assessment. The high-throughput screening process consists of three distinct steps: (i) determine the growth of each organism by measuring the optical density (OD), (ii) assess the metabolic activity via a resazurin assay, and (iii) determine biofilm formation on a glass or plastic surface using crystal violet staining [18]. The compounds used in this study are those that were effective at either killing or inhibiting Pa, Se, and/or Ab biofilms in the high-throughput screening. The compounds were divided into four groups for these experiments and are listed in **Table 1**. The first group is comprised of the compounds that were found to be effective at killing or inhibiting Pa, Se, and Ab biofilms; these are compounds 3, 7, 8, and 11. The second group is compounds that were effective at killing and/or inhibiting Pa and Ab biofilms, consisted of compounds 1, 2, 9, 10, 12, and 13. Compounds that were only effective on Pa biofilms are classified into the third group; these are compounds 14-16. Lastly, the fourth group, compounds Se1-Se16, contains the compounds that were effective only on Se biofilms. Structures of the compounds in Groups 1-4 are shown in **Figures 1, 2, 3, and 4**, respectively.
Many of the effective compounds are of the quinolone class [19]. Quinolones inhibit DNA-gyrase in Gram-negative bacteria and DNA-topoisomerase in Gram-positive bacteria [20]. The quinolone ciprofloxacin is commonly used as a treatment for patients with Pa infections [20]. Other highly represented classes include the diarylsulfonamides and ureas. Sulfonamides prevent folate synthesis by inhibiting dihydropteroate synthetase [21, 22], while ureas act by denaturing proteins and can inhibit bacterial cell wall synthesis [23, 24].
**Table 1.** List of compounds used in this study divided into four groups based on effectiveness against biofilms.

<table>
<thead>
<tr>
<th>Group</th>
<th>Effective against biofilms</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pa, Se, and Ab</td>
<td>3, 7, 8, 11</td>
</tr>
<tr>
<td>2</td>
<td>Pa and Ab</td>
<td>1, 2, 9, 10, 12, 13</td>
</tr>
<tr>
<td>3</td>
<td>Pa</td>
<td>14, 15, 16</td>
</tr>
<tr>
<td>4</td>
<td>Se</td>
<td>Se1, Se2, Se3, Se4, Se5, Se6, Se7, Se8, Se9, Se10, Se11, Se12, Se13, Se14, Se15, Se16</td>
</tr>
</tbody>
</table>
Fig. 1. The structures of group 1 compounds, which were effective against Pa, Se, and Ab biofilms. The compounds were divided into groups based on which biofilms they either killed or inhibited from formation. Compound 3 is an isothiazolone, while compounds 7, 8 and 11 are of the quinolone class.
<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>Benzimidazole</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>Furazan</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9.png" alt="Structure 9" /></td>
<td>Quinolone</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10.png" alt="Structure 10" /></td>
<td>Aryl Heterocycle</td>
</tr>
<tr>
<td>12</td>
<td><img src="image12.png" alt="Structure 12" /></td>
<td>Catechol</td>
</tr>
<tr>
<td>13</td>
<td><img src="image13.png" alt="Structure 13" /></td>
<td>Quinolone</td>
</tr>
</tbody>
</table>

**Fig. 2.** The structures of group 2 compounds, those originally effective against both Pa and Ab biofilms. Compounds 9 and 13 are quinolones, while the remaining compounds are a diverse group of a benzimidazole, furazan, aryl heterocycle, and catechol for compounds 1, 2, 10, and 12, respectively.
<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
<th>Class</th>
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</thead>
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<tr>
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<td><img src="image" alt="Quinolone" /></td>
<td>Quinolone</td>
</tr>
<tr>
<td>15</td>
<td><img src="image" alt="Mucochloric Acid" /></td>
<td>Mucochloric Acid</td>
</tr>
<tr>
<td>16</td>
<td><img src="image" alt="Benzimidazole" /></td>
<td>Benzimidazole</td>
</tr>
</tbody>
</table>

**Fig. 3.** The structures of group 3 compounds, those that were effective at killing or inhibiting only Pa biofilms. Compound 14 is another quinolone, while compound 15 is a mucochloric acid and compound 16 is of the benzimidazole class.
<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
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<tbody>
<tr>
<td>Se1</td>
<td>Diarylsulfonamide</td>
<td></td>
</tr>
<tr>
<td>Se2</td>
<td>Diarylsulfonamide</td>
<td></td>
</tr>
<tr>
<td>Se3</td>
<td>Urea</td>
<td></td>
</tr>
<tr>
<td>Se4</td>
<td>Quaternary Ammonium Salt</td>
<td></td>
</tr>
<tr>
<td>Se5</td>
<td>Hydrazone</td>
<td></td>
</tr>
<tr>
<td>Se6</td>
<td>Pyrazolopyrimidine</td>
<td></td>
</tr>
</tbody>
</table>

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<thead>
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<th>Structure</th>
<th>Class</th>
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<tbody>
<tr>
<td>Se7</td>
<td>Diarylsulfonamide</td>
<td></td>
</tr>
<tr>
<td>Se8</td>
<td>Sulfonamide</td>
<td></td>
</tr>
<tr>
<td>Se9</td>
<td>Quinolone</td>
<td></td>
</tr>
<tr>
<td>Se10</td>
<td>Urea</td>
<td></td>
</tr>
<tr>
<td>Se11</td>
<td>Urea</td>
<td></td>
</tr>
<tr>
<td>Se12</td>
<td>Hydrazone</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se13</td>
<td>Dye</td>
<td></td>
</tr>
<tr>
<td>Se14</td>
<td>Pyrrole</td>
<td></td>
</tr>
<tr>
<td>Se15</td>
<td>Diarylsulfonamide</td>
<td></td>
</tr>
<tr>
<td>Se16</td>
<td>8-Hydroxyquinoline</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 4.** Structures of group 4 compounds that were found to be effective at killing or inhibiting Se biofilms. Se1, Se2, Se7, and Se15 are diarylsulfonamides; Se3, Se10, and Se11 are ureas; and Se5 and 12 are hydrazones. The remaining compounds are a quaternary ammonium salt, a pyrazolopyrimidine, a sulfonamide, a quinolone, a dye, a pyrrole, and a 8-hydroxyquinolone for Se4, Se6, Se8, Se9, Se13, Se14, and Se16, respectively.
1.3 Assessing Compound Effectiveness at Killing and Inhibiting Pa and Se Biofilms

A combination of Confocal Laser Scanning Microscopy (CLSM) and Minimum Inhibitory Concentration (MIC) tests were used to assess the effectiveness of the compounds at killing and inhibiting Pa and Se biofilms and planktonic cells, respectively [9, 18, 25]. The high resolution biofilm images provided by CLSM allow for a visual comparison between healthy biofilms and those affected by the compounds.

Furthermore, the cytotoxicity of Group 1 compounds, those effective on Pa, Se, and Ab biofilms, was examined in order to assess medical use feasibility. The human macrophage and airway cell lines THP-1 and A549 were chosen to determine the potential toxicity of the novel compounds to the human body. THP-1 is a human acute leukemia monocyte cell line [26], while A549 consists of epithelial cells from a lung carcinoma [27]. Together, these two cell lines are commonly used for screening potentially hazardous materials [28-31]. For example, the cytotoxicity of nanoparticles was assessed on A549 and THP-1 cell lines because these represent cells that would be directly afflicted if nanoparticles were inhaled [29].

1.4 Purpose of Research

The purpose of this research is to confirm the high-throughput screening results and examine the effectiveness of these compounds on both preventing and killing Pa and Se biofilms. (Ab was tested in a parallel study; those results are not included here.)

These compounds could be used in a wide array of applications. Medically, they could be used as a coating on an implanted device, thereby preventing a biofilm from being able to attach and form. They could also potentially be used to treat an existing Pa or Se infection. These compounds also have a significant military application; biofilms not only cause corrosion on the
surfaces on which they form, but also cause the proliferation of planktonic bacteria when in an aqueous environment [18]. This means that not only are military vehicles such as the fuel tanks of aircraft at risk for deterioration, but fuel contained in a contamination fuel tank is also contaminated [18]. Both Pa and Se have been found in jet fuel tanks [32]. Thus, preventing these biofilms from forming and destroying them once they do form is an important problem, both for the medical field and the military.
2. Materials and Methods

2.1 Bacteria Growth Conditions

Planktonic, free-living *P. aeruginosa* PAO1 (Pa) was routinely grown aerobically in Lysogeny Broth (LB) [33] while *S. epidermidis* RP62A strain (Se) was grown in Tryptic Soy Broth (TSB) [34]. To initiate the growth of Pa biofilms, Pa was subcultured into 10% LB and grown at 37°C with shaking at 250 rotations per minute (RPM) for 2.5 hours, when the OD$_{600}$ reached 0.25. The bacteria was further diluted 1:100 into a modified 0.5X M63 minimum media with a higher concentration of FeSO$_4$ (50 mM KH$_2$PO$_4$, 7.5 mM (NH$_4$)$_2$SO$_4$, 9 μM FeSO$_4$, 0.5% casamino acids, 0.2% glucose, 1mM MgSO$_4$) (Appendix 1) [1, 9, 35]. Se biofilm cultures were prepared in the same way with the exceptions that TSB was used for the starting culture, the OD$_{600}$ reached 0.75, and the 1:100 dilution of the bacteria was subcultured into Phosphate Buffered Saline (PBS). Both Pa and Se biofilms were grown in 96-well plate confocal “friendly” chambers (MatTek Corp., Ashland, MA) and incubated for 18 hours in 37°C without shaking.

2.2 Effectiveness of Compounds at Inhibiting and Killing Biofilm Cultures

CLSM was used to investigate two different biofilm experiments: (i) the effect of each compound on inhibiting biofilm formation, and (ii) its ability to kill existing biofilms. All compounds used in this study were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. For the biofilm inhibition experiments, Pa or Se biofilms were grown in the presence of 10 μM of each compound for 18 hours at 37°C without shaking in appropriate media. For the biofilm killing experiments, biofilm cultures were grown without compound for the same time frame (18 hours). After washing once with PBS, the biofilm culture was further incubated for 24 hours at 37°C with various concentrations of compounds: 100, 200, and 300 μM for group 1, 300 or 500 μM.
μM for group 2, and 300 μM for groups 3 and 4. The flow chart summaries of these two experiments are shown in Appendix 2. Biofilm cultures were then washed twice with 100 μL of PBS and stained with a viability stain composed of SYTO 9 (green fluorescence) and propidium iodine (red fluorescence) (BacLight™; Invitrogen, Eugene, OR). The red dye, 20 mM propidium iodide, stained the dead bacteria, while the green dye, 3.34 mM SYTO 9, stained the nucleic acid of the live bacteria [36]. All plates were kept in the dark to protect them from light and incubated at room temperature for 15 minutes.

Finally, biofilm images were obtained using a Zeiss LSM510 META Confocal Microscope (Carl Zeiss, Inc., Germany). A 488 nm laser was used in conjunction with a Plan-Apochromat 100x/1.4 Oil DIC objective. Z-stacks were 8 bit and measured 127.3 μm x 127.3 μm x 16.0 μm in size. The filters used were 505-530 and 620-680 IR, as the excitation and emission wavelengths for the red fluorescence are 490 nm and 635 nm, respectively, and 480 nm and 500 nm for the green fluorescence [36]. The 3D images were generated using the “Projection” ability on LSM Image Browser software (v.1.4.0.121) (Carl Zeiss, Inc., Germany), and Live/Dead calculations were conducted on these images using MacBiophotonics ImageJ following the guidelines in the cell counting guide by Christine Labno at the University of Chicago Integrated Light Microscopy Core [37, 38].

2.3 Cytotoxicity

Cytotoxicity experiments were performed on only those compounds effective on Pa, Se, and Ab biofilms (group 1; compounds 3, 7, 8, and 11). The cell lines THP-1 and A549 were used to assess the toxicity of these compounds to the human body. THP-1 was maintained in 75cm² rectangular flasks with vented caps at 1-8x10^5 cells/mL in RPMI 1640 containing 25mM HEPES
and L-Glutamine (Thermo Scientific, HyClone Laboratories Inc., Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS) [26]. THP-1 cells were split every 2-3 days upon reaching a concentration of 5-8x10^5 cells/mL [26]. A549 cells were maintained in F12K containing L-Glutamine (ATCC, Manassas, VA) supplemented with 10% FBS and was allowed to grow until reaching approximately 80% confluence before splitting at 1:6 to 1:8 [27]. Cells were fed every 2-3 days [26, 27]. Cells were used within 30 passages for all experiments.

The cytotoxicity experiments were performed in 96-well plates. THP-1 cells were seeded at 80,000 cells/200 μL/well in RPMI 1640 supplemented with 10% FBS and incubated at 37°C, 5% CO₂ for 72 hours with 50 nM Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Co., St. Louis, MO) to stimulate differentiation of the cells into macrophages [29]. A549 cells were seeded at 30,000 cells/200 μL/well in F12K supplemented with 10% FBS and allowed to grow for 24 hours in the same conditions. The novel compounds were then added to differentiated THP-1 or A549 cells at 0-100 ng/μL after washing the wells with 200 μL of media [29]. DMSO was also added into the cells as a control. The 100 ng/μL concentration aligns to the highest concentration of 300 μM used in the biofilm culture killing experiments as shown in Table 2. The A549 and the differentiated THP-1 cells were also treated with 0-10 mM of Paraquat dichloride x-hydrate PESTANAL® (PQ) (Sigma-Aldrich Co., St. Louis, MO) as a positive control [31, 39]. PQ is an herbicide that is known to kill A549 and THP-1 [31, 39-41].

The cells were then incubated for an additional 48 hours with compound followed by a lactate dehydrogenase (LDH) assay, which was conducted according to the CytoTox-ONE Homogenous Membrane Integrity Assay Kit Instruction Manual [42]. LDH is released from dead cells, and the amount of LDH activity can be correlated to the percentage of dead cells. First, the 96-well plates were placed at room temperature for 30 minutes and 1% Triton-X-100 (Sigma-
Aldrich Co., St. Louis, MO) was added to the positive control DMSO-treated wells and incubated at room temperature for 15 minutes. Following this, a 1:1 ratio of media from treated cells (50 μL) and CytoTox-ONE™ Reagent (50 μL) was added together into a new 96-well plate. The plate was shaken for 30 seconds and then incubated at room temperature for 10 minutes. After adding 25 μl of Stop Solution, the plates were incubated again at room temperature for an additional 15 minutes. Finally, fluorescence was measured using an excitation wavelength of 560 nm and an emission wavelength of 590 nm following ten seconds of shaking [42]. All the samples were processed using a Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT). The results are shown as Relative Fluorescence Units (RFU), which was then calculated into a percentage of the cell death as compared to the DMSO-treated lysed cells.
Table 2. Concentrations of compound used, shown in ng/μL and μM.

<table>
<thead>
<tr>
<th>(ng/μL)</th>
<th>Compound (μM)</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>572.70</td>
</tr>
<tr>
<td>10</td>
<td>57.27</td>
</tr>
<tr>
<td>1</td>
<td>5.73</td>
</tr>
<tr>
<td>0.1</td>
<td>0.57</td>
</tr>
<tr>
<td>0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>
2.4 MIC

MIC experiments were performed under both aerobic and anaerobic conditions with the four compounds of group 1 (3, 7, 8, and 11), those compounds able to kill or inhibit Pa, Se, and Ab biofilms, in order to assess the effectiveness of each compound at killing planktonic living bacteria [25].

Both Pa and Se were grown in Mueller Hinton (MH) broth and incubated at 37°C for 18 hours with shaking at 250 RPM. Cultures were then diluted 1:100 into MH and supplemented MH, depending on the growth condition, as shown in Table 3, in the 96 well tissue culture flat-bottom plates (Becton Dickinson, Franklin Lakes, NJ). MH was supplemented with either 1% potassium nitrate (KNO₃) or 1% glucose, as Pa and Se cannot grow anaerobically in unsupplemented MH; Pa requires KNO₃, while Se requires either KNO₃ for cellular respiration or glucose for fermentation [8, 12]. The compounds were added in a two-fold dilution of concentrations from 50 μM down to 0.391 μM, in addition to DMSO, which was used as a control. The plates were then incubated at 37°C for 24 hours under either aerobic or anaerobic conditions (anaerobic chamber; Coy Laboratory Products Inc., Grass Lake, MI), after which their OD₆₀₀ was measured using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Sunnyville, CA) and used for the MIC calculations.
Table 3. Media used for Pa and Se in MIC experiments.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Media used in MIC experiments</th>
<th>Aerobic condition</th>
<th>Anaerobic condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MH</td>
<td></td>
<td>MH+1% KNO₃</td>
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<td></td>
<td>MH+1% KNO₃</td>
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<td>Se</td>
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<td></td>
<td>MH</td>
<td></td>
<td>MH+1% KNO₃</td>
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<td></td>
<td>MH+1% KNO₃</td>
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<tr>
<td></td>
<td>MH+1% Glucose</td>
<td></td>
<td>MH+1% Glucose</td>
</tr>
</tbody>
</table>
3. Results

3.1 Determining Biofilm Growth Conditions

Various media and incubation times were used to determine the conditions to grow either Pa or Se biofilms in aerobic conditions. The results show that the optimum media for Pa biofilms is M63 minimum media as compared to LB, 10% LB and 5% LB, in which majority of the bacteria were dead, as shown Fig. 5B-D. Moreover, 1X and 0.5X M63 minimum media in pH 7.0 and 7.3 were also investigated. Fig. 6D shows that Pa forms the healthiest and most packed biofilm in 0.5X M63 minimum media at pH 7.3. This media was further used to grow Pa biofilms at two different incubation time points: 18 and 20 hours. The results show that Pa forms a biofilm with a greater proportion of living cells at 18 hours compared to 20 hours when using stationary phase as a starting culture (Fig. 7D and E, respectively). Furthermore, the exponential phase culture formed a better Pa biofilm compared to both of the stationary phase cultures, as shown in Fig. 7F. Therefore, 18-hour exponential phase cultures were determined to be the best condition.

Se biofilm conditions for both inhibition and killing experiments were also assessed in various media: PBS, 10% TSB, 1% TSB and 0.2% TSB. The results show that the optimum media to grow a healthy and compact Se biofilm is PBS (Fig. 8 and 9). Although PBS lacks a carbon source, Se biofilms have been found to be able to grow in PBS for several days [14, 15, 43, 44].
**Fig. 5.** Pa biofilm growth in various media. Pa biofilms were grown in M63 minimum media, LB, 10% LB, and 5% LB at 37°C. The top view of the Pa biofilm is shown in A-D, while E-H is a sagittal view. M63 minimum media, LB, 10% LB, and 5% LB are shown in A/E, B/F, C/G, and D/H, respectively. Healthy, living cells are stained green, while dead cells are stained red. Biofilms are attached to the left surface as shown in the sagittal view, with a visible, thick line representing a compact biofilm. A healthy and packed Pa biofilm is shown in A/E, M63 minimum media.
**Fig. 6.** Pa biofilm growth in two concentrations of M63 minimum media at various pH. Pa biofilms were grown at 37°C in 0.5X or 1X M63 minimum media at either pH 7.0 or 7.3. The top view of the Pa biofilm is shown in A-D, while E-H is a sagittal view. A/E and B/F show the Pa biofilm grown in 1X M63 minimal media while C/G and D/H are 0.5X M63 minimum media. A/E and C/G display a Pa biofilm grown in media at pH 7.0 while B/F and D/H are at pH 7.3. All of the biofilms were grown at 37°C. Pa formed the healthiest and most packed biofilm in 0.5X M63 minimal media, pH 7.3, as shown in D/H.
**Fig. 7.** Pa biofilm growth in M63 minimal media. Pa biofilms were grown in M63 minimal media for either 20 or 24 hours at 37°C using either stationary or exponential phase bacterial cultures. A-C is the top view, while the sagittal view of the Pa biofilm is shown in D-F. Stationary phase bacteria was used as a starter to grow the Pa biofilm in A/D and B/E for 18- and 20-hour incubations, respectively, while exponential phase bacteria was grown for 18 hours, as shown in C/F. The optimal biofilm was grown using exponential phase culture.
**Fig. 8.** Se biofilm growth in various media. Se biofilms were grown in PBS, 10% TSB, 1% TSB, and 0.2% TSB at 37°C for either the inhibition or killing timeframe. A-H is the top view of the Se biofilm, while the sagittal view is shown in I-P. Se biofilms grown in PBS, 10% TSB, 1% TSB, and 0.2% TSB are shown in A/I and E/M, B/J and F/N, C/K and G/O, and D/L and H/P, respectively. The upper two rows display biofilm inhibition conditions; the Se biofilm was grown in various media for 18 hours. The lower two rows contain biofilm killing experiment conditions, wherein the Se biofilm was grown in various media for 18 hours, washed twice with PBS, and grown in the same media for an additional 24 hours. The most distinct Se biofilm is grown in PBS, as shown in A/I and E/M.
3.2 Effectiveness of Compounds on Inhibition and Killing of Pa and Se Biofilms

3.2.1 Compound Group 1

Compound group 1 is the compounds initially effective against Pa, Se, and Ab biofilms, compounds 3, 7, 8, and 11. The ability of these compounds to inhibit and kill biofilms was assessed for both Pa and Se. For inhibition of the biofilms, where 10 μM of each compound was co-cultured with the bacteria, the results show that compounds 3, 7, and 8 were effective at inhibiting both Pa and Se biofilm formation (Fig. 9 B-D and G-I, respectively), and most of the bacteria were dead. Compound 11 had no effect on the Pa biofilm, though it was able to inhibit the Se biofilm (Fig. 9 E/O and J/T, respectively.)

Among this group, only compound 3 was shown to significantly kill the Pa biofilm. Effective killing is defined as >50% dead bacteria, and compound 3 killed approximately 80-90% of the Pa biofilm as compared to the control (Fig. 10). Compounds 7, 8, and 11 showed a slight effect at killing the Pa biofilm; killing was approximately 20% more than shown in the control (Fig. 11-13). Even though compounds 7, 8, and 11 did not show significant effectiveness in killing an existing Pa biofilm, all of these compounds seemed to cause dispersion of the Pa biofilm, creating a loose and/or thinner biofilm. Both compounds 8 and 11 caused a thinner Pa biofilm at the highest concentration used in these experiments (300 μM) while compound 7 was more effective at the lowest concentration (100 μM) (Fig. 12H, 13H, and 11F, respectively). Moreover, compounds 7 and 8 also caused the Pa bacteria to change in morphology from a rod shape to an elongated, filamentous-like shape at all concentrations treated, while compound 11 showed this effect at higher concentrations (200 and 300 μM) (Fig. 11B-D, 12B-D, and 13C-D, respectively). However, none of compounds in this group were effective at either killing,
dispersing, or changing the morphology of the Se biofilm compared to the control, even at the highest concentration of 300 μM (Fig. 14-17).
Fig. 9. Compound group 1 inhibition of Pa and Se biofilm formation. Each compound was co-cultured with bacteria to determine the effectiveness of the compound at inhibiting biofilm formation. The images of the Pa biofilm in the presence of each compound are shown on the upper figure, while the lower figure shows the Se biofilm. A-J is the top view of the biofilm, and the sagittal view is shown in K-T. The control Pa biofilm is shown in A/K, while the control biofilm for Se is shown in F/P. Biofilms with compound 3, 7, 8, and 11 are shown in B/L and G/Q, C/M and H/R, D/N and I/S, and E/O and J/T, respectively. Compounds 3, 7, and 8 inhibited both Pa and Se biofilm formation, while compound 11 was only effective at inhibiting Se biofilm formation.
**Fig. 10.** Effectiveness of compound 3 at killing Pa biofilms. Pa biofilms were treated with compound 3 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Pa biofilm is shown in A/E, while Pa biofilm treated with 100 μM, 200 μM, and 300 μM of compound 3 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 3-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 3 was effective at killing Pa biofilms even at 100 μM.
Fig. 11. Effectiveness of compound 7 at killing Pa biofilms. Pa biofilms were treated with compound 7 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Pa biofilm is shown in A/E, while Pa biofilm treated with 100 μM, 200 μM, and 300 μM of compound 7 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 7-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 7 was not significantly effective at killing Pa biofilms.
Fig. 12. Effectiveness of compound 8 at killing Pa biofilms. Pa biofilms were treated with compound 8 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Pa biofilm is shown in A/E, while Pa biofilm treated with 100 μM, 200 μM, and 300 μM of compound 8 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 8-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 8 was not significantly effective at killing Pa biofilms.
**Fig. 13.** Effectiveness of compound 11 at killing Pa biofilms. Pa biofilms were treated with compound 11 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Pa biofilm is shown in A/E, while Pa biofilm treated with 100 μM, 200 μM, and 300 μM of compound 11 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 11-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 11 was not significantly effective at killing Pa biofilms.
**Fig. 14.** Effectiveness of compound 3 at killing Se biofilms. Se biofilms were treated with compound 3 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Se biofilm is shown in A/E, while Se biofilm treated with 100 μM, 200 μM, and 300 μM of compound 3 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 3-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 3 was not effective at killing Se biofilms.
**Fig. 15.** Effectiveness of compound 7 at killing Se biofilms. Se biofilms were treated with compound 7 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Se biofilm is shown in A/E, while Se biofilm treated with 100 μM, 200 μM, and 300 μM of compound 7 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 7-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 7 was not effective at killing Se biofilms.
**Fig. 16.** Effectiveness of compound 8 at killing Se biofilms. Se biofilms were treated with compound 8 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Se biofilm is shown in A/E, while Se biofilm treated with 100 μM, 200 μM, and 300 μM of compound 8 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 8-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 8 was not effective at killing Se biofilms.
**Fig. 17.** Effectiveness of compound 11 at killing Se biofilms. Se biofilms were treated with compound 11 at the various concentrations indicated. In the upper figure, A-D is the top view of the biofilm, while the sagittal view is shown in E-H. The control Se biofilm is shown in A/E, while Se biofilm treated with 100 μM, 200 μM, and 300 μM of compound 11 is shown in B/F, C/G, and D/H, respectively. The lower figure depicts the live/dead ratios of the compound 11-treated biofilms. The blue and red bars represent the percentages of living and dead bacteria, respectively. Compound 11 was not effective at killing Se biofilms.
3.2.2 Compound Group 2

Compound group 2 contains the compounds that were effective against Pa and Ab biofilms, compounds 1, 2, 9, 10, 12, and 13. However, the effectiveness of these compounds was determined only on Pa biofilms in this study. The inhibition experiment was performed with 10 μM compound concentration, the same as was used for group 1. The results show that compounds 1, 2, and 13 were effective at inhibiting Pa biofilm formation (Fig. 18 B/I, C/J, and G/N, respectively). Compound 9 caused a loose/unpacked Pa biofilm, while a slightly thinner Pa biofilm occurred in the presence of compound 12 (Fig. 18 D/K and G/N, respectively). Compound 10 did not show any effectiveness in inhibiting Pa biofilm formation, as shown in Fig. 18 E/L.

For Pa biofilm killing experiments, compounds 2, 10, and 12 were added to the biofilms at a concentration of 300 μM, while 500 μM of compounds 1, 9, and 13 were used. Even at the 500 μM concentration, none of these compounds were effective at killing the Pa biofilm, where effective killing is defined as >50% dead bacteria (Fig. 19). However, compound 9 caused a change in morphology from the rod shape to an elongated, filamentous-like shape, while compound 10 appeared to cause dispersion of the biofilm, as shown by a slightly thinner biofilm (Fig. 19 D/K and E/L, respectively).
**Fig. 18.** Compound group 2 inhibition of Pa biofilm formation. Pa was co-cultured with 10 μM of each compound. A-G represents the top view of the Pa biofilm, while the sagittal view is shown in H-N. The control Pa biofilm is shown in A/H. Biofilms in the presence of compound 1, 2, 9, 10, 12, or 13 are shown in B/I, C/J, D/K, E/L, F/M, and G/N, respectively. Compounds 1, 2, and 13 were effective at inhibiting the Pa biofilm.
**Fig. 19.** Compound group 2 killing of Pa biofilms. The biofilm was grown for 18 hours in M63 minimum media and then treated with each compound in this group. A-G is the top view of the Pa biofilm, while the sagittal view is shown in H-N. The control Pa biofilm is shown in A/H, while Pa biofilm treated with compound 1, 2, 9, 10, 12, or 13 is shown in B/I, C/J, D/K, E/L, F/M, and G/N, respectively. Compounds 1, 9, and 13 were added at a concentration of 500 μM, while compounds 2, 10, and 12 were added at 300 μM. No compounds were effective at killing the Pa biofilms, but compound 9 caused a change in morphology, and compound 10 caused dispersion of the biofilm.
3.2.3 **Compound Group 3**

Compound group 3 consists of compounds 14-16, those compounds that were only effective against Pa biofilms. Inhibition experiments were conducted in the presence of 10 μM of each compound. The results show that compound 14 was effective at inhibiting Pa biofilm formation, while compounds 15 and 16 were not (Fig. 20 B/J, C/K, and D/L, respectively).

Killing experiments on Pa biofilms were performed with a concentration of 300 μM of each compound. None of the compounds in this group showed a significant killing ability on Pa biofilms (Fig. 20 F/N, G/O and H/P). However, compound 14 caused slight killing and morphology change, as well as biofilm dispersion as evidenced by the thinness of the compound 14-treated biofilm compared to the control Pa biofilm (Fig. 20 F/N). Compound 15 also showed an impact on Pa biofilm dispersion (Fig. 21 O).
**Fig. 20.** Compound group 3 inhibition and killing of Pa biofilms. Pa bacteria was co-cultured with 10 μM of each compound in group 3 for inhibition experiments, as shown in the upper figure, while for killing experiments Pa biofilms were treated with 300 μM of compounds, as shown in the lower figure. A-H represents the top view of the Pa biofilm, while the sagittal view is shown in I-P. The control Pa biofilm is shown in A/I and E/M for inhibition and killing experiments, respectively. Biofilm formation in the presence of compound 14, 15 or 16 are shown in B/J, C/K and D/L, respectively, and biofilms treated with compound 14, 15, or 16 are shown in F/N, G/O, and H/P, respectively. Only compound 14 was effective at inhibiting the Pa biofilm, while compounds 15 and 16 were not. No compounds were effective at killing the Pa biofilms, although compounds 14 and 15 caused biofilm dispersion.
3.2.4 Compound Group 4

The compounds in group 4, compounds Se1-Se16, were only effective against Se biofilms. The same concentration of 10 μM was used to determine the effectiveness of these compounds on inhibiting the Se biofilm. The results are shown in two groups: the first group is composed of those compounds that were effective at inhibiting Se biofilm formation, which includes compounds Se3, Se4, Se9, and Se13-Se16 (Fig. 21). Compounds Se4, Se9, Se13, and Se14 also killed the majority of bacteria (Fig. 21 C/K, D/L, E/M, and F/N, respectively). The second group is the compounds that were ineffective, which is compounds Se1, Se2, Se5-Se8, and Se10-Se12, although compounds Se6 and Se12 did cause a slight thinning of the Se biofilm (Fig. 22 E/O and J/T, respectively).

For the Se biofilm killing experiments, all of the compounds were used at 300 μM. The results are again shown in two groups of compounds. The first group, compounds effective at killing the Se biofilm where effective killing is defined as >50% dead bacteria, includes compounds Se3, Se4, Se10, and Se12-Se15 (Fig. 23). The second group, which was ineffective at killing the Se biofilm, is composed of compounds Se1, Se2, Se5, Se6-Se9, Se11 and Se16 (Fig. 24). None of these compounds caused any dispersion of the Se biofilm.
**Fig. 21.** Group 4 compounds effective at inhibiting Se biofilm formation. Se was co-cultured with 10 μM of each compound in group 4. A-H represents the top view of the Se biofilm, while the sagittal view is shown in I-P. The control Se biofilm is shown in A/I. Se biofilm formation in the presence of 10 μM of compound Se3, Se4, Se9, Se13, Se14, Se15, or Se16 are shown in B/J, C/K, D/L, E/M, F/N, G/O, and H/P, respectively. All of these compounds were effective at inhibiting Se biofilm formation, and compounds Se4, Se9, Se13, and Se14 also killed the majority of bacteria.
**Fig. 22.** Group 4 compounds ineffective at inhibiting Se biofilm formation. Se bacteria was co-cultured with 10 μM of each compound in group 4. A-J represents the top view of the Se biofilm, while the sagittal view is shown in K-T. The control Se biofilm is shown in A/K. Se biofilm formation in the presence of 10 μM of compound Se1, Se2, Se5, Se6, Se7, Se8, Se10, Se11, or Se12 are shown in B/L, C/M, D/N, E/O, F/P, G/Q, H/R, I/S, and J/T, respectively. None of these compounds were effective at inhibiting Se biofilm formation, but compounds Se6 and Se12 did cause a slight thinning of the Se biofilm.
**Fig. 23.** Group 4 compounds effective at killing Se biofilms. Se biofilms were treated with 300 μM of group 4 compounds. A-H represents the top view of the Se biofilm, while the sagittal view is shown in I-P. The control Se biofilm is shown in A/I. Se biofilms treated with 300 μM of compound Se3, Se4, Se10, Se12, Se13, Se14, or Se15 are shown in B/J, C/K, D/L, E/M, F/N, G/O, and H/P, respectively. These compounds were all effective at killing the Se biofilms, where effective killing is defined as >50% dead bacteria.
**Fig. 24.** Group 4 compounds ineffective at killing Se biofilms. Se biofilms were treated with 300 μM of group 4 compounds. A-J represents the top view of the Se biofilm, while the sagittal view is shown in K-T. The control Se biofilm is shown in A/K. Se biofilms treated with 300 μM of compound Se1, Se2, Se5, Se6, Se7, Se8, Se9, Se11, or Se16 are shown in B/L, C/M, D/N, E/O, F/P, G/Q, H/R, I/S, and J/T, respectively. These compounds were not effective at killing existing Se biofilms.
3.3 Cytotoxicity

3.3.1 Determining THP-1 differentiation conditions

THP-1 differentiation into mature macrophages is a gradual process, and various experiments in literature use time frames from 24 hours to 5 days in the presence of PMA ranging from 10-400 ng/mL [45, 46]. Therefore, the THP-1 differentiation experiment was performed using either 50 nM or 100 nM of PMA with various incubation times: 24, 48, 72, and 96 hours. We observed that the cells began to differentiate at 24 hours and were fully differentiated by 48 hours. No significant difference was observed between the 48 hour, 72 hour, and 96 hour differentiated cells, nor between 50 nM and 100 mM when compared at the same time points. However, higher levels of PMA are known to upregulate genes, including TNF-α, IL-1β, IL-8, and MIP-1β; as this could affect the cytotoxicity experiment, 50 nM was used with a 72-hour incubation [45, 46].

3.3.2 Effects of DMSO on differentiated THP-1 and A549 cells

DMSO is known to have toxic properties to cells above as little as 2%, although the degree of effect depends on the cell line [47, 48]. As discussed previously, all of the compounds used in this study were dissolved in DMSO. In the cytotoxicity experiment, the concentration of DMSO added varied from compound to compound, ranging from 3-6%. Therefore, it was necessary to determine the toxicity of DMSO in order to exclude its effects from the toxicity of the compounds used in this study. The results show that 6% DMSO has about 20-30% toxicity on both A549 and differentiated THP-1 cells (Fig. 25).

Additionally, we observed that both differentiated THP-1 and A549 cells treated with 6% DMSO for 48 hours had smaller, more condensed cells compared to the untreated control cells,
and there appeared to be fewer total cells. Determination of LDH activity on lysed DMSO-treated cells supported the observation that there were fewer total cells in the 6% DMSO-treated cells compared to cells treated with 0.6% DMSO or less (Fig. 26). The amount of compound dissolved in DMSO added to the cells in this study ranged from approximately 0-6%. Therefore, 6% DMSO-treated cells were also included in the experiment to calculate the maximum LDH activity in order to eliminate the effects of DMSO from the effects of the compounds.
**Fig. 25.** Toxicity of DMSO on differentiated THP-1 and A549 cells. The toxicity of DMSO on both cell lines was determined in order to exclude its effect from the compounds used in this study. The blue line represents differentiated THP-1 cells, while the red line represents A549 cells. There is some toxicity by 6% DMSO on both cell lines, but not at 0.6% DMSO.

**Fig. 26.** LDH activity of lysed DMSO-treated cells. Lysed DMSO-treated cells were used to study LDH activity to indicate the amount of cells in each condition. The blue and red lines represent differentiated THP-1 and A549 cells, respectively. The total amount of LDH activity of both cells decreases with 6% DMSO; this indicates there are fewer total cells in this condition.
3.3.3 Cytotoxicity of group 1 compounds on differentiated THP-1 and A549 cells

Compounds 3, 7, 8, and 11, the four compounds of group 1, which were shown to inhibit or kill Pa, Se, and Ab biofilms, were tested to determine their cytotoxicity. The concentrations they were assessed at ranged from 0 μM to 260, 290, 300, and 570 μM for compounds 7, 11, 8, and 3, respectively. None of the compounds were shown to be significantly toxic (killed > 50%) to differentiated THP-1 or A549 cells at the highest concentrations used in this study (Fig. 27 and 28, respectively). Throughout four repetitions, the toxicity of compound 3 was higher at 570 μM than at 57 μM, which contain 6% and 0.6% DMSO, respectively, as the compound is dissolved in DMSO. This could be due to an effect similar to that of DMSO, where the number of cells is also decreased due to a property of the compound, and could explain why the number of cells at 570 μM appears to be negative compared to the count in the media only well. The same cytotoxicity experiment was also performed with PQ-treated cells as a positive control. PQ showed a strong cytotoxic effect on both differentiated THP-1 and A549 cells at 10 mM (Fig. 29 A and B, respectively) [31, 39].
Fig. 27. Cytotoxicity of group 1 compounds on differentiated THP-1 cells. The cytotoxicity of each compound was determined by an LDH activity assay. Various concentrations of compounds were used to treat the cells, and the cytotoxicity was calculated compared to the control. Compounds 3, 7, 8, and 11 are shown in A, B, C, and D, respectively. None of the compounds were significantly cytotoxic to this cell line.
**Fig. 28.** Cytotoxicity of group 1 compounds on A549 cells. An LDH activity assay was used to determine the cytotoxicity of these compounds on A549 cells. Various concentrations of compound were used in this experiment. The toxicity was calculated and compared to the control. Compounds 3, 7, 8, and 11 are shown in A, B, C, and D, respectively. None of the compounds were significantly cytotoxic to A549 cells.
Fig. 29. Cytotoxicity of PQ on differentiated THP-1 and A549 cells. The LDH activity assay was also performed on both cells in the presence of various concentrations of PQ as a positive control. PQ was cytotoxic at 1 mM and 10 mM in both differentiated THP-1 and A549 cells as shown in A and B, respectively.
3.4 MIC

The compounds of group 1, the same group as used in the cytotoxicity experiments, were also tested to determine the minimum concentration of compound required to inhibit the planktonic bacteria in both aerobic and anaerobic conditions [25].

The MIC results in Table 4 and Fig. 30 show that for Pa planktonic culture compound 7 was the most effective. It required only 4-5 μM to inhibit planktonic Pa from growing in all conditions. Compound 8 was less effective, requiring 7-21 μM, and compounds 3 and 11 were much less effective, requiring at least 25 μM. Significant differences in MIC between aerobic and anaerobic conditions in MH supplemented with KNO3 were achieved by compounds 8 and 11, which required a lower concentration in anaerobic conditions compared to aerobic conditions. No significant difference was found between aerobic and anaerobic conditions for compounds 3 and 7 in the same media. As far as differences in aerobic conditions between MH and MH supplemented with KNO3, compounds 3 and 8 were significantly different; MH supplemented with KNO3 was slightly sensitive to compound 3 and resistant to compound 8. From this reasoning, the results also show that Pa is more sensitive to compound 3 in anaerobic conditions than in aerobic conditions without KNO3.

The Se planktonic culture was most sensitive to compound 8; less than 1 μM was necessary to inhibit growth under all conditions. Compounds 7 and 11 were also effective, requiring from 4-10 μM depending on the media and condition used. Compound 3 was less effective and required 10-25 μM. Significant differences between aerobic and anaerobic conditions were caused by compounds 3 and 7, which were slightly lower in MIC for anaerobic conditions in MH supplemented with 1% KNO3, but there were no significant differences in
MIC for these compounds in MH supplemented with 1% glucose. There was no significant difference for compounds 8 and 11 between aerobic and anaerobic conditions for either media. No combination effect was determined in the presence of compounds 7, 8, and 11 with either glucose or KNO₃ for both aerobic and anaerobic conditions. However, compound 3 together with glucose in aerobic conditions resulted in a lower MIC compared to MH with or without KNO₃.

Among this group of compounds, compounds 7 and 8 were effective at inhibiting both Pa and Se planktonic cultures in both aerobic and anaerobic conditions, requiring less than 25 μM in all media, and for most conditions less than 10 μM. Compounds 3 and 11, while effective for Se planktonic cultures, required higher concentrations for planktonic Pa.
**Table 4.** MIC for planktonic Pa and Se bacteria in aerobic and anaerobic conditions.

<table>
<thead>
<tr>
<th></th>
<th>Pa</th>
<th>Se</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH</td>
<td>MH with 1% KNO₃</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50*</td>
<td>33.33±8.33</td>
</tr>
<tr>
<td>7</td>
<td>4.17±1.04</td>
<td>5.21±1.04</td>
</tr>
<tr>
<td>8</td>
<td>10.42±2.08</td>
<td>20.83±4.17</td>
</tr>
<tr>
<td>11</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

* The MIC was reported in μM
**Fig. 30.** MIC of group 1 compounds on planktonic Pa and Se bacteria. MIC experiments were conducted using 0-50 μM of each compound on planktonic Pa and Se cultures. MIC of group 1 compounds on planktonic Pa and Se are shown in the upper and lower figures, respectively.

† The MIC was determined to be more than 50 μM
4. Discussion

4.1 Effectiveness of compounds on Pa and Se bacteria and biofilms

In this study we confirmed the effectiveness of compounds from a 40,000-compound high-throughput screening in either inhibiting or killing Pa and Se biofilms. The optimum media used to grow each biofilm was determined, and the Pa and Se biofilms were grown in modified M63 minimum media and PBS, respectively. The compounds were classified into four groups based on the bacterial biofilms they were effective against, as shown in Fig. 1-4. Dispersion of the biofilm and changes in bacterial morphology were also assessed, but these abilities were not as important as overall effectiveness of the compound at either inhibiting the Pa or Se biofilm from forming or killing an existing biofilm.

The first group of compounds, which was effective against Pa, Se and Ab biofilms, was investigated only on Pa and Se biofilms. The results show that all compounds inhibited Se biofilm formation, but none killed or caused dispersion of the biofilm. Only compounds 3, 7, and 8 were able to inhibit Pa biofilm formation. Pa biofilm killing was only achieved with compound 3, though a dispersed Pa biofilm was found in biofilms treated with compounds 7, 8, or 11. The effectiveness of the compounds is summarized in Table 5. Furthermore, treating the Pa biofilm with certain compounds (compounds 7, 8, or 11) caused a change in cell morphology. Morphology change can be a result of the environment changing, the amount of oxygen, and sub-lethal antibiotic treatment [49-52]. This change was only shown in Pa, not Se, biofilms. This may be because of the difference in cell membranes between Gram positive and Gram negative bacteria; for example, (-)-Epigallocatechin-3-gallate causes different morphological changes to Pa, a Gram-negative bacteria, than Streptococcus mutans, which is Gram-positive [53].
Additionally, biofilm dispersion occurred in biofilms treated with compounds 7, 8, and 11. These compounds could be used in combination with a compound that kills Pa biofilms, such as compound 3, to lower the concentration necessary to kill the biofilm; the biofilm structure acts as a barrier for nutrients, oxygen, and antibiotics, and disrupting it could make it less resistant [49].

All of these results confirmed the high-throughput screening results, which indicated that these compounds were effective against growth, biofilm metabolism, or biofilm formation in Pa and Se biofilms. Compounds 7, 8, and 11 are in the quinolone class, while compound 3 is an isothiozolone. Quinolone is a family of synthetic broad-spectrum antibiotics that can be effective in both Gram positive and Gram negative bacteria, while isothiolone is an antimicrobial used to control bacteria, fungi, and algae in water cooling systems, fuel storage tanks, and personal products such as shampoo and hair products [20, 54]. In order to determine these compounds’ suitability for medical purposes, the cytotoxicity was investigated. None of these compounds were shown to have significant cytotoxicity on A549 or differentiated THP-1 cells. Even though DMSO showed some toxicity to both cells used in this study, the U.S. Food and Drug Administration has approved a 50% solution of DMSO to treat interstitial cystitis, while concentrations of DMSO in these experiments did not exceed 6% [55, 56].

In addition, MIC experiments were performed with these group 1 compounds to examine the effectiveness of compounds in both aerobic and anaerobic conditions. The results show that compound 7 inhibits both Pa and Se planktonic culture growth at a low concentration (10 μM or lower) in all conditions and media used in this study. Therefore, this compound prevented both bacteria from forming a biofilm in the biofilm inhibition experiments, and it also inhibited planktonic growth in both aerobic and anaerobic conditions. Compound 8 was similarly effective, but was more effective at inhibiting the growth of Se planktonic culture than Pa in both
aerobic and anaerobic conditions. Conversely, compound 3, which was effective at inhibiting both Pa and Se biofilm formation, and also killed existing Pa biofilms, showed a lower effectiveness in inhibiting bacteria planktonic grown in all media and conditions used in this study (higher than 10 μM). Finally, even though compound 11 was very effective in inhibiting Se planktonic growth and biofilm formation, it was not effective at inhibiting Pa biofilm formation, and had the highest MIC among this group of compounds to inhibit growth of Pa planktonic culture.

Compound group 2, which was effective on both Pa and Ab biofilms, was only investigated in this study on Pa biofilms. The effectiveness of these compounds is summarized in Table 6. The results show that compounds 1, 2, and 13, a benzimidazole, furan, and quinolone, respectively, showed inhibition of the Pa biofilm, while compound 9, a quinolone, caused a morphology change and compound 10, an aryl heterocycle, caused some dispersion of the biofilm. No compounds in this group were effective at killing the Pa biofilms; this may be because of the difference in media and material surface used between the high-throughput screening and CLSM experiments.

The experiments conducted on compound group 3, those compounds effective only on Pa biofilms, revealed that only compound 14 effectively inhibited the Pa biofilm. The effectiveness of the group 3 compounds is summarized in Table 7. However, compounds 14 and 15, which are a quinolone and mucochloric acid, respectively, were effective at dispersing the biofilm. Mucochloric acid is a highly functionalized molecule and can be used for several synthetic purposes; and some of these synthetic compounds are antimicrobial [57, 58]. For example, chemical databases of different mucochloric acid syntheses have been created and screened against multiresistant \textit{Staphylococcus aureus} with successful inhibition of planktonic bacteria as
determined by MIC results [59]. Alternatively, compound 16 was not effective in any experiment done in this study.

Compound 4, the last group of compounds examined in this study, was only effective on Se biofilms. The effectiveness of the compounds is summarized in Table 8. We divided inhibition and killing results into four groups: first, those compounds effective at both killing and inhibition; these are compounds Se3, Se4, Se13, Se14, and Se15. These compounds are classified as a urea, quaternary ammonium salt, dye, pyrrole, and diarylsulfonamide, respectively. Ureas are used as fertilizers; however, they can also inhibit bacterial cell wall synthesis and denature proteins [23, 24]. Quaternary ammonium salts are commonly used as disinfectants but also possess antimicrobial activity [60]. Dyes are a broad group of chemicals, most notable for their color staining. Pyrroles are heterocyclic aromatic organic compounds used as an additive to cigarettes [61]. Sulfonamides, commonly called sulfa drugs, are antimicrobials that prevent folate synthesis by inhibiting dihydropteroate synthetase [21, 22].

Second, those compounds that only inhibited the Se biofilm, but did not show killing: these are compounds Se9 and Se16, a quinolone and an 8-hydroxy-quiniline, respectively. 8-hydroxy-quinilines are used as liquid bandages as well as antiseptics and disinfectants, and they also contain pesticide properties [62, 63]. Third, there were two compounds in this group that were only able to kill the Se biofilm: Se10 and Se12, which are classified as a urea and hydrozone, respectively. Hydrozones are used in medical biotechnology to couple drugs to targeted antibodies [64]. Lastly, compounds Se1, Se2, Se5, Se6, Se7, Se8 and Se11 did not show any effect in either biofilm formation or killing.
In summary, most of the compounds effective at inhibiting and/or killing Pa biofilms are classified as quinolones, and while some of the compounds inhibiting and/or killing Se biofilms are quinolones, there is a wide variety of chemicals. Of all of the compounds analyzed in this study, compound 3 shows the most potential for future use against both Pa and Se biofilms, as it was able to inhibit Se biofilm formation, inhibit Pa biofilm formation, and kill existing Pa biofilms. Compounds 7 and 8 are also very promising, as they were able to inhibit both Pa and Se biofilm formation and were the most effective in the MIC experiments. Finally, Se3, Se4, Se13, Se14, and Se15 are the most favorable against Se infections, as they were the only compounds able to both inhibit Se biofilm formation and kill existing Se biofilms. While all of the compounds that were effective at inhibiting or killing Pa and Se biofilms should be further tested, these eight compounds should be the first investigated.
**Table 5.** Summary of compound group 1 effectiveness on Pa and Se biofilms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
<th>Killing</th>
<th>Dispersion</th>
<th>Morphology change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pa</td>
<td>Se</td>
<td>Pa</td>
<td>Se</td>
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<tr>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>O</td>
</tr>
<tr>
<td>7</td>
<td>X</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>8</td>
<td>X</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>11</td>
<td>O</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

**Table 6.** Summary of compound group 2 effectiveness on Pa biofilms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
<th>Killing</th>
<th>Dispersion</th>
<th>Morphology change</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pa</td>
<td>Se</td>
<td>Pa</td>
<td>Se</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>9</td>
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<td>10</td>
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<td>X</td>
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</tr>
<tr>
<td>12</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>13</td>
<td>X</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

**Table 7.** Summary of compound group 3 effectiveness on Pa biofilms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
<th>Killing</th>
<th>Dispersion</th>
<th>Morphology change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pa</td>
<td>Se</td>
<td>Pa</td>
<td>Se</td>
</tr>
<tr>
<td>14</td>
<td>X</td>
<td>O</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>15</td>
<td>O</td>
<td>O</td>
<td>X</td>
<td>O</td>
</tr>
<tr>
<td>16</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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</table>
Table 8. Summary of compound group 4 effectiveness on Se biofilms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
<th>Killing</th>
<th>Dispersion</th>
<th>Morphology change</th>
</tr>
</thead>
<tbody>
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<td>Se1</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se2</td>
<td>O</td>
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<td>O</td>
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<tr>
<td>Se3</td>
<td>X</td>
<td>X</td>
<td>O</td>
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<td>Se4</td>
<td>X</td>
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<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se5</td>
<td>O</td>
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<td>O</td>
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</tr>
<tr>
<td>Se6</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se7</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se8</td>
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<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se9</td>
<td>X</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se10</td>
<td>O</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se11</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se12</td>
<td>O</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se13</td>
<td>X</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se14</td>
<td>X</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se15</td>
<td>X</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Se16</td>
<td>X</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>
4.2 Future directions

All the biofilm killing and inhibition experiments performed in this study were grown on glass surfaces. However, both Pa and Se biofilms are naturally found on many different materials. Further studies should be conducted to determine the effectiveness of these compounds at inhibiting and/or killing biofilms on other surfaces, such as medical tubing, bone, implants, or fuel tank. Moreover, the effectiveness of all of these compounds could be determined in different growth conditions, such as flow cell biofilms or anaerobic biofilms, as both Pa and Se are able to grow in such conditions.

While this study focused on the capacity of each individual compound to kill biofilms or inhibit biofilm formation, those compounds that caused dispersion or change in morphology may make the biofilms more sensitive to treatment with other compounds. Further studies could determine the effectiveness of treating biofilms with combinations of these compounds, or combining a compound treatment with an existing antimicrobial.

In addition, those compounds that show an effectiveness on both Pa and Se biofilms should be investigated in co-biofilm cultures of Pa and Se, as well as on other bacteria. Patients can be afflicted with multiple strains of bacteria; for example, Pa and *Staphylococcus aureus* are found to co-exist in the airways of CF patients [65]. These compounds should be tested on a wide range of different biofilm-forming bacteria to determine their ability to inhibit or kill all of these biofilms.

Using all of these additional studies, we should be able to discover compounds effective against wide varieties of bacteria, both single culture and co-cultures; this will benefit both prevention and treatment of these afflications.
References


42. CytoTox-ONE Homogeneous Membrane Integrity Assay, 2009, Promega Corporation.


Appendix 1. M63 minimal media as used in Pa biofilm CLSM experiments.

5X M63 (500 mL)

34 g KH$_2$PO$_4$
5 g (NH$_4$)$_2$SO$_4$
0.0125 g FeSO$_4$

Add deionized water for final volume of 500 mL (approximately 300 mL to start)

Adjust pH to 7.3 using 1M KOH or KOH pellets

Autoclave or filter

0.5X M63 (50 mL)

5 mL 5x M63
5 mL 5% casamino acids
500 μL 20% glucose
39.5 mL sterile distilled water
50 μL MgSO$_4$

Final pH should be approximately 7.5
Appendix 2. Timelines of CLSM, cytotoxicity, and MIC experiments.

1. Illustration of timeframe for compound inhibition CLSM experiment.

- Bacteria from -80°C grown on LB plate
  - (20 hours) 37°C
- Exponential phase bacteria subcultured
  - (2.5 hours) 37°C, 250 RPM
- Bacteria seeded with compound on 96 well plates
  - (18 hours) 37°C
- Live/Dead fluorescent dye added
  - (15 min) 22°C, protect from light
- Images taken using CLSM

2. Illustration of timeframe for compound killing CLSM experiment.

- Bacteria from -80°C grown on LB plate
  - (20 hours) 37°C
- Exponential phase bacteria subcultured
  - (2.5 hours) 37°C, 250 RPM
- Bacteria seeded on 96 well plates
  - (18 hours) 37°C
- Washed twice with PBS, then added compound
  - (24 hours) 37°C
- Live/Dead fluorescent dye added
  - (15 min) 22°C, protect from light
- Images taken using CLSM
3. Illustration of timeframe for cytotoxicity experiments for differentiated THP-1 cells.

THP-1 seeded with PMA
(72 hours) → 37°C
Washed once with cell media, then added compound
(48 hours) → 37°C
LDH activity assay

4. Illustration of timeframe for cytotoxicity experiments for A549 cells.

A549 seeded
(24 hours) → 37°C
Washed once with cell media, then added compound
(48 hours) → 37°C
LDH activity assay

5. Illustration of timeframe for MIC testing.

Bacteria from -80°C grown on LB plate
(20 hours) → 37°C
MH broth inoculation
(24 hours) → 37°C, 250 RPM
Bacteria seeded with compound on 96 well plates
(24 hours) → 37°C (aerobic/anaerobic)
OD\textsubscript{600} measurement