SYNERGISTIC EFFECTS OF MONOCULTURE BIOFILM DISPERSION AND ANTIBIOTIC TREATMENT

by

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DEDICATION

I dedicate this work to every determined little girl with an unanswered question.

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LIST OF ABBREVIATIONS

Abbreviation	Description
CAUTI	Catheter Associated Urinary Tract Infection
c-di-GMP	Cyclic Dimeric Guanosine Monophosphate
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
eDNA	Extracellular DNA
ECM	Extracellular Matrix
GASP	Growth Advantage in Stationary Phase
LB	Luria-Bertani
mAUM	modified Artificial Urine Media
MIC	Minimum Inhibitory Concentration
N	Nitrofurantoin
OD600	Optical Density at 600 nm
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
T	Tobramycin

ABSTRACT

Bacterial biofilms have been identified as the causative agent of many infections, including catheter associated urinary tract infections (CAUTIs). While in a biofilm state, bacterial cells are less susceptible to antibiotic therapy. However, once released from a biofilm, antimicrobial susceptibility returns. In an aim to increase antibiotic efficacy of CAUTI treatment, uropathogenic *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* F11 biofilms were cultured on silicone disks, modeling the surface of urinary catheters. These biofilms were then treated with two potential dispersal agents and challenged with antibiotics. Antibiotics selected were tobramycin and nitrofurantoin due to their clinical use against urinary tract infections. Both potential dispersal agents showed the most effect when used to treat *P. aeruginosa* biofilms cultured in Luria-Bertani broth (LB). There was no increased dispersal or improved antibiotic efficacy after treatment of modified artificial urine media (mAUM)-cultured *P. aeruginosa* biofilms.

I. INTRODUCTION

Bacteria can exist as suspended (free floating, individual cells) or as surface-adherent sessile biofilm communities. While biofilms can occur on air-liquid interfaces (1, 2), where they are sometimes referred to as microbial mats or pellicles, on medical devices they occur at solid-liquid interfaces (3). Biofilm formation affords protection from environmental stressors such as antimicrobial agents (4, 5) and desiccation (6). Catheter associated urinary tract infections (CAUTIs) have been noted as the most common healthcare associated infections. Uropathogenic *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are recorded as the most prominent cause (7, 8). Biofilm formation occurs in five stages: reversible attachment, irreversible attachment, maturation I and maturation II and dispersion (9, 10). Bacteria in biofilms undergo phenotypic adaptations to include altered gene expression (11, 12) and increased quorum signaling (13, 14). Two quorum signaling systems have been identified in *Pseudomonas aeruginosa*: *las* and *rhl* (15, 16). These systems have been identified in production of virulence factors such as rhamnolipids (17, 18) and are also involved in twitching motility, a phenotype conditionally involved in *P. aeruginosa* biofilm establishment (14, 19).

Biofilm Attachment

Biofilm formation begins with reversible attachment, during which bacterial cells transition from a planktonic to a sessile state. *Escherichia coli* biofilm establishment has been linked to the production of curli fimbriae, amyloid fibers associated with pathogenesis and adhesion (20–22). Genes involving the synthesis of the outer membrane (23), three two-component regulatory systems (23–25) and environmental conditions

(26–28) are involved in curli biosynthesis. *P. aeruginosa* initial biofilm attachment has been linked to flagella (19), type IV pili (14, 29) as well as Pel (30, 31) and Psl polysaccharides (32, 33).

Second messenger cyclic dimeric guanosine monophosphate (c-di-GMP) is involved in the shift in the transition of gram-negative bacterial cells from planktonic to a sessile state (34, 35). Elevated c-di-GMP may promote irreversible attachment by hindering motility (36, 37), enhancing adhesin production (37, 38), and regulating production of the extracellular matrix (39, 40).

Biofilm Maturation

During the maturation phases of biofilm development, a thick extracellular matrix (ECM) composed of exopolysaccharides, proteins and extracellular DNA (eDNA)– begins to develop (41, 42). The exact ECM composition is dependent upon attachment surface, community composition, and nutrient availability (42, 43). Alteration in the cellulose-curli network associated with *E. coli* biofilm matrix, results in decreased biofilm formation (44), and integrity (45). An analysis of the *P. aeruginosa* genome identified 35 genes that are upregulated by high c-di-GMP, including PA4624 and PA4625 (46, 47). Deletion of PA4625, designated *cdrA*, resulted in a decreased biofilm biomass and structural integrity (48) further supporting the importance of ECM components in maintaining the structural integrity of biofilms.

Extracellular DNA is a major structural component of *P. aeruginosa* biofilms (49), and forms in organized patterns, possibly linked to quorum sensing (50). Treatment of *P. aeruginosa* biofilms with DNase, a DNA degrading enzyme (51), was shown effective at inducing bacterial biofilm dispersal (52). DNase was also effective at

inducing *E. coli* biofilm dispersal (53, 54) as well as other gram-negative (41, 55) and gram-positive (41, 56) bacterial biofilms. Additionally, proteinase (57, 58), and surfactant treatments (59) have shown to induce biofilm dispersal by binding to ECM proteins.

Biofilm Dispersion

Biofilm dispersal can be classified as active or passive. Active, or seeding, dispersion occurs as a result of environmental dispersal triggers (60, 61) as opposed to passive dispersal which is described as physical removal of the biofilm (62, 63). Active dispersal can be initiated by change in nutrient availability (60), the development of an unfavorable microenvironment due to waste accumulation or the production of anaerobic metabolic byproducts (64, 65), as well as decreased c-di-GMP (36, 66). Active dispersal can also be induced by enzymes (52, 58) and alteration of nutrient conditions, notably pyruvate depletion (67).

Seeding dispersal has been correlated with an increased pathogenicity as the planktonic bacterial cells disperse and form subsequent biofilms in other areas (68). Once bacterial cells have returned to a planktonic state, susceptibility to antimicrobial therapy does return (69). This observation provides support that phenotypic adaptations, the presence of ECM and altered metabolic rates, may contribute to observed antibiotic tolerance, as opposed to genetic mutations (70, 71). Induction of microbial biofilm dispersion has thus become a prevalent topic in microbiology research (72–74).

Boric acid is a weak acid that dissociates to borate in aqueous solution (75), by acting as a Lewis acid (76), with pKa reported 9.24 at 25°C in fresh water (77, 78). The dissociation of boric acid ($B(OH)_3$) into the borate ion ($B(OH)_4^-$) varies with pH and temperature (77–79). At a concentration of 0.75% (w/v), boric acid was shown to have

antibacterial properties when used to irrigate dental plaques (80). Additional studies showed 0.50% (w/v) boric acid may be effective at inducing dispersion of naturally occurring polymicrobial biofilms (81). While the mechanism of dispersal is currently unknown, boric acid has been found to form non-intercalative covalent bonds to DNA, possibly by binding to deoxyribose (82). Given these findings, it is possible that boric acid may bind to eDNA present in the ECM of bacterial biofilms. The molecular interactions and involvement of eDNA in the structure of the biofilm matrix is not well known, however the DNase induced biofilm dispersal (52) suggests that degradation of eDNA in the matrix leads to matrix destabilization. While boric acid has not been found to degrade DNA at concentrations less than 5 M (82), boric acid binding to eDNA may disrupt interactions between eDNA and other matrix components, such as proteins and polysaccharides, without degrading the eDNA. Disrupting the interactions between the structural components of the ECM may then destabilize the biofilm matrix, and result in biofilm dispersal. This proposed mechanism is summarized in **Figure 1.**

Rhamnolipids, biosurfactants produced by *P. aeruginosa* have been found to be effective at preventing biofilm establishment and disrupting present biofilms (59, 83). Sodium dodecyl sulphate (SDS) (also referred to as sodium lauryl sulfate) binds to proteins via hydrophobic interactions and causes unfolding of protein tertiary structures when at concentrations less than the critical micelle concentration, $8.4X10^{-3}$ M in aqueous solution at 25°C (84, 85). At higher concentrations, charge-charge repulsion causes hydrophobic regions of the protein to expand (84), resulting in protein denaturation (86, 87). Further, SDS at a concentration of $2.77X10^{-3}$ M was found to induce dispersal of *P. aeruginosa* biofilms cultured in a BioFlux channel (59). Given the effects of SDS and

rhamnolipids, we hypothesized that a mixture of anionic surfactants, such as Dawn Dish Soap, may induce similar dispersal effects.

The efficacy of boric acid and Dawn Dish Soap to induce dispersal of monoculture uropathogenic *E. coli* and *P. aeruginosa* biofilms was investigated. Biofilms were cultured on silicone disks, modeling the substrate of urinary catheters and then challenged with nitrofurantoin and tobramycin. Tobramycin and nitrofurantoin are antibiotics effective against *P. aeruginosa* and *E. coli* and are clinically used in the treatment of urinary tract infections (88, 89). Tobramycin is of considerable interest due to the reported resistance of uropathogenic *P. aeruginosa* to this antibiotic (90). The goal of this study was to investigate the combination of a dispersal agent and an antibiotic as a potential treatment of CAUTIs.

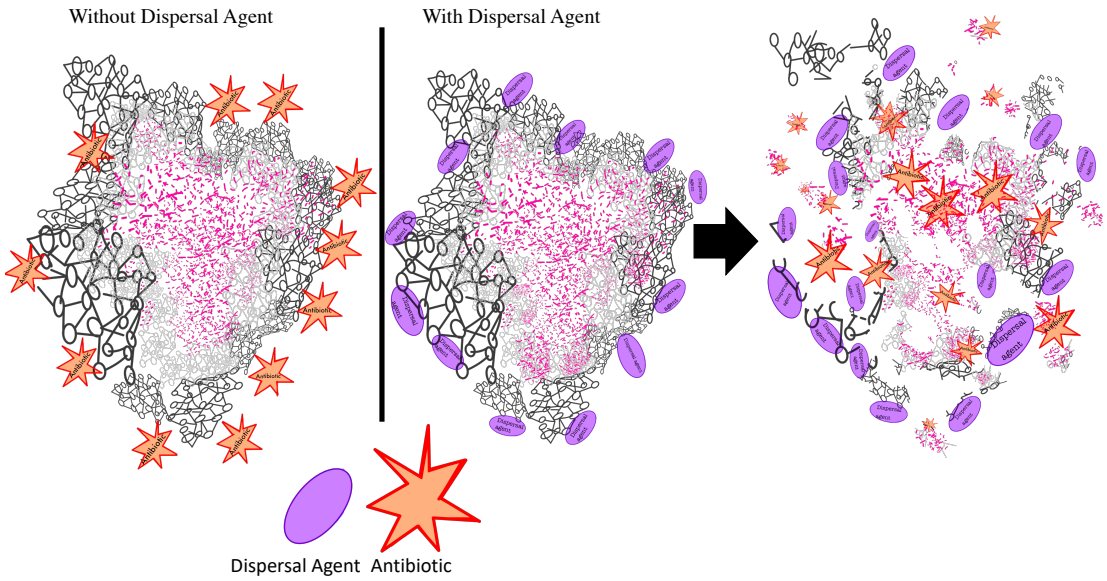

Figure 1. Potential biofilm dispersal mechanism of a combination treatment containing a dispersal agent and antibiotic.

II. MATERIALS AND METHODS

Strains and Cultures

The strains used in this study were *Pseudomonas aeruginosa* PAO1, carrying *gfp* (91) and uropathogenic *Escherichia coli* F11, carrying *mcherry* (92). Both strains were stored in 12.5% (v/v) glycerol at -80°C. Prior to each experiment, the frozen strains were revived by streaking onto Luria-Bertani (LB) agar, then incubating in a stationary 37°C incubator for 18-24 hours. After incubation, the plate cultures were stored in a 4°C refrigerator for up to one week. Starter cultures were made by inoculating 5 mL LB broth or 5 mL modified artificial urine media (mAUM) from the revived plate cultures, then the starter cultures were incubated in a 37°C water bath, shaking (150 RPM) for 18 to 24 hours.

Modified Artificial Urine Media (mAUM)

Urine composition is known to vary in relation to a person's hydration status and diet (93–95). To control for these discrepancies, artificial urine media was used to model urinary conditions.

Modified artificial urine media (mAUM) was prepared as previously described (Kim et al 2013). All stock solutions were prepared, sterilized by filter sterilization or autoclaving, and stored in sterile airtight bottles, excluding RPMI amino acids (Sigma-Aldrich #R7131-100 mL) and L- Glutamine (VWR RL0131-0100), as these components were diluted from sterile commercial stock solutions. Given the limited shelf life of urea, this solution was prepared fresh each time mAUM was prepared. All other stock solutions were stored for a maximum of eight months.

Table 1. Modified Artificial Urine Media (mAUM) recipe. Final pH 7.0.

Component	Concentration (mM)
Citric Acid	2
Lactic Acid	1
Sodium Chloride	90
Ammonium Chloride	25
RPMI 1640 Amino Acids (without L-Glutamine)	20 mL/L
Urea	170
Uric Acid	0.4
Creatine	7
Calcium Chloride	0.25
Magnesium Sulfate Septahydrate	10
Sodium Sulfate, Anhydrous	10
Sodium Bicarbonate	25
Sodium Nitrate	0.6
Ferrous Sulfate	0.005
Monopotassium Phosphate	18
Dipotassium Phosphate	18
L-Glutamine	2

Growth Curves

Starter cultures of *E. coli* and *P. aeruginosa* were cultured in LB and mAUM, as described above.

Each well in a 96 well plate was filled with 200 µL of mAUM or LB broth pre-inoculated with $8.38 \pm 2.5X10^7$ CFU/mL (LB broth) or $6.44 \pm 0.19X10^6$ CFU (mAUM) *E. coli*, or $4.98 \pm 2.60X10^7$ (LB broth) or $2.36 \pm 0.96X10^7$ (mAUM) *P. aeruginosa* per well. Negative control wells were filled with 200 µL sterile media to serve as a contamination control. The plate was incubated at 37°C in a Biotek Powerwave XS2 with 20 seconds of gentle shaking prior to each optical density at 600 nm (OD600) reading. Readings were taken every 20 minutes for seven days.

Cell Viability

E. coli starter cultures were diluted in fresh LB to $4.19 \pm 1.25X10^{8}$ CFU/mL and mAUM to $3.22 \pm 0.954X10^{7}$ CFU/mL. *P. aeruginosa* cultures were diluted in sterile LB broth and mAUM to $2.49 \pm 1.30X10^{8}$ CFU/mL and $1.18 \pm 4.82X10^{7}$ CFU/mL, respectively. All cultures were incubated in a shaking water bath at 37°C, 150 RPM for seven days. Samples were collected at 0, 3, 5, and 7 days, serially diluted in sterile phosphate buffered saline (PBS) and plated on standard LB agar, for viable cell counting.

Minimum Inhibitory Concentration

E. coli

Nitrofurantoin stock solutions were made by dissolving nitrofurantoin powder (Signa 67-20-9) in dimethyl sulfoxide (DMSO) to 20 mg/mL. A working solution was made by diluting the stock solution into DMSO to a final concentration of 0.1 mg/mL on the day of use. Stock solutions were stored in a dark box at -20°C for up to one year.

For initial range finding, 15 mL of fresh LB broth was inoculated with 150 µL of *E. coli* LB broth starter culture. A 96 well plate was filled with diluted sample and nitrofurantoin, with the concentration of nitrofurantoin from 1 mg/mL to $1X10^{-4}$ µg/mL, decreasing by a factor of 10 as well as concentrations 100 µg/mL to 0.097 µg/mL, decreasing by a factor of 2 each time. The positive control wells contained LB broth and inoculum only and negative control contained only media and nitrofurantoin at each concentration. The plate was incubated at 37°C in a stationary incubator for 18 to 24 hours. After incubation, the plate was visually checked for turbidity. The concentration range was decreased due to no turbidity observed in wells with greater than 25 µg/mL of nitrofurantoin.

E. coli starter cultures were prepared as detailed above; 50 µL of starter culture was used to inoculate sterile LB broth containing nitrofurantoin at concentration 12-18 µg/mL (decreasing in increments of 2 µg/mL) and 20-25 and 30 µg/mL (decreasing in increments of 1 µg/mL). Cultures were incubated in a shaking water bath (150 RPM) at 37°C for 18-24 hours. Test tubes were visually inspected for turbidity. *E. coli* cultured in LB broth with nitrofurantoin 18 µg/mL was serially diluted in PBS and plated on standard LB agar for viable cell counting, to determine if nitrofurantoin exhibited a bacteriostatic versus bactericidal effect. Plates were incubated at 37°C for 18-24 hours and were visually inspected for colony growth.

Starter cultures of *E. coli* mAUM were prepared as described above, then inoculated into 5 mL of mAUM containing nitrofurantoin ranging from 5 to 30 µg/mL. The cultures were incubated for 18 to 24 hours at 37°C in a rotary water bath (150 RPM) and visually checked for turbidity. Since nitrofurantoin is highly pigmented in mAUM, turbidity was confirmed with OD600 readings, using 500µL of inoculated mAUM containing 30 µg/mL nitrofurantoin as a blank. This was done to account for absorbance readings of *E. coli* cells, components of mAUM and nitrofurantoin at the highest concentration tested. *E. coli* cultured in mAUM in the presence of 20 µg/mL of nitrofurantoin was not turbid. To determine bacteriostatic versus bactericidal effect, this sample was serially diluted in PBS and plated onto LB agar. The plates were incubated at 37°C for 18-24 hours and visually inspected for evidence of colony growth.

P. aeruginosa

Tobramycin stock solution was made by dissolving tobramycin powder (TCI T2503) in water to concentration 10 mg/mL. A working solution was made by diluting

stock solution in water to final concentration 0.1mg/mL. Stock solution was stored in a dark box, at -20°C for up to one year. Working solutions were prepared and used within 24 hours.

Starter cultures of *P. aeruginosa* PAO1 were prepared as described above in both LB broth and mAUM. Starter cultures were then diluted in 5 mL of LB broth or mAUM to $2.49 \pm 1.30 \text{ X}10^8$ or $0.118 \pm 4.82 \text{ X}10^7$ CFU, respectively, containing tobramycin ranging from 0 to 10 μg/mL. Cultures were incubated at 37°C in a rotary water bath (150 RPM) for 18-24 hours, and then visually inspected for turbidity. Samples treated with 2 μg/mL appeared transpicuous. These samples were serially diluted in PBS, plated on standard LB agar and incubated for 18-24 hours at 37°C. To determine bactericidal versus bacteriostatic effect, the plates were visually inspected for evidence of colony growth.

Determination of Dawn Concentration

Dawn Dish Soap, Ultra Concentrated, blue, original scent (99380720) was used to evaluate the efficacy of Dawn Dish Soap as a potential dispersal agent. The exact chemical composition of this formulation is protected under multiple US patents. The general composition of Dawn is an aqueous mixture of anionic surfactants including sodium dodecyl sulfate, sodium laureth sulfate and lauryl amine oxide.

To determine the concentration of Dawn that resulted in biofilm dispersion, biofilms were cultured in 96-well microtiter plates. Starter cultures of *E. coli* and *P. aeruginosa* were prepared as previously described above and diluted in fresh LB $8.38 \pm 2.5\text{X}10^7$ CFU and $4.98 \pm 2.60\text{X}10^7$ CFU, respectively. Wells in a 96 well plate were filled then with 200 μL of the diluted culture. The negative control wells were filled with

200 µL of uninoculated media. The plates were incubated at 37°C in a rotary shaker (150 RPM) for 72 and 96 hours. Following incubation, the wells were emptied using a multichannel pipettor, being cautious to avoid contact with the bottom and sides of the wells to avoid disrupting the biofilms. Treatment wells were filled with 200 µL of Dawn ranging from 0.01 to 0.001% (v/v). Positive control wells contained only the diluted culture and the negative wells contained only Dawn at concentrations ranging from 0.1% to 0.001% (v/v). The remaining wells in the 96-well plate contained only uninoculated LB broth, to serve as a contamination control. The plate was incubated in the same conditions described above, for 18-24 hours. After incubation, treatment was removed and 200 µL of fresh LB broth was added to each well. The plate was incubated at 37°C in a rotary shaker for an additional 18-24 hours. A single OD600 reading was taken using a Biotek Powerwave XS2 plate reader.

Boric Acid and Antimicrobial Combination Treatment of Preformed Biofilms

Biofilm Culturing

All biofilms were cultured using a silicone disk as an attachment substrate measuring 7 mm in diameter, 3 mm in thickness, and were cut from sheets of silicone using a borer.

Starter cultures were prepared as described above in LB or mAUM. Sterile 250 mL flasks containing 10 silicone disks were filled with 25 mL of LB or mAUM. Starter cultures were diluted in 25 mL of sterile media (**Table 2)**. The flasks were incubated at 37°C in a rotary water bath (150 RPM) for 72 hours or 7 days.

Table 2. Inoculum dose and treatment time of monoculture *P. aeruginosa* PAO1 and *E. coli* F11 biofilms after culturing for 72 hours and 7 days in LB broth or mAUM.

Culture	Inoculum Dose	Media	Treatment Time (Hours)	
			72 Hour	7 Day
P. aeruginosa PAO1	$2.49\pm1.30X10^{8}$	LB	1.5	0.5
	$0.118\pm4.82X10^{7}$	mAUM	0.5	
E. coli F11	$4.19\pm1.25X10^{8}$	LB	2.5	2
	$0.32\pm9.54X10^{7}$	mAUM	0.5	0.5

Biofilm Treatment

Each disk was removed from the flask by puncturing the center of the disk with a sterile 27-gauge needle. The disks were rinsed three times in 5 mL of PBS and transferred to a scintillation vial for treatment (**Table 3)**. The PBS wash vial was changed after each disk to minimize any risk of contamination. The disk was removed from the needle by holding the sides with ethanol sterilized forceps. The treatment vials were incubated stationary at 37°C. The treatment time started when scintillation vials were placed into the incubator. Treatment time was not lowered beyond 30 minutes due to the length of the protocol.

Table 3. Treatment conditions. All treatments were performed in sterile scintillation vials at 37°C, in the presence or absence of antibiotic at MIC.

Treatment	Concentration
PBS (Control) (M)	$1.36X10^{-1}$ NaCl
	$2.68X10^{-3}$ KCl
	$1.01X10^{-2}$ Na_2HPO_4
	$1.76X10^{-3}$ KH_2PO_4
	pH 7.4
Boric Acid (w/v)	0.25%
	0.50%
	0.75%
Dawn Dish Soap (v/v)	0.005%

The disks were removed from the treatment vials using 27-gauge needles, rinsed three times in 5 mL of sterile PBS, then transferred to a sterile scintillation vial containing 5 mL of PBS and sonicated at 120 volts for 5 minutes (Fisher Scientific, FS20).

After sonication, the disks were removed from the scintillation vials using ethanol sterilized forceps. The vials were then vortexed for 15 seconds, serially diluted to a final dilution factor of 10^{-3} in sterile PBS and plated on standard LB agar for viable cell counting.

Analysis

Statistical analysis was performed using R version 3.6.1 on a Mac platform, utilizing ggplot2 (96) and reshape2 (97) packages.

III. RESULTS AND DISCUSSION

Cell Viability in LB broth and mAUM

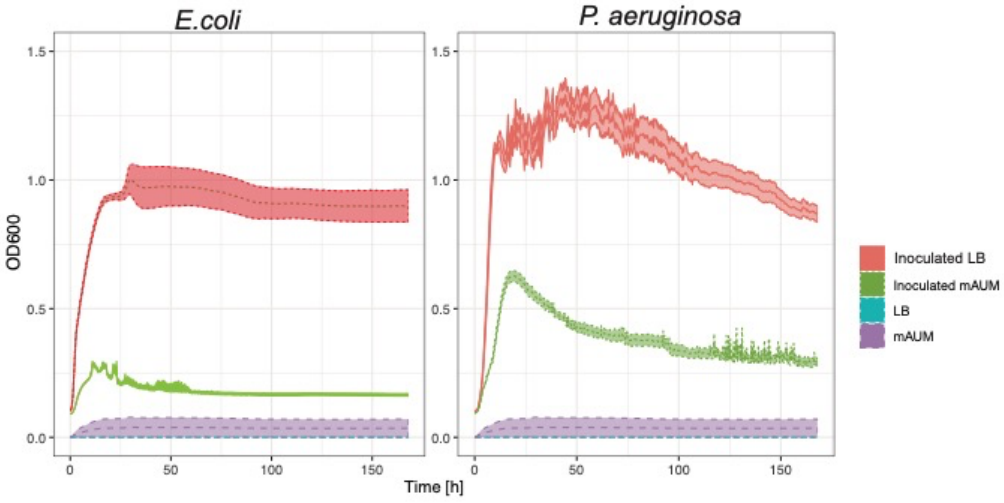

Figure 2. Growth curves. *P. aeruginosa* and *E. coli* cultured in LB or mAUM for seven days. OD600 readings were taken after 20 seconds of gentle agitation, every 20 minutes. Control wells contain uninoculated media. n=3 Shading represents standard error.

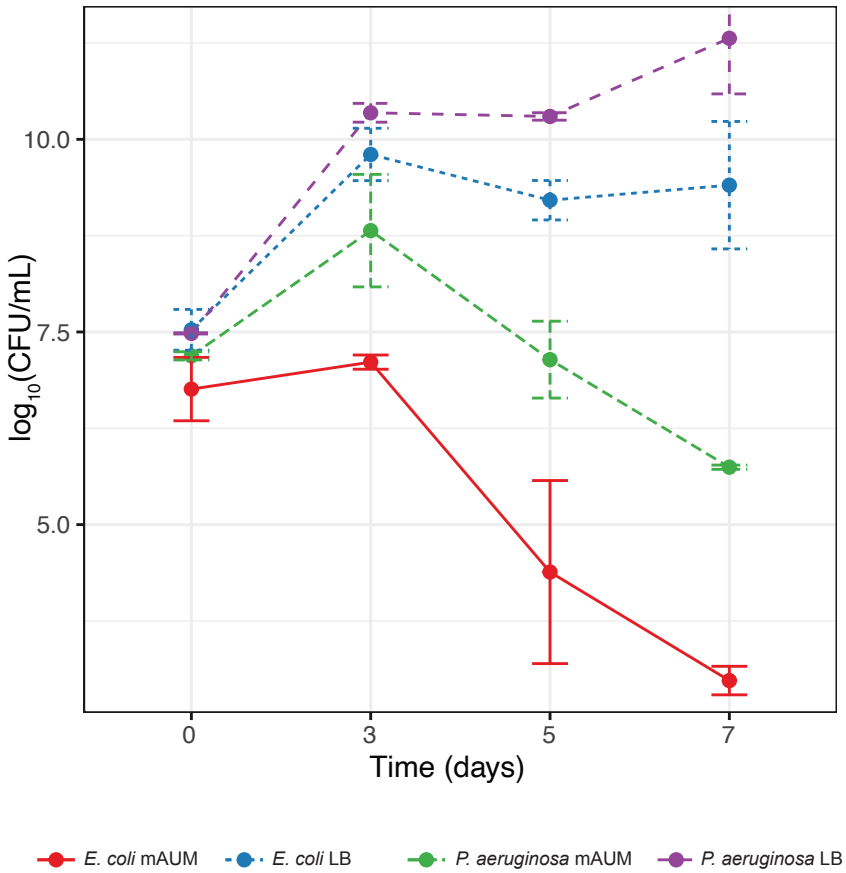

Figure 3. Cell viability. Cell viability was quantified by viable cell counting. The viability of LB cultured *P. aeruginosa* and *E. coli* was stable at 3 and 5 days, with an increased viable cell count recorded at 7 days of growth. *P. aeruginosa* and *E. coli* cultured in mAUM viability decreased after three days in media. Error bars represent standard deviation. n=3.

P. aeruginosa PAO1 and *E. coli* F11 were cultured in mAUM and LB for seven days. OD600 readings revealed PAO1 reaching stationary growth after approximately 48-50 hours when cultured in LB and mAUM. *E. coli* appears to enter stationary growth after approximately 48-50 hours when cultured in LB (**Figure 2**). Absorbance of uninoculated mAUM increased over time reaching above 0.200 after approximately 72 hours. This may be the result of precipitation of unused media components, as opposed to contamination, as the absorbance stabilized after initial increase. Contamination of the

control wells would be expected to continue to increase over time, following a similar pattern as the inoculated wells.

Viability of *P. aeruginosa* and *E. coli* in LB and mAUM after seven days was confirmed by viable cell counts. In LB, *P. aeruginosa* viable cell counts began to stabilize between three to five days of growth and then increase at seven days (**Figure 3**). In response to starvation, *P. aeruginosa* cells have been shown to fragment, or divide without growing, resulting in an increase in cell number and decrease size (98). This adaptation may aid in nutrient acquisition (98, 99). *P. aeruginosa* has also been shown to exist as cellular aggregates in batch media and disperse into single cells in response to a detrimental growth conditions, such as nutrient deprivation (100). It is possible that the increase in viable cell counts at seven days (**Figure 3**) is the reflection of aggregate dispersal or cell fragmentation. When cultured in mAUM, *P. aeruginosa* cell counts began to decrease after three days of growth and continued to decrease between three to seven days (**Figure 3**). LB is a complex, general growth media that contains peptone and yeast extract, serving as a source of amino acids, peptides and proteins, and sodium chloride. In contrast, mAUM is a defined media consisting of salts, and nitrogen-containing compounds such as amino acids and uric acid (**Table 1**). The difference in *P. aeruginosa* viability is possibly due to the differences in the nutrient composition of the growth media. Future work should be aimed at investigating *P. aeruginosa* growth in different media, to establish possible nutrient requirements as well as environmental stimuli involved in aggregative growth and fragmentation.

E. coli followed similar patterns as *P. aeruginosa* when cultured in LB and mAUM (**Figure 3**). The increased viable cell counts when cultured in LB broth may be

due to utilization of bacterial cell detritus (101, 102). This increase was not seen when *E. coli* was cultured in mAUM. One sample was excluded from seven-day mAUM cultured *E. coli* F11, as this reading was at not at a detectable level. When cultured in mAUM, there was a steady decrease in viable cell counts after three days of growth (**Figure 3)**. This may be indicative of depletion of readily available nutrients, or an inability to utilize cell detritus in this media. Cell growth during starvation has been termed growth advantage in stationary phase (GASP). The GASP phenotype has been observed in multiple *E.coli* strains and has been linked to increased stress sigma factor σ^S, the product of *rpoS* expression (103–105). The increase in stress sigma factor is due to an increased expression of *rpoS* and decreased degradation of σ^S during the stationary phase(106). Cells expressing the GASP phenotype are able to utilize substrates released by dying cells, notably amino acids (105). It may be possible that when cultured in mAUM the expression of *rpoS* or the stability of σ^S is reduced, prohibiting the GASP phenotype expression, resulting in the noted continuous decrease in viable cell counts. Alternatively, nutrients may have been depleted faster in mAUM as compared to LB, resulting in *E. coli* utilization of detritus occurring between the time of inoculation and the three-day sample collection. Further studies regarding uropathogenic *E. coli* F11 viability in mAUM should be performed to investigate this finding. Genetic analysis investigating the potential regulation of genes involved in *E. coli* F11 metabolism, when cultured in LB and opposed to mAUM could offer insight on nutrient utilization.

Viable cell counts of *E. coli* F11 cultured in mAUM (**Figure 3**) are not consistent with OD600 readings, which revealed decreased absorbance over time, in wells containing *E. coli* inoculated mAUM and increased absorbance of uninoculated mAUM

(**Figure 2).** The elevated OD600 readings of uninoculated mAUM may be due to the precipitation of ingredients. Precipitation of uninoculated mAUM used in this study was noted during storage at 4°C as well as 30°C. Precipitation was not visually apparent in inoculated mAUM. This suggests bacterial cell utilization of nutrients may be involved in the prevention of nutrient precipitation. When precipitation of un-inoculated mAUM became apparent, the media was discarded, and fresh media was made for experimental use. Analysis on the precipitant was not performed, however, precipitation may be due to the interaction between calcium chloride and dipotassium phosphate forming calcium phosphate. The modified artificial urine recipe used in this study contained a reduced concentration of calcium chloride to prevent precipitation during storage (107). Future work should be aimed at optimizing storage of this media.

Minimum Inhibitory Concentrations

The minimum inhibitory concentration (MIC) of nitrofurantoin differed when *E. coli* was grown in LB broth or mAUM. When cultured in LB broth MI of nitrofurantoin was found to be 18 μg/mL, as opposed to 20 μg/mL required when *E. coli* was cultured in mAUM (**Table 4**). The MIC of tobramycin against *P. aeruginosa* was found to be 2 μg/mL; this did not change when *P. aeruginosa* was cultured in LB broth or mAUM (**Table 4**).

Table 4. MIC of tobramycin against *P. aeruginosa* PAO1 and nitrofurantoin against *E. coli* F11 when cultured in mAUM or LB broth.

Culture	Antibiotic	Media	MIC (μg/mL)
P. aeruginosa PAO1	Tobramycin	LB mAUM	2
E. coli F11	Nitrofurantoin	LB mAUM	18 20

Dawn Treatment Concentration

Dispersal of *P. aeruginosa* and *E. coli* biofilms grown for 72- and 96-hours after exposure to Dawn for 24 hours was quantified using OD600 (**Figure 4**). After 18-24 hours of treatment, Dawn and planktonic cells were removed. Increased dispersal would result in an increased number of planktonic cells removed with the treatment, and a decreased number of bacterial cells remaining in the wells when fresh media was added. Therefore, increased efficacy of Dawn as a dispersal agent was quantified as decreased OD600 after the addition of fresh media. The effect persisted despite an increase in biofilm culture time. Dawn at a concentration of 0.005% (v/v) exhibited a consistent dispersal effect of both *P. aeruginosa* and *E. coli* biofilms after 72- and 96-hours of growth ($p < 0.005$) (**Figure 4**). A similar dispersal effect of both cultures at both time points, was seen when Dawn concentration was as low as 0.001%, (v/v) (**Figure 4)**. Ultimately, the decision was made to proceed with treatment of silicone adhered biofilms with Dawn 0.005% (v/v) as this was the median effective treatment concentration ($p < 0.005$) (**Figure 4**), and the efficacy of treatment was expected to vary in response to the change in the biofilm attachment surface.

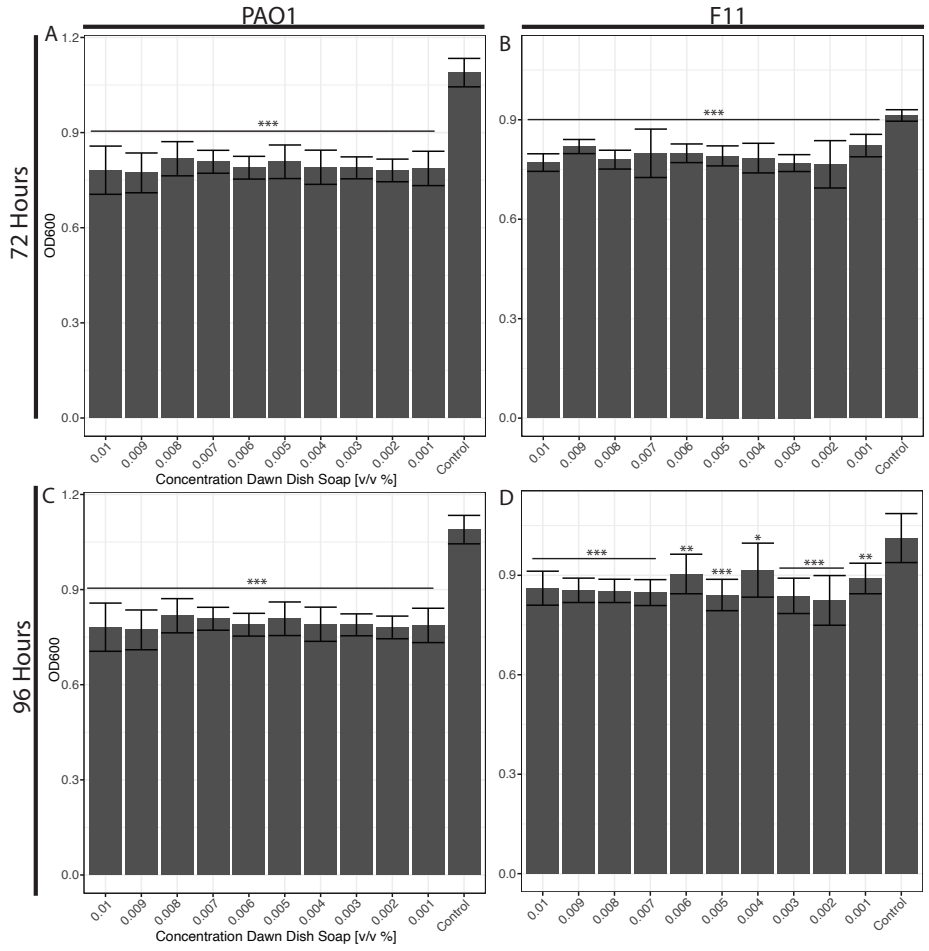

Figure 4. Dawn Dish Soap dispersal efficacy. *P. aeruginosa* (**A, C)** and *E. coli* (**B, D**) growth in LB after 72 (**A, B**) and 96-hour (**C, D**) biofilm treatment with Dawn for 18-24 hours. Treatment with all of the tested concentrations of Dawn resulted in biofilm dispersal, quantified as lowered OD600 value. At a concentration of 0.005% (v/v) Dawn treatment resulted in biofilm dispersal of *E. coli* F11 72-hour **A**.) ($p= 3.0X10^{-6}$) and 96-hour **B)** ($p= 2.3X10^{-6}$), as well as *P. aeruginosa* 72-hour **C.)** ($p< 2.0X10^{-16}$) and 96-hour **D.)** ($p=1.2X10^{-15}$) biofilms. (* $p\leq0.05$, ** $p\leq0.005$, *** $p\leq0.0005$). Error bars represent standard deviation. Dunnett's n= 3.

Dawn is a mixture of sodium dodecyl sulfate, sodium laureth sulfate and lauryl amine oxide. Sodium laureth sulfate and SDS are anionic surfactants, carrying a negative charged head (108). *P. aeruginosa* has been found to survive using SDS as a sole carbon source (109) and further work has identified the expression of the *lao* operon and *sdsA1* is induced by SDS (110). *P. aeruginosa* has been found to form macroscopic cellular

aggregates, or pellicles, in response to SDS exposure, as a possible survival strategy (109). In the present study, there were no obvious signs of cellular aggregation after exposure to Dawn. This may be due to the fact that a surfactant mixture, with the exact SDS concentration unknown, was used as opposed to SDS (3.5 mM) used in prior studies (107).

The Efficacy of Combination Treatment

Sonication has been used to dislodge biofilms from removed implants for diagnostic analysis (111) and has been found to be a more effective removal strategy than scraping (112). After treatment, sonication was performed to dislodge any remaining biofilm on the silicone disks. The sonicated vials were then used for viable cell plating. A low colony count after viable cell plating was correlated with an increased dispersal effect, as this indicated more of the initial biofilm was removed by boric acid or Dawn treatment. Any bacterial cells remaining in the undispersed biofilm could act as a seed for more biofilm growth after the boric acid treatment. This would render the treatment of biofilm associated infections with dispersal agents ineffective. Therefore, the efficacy of combination treatment was quantified as a lower counted colony after viable cell plating of the sonicated samples.

Boric Acid Treatment with Antibiotic Challenge

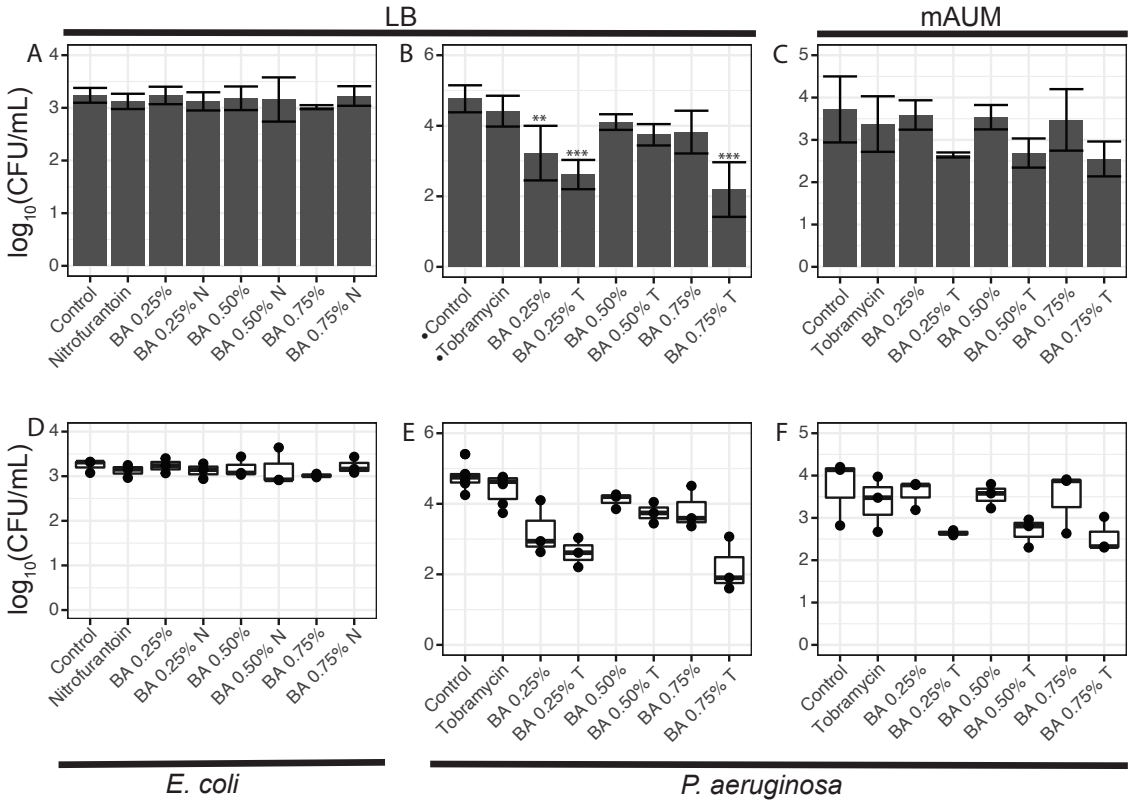

Figure 5. Boric acid treatment and antibiotic challenge. Biofilms of E. *coli* (**A, D**) and *P. aeruginosa* (**B, C, E, F**) cultured for 72 hours in LB (**A, B, D, E**) or mAUM (**C, F**) after treatment with boric acid and antibiotic challenge. **B, E)** *P. aeruginosa* cultured in LB showed increased susceptibility to tobramycin (T) after treatment with 0.25% (w/v) boric acid (p=0.0008) and 0.75% (w/v) boric acid (p=0.00005). **A, D)** The efficacy of nitrofurantoin (N) treatment of *E. coli* F11 did not improve in the presence of boric acid (p= 0.896). Error bars represent standard deviation. Tukey's HSD, n=3, •denotes treatments with n=6. Error bars indicate standard deviation.

P. aeruginosa

Monoculture *P. aeruginosa* biofilms cultured in LB exhibited an increased susceptibility to tobramycin when exposed to 0.25% (p= 0.0008) and 0.75% (w/v) (p=0.00005) boric acid in combination with tobramycin for 1.5 hours, in comparison to control samples treated without a dispersal agent (**Figure 5. B, E**). Treatment of

preformed LB-cultured *P. aeruginosa* biofilms with 0.50% (w/v) boric acid was performed independent of other treatments, resulting in 6 biological replicates of control treatments (**Figure 5. B, E**).

Viable cell counting revealed significantly less biofilm remained on the silicone disk after LB cultured *P. aeruginosa* biofilms were treated with 0.25% (w/v) boric acid than when treated with PBS (p=0.004) (**Figure 5. B, E**). This suggests boric acid at a concentration of 0.25% (w/v) may have been effective at inducing biofilm dispersal. The penetration of tobramycin into *P. aeruginosa* biofilms has been found to be hindered by tobramycin interactions with negatively charged components of the ECM, notably alginate (113) and possibly eDNA (114, 115). Inducing dispersal of *P. aeruginosa* biofilms can increase the efficacy of tobramycin treatment by overcoming the inhibitory effects of the ECM, increasing access of tobramycin to the bacterial cells. Thus, the increased efficacy of tobramycin when LB-cultured *P.* aeruginosa biofilms were treated with 0.25% boric acid (p=0.0008) (**Figure 5. B, E)** may be due to increased dispersal and subsequent increased access of tobramycin to *P. aeruginosa* cells. This data implies a synergistic effect between boric acid and tobramycin may exist, in which tobramycin efficacy is increased due to the induced dispersal effect of boric acid treatment, as portrayed in **Figure 1**.

In comparing the dispersal effect of 0.25% to 0.75% (w/v) boric acid on LB cultured *P. aeruginosa* biofilms, there was no statistically significant difference (p=0.815) (**Figure 5. B, E**). There was also no significant difference in the improved efficacy of tobramycin treatment in combination with 0.25% or 0.75% (w/v) boric acid (p=0.961) (**Figure 5. B, E**). This finding may be suggestive of a possible dispersal effect

of 0.75% (w/v) boric acid, that is correlated to the increased efficacy of tobramycin in combination with this treatment (**Figure 5. B, E)**. However due to the relatively small sample size (n=3) in combination with data variability, the statistical power may be too small for statistical analysis to reflect this. Alternatively, there may be a synergistic effect between 0.75% (w/v) boric acid and tobramycin that is unrelated to boric acid induced biofilm dispersal. Tobramycin has been theorized to be sequestered in the outer layers of the biofilm matrix by binding to eDNA (114, 115). Binding to eDNA is thought to be a possible mechanism responsible for the inefficacy of tobramycin treatment of biofilms (114). It may be possible that the increase in tobramycin efficacy found in this study is due to boric acid binding to eDNA and thus overcoming the biofilm inhibition of tobramycin, while simultaneously destabilizing the biofilm matrix (**Figure 1).** This destabilization may be further amplified by the simultaneous binding of tobramycin and boric acid to eDNA (114). Extracellular DNA has shown to be an effective target for induction of biofilm dispersal and sensitization of biofilms to antibiotics (41). The simultaneous binding of tobramycin and boric acid to eDNA may explain the statistically insignificant dispersal induced by 0.75% (w/v) boric acid in the absence of tobramycin, but increased efficacy of tobramycin with 0.75% (w/v) boric acid treatment (**Figure 5. B, E).** Tobramycin at extracellular concentrations ≥8 mg/L disrupts the outer membrane of *P. aeruginosa*, resulting in a bactericidal effect (116). It is unlikely that the increased the efficacy of tobramycin against *P. aeruginosa* PAO1 used in this study was related to outer membrane disruption, as tobramycin concentrations were kept below 8 mg/L and a bacteriostatic effect was confirmed via viable cell plating. Treatment with 0.50%(w/v) boric acid did not result in an increased dispersal of 72-hour LB-cultured *P. aeruginosa*

biofilms (p=0.579) (**Figure 5. B, E**). There was no increased efficacy of tobramycin when used in combination with 0.50% (w/v) boric acid either (p= 0.561) (**Figure 5. B, E**). This suggests the dispersal effect of boric acid may be concentration dependent.

P. aeruginosa biofilms cultured in mAUM did not show increased dispersal or tobramycin susceptibility when treated with 0.25%, 0.50%, 0.75% (w/v) boric acid (**Figure 5. C, F**). There may be an increase in tobramycin efficacy when used in combination with 0.25% (w/v) boric acid, as indicated by a log-fold reduction in attached viable cell number, but this was not confirmed by statistical analysis (p= 0.6519). This may be due to the variability in data as supported by standard deviation and poor statistical power. Variability in the data may be the result of *P. aeruginosa* cellular aggregation (109). *P. aeruginosa* biofilms cultured in mAUM were treated for 30 minutes as compared to the 90-minute treatment time of LB cultured biofilms in which 0.25% (w/v) boric acid was effective (**Figure 5. B, E**). Treatment times were not decreased beyond 30 minutes due to the time cost of transferring disks to treatment vials. This suggests the effect of 0.25% (w/v) boric acid may be time dependent. The inconsistent efficacy of boric acid treatment may also be consequent of different growth conditions, as growth conditions have been shown to influence the composition of the ECM (117, 118). This study did not evaluate the differences in ECM composition resulting from different growth conditions. If the target of boric acid action is a component of the ECM, as we suspect, the efficacy of dispersal would likely be dependent on the available binding sites. Further studies focused on optimization of treatment time and evaluation of *P. aeruginosa* ECM composition when cultured in mAUM and LB broth need to be performed for complete evaluation of the efficacy of boric acid treatment.

E. coli

Boric acid was not effective at inducing dispersal of LB broth cultured-*E. coli* F11 biofilms and nitrofurantoin efficacy was not increased (p=0.896) (**Figure 5. A, D**). In an attempt to optimize treatment time, 72-hour *E. coli* biofilms cultured in LB broth were treated for 3 hours. After treatment and subsequent sonication, biofilms treated with boric acid (0.25%, 0.50%, and 0.75% (w/v)) were not consistently detectible by viable cell counting. Viable cell counts of control disks were at statistically detectable levels, supporting biofilm attachment was likely successful and thus treatment with boric acid may be the cause of low viable cell counts after sonication. This data suggests the effect of boric acid on 72-hour LB cultured *E. coli* biofilms may be time dependent and the sensitivity of this study was not appropriate to detect this effect.

Boric acid treatment of mAUM-cultured *E. coli* biofilms for 30 minutes showed no biofilm remaining on the disks as detected by sonication and viable cell counting. This was true for control disks as well, possibly indicating no biofilm was present on the silicone disks prior to treatment. Alternatively, sonication may have resulted in bacterial cell death (119, 120), as *E. coli* has been shown to be more susceptible to ultrasound when collected from a population in stationary phase, as opposed to exponential growth phase (121). The lack of remaining biofilm after sonication was surprising given the uropathogenic nature of this *E. coli* strain. However, Hancock *et al.* found biofilm production of uropathogenic *E. coli* strains to be lower than that of asymptomatic bacteriuria strains when cultured in human pooled urine on static microtiter plates and continuous flow chamber systems (122). The *E. coli* F11 strain was isolated from a healthy patient without symptoms of a UTI (123) and was not associated with a catheter.

Prior work confirmed biofilm production by *E. coli* F11 cultured in mAUM (92) under different growth conditions. The differing growth conditions may have inhibited biofilm formation. In this study, biofilms were cultured at 37°C in a shaking incubator, as opposed to the previous study in which, the biofilms were cultured statically at ambient temperature (92). Decreased bacterial adhesion and increased detachment, have been related to increased turbulent flow and liquid temperature (124, 125). The increased detachment of biofilms when exposed to turbulent fluid flow is theorized to be caused by the multidirectional exertion of shear force (126–128). Given these findings, it is possible that the shaking condition and elevated temperature in relation to the previous study, inhibited *E. coli* F11 biofilm production when cultured in mAUM. One other notable difference is that the biofilms in the previous study were cultured on stainless steel and Teflon substrates (92), while biofilms in this study were cultured on silicone. Biofilm formation has also been shown to be surface dependent (117, 129, 130), and thus biofilm formation on silicone may be reduced in this strain. Biofilms were present on disks cultured in LB broth under the same incubation conditions. This is indicative that the decreased biofilm growth when cultured in mAUM may be due to nutrient conditions, as opposed to increased growth temperature or shear force. Viable cell counting confirms *E. coli* F11 was viable in mAUM for up to seven days, with reduced cell number after 3 days (**Figure 3**). This is indicative that the population may be entering the death phase between three to five days of incubation (131, 132). It is possible that mAUM is not suitable for *E. coli* F11 biofilm establishment on silicone after three days of incubation without media refreshment. The decrease in biofilm establishment may also be a multifactorial effect of overall growth conditions. Follow up studies should utilize a

continuous culture system, such as a Stovall flow cell, to determine if a constant supply of fresh media will increase biofilm persistence.

Dawn Dish Soap Treatment with Antibiotic Challenge

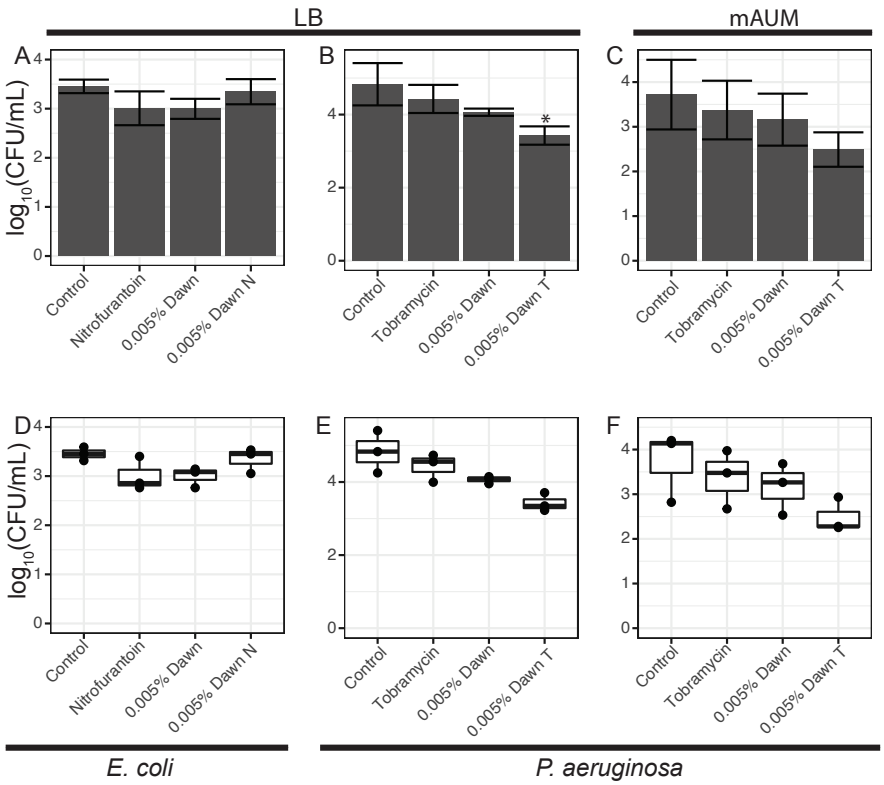

Figure 6. Dawn Dish Soap treatment and antibiotic challenge. 72-hour *E. coli* (**A, D**) or *P. aeruginosa* (**B, E, C, F)** biofilms cultured in LB (**A, D, B, E**) or mAUM (**C, F**) after treatment with Dawn and challenged with nitrofurantoin (N) or tobramycin (T). **B, E)** *P. aeruginosa* cultured in LB exhibited increased susceptibility to tobramycin treatment in the presence of Dawn (p=0.044). Dawn in the absence of tobramycin challenge was ineffective at inducing dispersal (p=0.131). Tukey's HSD. n=3. Error bars represent standard deviation.

At a concentration of 0.005% (v/v) Dawn was not effective at increasing the efficacy of nitrofurantoin against *E. coli* F11 biofilms cultured in LB for 72 hours (**Figure 6. A, D**) (p=0.903). *Pseudomonas aeruginosa* biofilms cultured in LB exhibited increased susceptibility to tobramycin treatment in the presence of Dawn (p= 0.044), but in the absence of tobramycin, Dawn was ineffective at inducing biofilm dispersion (p=0.131) (**Figure 6. B, E**). Dawn at 0.005% (v/v) was not an effective dispersal agent of

mAUM cultured *P. aeruginosa*. When combined with Dawn (0.005% (v/v)) the efficacy of tobramycin treatment of *P. aeruginosa* biofilms cultured in mAUM was not improved (p=0.359) (**Figure 6. C, F**).

The dispersal effect of Dawn (0.005% (v/v)) alone was insignificant against both LB cultured *E. coli* (p=0.186) and *P. aeruginosa* (p=0.131) biofilms when cultured on silicone disks (**Figure 6. A, D and B, E)**. This is inconsistent with preliminary data that showed evidence that 0.005% (v/v) Dawn induced dispersal of 72- and 96-hour *E. coli* and *P. aeruginosa* biofilms when cultured in LB (**Figure 4).** However, preliminary studies were done using biofilms cultured in 96 well microtiter plates. Biofilm attachment has been shown to vary based on the attachment surface (133). Given that preliminary studies varied only in the attachment surface (polystyrene microtiter plates) and not media conditions, the discrepancy of Dawn dispersal activity is possibly due to differences between biofilm attachment on the silicone disk and attachment to polystyrene. Future studies investigating increased Dawn concentration and other attachment surfaces would be needed to further evaluate potential antibiofilm characteristics of Dawn. Interestingly, tobramycin efficacy against *P. aeruginosa* biofilms cultured in LB, was increased when combined with 0.005% (v/v) Dawn (p=0.044) (**Figure 6. B, E).** It is possible that tobramycin binding to eDNA, as proposed by Chiang *et al.*, and Dawn simultaneously binding to proteins present in the ECM caused disruption of the extracellular matrix, resulting in biofilm dispersal. Dispersal of the biofilm would expose planktonic bacterial cells to extracellular tobramycin, thus increasing the efficacy of treatment. This could be further evaluated via confocal microscopy.

Treatment Efficacy in Relation to Biofilm Age

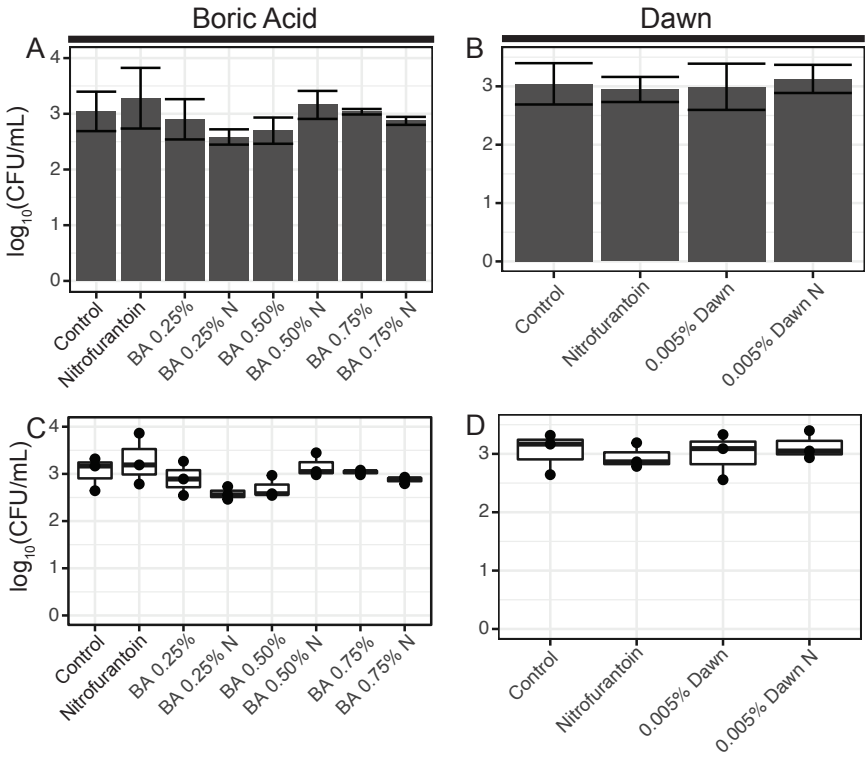

Figure 7. Treatment of seven-day biofilms with antibiotic challenge. *E. coli* F11 cultured in LB for seven days treated with boric acid (**A, C**) or Dawn (**B, D**) with nitrofurantoin (N) challenge. Tukey's HSD. n=3. Error bars represent standard deviation.

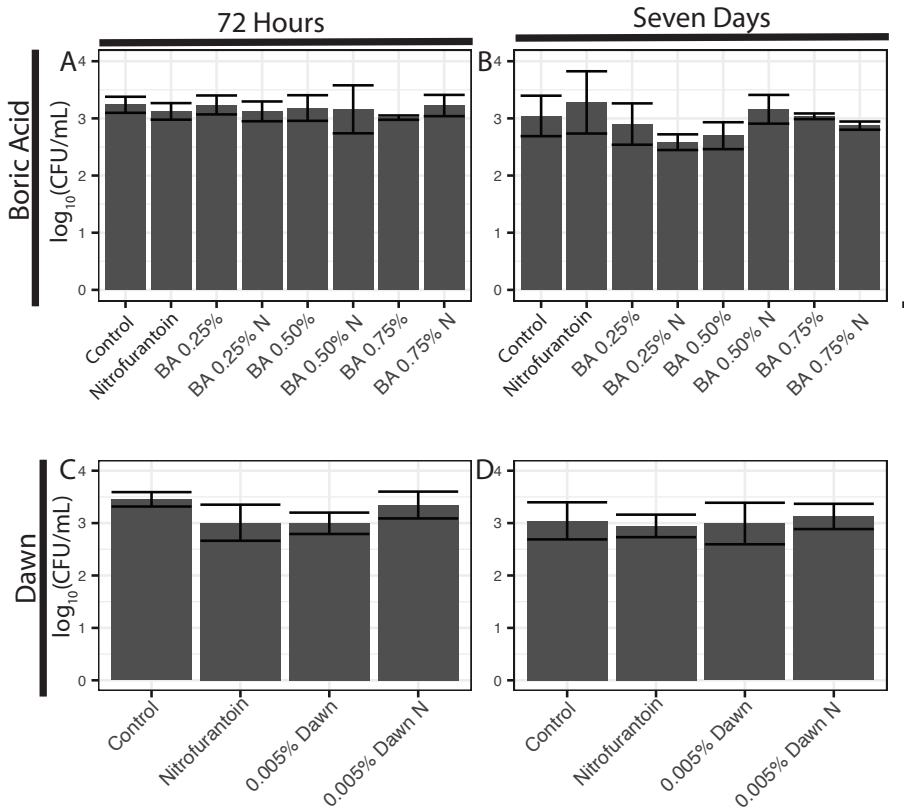

Figure 8. Comparison of treatment efficacy of three- and seven-day biofilms. Three (**A, C**) and seven-day (**B, D**) LB-cultured *E. coli* F11 treated with Dawn (**C, D**) and boric acid (**A, B)**. This is compilation of data from **Figures 5** and **6.**

Boric acid treatment of seven-day LB broth cultured *E. coli* biofilms did not induce dispersal or increase the efficacy of nitrofurantoin at 0.25%, 0.50% or 0.75% (w/v) boric acid (p=0.147) (**Figure 7. A, C)**. Similarly, treatment with Dawn at a concentration of 0.005% (v/v) resulted in no significant dispersal or increased nitrofurantoin susceptibility (p=0.903) (**Figure 7. B, D)**. This is consistent with the results found after treatment of three-day *E coli* biofilms cultured in LB broth (**Figure 8**). This indicates LB broth cultured *E. coli* biofilms are not susceptible to the tested concentrations of boric acid and Dawn, and this appears to be unrelated to biofilm age. The mechanism of action of boric acid is currently unknown, therefore we cannot conclude why this compound was ineffective at inducing *E. coli* dispersion. Dawn at a

concentration of 0.005% (v/v) was effective at inducing dispersal of 72 and 96-hour *E. coli* biofilms when cultured in LB broth on polystyrene (**Figure 4. B, D)**, but ineffective at dispersing the biofilms cultured on silicone disks in the same media for three or seven days (**Figure 8. C, D**). This may be due to differences in the substrate as well as the prolonged biofilm age. Rhamnolipids, a biosurfactant produced by *P. aeruginosa* was found to induce selective permeability of *E. coli* biofilms and in effect complement N-(3-oxo-dodecanoyl) homoserine lactone signaling, causing dispersal of *E. coli* biofilm (134). However, when studied in the absence of N-(3-oxo-dodecanoyl) homoserine lactone, rhamnolipids were shown to be ineffective at inducing *E. coli* biofilm dispersal (134, 135). This suggests the biofilm matrix of *E. coli* may not be conducive to surfactant induced dispersal in the absence of other compounds. Future studies evaluating the matrix composition of *E. coli* F11 under various culture conditions would be valuable in determining the effect of Dawn and boric acid on the extracellular matrix.

After treatment for 30 minutes seven-day *E. coli* biofilms cultured in mAUM showed no remaining biofilm, as evident by statistically undetectable counts after viable cell counting after sonication. This is consistent across control and treatment conditions, in three biological replicates and with findings after treatment of three-day *E. coli* biofilms cultured in mAUM. This lends support that the lack of biofilm remaining on the silicone disk after treatment of three-day biofilms, was likely not due to growth time. It is likely that biofilm formation was prevented by a combination of growth conditions possibly including shear forces combined with reduced nutrient conditions and the silicone substrate, as discussed above. Boric acid and Dawn treatment of seven-day *P. aeruginosa* PAO1 biofilms cultured in LB broth or mAUM for 30 minutes showed no

biofilm remaining on the silicone disks as detected by sonication and viable cell counting. This was consistent across control and treatment conditions in three biologic replicates. Data indicated that *P. aeruginosa* PAO1 was able to form biofilms on silicone disks in LB broth and mAUM when grown for 72 hours (**Figure 6. B, E** and **Figure 5. B, E)**. This further indicates that the lack of biofilms remaining on silicone after treatment and sonication of seven-day biofilms was likely the result of prolonged batch culture incubation as opposed to a physical inability to form biofilms. *Pseudomonas aeruginosa* biofilm dispersal has been shown to result from glucose depletion (136), an increase in media pH, and rhamnolipid production (14, 137, 138). Rhamnolipids are synthesized by the *rhlAB* operon (18) and linked to quorum signaling (139). In mature biofilms, this quorum signaling has been found to be upregulated (14, 137), resulting in increased rhamnolipid production that is thought to be linked to biofilm dispersal (83, 140). Rhamnolipids have also been found to interfere with cell-surface interaction and thus prevent biofilm establishment (141, 142). A previous study investigating the impact of rhamnolipids on biofilm dispersal found *P. aeruginosa* PAO1 biofilms, when cultured on glass cover slips, collapse after approximately 50 hours of batch culture incubation, with no noted biofilm formation with further incubation (83). Spent media collected after 48-hour of biofilm incubation was further found to disrupt initial biofilm formation of *P. aeruginosa* PAO1, this was correlated with increased rhamnolipids in the media (83). The lack of biofilm remaining after treatment and sonication of *P. aeruginosa* biofilms cultured for seven days, may be caused by biofilm dispersal and high amounts of rhamnolipids present in media preventing further biofilm establishment. Future studies

utilizing continuous media replacement would be beneficial to investigate boric acid treatment of *P. aeruginosa* biofilms cultured for various times.

IV. CONCLUSION

Final Conclusions

The effect of boric acid treatment was found to be dependent on culture, exposure time and growth conditions. Boric acid at concentration 0.25% (w/v) showed evidence of dispersal and increased efficacy of tobramycin in the treatment of *P. aeruginosa* PAO1 biofilms, cultured in LB on silicone disks (**Figure 5. B, E**). However, these results were not consistent when used in treatment of *P. aeruginosa* PAO1 biofilms cultured in mAUM (**Figure 5. C, F**) or *E. coli* F11 biofilms cultured in LB (**Figure 5. A, D**). Dawn at a concentration of 0.005% (v/v) was ineffective at inducing biofilm dispersal of *E. coli* F11 biofilms when cultured in LB broth for 72-hours **(Figure 6. A, D**) or 7 days (**Figure 7**). It was also ineffective at inducing dispersal of *P. aeruginosa* PAO1 biofilms when cultured in LB (**Figure 6. B, E**) or mAUM (**Figure 6. C, F**) for 72-hours. However, *P. aeruginosa* biofilms cultured in LB for 72-hours did show increased susceptibility to tobramycin in combination with 0.005% (v/v) Dawn treatment (**Figure 6. B, E**). This may be due to the binding of tobramycin to eDNA while surfactants contained in Dawn denatured proteins present in the ECM, inducing a synergistic disruption of the biofilm.

One inconsistent finding was the increased efficacy of tobramycin against *P. aeruginosa* biofilms cultured in LB, when treated with 0.75% (w/v) boric acid, as 0.75% (w/v) boric acid did not exhibit any significant effects in the absence of tobramycin (**Figure 5. B, E**). There may be a dispersal effect of 0.75% (w/v) boric acid that was not reflected in statistical analysis. Alternatively, the increased effect of tobramycin in this treatment combination could possibly be due to simultaneous binding of boric acid and

tobramycin to eDNA, causing disruption of biofilm matrix at a greater extent than either compound alone.

The effect of boric acid or Dawn treatment of *E. coli* F11 cultured in mAUM on silicone disks could not be determined, due to statistically undetectable viable cell counts after treatment. This could be due to *E. coli* F11 inability to establish biofilms on this substrate. This strain was previously shown to form irreversibly attached biofilms on stainless steel and Teflon when cultured static at 30°C (92). The inability of this strain to form established biofilms on silicone is possibly a multifactored result of overall growth conditions, including the substrate. Further studies investigating the production of adhesion factors under the studied conditions and optimizing growth conditions, would be beneficial in the investigation of uropathogenic *E. coli* treatment.

The overall results of this study show boric acid to exhibit dispersal effect when used to treat monoculture *P. aeruginosa* biofilms that was not shown when used against *E. coli* biofilms (**Figure 5 B, E**).

Future Directions

Boric acid has been shown to be effective at dispersing environmental polymicrobial biofilms (81), and this study showed boric acid to have a culture dependent dispersal effect. Given these findings and the polymicrobial nature of many CAUTIs (143), it would be beneficial to evaluate the efficacy of boric acid in combination with antibiotic treatment of polymicrobial biofilms containing *P. aeruginosa* at different concentrations. To further investigate the potential of boric acid in treatment of CAUTI's, polymicrobial communities should include uropathogenic strains of *E. coli, P. aeruginosa, Klebsiella pneumoniae* and *Proteus mirabilis* given their involvement in

CAUTIs (144). During culturing, different inoculation doses of each microbe should be used to investigate the potential of interspecies competition as well as the potential variation in biofilm matrix composition.

The current methods tested the potential of boric acid and Dawn as dispersal agents against cultures grown in batch media and treatment was performed at the optimal growth temperature of the tested organisms. These conditions, while optimal for bacterial growth, do not represent CAUTI treatment. Bacterial biofilms formed on the inner lining of catheters are exposed to an influx of fresh urine as the patient's bladder is voided. The presented methods evaluated bacterial biofilms cultured in batch media, in which nutrients were not replenished. To more closely model the conditions of a CAUTI, the presented methods should be modified to incorporate continuous media replacement. This can be done using a Stovall flow cell, with the flow rate set to a rate that is consistent with the average void rate of a catheterized bladder. Modeling biofilm formation can be done using the flow cell to introduce fresh, uninoculated media into a flask containing silicone disks as described in the present methods. Alternatively, inoculated media can be filtered through the flow system and allowed to drain into a collection flask. This model would promote biofilm formation in the inner lining of the silicone tubing, similar to that of urinary catheters. Following biofilm culturing time, the inoculated media can be replaced with treatment solution and filtered through the tubing. This method would potentially model treatment in a more appropriate manner than was done in the present study, as this could account for the sheer force of washing the catheter tubing in addition to the treatment solution. Treatment temperature should also be investigated. The present study treated cultured biofilms at 37°C, the optimal growth

temperature for *E. coli* and *P. aeruginosa,* and the core human body temperature. Catheter tubing is largely external and subjected to ambient temperatures. Modeling biofilm treatment at various temperatures (potentially 37°C, 35°C, 30°C) would provide additional information on the potential treatment efficacy of antibiotic treatment in combination with boric acid and Dawn.

Based on previous findings of boric acid binding to calf thymus DNA (82), we hypothesize a similar mechanism of action, in boric acid binds to the eDNA of the biofilm matrix. If this is correct, the variation in boric acid treatment of *E. coli* and *P. aeruginosa* biofilms may be due to differences in eDNA concentration in the matrix. Dawn is a surfactant mixture containing SDS, and other anionic surfactants. We hypothesize a potential mechanism of action associated with Dawn induced biofilm disruption to involve binding to extracellular proteins in the biofilm matrix. These proposed mechanisms should be further studied utilizing confocal microscopy. Concanavalin A is available commercially pre-conjugated to a variety of fluorophores and can allow visualization of the *P. aeruginosa* biofilm matrix using confocal microscopy, as this will bind to mannose subunits of the Psl component of the *P. aeruginosa* biofilm matrix (30, 145). This staining technique has been successfully utilized for visualization of this strain of *P. aeruginosa* PAO1 cultured in mAUM, in prior studies (92). *P. aeruginosa* PAO1 contains a GFP insertion (91) and will be excited at 488 nm, further allowing conclusions to be made regarding bacterial cell presence and density. The strain of *E. coli* F11 used in this studies carries *mCherry* under control of a constitutive promoter which was inserted into the *lacZ* gene of the lac operon (92). The expression of *mCherry* can provide further insight into the effect of boric acid and Dawn

treatment of *E. coli* F11 by allowing visualization of bacterial cell presence and cell density. Previous studies using mAUM-cultured biofilms of the modified *E. coli* F11 strain used in this study, showed evidence of cellulose in the *E. coli* biofilm matrix by utilization of calcofluor white matrix staining (92). The mCherry excitation peak is 585 nm and emission peak at 610 nm (146, 147) while calcofluor white excitation peak 340 nm and emission peak 432 nm (148).Given the large difference in excitation and emission peaks of these fluorescent molecules, utilization of these fluorescent molecules concurrently will allow for visualization of viable *E. coli* cells as well as biofilm matrix and allow for further investigation of the proposed dispersal mechanisms. In combination with microscopy, further evaluation of c-di-GMP concentration in the substrate of biofilms treated with boric acid or Dawn would aid in confirming a dispersal effect of boric acid.

The results of this study do not indicate a statistically significant dispersal effect of Dawn Dish Soap under the tested conditions. However, when used in combination with Dawn the efficacy of tobramycin, against *P. aeruginosa* cultured in LB broth, was improved. Follow up microscopical investigation of this effect would provide valuable insight into the treatment of silicone adhered biofilms. Further research into the potential of Dawn Dish Soap as a dispersal agent of silicone adhered biofilms should be aimed at higher concentrations of Dawn. This would, result in an overall increase in the concentration of surfactants used in treatment. It would also be interesting to include treatment with cationic surfactants or salts, such as sodium chloride, to evaluate potential charge-charge interactions between components of the ECM, such as negatively charged DNA, and treatment compounds.

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