INFLUENCE OF INDOLE AND MIXED CULTURE GROWTH ON *PSEUDOMONAS AERUGINOSA* BIOFILM STRUCTURE

by

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DEDICATION

I would like to dedicate this work to the late James Daniel Koonce Jr. for his love of education, people, and most importantly life. You inspire me daily, and helped me in understanding that each day is a gift and should be lived inspiringly.

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ABSTRACT

When microorganisms are studied within their natural environments, they are not commonly found as single species. Microbes are usually found as clusters or multitudes of aggregates within surface-attached biofilm communities. The discovery of this sessile lifestyle has had a resounding effect on the scientific community and the approach toward studying anti-microbial resistance (AMR). Past studies showed that quorum sensing (QS) mutants of *Pseudomonas aeruginosa,* unable to produce N-acylated homoserine lactone (AHL) signals had altered biofilm structure. During mixed culture growth of *P. aeruginosa* with *Escherichia coli,* we found that *E. coli* production of the organic compound, indole, inhibited AHL-regulated genes in *P. aeruginosa.* The current study was conducted to see if mixed culture growth of *P. aeruginosa* with indole-producing *E. coli* affected biofilm structure. Two *wt Pseudomonas aeruginosa* strains, PA14 and PAO1 were co-cultured with *wt Escherichia coli* BW25113 (indole producing), and *E. coli* tryptophanase mutant *tnaA* (non-indole producing). Comparably, exogenous indole was added in multiple concentrations (mM) in an effort to monitor the response of the *P. aeruginosa* strains. *P. aeruginosa pqs* mutant strains where also studied to see if indole induced similar effects in a non-AHL mediated QS system. At lower concentrations (0.1mM), indole induced a spike in growth rates and genes geared toward virulence mechanism of *P. aeruginosa*. In contrast, higher concentrations (0.5, 1mM), overall

induced downregulation of virulence mechanisms controlling pyocyanin, rhamnolipids, and elastase production, inhibiting quorum sensing.

I. INTRODUCTION

In nature, bacteria generally possess two modes of existence, planktonic which are mobile, freely moving cells and sessile, which are polymicrobial (multi-species) communities termed biofilms. A wide variety of bacteria grow into polymicrobial biofilms in equally diverse environments, including the human body, and can play a role in human disease. Biofilms can form on biotic or abiotic surfaces, subject to the presence of the correct environmental signals (Goller & Romeo, 2008). The formation of a microbial biofilm is an endless cycle, in which organized communities of bacteria are encased in a matrix of extracellular polymeric substances (EPS) that hold microbial cells together to a surface (Rasamiravaka, Labtani, & Duez, 2014). By including multiple bacterial and/or fungal species in a single community, biofilms obtain numerous advantages, such as passive resistance, metabolic cooperation, byproduct influence, quorum sensing systems (QS), an enlarged gene pool with more efficient DNA sharing, and many other synergies, which give them a competitive advantage (Wolcott, Costerton, Raoult, & Cutler, 2012). Bacterial adhesion is one of the most important steps during biofilm formation. This process is influenced by several factors, such as temperature

and ph., it is important to highlight the chemical composition, hydrophobicity, electrostatic charge, and surface roughness (Pereira, Bonatto, Lopes, Periera, & Silva, 2015).

These environmental factors are also dependent upon the growth (phase) rate of the planktonic bacterial culture. When these two factors are combined, the planktonic culture reaching a certain growth threshold, and being presented the proper environmental cues, then the adhesion process begins. The transition from the bacterial planktonic single-cell state to the biofilm state is dependent on the production of adhesins and extracellular matrix components by the bacteria. Microscopy and biochemical studies (reviewed in Costerton et al., 1987) showed biofilms to consist of surface-attached microbial communities surrounded by extracellular polymeric substance (EPS) containing polysaccharides, and other substances such as DNA and proteins. The cell density within mature biofilms is quite high.

Given the high cell density within biofilms it is not surprising that QS also affects biofilm formation. QS was first described in the bioluminescent bacterium, *Vibrio fischeri*. The N-acylated homoserine lactone (AHL) signal is produced by an AHL-synthase, encoded by *luxI* gene, which can diffuse through the cell membrane. Above a population threshold, the AHL signal reenters the cell and interacts with the *luxR* gene product, which encodes a transcriptional regulator. AHL-based QS is common in the Proteobacteria and the genes associated with this feature are referred to as *luxI* and *luxR* homologs (reviewed in Ng and Bassler, 2009). One of the most widely researched model organisms known for its biofilm mode of existence is *Pseudomonas aeruginosa*. Due to its relatively large genome and flexible metabolic capabilities, this organism exploits

numerous environmental niches. *P. aeruginosa* is an opportunistic pathogen that can set upon the human host when the normal immune defenses are disabled (Mulcahy, Isabella, & Lewis, 2014). This organism is also known to be an important human, animal, and plant pathogen that produces several virulence factors. Its QS systems are presumably the best characterized among Gram-negative bacteria (Rasamiravaka, et al., 2015). The discovery that *P. aeruginosa* produces at least two extracellular signals involved in cell-to-cell communication and cell density dependent expression of many secreted virulence factors suggests cell-to-cell signaling could be involved in the differentiation of *P. aeruginosa* biofilms, much as cell-to-cell signaling is involved in the development of specialized structures (Davies, et al., 1998). *P. aeruginosa* QS consists of two acyl homoserine lactone based systems, Las and Rhl, encoded by the *luxI* and *luxR* homologs (*lasI, lasR, rhlI,* and *rhlR,* and a quinolone based system, PQS, encoded by the *pqs* operon. In 1998, Davies et al. found that the complex biofilm structures in *Pseudomonas aeruginosa* were altered in *lasI* mutants, which were unable to synthesize 3-oxo-dodecanoyl homoserine lactone (3-o-C12 HSL) signal, but their structure in *lasI* mutants could be restored if 3-o-C12 HSL was restored (Mashburn-Warren & Whiteley, 2006) (Schuster, Sexton, Diggle, & Greenberg, 2013).

The Rhl QS regulates rhamnolipid production while the PQS regulates the generation of the eDNA matrix (Rybtke, Hultqvist, Givskov, & Tolker-Nielsen, 2015). Rhamnolipids are well-known biosurfactants produced as one of many virulence factors of *Pseudomonas* species, produced predominantly by *P. aeruginosa* (Pinzon & Ju, 2009). It is one of the key contributors towards the adhesive lifestyle phase of *Pseudomonas* biofilms. Rhamnolipids are essential for swarming motility, biofilm formation

(structure), and act as hemolysins. They enhance the uptake of hydrophobic substrates and play a role in shielding *P. aeruginosa* cells from host defense (Wittgens, et al., 2016). A secondary messenger, cyclic diguanosine-5'-monophosphate (c-di-GMP), which is a general key regulator of the bacterial biofilm lifecycle. High levels of c-di-GMP induce the production of adhesins and extracellular matrix components which enable bacteria to form biofilms, low c-di-GMP levels downregulate the production of adhesins and extracellular matrix components and lead biofilm bacteria into dispersal to undertake a planktonic mode of growth (Fazil, et al., 2014).

There are numerous studies focusing on methods toward disruption of the QS system of *P. aeruginosa* instead of solely focusing on individual virulence factors in order to disintegrate biofilm formation through structural disruption. The hemi synthetic compound azithromycin (AZM, a macrolide antibiotic) was found to present both QS and biofilm inhibitory effects in *P. aeruginosa* when used at sub-bactericidal concentration [2.67 µM] (Rasamiravaka, et al., 2015). A number of natural compounds were shown to affect QS-controlled gene expression in *P. aeruginosa* and to reduce biofilm production; these include, among recently reported, 6-gingerol [at 10µM], eugenol from clove [at 50µM], ajoene [at 100µM], S-phenyl-L-cysteine sulfoxide [at 1µM], and some flavonoids (the flavan-3-ol catechin) [at 4mM] and naringenin [at 4mM] (Rasamiravaka, et al., 2015). Another well-known QS disruptive molecule that is produced naturally by many pathogenic bacterial species, both Gram-negative and Gram-positive, is indole. For indole producing bacteria, the primary resource for indole production is the amino acid tryptophan. An enzyme tryptophanase encoded by gene *tna*A reduces tryptophan to pyruvate, ammonia, and indole in a reversible reaction (Hu, Zhang, Mu, Shen, & Feng,

2011). Indole is excreted as a byproduct when tryptophan is reduced through a reductive deamination process.

When environmental cues are conducive for bacterial aggregation, the biofilm commonly consists of multiple species. Within these multi-species biofilms, a hierarchy is developed through competition for available resources. *P. aeruginosa* occupies different positions within the community in comparison with other Gram-negative bacteria, Gram-positive bacteria, and fungi. The incubation of *P. aeruginosa* planktonic cells with *Roseobacter dentrificans* (Gram-negative) decreased the expression of pyocyanin and thiosulfate biosynthesis genes. In addition, *P. aeruginosa* and *Candida* cells in a mixed multi-species environment mutually suppress biofilm development in general (Kuznetsova, Maslennikova, Karpunina, Nesterova, & Demakov, 2013). One particular microorganism commonly found in several niches with *P. aeruginosa* is *E. coli*. These two species can be found in low-nutrient environments, such as drinking water, groundwater, surface water, and wastewater, and on abiotic surfaces such as medical devices, urinary tract, and wounds. Areas of interest include, multi-specie environments (biofilms), quorum sensing (QS), and natural processes that lead to disruption of the QS systems, Quorum sensing Inhibition (QSI). A study in (2011) found that when *P. aeruginosa* is co-cultured with *E. coli*, indole affects certain quorum sensing virulence factors by down regulating them. They measured pyocyanin production by *P. aeruginosa* in pure and mixed cultures and found that under all conditions tested, pyocyanin production was abolished in the presence of indole (Chu, et al., 2012). Based on these and other findings we believe that indole produced during microbial competition could potentially influence the biofilm structure of *P. aeruginosa* analogous to the effects

seen in *P. aeruginosa lasI* strains (Davies et al., 1998). Regulation of the virulence factors elastase (lasB), rhamnolipids, and pyocyanin are controlled by the hierarchically organized systems, *las*, *rhl*, and *pqs*. However, the *las* system initiates both other QS systems (*rhl*, and *pqs*) for *P. aeruginosa* (Schaadt, Steinbach, Hartmann, & Helms, Rule-based regulatory and metabolic model for Quorum sensing in P. aeruginosa, 2013). The microbial world is a vast, ever-changing community of multi-species environments where the primary mode of existence is within a multi-cellular biofilm. A biofilm is a highly complex structure comprised of a self-induced extracellular matrix. In order to switch from a free-floating planktonic lifestyle to the communal biofilm lifestyle requires a change in the bacteria in order to initiate the production of adhesins and extracellular matrix compounds which interconnect them in the biofilm. This creates a type of structural scaffolding ideal for cell to cell communication (Whiteley, Mukherjee, & Ghannoum, 2015). Quorum sensing (cell-to-cell) communication can be advantageous, particularly in the contexts of sex, niche adaptation, and production of secondary metabolites for growth and defense purposes, which would also be beneficial for survival amongst all species involved creating mutualistic symbiotic relationships within the biofilm as well as with its host.

Microbial biofilms can be found nearly everywhere in nature and can adhere to nearly any surface if provided the proper environmental conditions and have often been a major topic of discussion, especially within the medical community. For example, formation of biofilms on medical devices, such as catheters, or implants often result in difficult to treat chronic infections. Some infections are associated with biofilm formations on human surfaces such as teeth, skin, and the urinary tract (Lopez, Vlamakis,

& Kolter, 2010). A more notable biofilm formation associated chronic infection amongst humans is cystic fibrosis. It has been well documented that the cystic fibrosis airway is a polymicrobial environment primarily dominated by *Pseudomonas aeruginosa*. However, a recent study discovered that an oral commensal *Streptococci* exploits the production of *P. aeruginosa* exopolysaccharide in order to enhance biofilm formation while simultaneously restricting biofilm formation by *P. aeruginosa* (Scoffield, Duan, Zhu, & Wu, 2017).

These adaptations and restrictions are managed through cell-to-cell communication (quorum sensing) within the structural matrix (scaffolding) of the biofilm. Quorum sensing is an integral component of the global gene regulatory networks which are responsible for facilitating adaptation to environmental stress. This communication is correlated through small diffusible molecules called autoinducers or "pheromones". In contrast to hormones, pheromones are secreted outside the producer organism and facilitate communication between individual organisms (Williams, Winzer, Chan, & Camara, 2007). The signal molecules most often used by Gram-negative bacteria are N-acylhomoserine lactones (AHLs).

Various pathogenic Gram-negative bacteria produce virulence factors, biofilm formation and maturation which are (co-)regulated by QS (Christiaen, et al., 2014). A prime exemplary Gram-negative pathogenic organism is *Pseudomonas aeruginosa*. *P. aeruginosa* uses two hierarchical QS regulatory systems (Las and Rhl) to regulate the expression of many of its virulence factors, including elastase, proteases, exotoxin A, pyocyanin, and siderophores (Dekimpe & Deziel, 2009).

Disruptive mechanistic approaches towards cell-to-cell communication becomes a viable concept in creating changes within the structural integrity of multi-specie biofilm formations. This approach has been termed "quorum signal inhibition" (QSI), which focuses more on the reduction of virulence factors rather than the killing of the targeted microorganism. Numerous QSI agents have been discovered with the antibiotic azithromycin being the most commonly used one amongst them. This antibiotic was shown to inhibit important QS targets in *Pseudomonas aeruginosa* such as *lasl* and *rhlA*. Other key agents used include tannic acid, iberin, 14-alpha-lipoyl andrographolide and indole (Perez-Perez, Jorge, Rodriquez Perez, Pereira Olivia, & Lourenco, 2017). A 2015 study found that an enzyme, acylase was able to degrade several AHL model compounds. Among them, 3-oxo-C12-L-HSL was of main importance as the signal was found to play a key role in *P. aeruginosa* biofilm formation, one of the main UTI causing pathogens in catheterized hospital patients (Ivanova, Fernandes, Mendoza, & Tzanov, 2015).

II. METHODOLOGY

2. 1 Bacterial strains, media, chemicals

Bacterial strains used in this study are found in Table 1. The strains were maintained in Luria-Bertani (LB) medium. For long-term preservation, liquid cultures were frozen at -80°C using glycerol (12.5 % v/v) as a cryoprotectant. Prior to experimentation, cultures were revived from frozen stocks, and cultured overnight in LB agar. The cultures were then subcultured in LB broth from LB plates and incubated overnight at 37°C with aeration (100 rpm). Unless stated otherwise, during experimentation the turbidity of the overnight cultures was adjusted to OD_{600} of 0.1 [$1.1 \pm 0.2 \times 10^5$ CFU/ml] using sterile LB broth. During mixed culture experiments, we used LB plates with ampicillin (100 μg/ml) to select for *P. aeruginosa* or LB plates with cefsulodin (20 μg/ml) to select for *E. coli* (Chu, et al., 2012). Stock solutions of Kovacs reagent and indole (Alfa Aesar) were prepared in deionized water and in dimethyl formamide (DMF), respectively. All stock solutions were filter sterilized using 0.22-μm-poresize sterile filters and stored at 4°C in 15-50 ml conical falcon tubes.

Rhamnolipid medium: This medium required glucose or 20g glycerol, 0.7g KH_2HPO_4, 0.9g Na_2HPO_4, 2g $NaNO_3$, 0.4g $MgSO_4 \cdot H_2O$, 0.1g $CaCl_2 \cdot 2H_2O$, per 1L with 2ml of trace element solution (2g $FeSO_4 \cdot 7H_2O$, 1.5g $MnSO_4 \cdot H_2O$ and 0.6g $(NH_4)6Mo_7O_24 \cdot 4H_2O$) mixed in. In order to complete the (Sigmund and Wagner) SW agar 0.2g cetyl trimethylammonium bromide (CTAB), 0.005g Methylene Blue (MB) and 12g agar was added to 1L of the above protocol (Pinzon & Ju, 2009). Three-wells were then molded into the agar using the tip of a sterilized 5mL Eppendorf pipette. SW agar was stored at 4°C until used.

Molecular Probes: An Invitrogen labeling and detecting LIVE/DEAD® BacLight™ Bacterial Viability Kit was used to monitor the viability of bacterial biofilm populations as a function of the membrane integrity of the cell. The bacterial viability kit manufacturer instructions were used as the protocol for the staining procedure.

2.2 Competitive Experiments

2.2.1 Competitiveness of indole and *E.coli BW25113 strains*

We investigated the competitiveness of several *P. aeruginosa* strains during growth in mixed culture with *E. coli BW25113,*and *E. coli BW25113* tryptophanase *tnaA* mutant. Cells were revived from frozen stock cultures, subcultured, and the OD600 adjusted to 0.1 as previously described. For mixed culture analysis, 100 µL inocula from each strain of *P. aeruginosa* were mixed with 100 µL of *E. coli* in 50 ml sterile LB broth. For biofilm analysis, eight sterile silicon discs (7 mm diameter) were placed into the flasks as biofilm colonization substrata. The experiment was conducted for 72 h at 37 °C with a shaking speed of 150 rpm. Every 24 h, 500µL (for the planktonic) and a pair of silicon discs (for the biofilm) were taken for bacterial and chemical analyses. The sonication and serial dilution protocol for biofilm analysis and dilution plating protocol for planktonic populations are described previously (Chu, et al., 2012). In addition to the mixed culture analysis, pure culture experiments for each strain were also conducted as controls. Chemical analysis is described below. Each experiment was replicated a minimum of three times.

2.2.2 Exogenous addition of indole

To determine whether exogenous addition of indole gave *E. coli* a benefit in overall competitiveness of the mutant strains of *P. aeruginosa* in mixed culture growth, indole from a 100mM stock solution was added to the culture medium at different concentrations (0.1, 0.5 and 1mM), and the competition experiments were conducted as described in section (2.1).

2.3 Indole Assay

2.3.1 Degradation (Wild type and Mutant strains)

P. aeruginosa wt and mutants were subcultured and adjusted to a 0.1 (OD_{600}) concentration as previously described. Cultures were grown at 37°C in 40mL LB shaking at 150 rpm. 1ml samples were extracted every 24 hrs up to 72 hrs and added to a falcon tube and centrifuged for 15 min at 3000 rpm at 4°C. Supernatant was extracted and added to a separate falcon tube and 400µl of Kovacs reagent (Hidalgo-Romano et al., 2014) was added and measured at OD540.

2.3.2 Exogenous addition of indole

To investigate whether addition of exogenous indole affected the competitiveness of *P. aeruginosa* wild-type and mutant strains in mixed culture growth, indole was added to the culture medium at different concentrations (0.1, 0.5, and 1mM) and experiments conducted as described in section (2.1).

2.4 Pyocyanin assay

2.4.1 Pyocyanin extraction

Prior to experimentation, cells were subcultured from frozen stock cultures. *P. aeruginosa* cultures were extracted whole with an equal volume of chloroform. Extractions were vortexed profusely for 1 min, and allowed to sit for 5 min in order for aqueous layers to separate. Chloroform (bottom) layer was then extracted with a half a volume of .1N HCl and placed in a 15 ml falcon tube and centrifuged at 13000 rpm for 10 min. Aqueous (middle) layer was extracted three-times and absorbance of 520nm determined.

2.4.2 Exogenous addition of indole

To investigate whether exogenous addition of indole had an effect on the QS virulence factor of *P. aeruginosa* in mixed culture growth, indole was added to the culture medium at a concentration of (0.5mM) and experiments conducted as described in section (2.1).

2.5 Rhamnolipid assay

2.5.1 Rhamnolipid test

P. aeruginosa and *E. coli* strains were subcultured in LB medium and adjusted to 0.1 (OD_{600}) concentration. After 24 h, cultures were centrifuged for 10 min at 1200 rpm. Supernatant was extracted and filtered (0.22µm pore-size filter) and added to a sterile 15 ml falcon conical tube. Ten µl of filtrate was added into each well on the SW agar medium. The plates were incubated for 48 h at 37°C and stored in at 4ºC for 48 h. Cold storage darkens the blue color making rings more visible (Pearson, Pesci, & Iglewski, 1997) (Pinzon & Ju, 2009).

2.5.2 Exogenous addition of indole

This procedure was conducted in order to see if the addition of exogenous indole had an effect on the QS (*rhl*) system responsible for rhamnolipid production in *P. aeruginosa* during mixed culture growth. Indole from a 100mM stock solution was added to the culture medium at a concentration of (0.1 and 0.5mM).

2.6 Elastase assay

2.6.1 Elastolysis test

Elastolytic activity in *P. aeruginosa* culture fluids was determined by a dye-release, elastin Congo red (ECR) assay (Pearson, Pesci, & Iglewski, 1997), with modifications. Briefly, cells from mid-log phase cultures grown in LB were washed and resuspended in LB to an OD_{600} of 0.05. After 21 h at 37°C shaking at 150 rpm culture supernatants were filtered (0.22-µm pore-size filter) and stored at -80°C. Triplicate 50-µl samples of culture filtrates were added to tubes which contained 20 mg of ECR (Sigma) and 1 ml of buffer (0.1 M Tris [pH7.2], 1mM $CaCl_2$). Tubes were incubated 18 h at 37°C with rotation and then were placed on ice 0.1 ml of 0.12 EDTA. Insoluble ECR was removed by centrifugation, and the OD_{495} determined. (Pearson, Pesci, & Iglewski, 1997).

2.6.2 Exogenous addition of indole

To investigate whether exogenous addition of indole had an effect on the QS virulence factor of *P. aeruginosa* in mixed culture growth, Indole from a 100mM stock solution was added to the culture medium at a concentration of (0.1 and 0.5mM).

2.7 Confocal and fluorescent microscopy

2.7.1 Confocal and fluorescent microscopy on biofilms

P. aeruginosa wt and mutants were sub-cultured. Cultures were adjusted and inoculated in 10 ml LB in a sterile petri-dish containing a sterile glass microscope slide. Inoculated sterile glass petri-dishes were then grown at 37°C rotating at 50 rpm for 24, 36, and 48 h (Fig 9A). The glass slides were aseptically removed and rinsed gently with 10 mM phosphate buffer solution (PBS). Next the slides were stained using LIVE/DEAD

Baclight viability kit. 100 µl of a 1:1 ratio of component A (Syto 9) and component B (Propidium iodine) both diluted 2X was added to the slides and allowed to site for 15 min in the dark at room temperature. Slides were gently rinsed once more in 10 mM PBS and 5 µl 90% glycerol added and covered with a glass cover slip and adhered by nail enamel (Fig 9B). Slides were then viewed using confocal laser scanning microscopy (SCLM), images will be assessed using COMSTAT software, a program for quantification of three-dimensional biofilm. Confocal microscopy assays were conducted in order to assess if indole is a causative factor affecting *P. aeruginosa* biofilm structural integrity.

2.7.2 Exogenous addition of indole

To investigate whether exogenous addition of indole had an effect on *P. aeruginosa* biofilm structure in mixed culture growth, indole was added to the culture medium at a concentration of (0.5, 1.0 and 1.5 mM).

III. RESULTS

3.1 Competitiveness of *P. aeruginosa* wt strains PA14 and PAO1 in mixed culture

3.1.1 Competitiveness in mixed culture with *E. coli* wt strain BW 25113

Two *P. aeruginosa wt* strains PA14 and PAO1 were each individually combined with *E. coli* BW 25113 to determine if indole being naturally produced by *wt E. coli* had an effect on the QS systems of *P. aeruginosa*. Both the planktonic culture and biofilm cultures were monitored and bacterial counts recorded. When PA14 and BW25113 are combined, both organisms show an initial increase in growth rates in planktonic (Fig 1A) and biofilm (Fig 2A) populations. PAO1 combined with BW 25113 also yielded similar results in both planktonic (Fig 1B) and biofilm (Fig 2B) populations. Both PA14, PAO1, and BW 25113 all seemed to benefit from the presence of one another by displaying initial increases in growth rates (population numbers) when compared to the pure culture growth rates. However, *Pseudomonas* consistently remained dominant while in competition for resources. BW 25113 had a slight advantage was when combined with PAO1 in biofilm (Fig 2B) culture.

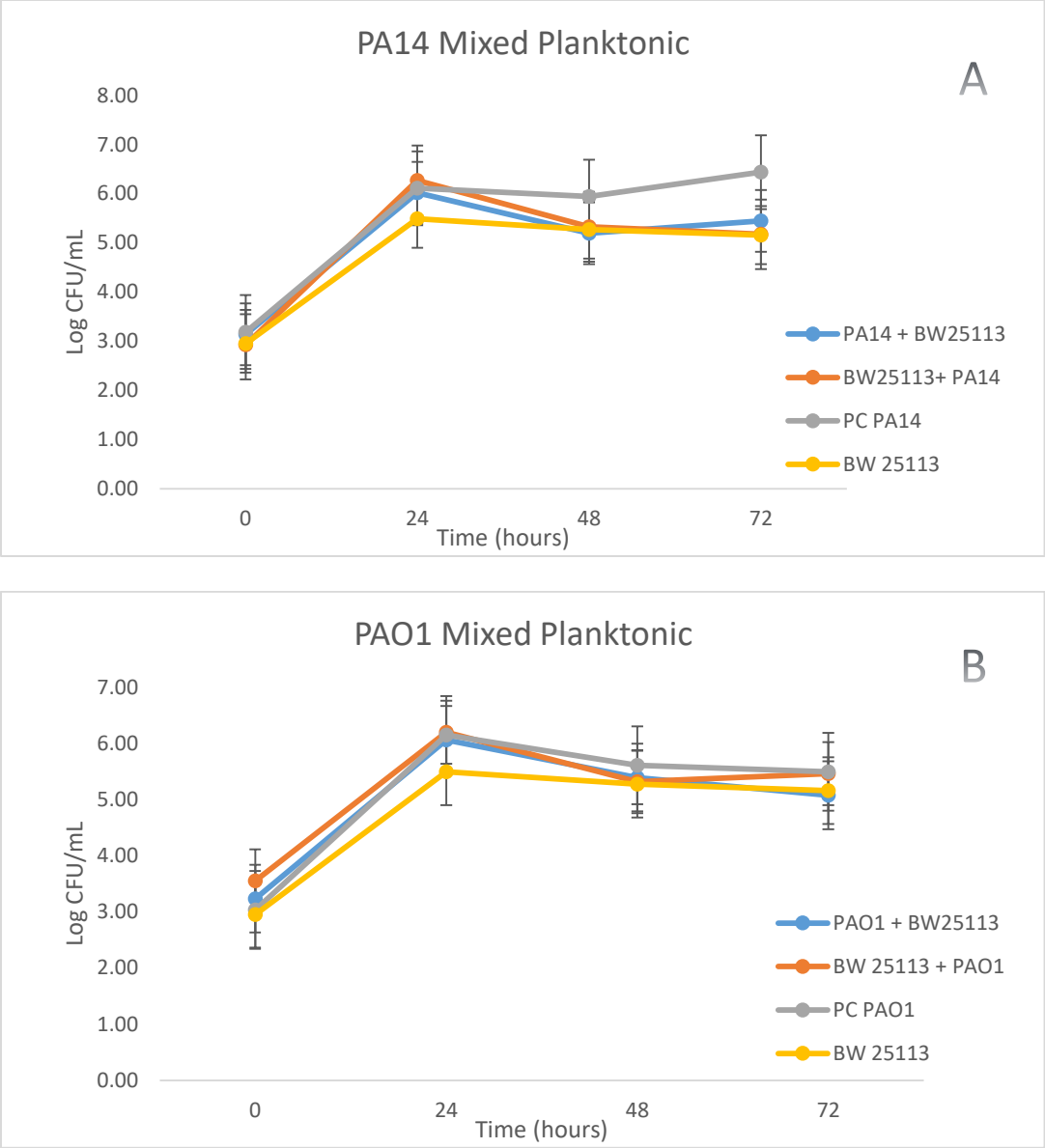
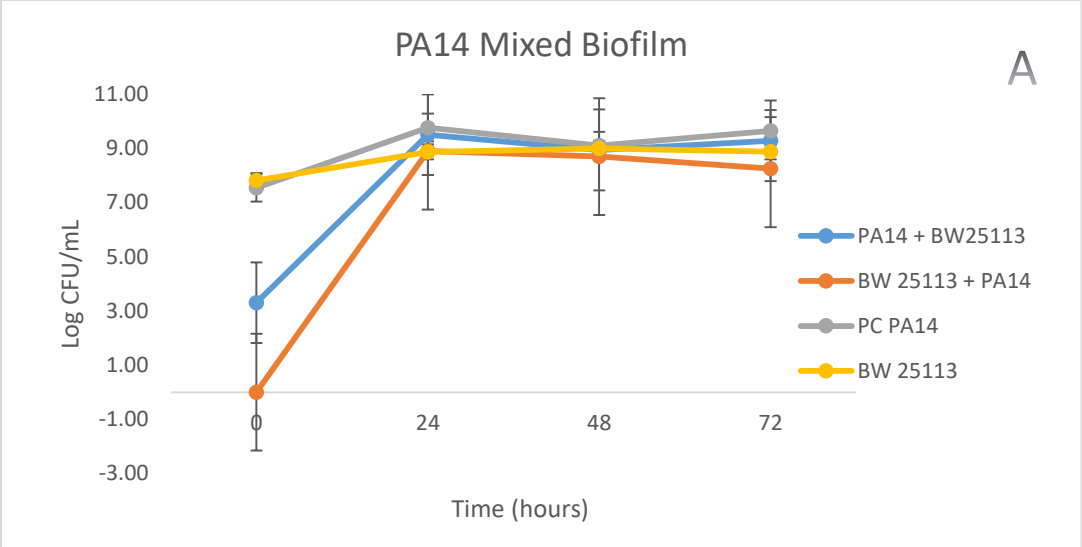


Figure 1 – Influence of *E. coli* BW 25113 grown in planktonic co-culture with *P. aeruginosa* PA14 (Fig 1A). Influence of *E. coli* BW 25113 grown in planktonic co-culture with *P. aeruginosa* PAO1 (Fig 1B).



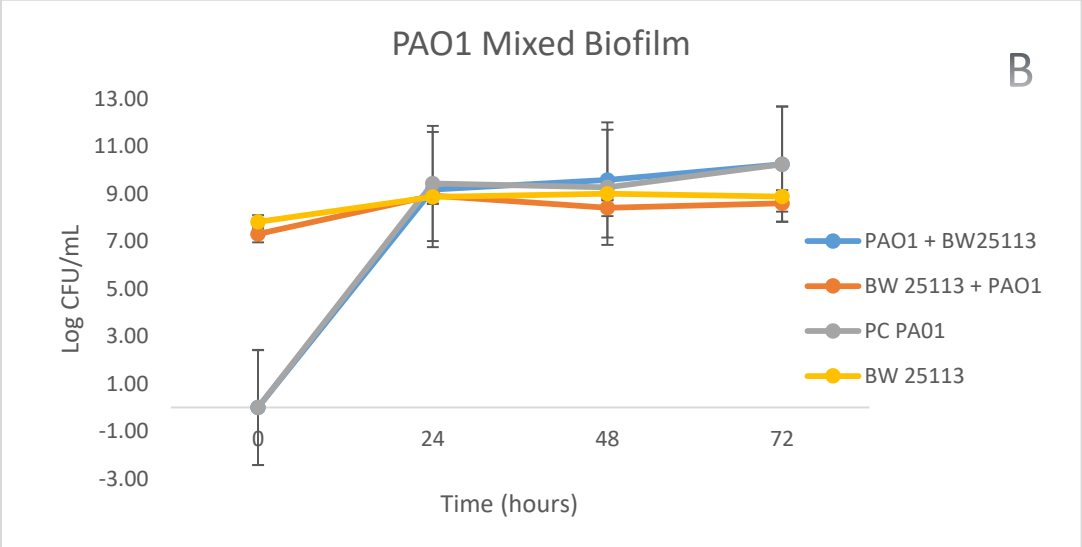
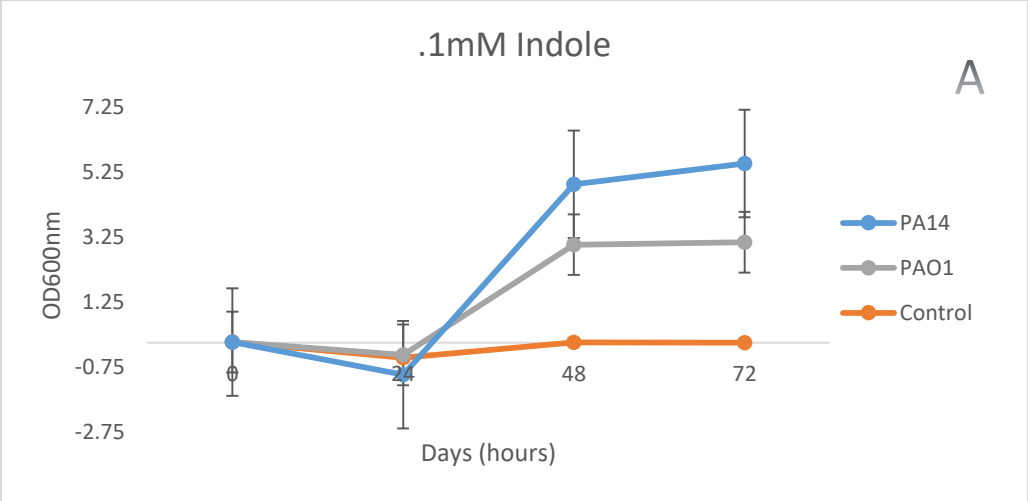


Figure – 2 Influence of *E. coli* BW 25113 co-culture biofilm with *P. aeruginosa* PA14 (Fig 2A). Influence of *E. coli* BW 25113 grown in co-culture biofilm with *P. aeruginosa* PAO1 (Fig 2B).

3.2 Competitiveness of *P. aeruginosa wt* Strains PA14 and PAO1 against Indole

3.2.1 Competitiveness with the addition of exogenous indole

To test the effect indole created in co-cultures with PA14 and PAO1 exogenous indole was added to cultures of the wild-type *P. aeruginosa* strains. Figure 3, (Fig 3A) PA14 and PAO1 *wt* strains in the presence of 0.1mM exogenous indole showed an increased rate of growth after a 24 hour time period, results demonstrated both *wt* strains were able to degrade indole based on the population recovery after 48 h. These results are because *P. aeruginosa* metabolizes indole as a source of energy or that the presence of the chemical compound could influence upregulation of virulence factors in both PA *wt* strains increasing competitiveness. Adversely, when the concentration of indole is increased to 0.5mM (Fig 3B) both PA *wt* strains showed a decrease in growth rates before showing a recovery in the population, this concentration seemed to produce a more normalized response. The results closely resemble or mimic the amount of natural indole produced when co-cultured with BW 25113 (Chu, et al., 2012).



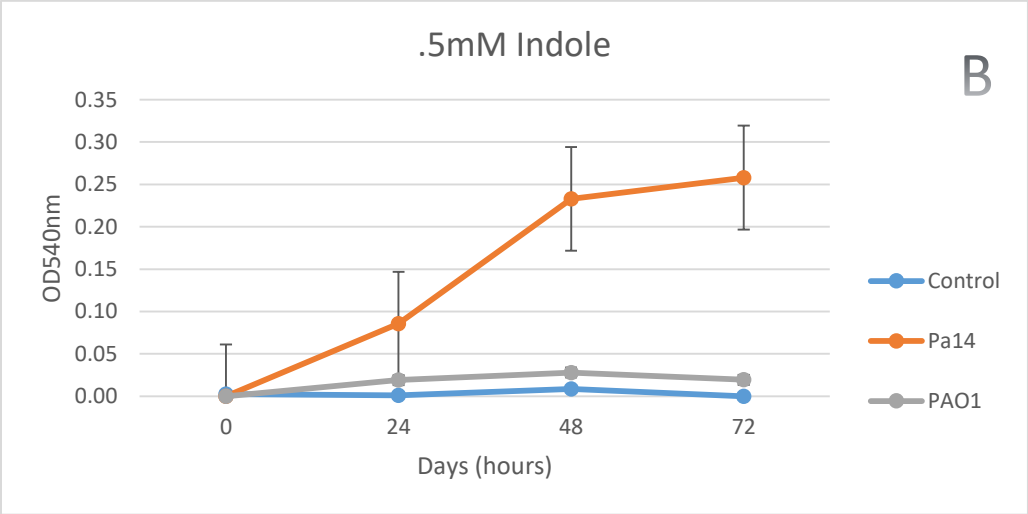
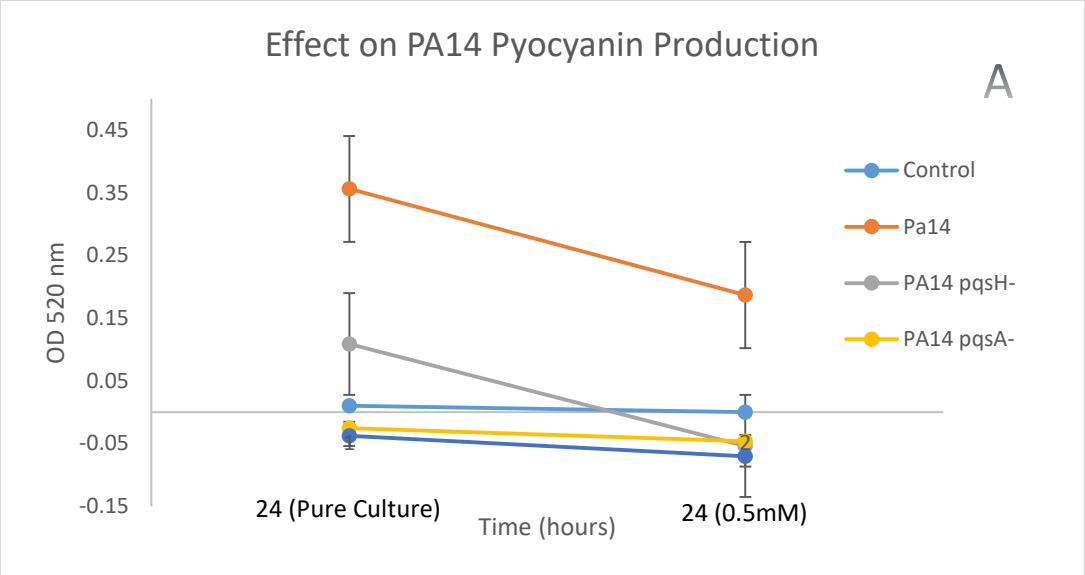


Figure 3 – Influence of 0.1mM concentration of exogenous indole on *P. aeruginosa* PA14 and PAO1 in planktonic culture (A). Influence of 0.5mM concentration of exogenous indole on PA14 and PA01 in planktonic culture (B).

3.3 Pyocyanin Assay

3.3.1 Effect on pyocyanin production with the addition of exogenous indole

Based on the results in sections 3.1 and 3.2, the presence of indole at lower concentrations generated an increase in growth rates while higher concentrations demonstrated a decrease in population growth until the population was able to recover after 48 h. Previous studies determined that indole was able to downregulate production of pyocyanin, both the *wt* and *Pseudomonas* quinolone signal (PQS) strains of PA14 and PAO1 were tested (Fig 4) in order to determine the effect (0.5mM) normalized concentration of indole had toward downregulating pyocyanin production. Two sets of *Pseudomonas* (pure culture and 0.5mM exogenous indole) were grown for 24 hours, then the pyocyanin extraction procedure, section 2.4.1, was conducted in order to obtain sample measurements. A significant decrease in pyocyanin production occurred in *wt* PA14 when indole was present along with PQS strain PA14 *pqsH*- (Fig 4A). Both *wt* PAO1 and PQS mutant PAO1 *pqsH*- also resulted in a significant decrease in pyocyanin production when indole is present (Fig 4B).



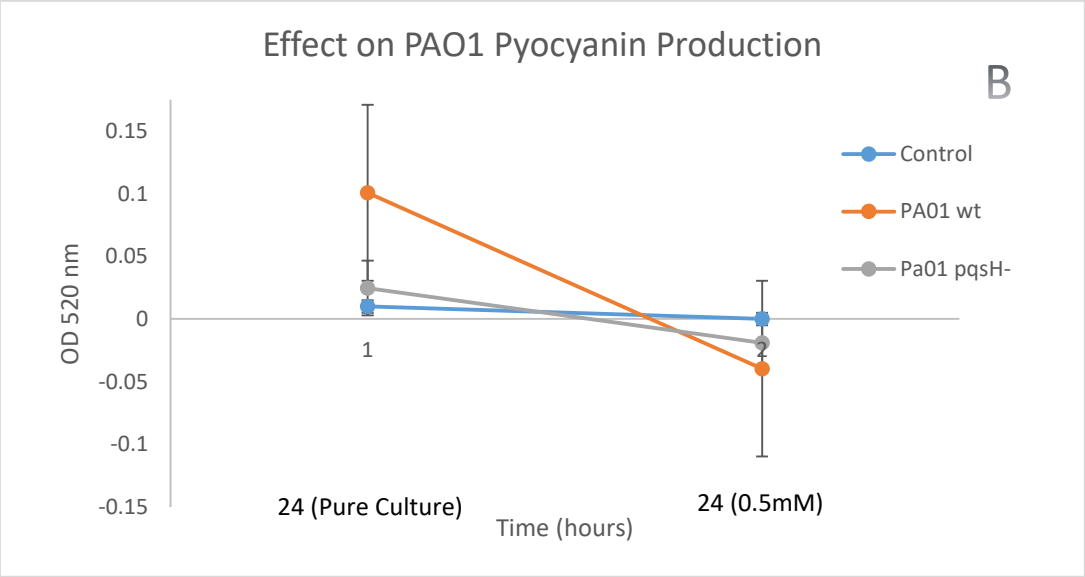


Figure 4 – Influence of 0.5mM exogenous indole on pyocyanin production of *Pseudomonas aeruginosa wt* PA14, PAO1 and QS mutant strains. All strains showed a decrease in pyocyanin production over a 24 hour period. PA14 and the PQS mutant strains all showed a decrease in pyocyanin production (Fig 4A). PAO1 and the PQS mutant strain also showed a decrease in pyocyanin production (Fig 4B).

3.4 Rhamnolipid Test

3.4.1 Effect on rhamnolipid production with the addition of exogenous indole

The effect of multiple concentrations of exogenous indole on rhamnolipid production in *wt* PA14 and PAO1, and mutant strains were tested (Figures 6, 7). The inner most ring, the bluest halo was measured (Blue layer area) and the combination of all rings present was measures as the total (Total area) (Fig 5). PA14 *wt* showed parallel increases in rhamnolipid production when exposed to both 0.1mM and 0.5mM concentrations of indole (Fig 6A). Whereas PAO1 *wt* showed a slight increase in production when exposed to 0.1mM of indole and then a slight decrease with 0.5mM added (Fig 6B). Overall, both PA14 and PAO1 *wt* yielded an upregulation in production of rhamnolipids. *PA14* mutants *pqsA*- and *pqsA-/H*- both yielded an upregulation in production with 0.1mM indole exposure and a moderate downregulation at 0.5mM (Fig 7A). The *pqsH*- mutant was variable between the inner blue layer versus the total area when exposed to 0.1mM indole. However, unlike the other mutants, *pqsH*- showed upregulation at 0.5mM exposure (Fig 7A). PAO1 mutant *pqsH*- similarly to the PA14 *pqsH*- mutant above yielded a decrease in production when exposed 0.1mM indole, but an increase at 0.5mM (Fig 7B). Overall every *Pseudomonas* mutant yielded an increased production in rhamnolipids.

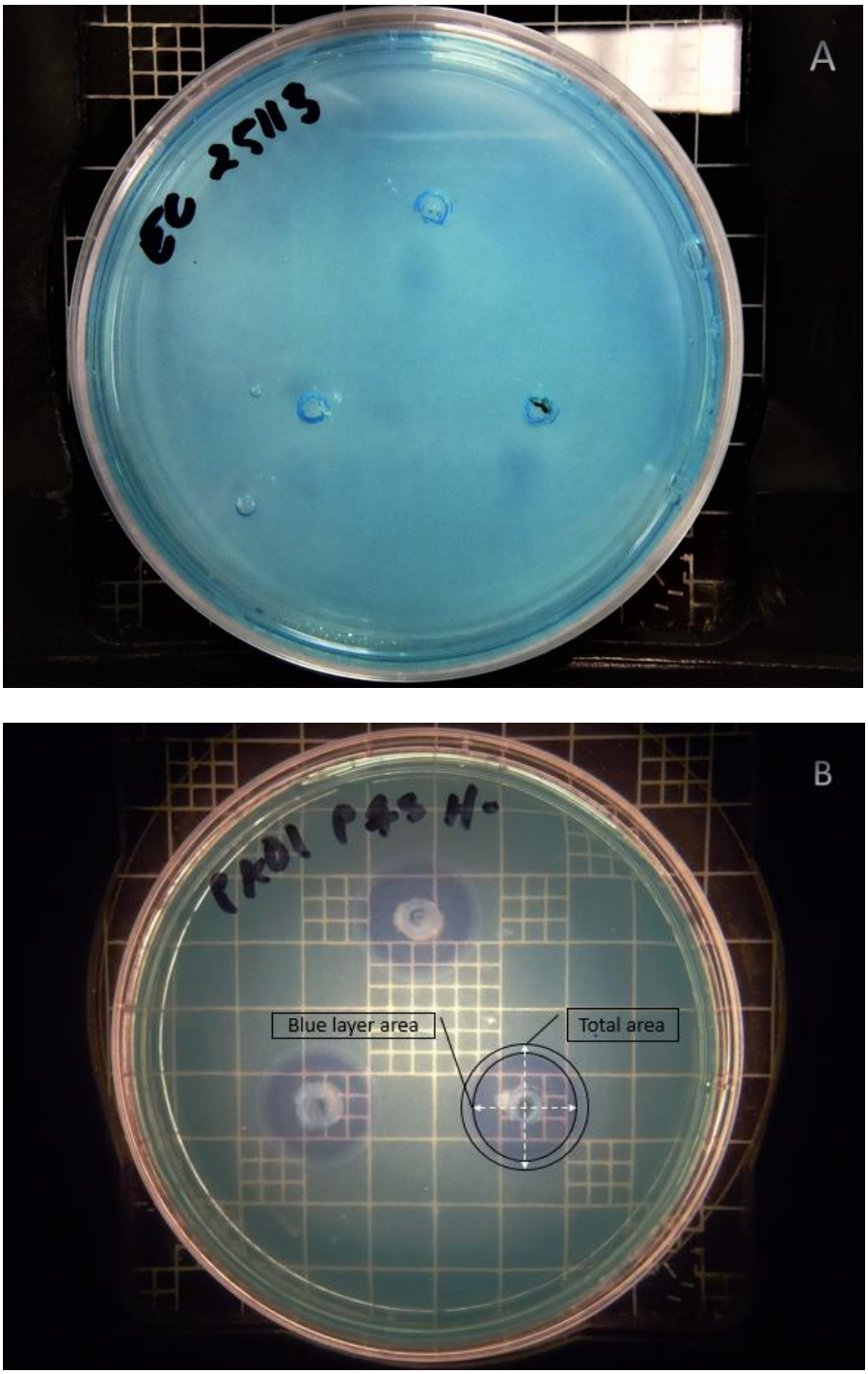
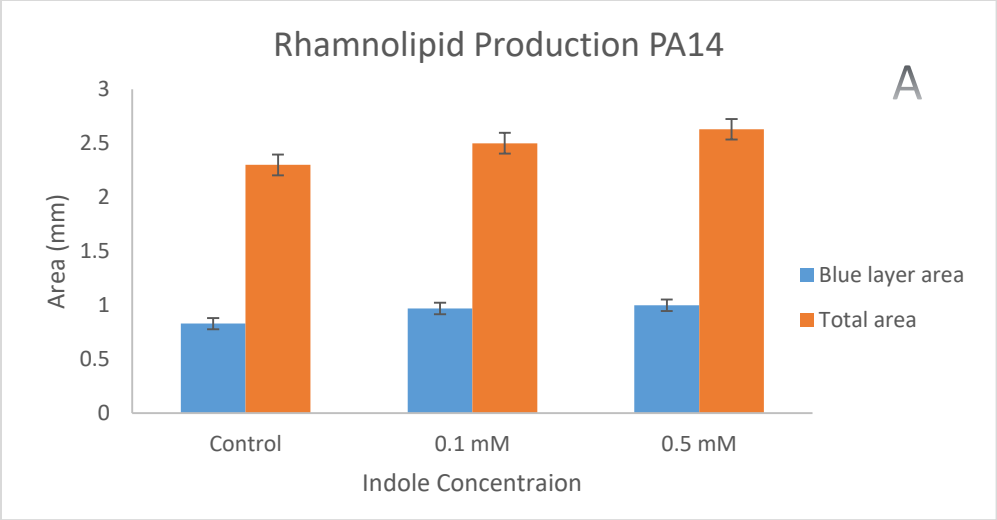


Figure 5 – *E.coli* is incapable of producing rhamnolipids (Fig 5A). *P. aeruginosa* produces rhamnolipids, which are key in structural biofilm formation. The inner most ring (Blue layer area) and outer ring (Total area) were measured with and without the addition of exogenous indole (Fig 5B).



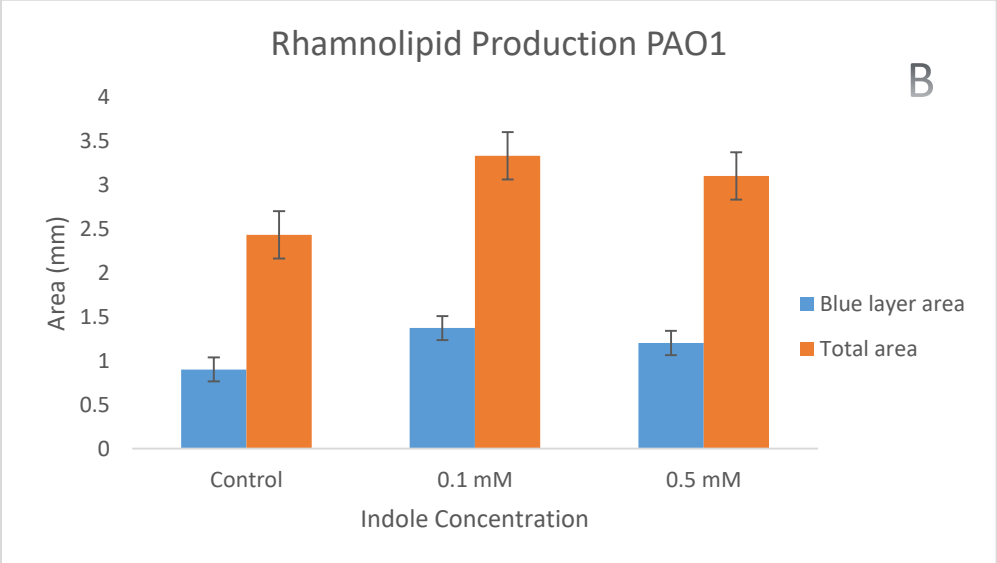


Figure 6 – The effect of exogenous indole on rhamnolipid production in *Pseudomonas wt* strains PA14 and PAO1. PA14 appeared to increase production of rhamnolipids with a higher indole concentration (Fig 6A). Whereas PAO1 decreased in productivity after 0.1mM, but still increased in overall production of rhamnolipids (Fig 6B).

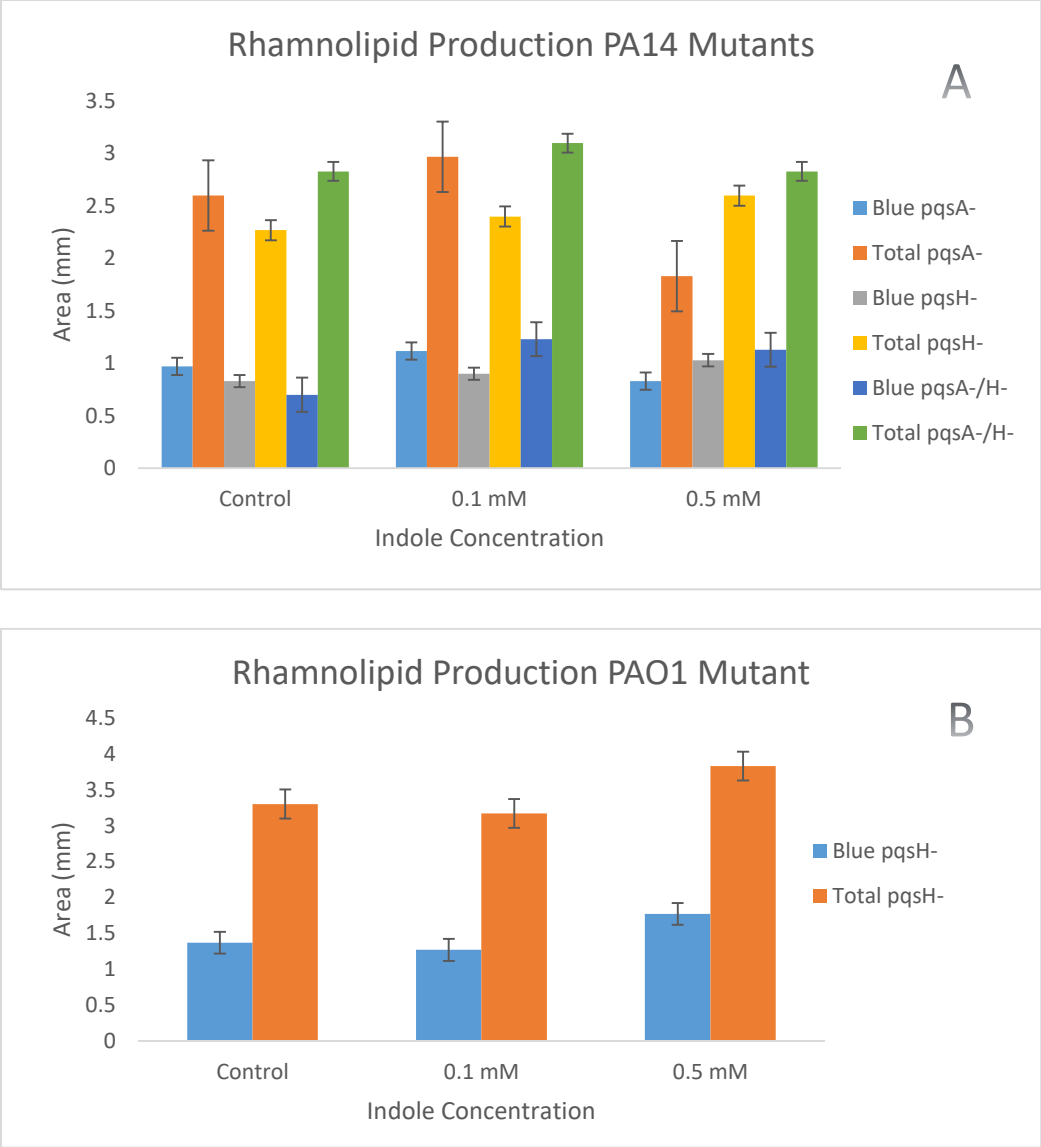
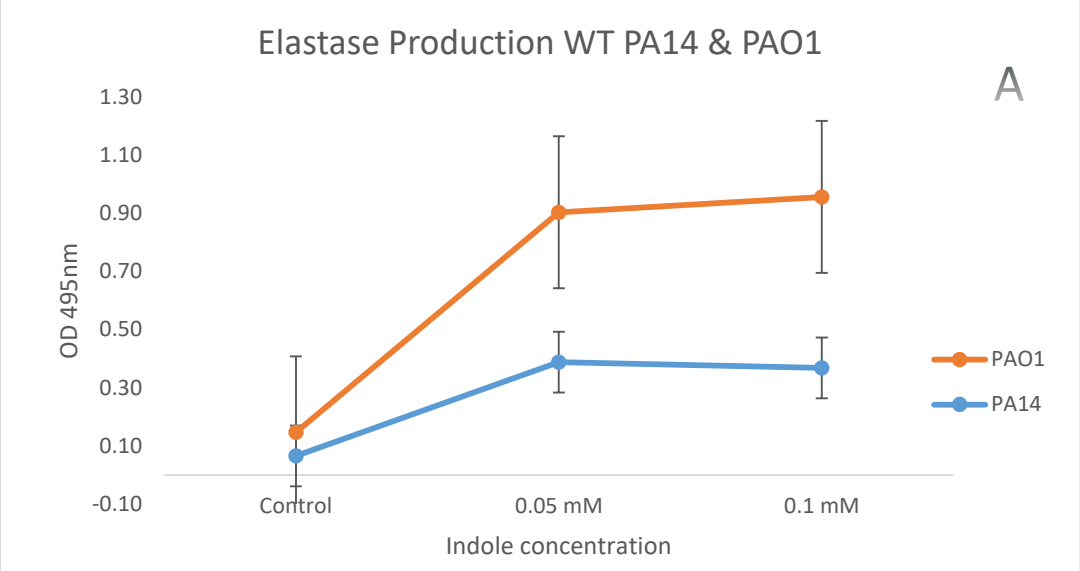


Figure 7 – Effect of exogenous indole on rhamnolipid production in PA14 and PAO1 mutant strains. PA14 mutants overall showed an increase in production with 0.1mM indole, but decreased at 0.5mM with the exception of *pqsH-* and *pqsA-/H-* (Fig 7A). PAO1 mutant showed parallels decreasing with 0.1mM and overall increasing at 0.5mM (Fig 7B).

3.5 Elastolysis Assay

3.5.1 Effect on Elastolysis production with the addition of exogenous indole

Elastase is one of the virulence factors produced by *Pseudomonas aeruginosa* that is linked to immune evasion, elastin belongs to the class of proteases that break down protein. Both *wt* PA strains, PA14 and PAO1 showed a significant spike in production when indole was introduced at 0.05mM. Upregulation moderately levels off from 0.05mM to 0.1mM (Fig 8A). All three PA14 mutants, *pqsA-*, *pqsH-*, and *pqsA-/H-* showed upregulation when indole was present. PA14*pqsA-* and *pqsA-/H-* both decreased with the 0.1mM exposure while PA14*pqsH-* showed slight increase when exposed to 0.1mM. PAO1 mutant PAO1*pqsH-* just like PA14*pqsH-* showed increases with both the addition of 0.05 and 0.1mM indole (Fig 8B). *wt* PAO1 had a much greater increase in production than PA14 *wt*, but both had an overall positive increase in production. Results wise the mutants PA14*pqsA-* and PA14*pqsH-* showed an increase in elastase production. PA14*pqsA-/H-* showed a miniscule decrease and PAO1*pqsH-* showed a significant increase of nearly 6 times more in the presence of indole.



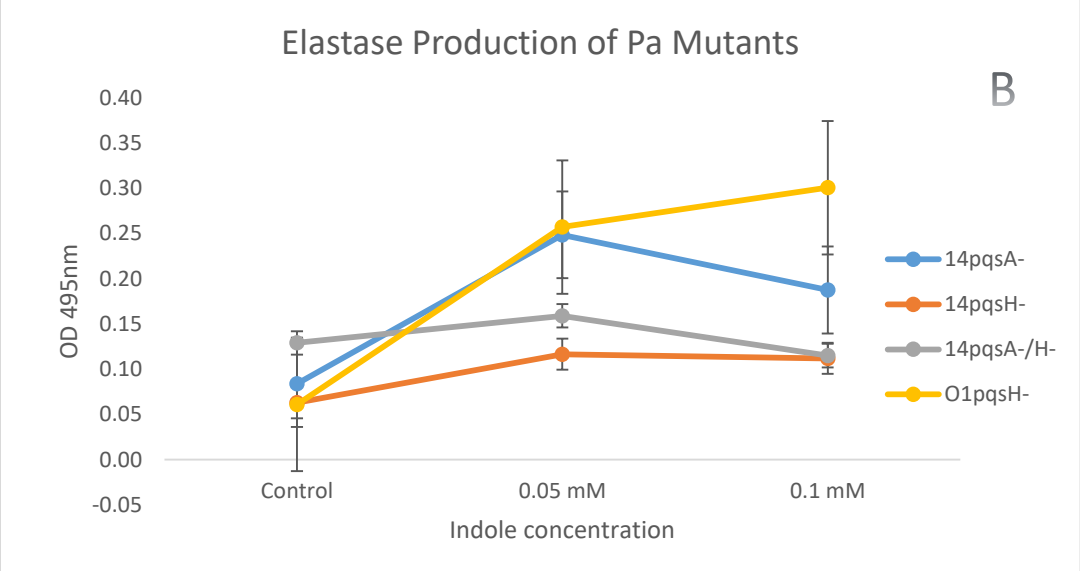


Figure 8 – The effect of exogenous indole on elastase production in *Pseudomonas wt* PA14, PAO1 and their *pqs* mutant strains. Both *Pseudomonas wt* strains showed increases in the presence of indole (Fig 8A). PA14 mutant strains *14pqsA-*, *14pqsH-* showed increases while 14*pqsA-/H-* showed a decrease. PAO1 mutant O1*pqsH-* showed a significant upregulation in elastase production with indole present (Fig 8B).

3.6 Confocal and Fluorescent Microscopy

3.6.1 Effect of exogenous indole on biofilm structure

Cultures of *E. coli wt* strain BW25113, *E. coli tnaA* mutant, *P. aeruginosa wt* strains PA14 and PAO1 were grown in various combinations, stained and observed on a fluorescent microscope. Individual (pure) cultures were grown of all four strains to use as a control measurement. Co-cultures were grown combining, (1) BW25113 + PA14, (2) BW25113 + PAO1, (3) PA14 + *tnaA*, (4) PAO1 + *tnaA*, these co-cultures were grown without indole and with the addition of indole at varied concentrations, 0.5mM, 1.0mM, and 1.5mM. The two *wt* co-cultures (1 & 2) were grown in order to replicate a natural environmental biofilm formation between the two isolates with indole produced naturally by *E. coli*. The *E. coli tnaA* mutant, a non-indole producing mutant strain was combined with both PA *wt* (3 & 4) in order to compare the biofilm formation with that of indole producing strain *E.coli* BW 25113 (1 & 2). Each co-culture (1, 2, 3, & 4) was inoculated with exogenous indole added at three concentrations, 0.5mM, 1.0mM, and 1.5mM. This procedure was conducted in order to help determine structural comparisons and structural differences within biofilm formations, and indole concentrations. All of the cultures were incubated for 48 hours, with biofilm samples collected at 24, 36 and 48 hours. The samples were then gently rinsed in 10mM phosphate buffer solution (PBS), and exposed to LIVE/DEAD fluorescent red (Propidium iodide) and green (syto 9) components and fixed as a sealed wet mount to be observed and imaged on a fluorescent microscope. *P. aeruginosa wt* PA01 (pure) culture (Fig 10) was grown to use as a control for comparison against co-cultures and cultures with exogenous indole added. *wt* PAO1 was inoculated in co-culture with *E. coli wt* BW 25113 (Fig 11), co-cultured with mutant *tnaA* (Fig 12),

and with mutant *tnaA* and added (0.5mM) exogenous indole (Fig 13). The samples collected from all three co-cultures (24, 36, and 48 h) all closely resemble that of their respective co-culture time cohort (Fig 11, 12 and 13). Figure 14 shows *wt* PA01 pure culture biofilm compared to that of *wt* PA01 with 1.0mM exogenous indole added. 24 h samples all yielded similar results (Fig 14A, D). In contrast, the 36 h sample showed an increased rate of growth in the PA01 sample with 1.0mM (Fig 14E) when compared to the 36 h pure culture sample (Fig 14B). At 48 h, the PA01 plus 1.0mM sample showed a significant decrease in population over the course of the 48 h exposure to 1.0mM indole (Fig 14F) when compared to the pure culture sample (Fig 14D) that showed moderate change in growth from 24 to 48 hrs. When *E. coli tnaA* mutant is co-cultured *wt* PA01 and exogenous indole added (0.5, 1.0, 1.5mM), at 24 h there is a noticeable difference from 0.1mM to 1.5mM, gradual decreases in the population as the concentrations increase (Fig 15), this could be representative of mild toxic effect at the higher concentrations. Figure 16 is a comparison between BW 25113, *TnaA*, and *tnaA* plus exogenous at a 0.5mM concentration at 36 h. There was no significant changes noticed between the confocal samples.

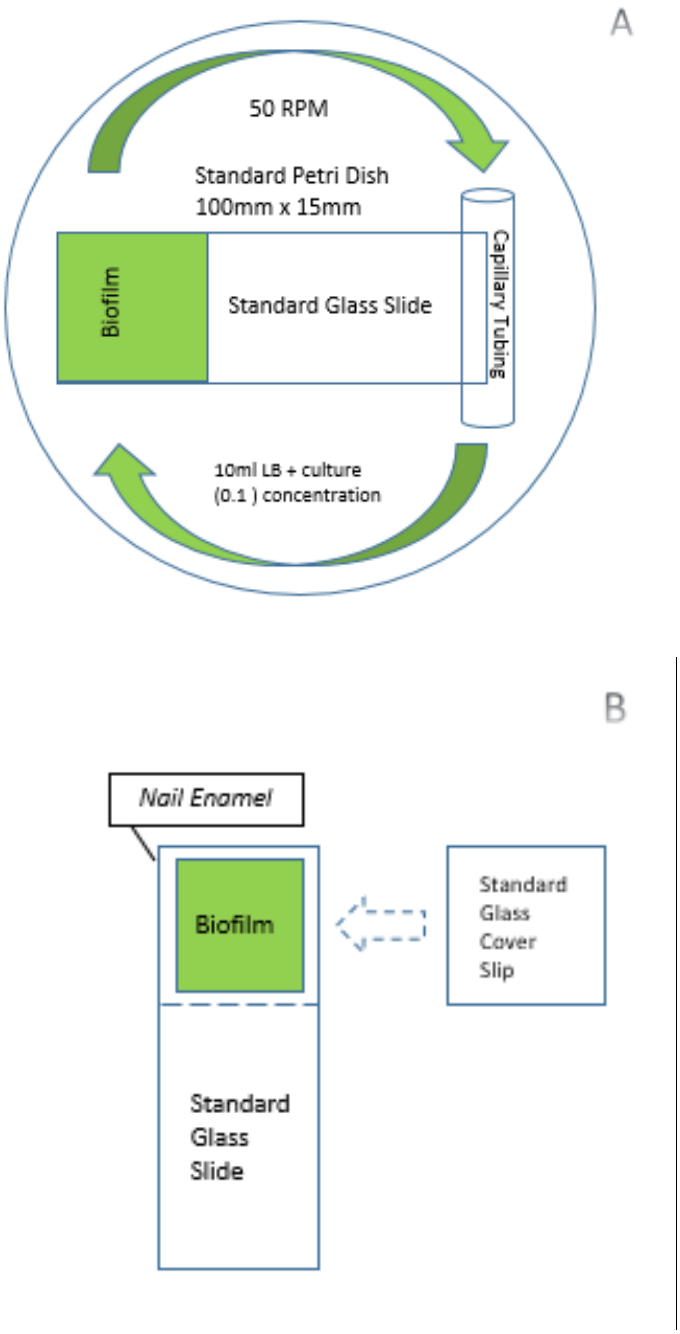


Figure 9 – Diagram for how biofilms were cultivated on glass slides for confocal imagery (Fig 9A). Diagram of how biofilms were maintained for observation and confocal imagery (Fig 9B).

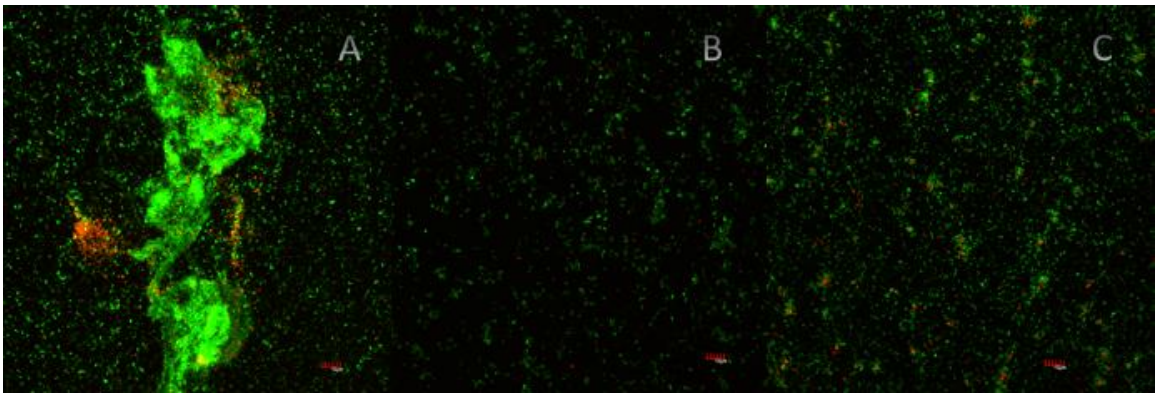


Figure 10 -- *wt P. aeruginosa* PA01 at 24 h biofilm (Fig 10A), 36 h biofilm (Fig 10B), and 48 h biofilm (Fig 10C).

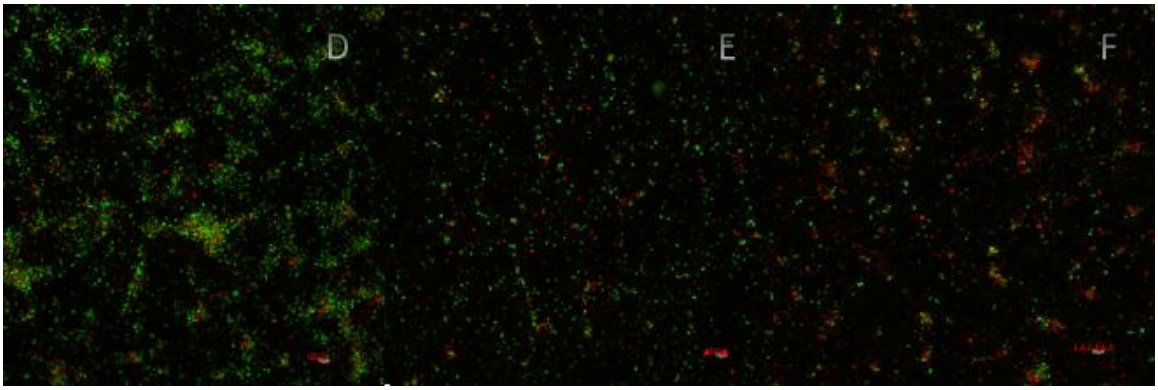


Figure 11 – *wt E. coli* BW 25113 co-cultured with *wt* PA01 biofilms at 24 h (Fig 11D), 36 h (Fig 11E), and 48 h (Fig 11F).

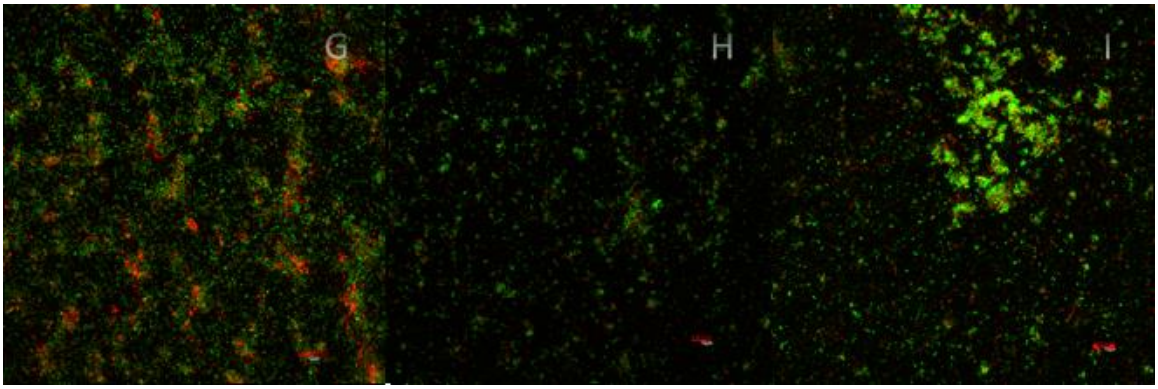


Figure 12 – *E. coli* tryptophanase *tnaA* mutant co-cultured with *wt* PA01 biofilms at 24 h (Fig 12G), 36 h (Fig 12H), and 48 h (Fig 12I).

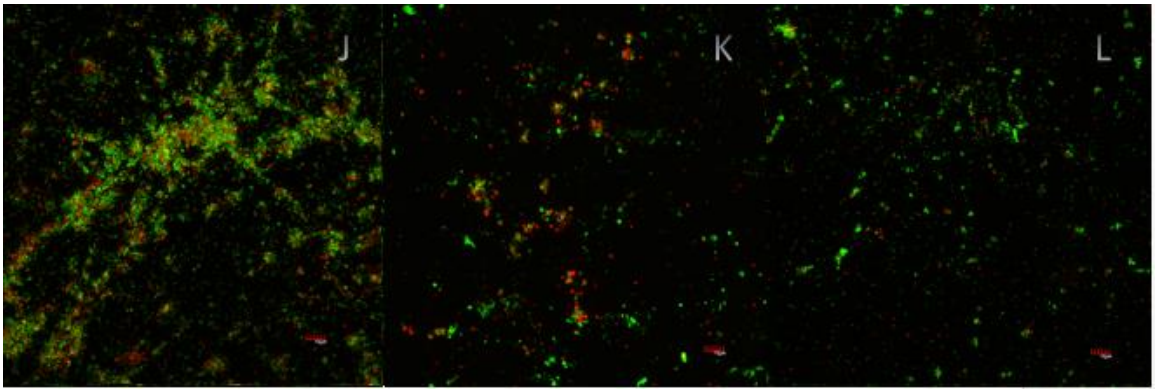


Figure 13 – *E. coli* tryptophanase *tnaA* mutant co-cultured with *wt* PA01 plus the addition of exogenous indole (0.5mM) biofilms at 24 h (Fig 13J), 36 h (Fig 13K), and 48 h (Fig13L).

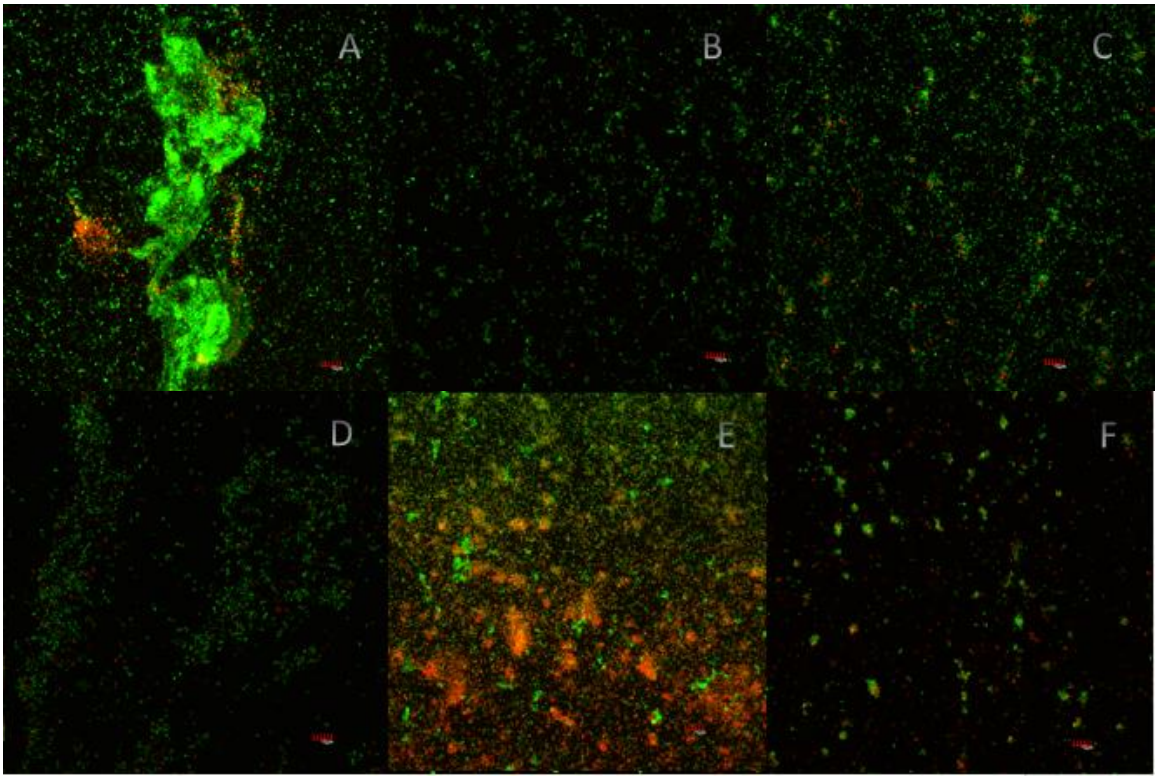


Figure 14 -- *wt P. aeruginosa* PA01 at 24 h biofilm (Fig 10A), 36 h biofilm (Fig 10B), and 48 h biofilm (Fig 10C). Compared against *wt P. aeruginosa* PA01 with the addition of exogenous indole (1mM) biofilms at 24 h (Fig 14D), 36 h (Fig 14E), and 48 h (Fig 14F).

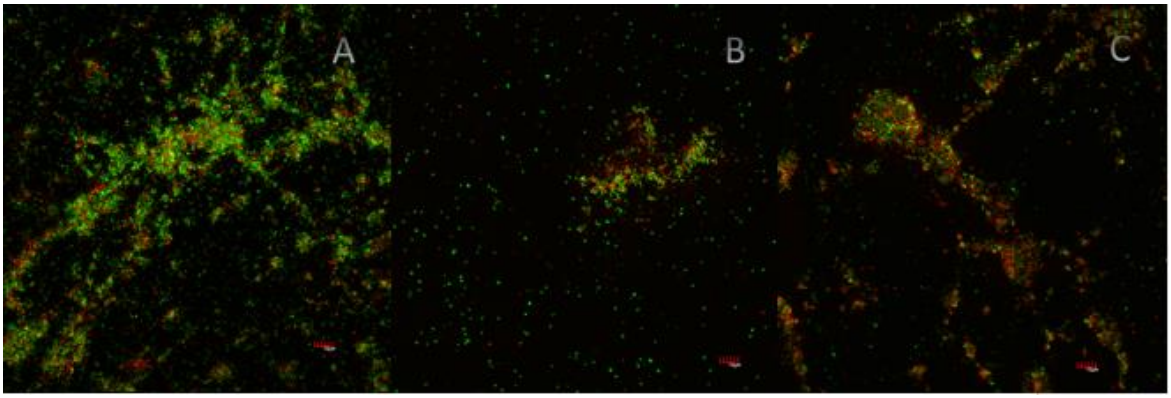


Figure 15 – *E. coli* tryptophanase *tnaA* mutant co-cultured with *wt* PA01 biofilms plus the addition of exogenous indole at different concentrations at 24 h, 0.5mM (Fig 15A), 1.0mM (Fig 15B), and 1.5mM (Fig 15C).

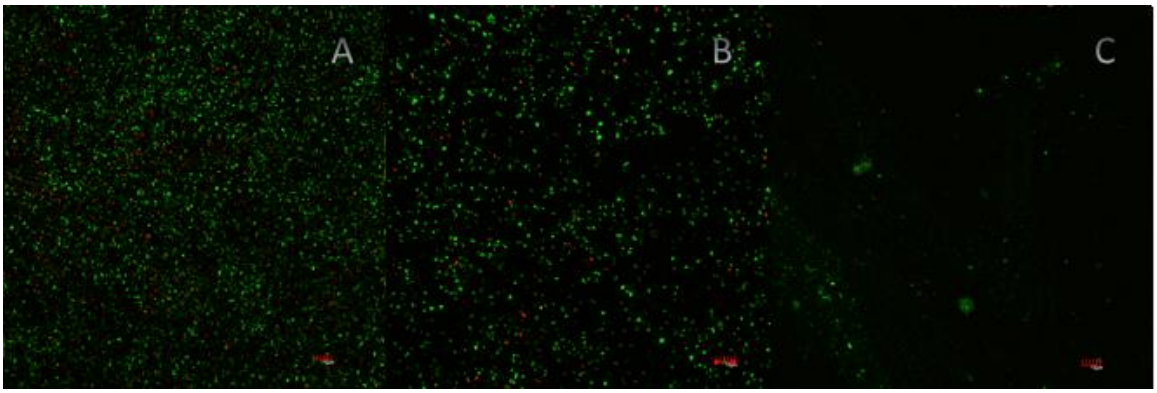


Figure 16 – Pure biofilm cultures at 36 h of *wt E. coli* BW 25113 (Fig 16A), *E. coli tnaA* mutant (Fig 16B), and *E. coli tnaA* with the addition of exogenous indole (0.5mM) (Fig 16C).

IV. DISCUSSION AND CONCLUSION

Past studies provided evidence that indole exhibited QSI components toward *P. aeruginosa* when co-cultured with *wt E. coli* (indole producing). There were noticeable downregulations of certain relevant virulence factors, most prominently pyocyanin (Fig 4). When *wt P. aeruginosa* is co-cultured with *wt E. coli* we find that *wt P. aeruginosa* becomes slightly more competitive as the concentration of indole is increased to 0.5mM from 0.1mM. The results were also very similar for the elastase and rhamnolipid production (Figures 6 & 7), at lower concentrations there were hardly any significant changes, but when increased there were decreases in production of both virulence factors. This upregulation in production is more than likely a counter effect to the stress created by indole; an environmental factor cueing a competitiveness for viable space and resources. It was suggested that indole may also decrease production of elastase and rhamnolipids along with pyocyanin (Chu, et al., 2012). The results in this study would correlate with those findings, with production of virulence factors being increased with the presence of indole at lower concentrations and decreased at higher concentrations.

Chu (2012) found that the concentration of indole needed in order for the compound to effectively alter and create a QSI effect in *P. aeruginosa* was 1mM. A similar study also found that the presence of indole initially stimulated biofilm production (same as in this study) and at higher concentrations would create QSI effects and decrease production of pyocyanin, elastase, and rhamnolipids in *P. aeruginosa* (Lee, Attila, Cirillo, Cirillo, & Wood, 2009).

The results of this study agree with the findings of the two studies mentioned above. That when indole is present at lower concentrations there is an initial upregulation in production of virulence factors, and when increased to a normalized concentration there is a decrease in pyocyanin, rhamnolipid and elastase production. The consistent indole concentrations used in this study were 0.1mM and 0.5mM. The initial response to the lower (0.1mM) concentration was a stimulation in production of virulence factors and growth rates, but once exposed to the higher concentration (0.5mM) a decrease became common in majority of the assays conducted, even amongst the *P. aeruginosa pqs* mutants. This would show consistency with the trend in downregulation of pyocyanin, elastase, rhamnolipids genes and biofilm production with the gradual increase in indole concentration. If the concentrations of indole were increased it is highly probable the experiments would yield results similar to those produced in previous studies.

In summary study demonstrates the organic compound indole does in fact act as a QSI agent. Study demonstrates indole does in fact decrease production of mechanisms of elastase, mechanisms of cytotoxicity (pyocyanin), and mechanisms of biofilm structure and dynamins (rhamnolipids). These results would strengthen the theory that indole may alter the structural scaffolding within a biofilm formation when present. Further evaluation of other key *Pseudomonas aeruginosa* virulence mechanisms such as motility, antibiotic resistance, and iron scavenging would be strong support in showing difference among the biofilm matrix.

Confocal microscopy demonstrated there were no significant alterations in biofilm structure (scaffolding). The cultures did not seem to be affected by the presence

or absence of indole in pure cultures. Similarly, the co-culture (*E. coli* and *P. aeruginosa*) biofilms did not seem to be affected regardless if *wt E. coli* or *E. coli tnaA* was present. Earlier studies observed *P. aeruginosa* was capable of degrading indole within a mixed population (Chu, et al., 2012), it is possible that *P. aeruginosa* has other mechanisms (possibly gene up or downregulation related toward indole removal) that enable this organism to preserve its biofilm structure.

This is a key factor in providing evidence that QS is in fact disrupted by the presence of indole. By inhibiting QS virulence factors in *Pseudomonas* such as pyocyanin, creates the inference that other QS components may be downregulated or upregulated as well, leading to the hypothesis that if there is interference in a microbial population altering particular threshold densities due to QS interference from co-inhabitants then that could cause structural changes in biofilm formations. To further support this hypothesis, a study in 2014 found that certain bacteria are capable of producing and secreting small compounds that inhibit QS, while others quench QS by enzymatic degradation of *N*-acylhomoserine lactones (AHLs), both resulting in an effect on the biofilm formation of *Pseudomonas* PAO1 (Christiaen, et al., 2014).

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