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The Effects of Treating Multispecies Biofilms with Bacteriophage

Alexandria Francis Cooper
Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

THE EFFECTS OF TREATING MULTISPECIES BIOFILMS WITH BACTERIOPHAGE

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN BIOLOGY

BY

ALEXANDRIA F. COOPER

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ABSTRACT

Biofilms can be found in any environment within proximity to water and are problematic in an assortment of industries. Numerous efforts have been employed to dislodge biofilms including bacteriophages (viruses that infect bacteria). Phage remediation is a promising solution for combatting biofilms that form on catheters in long term use patients. These biofilms often result in catheter-associated urinary tract infections (CAUTIs) and are the most common type of healthcare related infection reported. Additionally, they result in longer hospital stays and increased morbidity. To date, most of the research on the topic focuses on single species biofilms, despite their rarity in nature. Here we assess the efficacy of five phages and a phage cocktail to treat both homogeneous as well as heterogeneous, multispecies biofilms. The effects of phage treatment were assessed looking at both the absorbance and colony counts of the biofilm as well as the planktonic fraction. Two phages, Greedy and phiKZ, had the greatest success in lysing bacterial cells and were thus selected for a phage cocktail treatment regimen. By evaluating the effects on both monoculture and heterogeneous biofilms, phage species can more accurately be assessed for their potential use in treating CAUTIs.

CHAPTER ONE

INTRODUCTION

Biofilms

A biofilm is an aggregation of microbes and substrates from the surrounding environment that are encased in a microbe-produced complex polysaccharide matrix often adhered to a solid surface (see review Flemming *et al.* 2016). There are several phases of biofilm formation. First, a primer film composed of microbial and environmental molecules forms on the surface of the substrate (Chondki *et al.* 2011). Hydrophobic interactions either repel or attract microbes to the surface, in which attracted bacterial cells bind weakly to each other (see review Kaplan 2011). Next, in the growth phase, irreversible attachment occurs when the weak bonds are reinforced by stronger bonds to the cell wall or pili (Rosan and Lemont, 2000). In maturity, the initially attached bacteria attract other bacteria through means such as cell to cell communication through chemical signals (quorum sensing). This, in conjunction with cell multiplication and the secretion of polysaccharides (generating the matrix), results in organization of the biofilm and the shift from an aerobic environment to a facultative anaerobic environment. When the biofilm reaches a critical mass, the last phase in biofilm development occurs: dispersal; bacteria are dispersed into the environment to colonize new areas. Figure 1 illustrates this process.

The microbes within a biofilm differ from their counterparts in the liquid surrounding the biofilm (the planktonic fraction) in many ways. They may develop an altered phenotype, including different growth rates (see review Flemming *et al.* 2016) and transcription profiles

(see review Socransky and Haffajee, 2002). Additionally, communities within biofilms often display increased gene exchange rates and tolerance to antimicrobials (Flemming *et al.* 2016). The polysaccharide matrix provides channels useful for nutrient acquisition and chemical signaling (Flemming *et al.* 2016). It also provides physical protection of microbes deeper in the matrix. These altered properties are unique to biofilms and are thus unpredictable by typical liquid culture based studies (Flemming *et al.* 2016). These advantageous effects are compounded in multispecies (heterogeneous) biofilms when compared with single species (homogenous) biofilms.

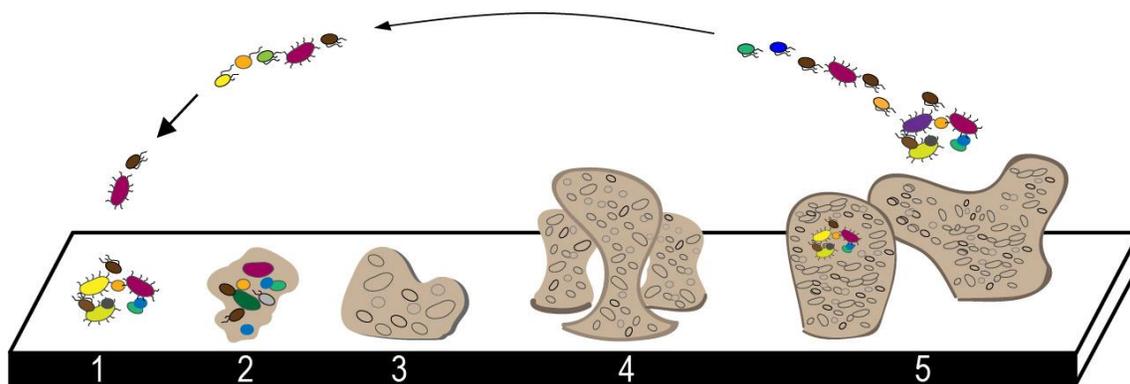


Figure 1. Five phases of biofilm development: (1) initial attachment, (2) irreversible attachment, (3) initial maturation, (4) later maturation, and (5) dispersion.

Studies have shown that multispecies biofilms, in comparison to their single species counterparts, have increased mass and resistance to antimicrobials (Lee *et al.* 2014, Burmølle *et al.* 2014). Due to the microscopic matrix structure, members of the microbial community within a biofilm interact both physically and socially. These social interactions may be cooperative. For example, microbes within biofilms have been observed to share resistance to, e.g., antimicrobials, antibiotics, and toxins (Lee *et al.* 2014, Burmølle *et al.* 2014, Chandki *et al.* 2011); often if a member of the biofilm community is resistant to an antibiotic, the entire

community becomes resistant. Instances of metabolic cooperation (syntrophy) have also been observed amongst microbes within biofilms (Lee *et al.* 2014, Ren *et al.* 2014). Alternatively, these microbial social interactions may be antagonistic such as producing chemicals toxic to other microbes and resource composition (Rao *et al.* 2005).

Biofilms are a key constituent of the microbial world. They are ecologically important and can be found anywhere within proximity to water. Biofilms found on rocks in streams provide sustenance to bottom feeders, indicating their essential role in the food web of streams (see review Battin *et al.* 2016). They likewise have immediate benefits for human health. For instance, within the human microbiota, biofilms in the gut play an important role in degradation of substrates (de Vos 2015). Biofilms also benefit human health through their important role within the wastewater treatment process. In fact, three types of biofilms, distinguished by their structure, are typically included within this process: static biofilms, particulate biofilms, and flocs (see review Nicolella 2000). Research has found that the presence of a biofilm in the activated sludge stage of water treatment improves processing performance (Gebara 1999). Yet another industrial use of biofilms includes their potential applications for bioremediation, e.g. crude oil degradation (Dasgupta *et al.* 2013). Biofilm reactors have the potential to revolutionize production of biological materials (e.g. antibiotics) and even electricity (see recent review Todhanakasem 2016). While a few of the documented beneficial uses of biofilms are listed here, this is far from a comprehensive list.

Despite the many ecological benefits of biofilms, they can also be detrimental. Problematic biofilms include, for example, those that form on the bottom on boats or in fuel tanks, food processing centers, municipal drinking water systems, or sewer systems in large cities (e.g. Drake *et al.* 2005, Hvitved-Jacobsen *et al.* 2013, Douterelo *et al.* 2016). Biofilms can

also be detrimental to human health (Macfarlane and Dillon 2007). In cystic fibrosis patients, *P. aeruginosa* biofilms form in the lungs and are extremely difficult to treat due to the protective nature of biofilms (Høiby *et al.* 2010). Biofilms that form on the teeth of animals and humans (plaque) cause periodontal disease (Chandki *et al.* 2011). Infection and biofilm formation within the human body can have severe and even lethal effects and is thus an important area of research within the biomedical community.

Phage Therapy

Given the many detrimental effects biofilms have on human health, strategies for combating their formation as well as their destruction are of critical importance. Biofilm treatment using bacteriophage (phage therapy) is a particularly promising approach. Bacteriophages or phages are viruses that infect bacteria; they are nature's biological weapon against bacteria. Here we will briefly review the aspects of bacteriophage biology of relevance to our study and refer the reader to the many excellent available reviews, including Clokie *et al.* (2011), for further details about phages. When lytic phages encounter a bacterial cell host, they inject their DNA or RNA genome. Once inside the host, the phage replicate and eventually trigger the bacterial host cell to "burst." Phage progeny are released with this burst to hunt new hosts (bacteria). This process, called lysis, has been found to play a significant role in bacterial death in several ecosystems (e.g. Berdjeb *et al.* 2011).

Bacteriophage remediation is also a promising approach as phages can be grown within the laboratory at minimal expense. In fact, some phages produce specific enzymes for the degradation of the polysaccharide matrix that is central to the stability of biofilms (Harper *et al.* 2014). Moreover, phages typically have a narrow host range; they are often only capable of

successfully infecting and lysing a single bacterial species or strain. Thus, phages can be used to specifically target a bacterial infection with high efficacy (Koskella and Meaden 2013).

Phage therapy, however, does raise reasonable concerns. For instance, when phage lyse (kill) bacteria, toxins may be released into the body and/or encoded by the phage itself (Loc-Carrillo and Abedon 2011). Lysis also can lead to the rapid release of lipopolysaccharides from the bacterial cell wall that, when at high concentrations, can lead to cytokine cascades (Nilsson 2014). Furthermore, phage treatment of internal organs, many of which are not natural environments for phages, has been shown to activate an immune response (Nilsson 2014). In targeting one biofilm, it is important to remember that the human body is composed of many beneficial bacteria that support health (see review Lloyd-Price *et al.* 2016). Thus, phage therapy may lead to subsequent infections or dysbiosis.

In addition to these concerns, there are several challenges for phage therapy. First and foremost is the ability to administer phage treatment. As phage treatment would be most effective if introduced in close proximity to the bacterial infection, this is a hurdle when the area or organ being treated cannot be easily accessed. It is for this reason that phage therapy has been most successful for “easy-to-reach” infections, e.g. acne (Jonńczyk-Matysiak *et al.* 2017). The greatest obstacle facing the wide-spread adoption of phage therapy, however, is evolution itself. While phages can infect and kill bacteria, they are in a constant arms race with bacteria, which are continually evolving resistance to infection from phages (Dennehy 2012). This race is expedited by the short generation times of bacteria and phage.

To combat this problem, research has focused on phage cocktails (combining multiple phages for treatment), and using phage treatment in combination with antibiotics or chemicals. One study found a cocktail of seven phages to be effective at inhibiting *Staphylococcus aureus*

biofilms (Kelly *et al.* 2012). The phage Φ Pan70 was successful at treating *Pseudomonas aeruginosa* biofilms in mice with an animal survival of 80-100% (Holguin *et al.* 2015). Quorum sensing enzymes secreted by a modified T7 phage inhibited biofilm formation in heterotrophic *Escherichia coli* and *P. aeruginosa* biofilms (Pei and Lamas-Samanamud 2014). Two phages were used to reduce *Campylobacter jejuni* biofilms on glass slides; *C. jejuni* biofilms are problematic in the digestive tract and water pipes (Siringan *et al.* 2011). In the work of Chaundry *et al.* (2017), *P. aeruginosa* biofilms were more effectively treated with a combination of phage and antibiotics than either treatment alone. These are but a few studies exploring phage remediation of biofilms. The rise in antibiotic-resistant bacteria has impelled research efforts to identify phage species capable of combating a variety of common infections.

Catheter-Associated Urinary Tract Infections

Biofilms that form in long-term use urinary catheters are particularly problematic. These biofilms, if left untreated, can lead to catheter-associated urinary tract infections (CAUTIs). According to the Center for Disease Control and Prevention (CDC), CAUTIs are the number one urinary tract infection (UTI) contracted in the hospital (CDC 2017). Furthermore, per a March 2016 CDC press release, one in ten CAUTIs are caused by urgent or serious antibiotic-resistant bacteria (CDC 2016). For this reason, much research has gone into CAUTI treatment and reducing the occurrence of CAUTIs (see reviews Siddiq and Darouiche 2012, Tenke *et al.* 2017). Chemical treatment of catheters with Triclosan (Jones *et al.* 2006), EDTA (Percival *et al.* 2009) or Fluorofamide (Morris and Stickler 1998) has yet to show a sufficient reduction in CAUTI cases. Investigations into other materials (glass, polystyrene, and plastics) used in catheter construction have unfortunately not produced a reduced incidence of CAUTIs (see review Tenke *et al.* 2017). Catheters fitted with silver electrodes were successful in decreasing the rate of

biofilm formation when electric currents were applied (Chakravarti *et al.* 2005). This study and a subsequent study (Voegelé *et al.* 2015), however, have been limited to *in vitro* models. Of particular recent interest, the application of phages has also been explored for thwarting catheter-associated biofilm formation and CAUTIs (see review Siddiq and Darouiche 2012) and will be discussed in greater depth in a subsequent subsection of this chapter.

While several bacteria have been associated with CAUTIs, the most prevalent is *Proteus mirabilis*. *P. mirabilis* is a quorum sensing bacteria found in soil, water, and the digestive tracts of multiple animals. Quorum sensing (QS) bacteria send out chemical signals as a means of communicating with other members of its community, an ability which can be quite advantageous to the bacterium, particularly during biofilm formation. With *P. mirabilis*' capacity for both swarming and swimming motility (Williams *et al.* 1978), coordination between the two processes is of particular interest (Rossman *et al.* 2015). Research has found that *P. mirabilis* can detect cues in the surrounding environment triggering its switch from swimming to swarming or vice versa (Armbruster *et al.* 2013). Swarming cues, including L-arginine, DL-histidine, L-glutamine, malate, and DL-ornithine, are all present in normal human urine and can thus initiate the establishment of biofilms within catheters (Armbruster *et al.* 2013). Furthermore, *P. mirabilis* is also able to form crystalline biofilms as a result of urease activity, which in humans can result in the occurrence of CAUTIs (Moblely and Warren 1987, Stickler *et al.* 1993, Holling *et al.* 2014).

Other common species associated with CAUTIs include *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Morganella morganii* (Gravey *et al.* 2017). Herein we will focus on just two of these taxa as they are amongst the best studied in microbiology: *P. aeruginosa* and *E. coli*. Like *P. mirabilis*, *P. aeruginosa* is a QS bacterium.

It is commonly found in the skin flora, soil, and water. *P. aeruginosa* is also an opportunistic human pathogen associated with pulmonary infections, particularly in cystic fibrosis patients, as well as infections of burn wounds and chronic wounds (Mulcahy *et al.* 2014). *P. aeruginosa* utilizes its unipolar flagella for swarming and swimming motility. Additionally, *P. aeruginosa* siderophore activity could have an important effect on community structure and dynamics within a biofilm (Cornelis and Dingemans 2013). The second bacterium of interest is *E. coli*, which can also be a constituent of catheter biofilms and is the most common cause of UTIs (Hooton *et al.* 2010). *E. coli*, while non-quorum sensing, utilizes its type I pili for biofilm initial attachment and potentially the spread of biofilm growth across a solid surface (Pratt and Kolter 1998). Efficient biofilm formation has been observed for *E. coli* species in environments replicating the bladder and catheters (Azevedo *et al.* 2014).

Bacteriophages of the Urinary Microbiota

In many areas of the human microbiome, most notably the gut (Manrique *et al.* 2017), overall microbial community structure is largely dictated by phages. Changes in the “native” human virome (viral community - both eukaryotic viruses that infect human cells and phages) have been associated with diseases and perturbations, e.g. inflammatory bowel (Norman *et al.* 2015, Manrique *et al.* 2016), periodontal disease (Ly *et al.* 2014), and the spread of antibiotic resistance (Modi *et al.* 2013), among others. Phages are also present within the microbiota of the bladder. Sequencing of voided urine from individuals with and without a UTI revealed the urinary virome (Santiago-Rodriguez *et al.* 2015). Similar to that observed within the gut microbiota, the urinary virome study found that phages vastly outnumber eukaryotic viruses in the urinary virome (Santiago-Rodriguez *et al.* 2015). In addition to phage detection via large-scale whole genome sequencing efforts (Rani *et al.* 2016, Santiago-Rodriguez *et al.* 2015), phage

species have been isolated directly from the urinary microbiota and characterized in the lab, including seven isolated in the Putonti lab (Malki *et al.* 2016).

In contrast with other areas of the human microbiome, the bacteria and phages present within the bladder are just now beginning to be explored. It was long believed that the urinary tract of healthy (asymptomatic) individuals was sterile. It was for this reason that during the Human Microbiome Project, which cataloged the bacterial species present within the human body, the bladder was omitted. This longstanding belief, however, was overturned when the bladder was found to include a variety of bacterial species (Wolfe *et al.* 2012). Prior work in the Putonti lab found that latent (dormant) phages are prevalent within bacterial species from the female bladder. Analysis of the genomes of over 200 bacteria isolated from the female urinary microbiota has found >80% harbor phage sequences (Miller-Ensminger *et al.*, unpublished). This initial work and focused studies on bacterial strains from the bladder (Malki *et al.* 2015) suggests that phages are highly prevalent within the urinary microbiota. Details regarding phage host range and the role that phages play in shaping the bacterial populations within the bladder, however, have yet to be determined.

Phage Therapy for Catheter-Associated Biofilms

The use of phage treatment to inhibit and reduce biofilm formation began in 2006 when Curtin and Donlan showed that Bacteriophage 456 could be used to treat catheter-associated *Staphylococcus epidermis* biofilms (Curtin and Donlan 2006). Several phages were found to be 90% effective in disrupting the formation of homogenous *E. coli* and *P. mirabilis* biofilms in comparison to untreated controls (Carson *et al.* 2010). With the development of a catheter model system, phage M4 and four other phages were tested to treat *P. aeruginosa* biofilms (Fu *et al.* 2010). While a *P. aeruginosa* colony resistant to all five phages was uncovered, it had a reduced

growth rate (Fu *et al.* 2010). The bacteriophage T7 was also found to be successful in inhibiting biofilm formation of more complex communities including both *E. coli* and *P. aeruginosa* (Pei and Lamas-Samanamud 2014). Similarly, phage capable of inhibiting biofilm formation for other pairs of CAUTI-associated bacteria have been studied (Lehman and Donlan 2015).

One avenue of phage therapy specific to catheter-associated biofilm formation is the potential to pretreat catheters with specific phage(s). For instance, catheters pretreated with a phage cocktail showed inhibition of *P. aeruginosa* biofilm growth; this success, however, was short-lived as *P. aeruginosa* biofilm growth resumed at 24 hrs post-treatment (Fu *et al.* 2010). Other studies have been more successful. For example, two phages were used to treat catheters and showed significant reduction of *P. mirabilis* biofilms for up to 168 hrs after treatment (Melo *et al.* 2016). In another study, catheters were pretreated with a “benign” strain of *E. coli* and phage to successfully inhibit biofilm formation (Liao *et al.* 2012).

Prior research in phage therapy for catheter-associated biofilms has routinely found that the timing of phage treatment is vital to its effectiveness. While pretreatment of catheters with phage can inhibit biofilm formation, phage treatment of established biofilms has been less effective. While it may delay biofilm growth, as one study found, catheter blockage is often inevitable (Nzakizwanayo *et al.* 2015). Further testing of phages is thus needed to determine the viability of a phage therapy solution for the prevention and treatment of biofilms within catheters and resulting CAUTIs.

Specific Aims

While prior research exploring phage therapy for catheter-associated biofilms has largely focused on homogenous biofilms in the laboratory, biofilms from patients with CAUTIs can be significantly more complex, i.e. include multiple species (Saint and Chenoweth 2003). Thus,

phage therapies capable of disturbing complex biofilm communities, have a greater potential to reduce infection rates in patients. My study explores the efficacy of phage treatment on the biofilms of three bacteria commonly associated with catheter biofilms (*P. mirabilis*, *E. coli*, and *P. aeruginosa*), investigating both homogeneous (monoculture) biofilms as well as heterogenous (multispecies) biofilms. To the best of our knowledge, this is the first study considering phage therapy for three species biofilms of catheter-associated bacteria.

This work is aimed at answer three questions. First, which phage(s) reduce biofilms? Second, how does phage treatment affect bacterial growth and community dynamics? Third, how does phage treatment affect bacterial phage resistance?

CHAPTER TWO

METHODS

Bacteria and Bacteriophage Strains

Three bacterial species were used in this study: *Proteus mirabilis* CDC PR 14, *Pseudomonas aeruginosa* 1C, and *Escherichia coli* C. *P. mirabilis* and *P. aeruginosa* were obtained from the American Type Culture Collection, strains ATCC 29906 and ATCC 15692, respectively. *E. coli* C was obtained from Dr. Christina Burch (University of North Carolina Chapel-Hill). Each bacterium was propagated in liquid culture using LB (10.0 g/L tryptone, 5.0 g/L yeast, 10.0 g/L NaCl). Liquid cultures were grown at 37°C in a shaking (200 rpm) incubator.

Bacteriophages used in this study are listed in Table 1. Bacteriophages Habibi, Greedy, and Lust were isolated by our lab and have been described previously in the literature (Malki *et al.* 2015; Malki *et al.* 2016). Bacteriophage phiKZ was obtained from the Felix d'Herelle Reference Center for Bacterial Viruses (Quebec, Canada), strain designation HER153. DMS3 was obtained from the O'Toole Lab (Dartmouth University). As shown in Table 1, the phages utilized in this study vary in their natural habitat as well as host range. Habibi and phiKZ both belong to the family of viruses *Myoviridae*. This family of viruses are characterized by their contractile tail sheath (Maniloff and Ackermann, 1998). PhiKZ is one of the largest documented members of this family (Herdtvelt *et al.* 2005), as most *Myoviridae* phages have genome sizes on par with that of Habibi. Greedy, Lust, and DMS3 are *Siphoviridae* phages. In contrast to *Myoviridae*, *Siphoviridae* viruses have long, noncontractile tails (Maniloff and Ackermann,

1998). The phages Greedy and Lust were both isolated from the urinary microbiota (Malki *et al.* 2016) and thus have particular relevance to the study conducted here. Figure 2 shows electron microscope images for the five phage species.

Phage	Origin	Host	Family	Genome Size	GC%
Habibi	Lake Michigan	<i>E. coli</i> & <i>P. aeruginosa</i>	<i>Myoviridae</i>	65.73 kbp	54.9
Greedy	Bladder Isolate	<i>E. coli</i>	<i>Siphoviridae</i>	60.04 kbp	44.6
Lust	Bladder Isolate	<i>E. coli</i>	<i>Siphoviridae</i>	41.94 kbp	54.5
phiKZ	Sewage	<i>P. aeruginosa</i>	<i>Myoviridae</i>	280.30 kbp	36.8
DMS3	Clinical isolate	<i>P. aeruginosa</i>	<i>Siphoviridae</i>	36.42 kbp	64.3

Table 1. A summary of information about the five phages used in this study.

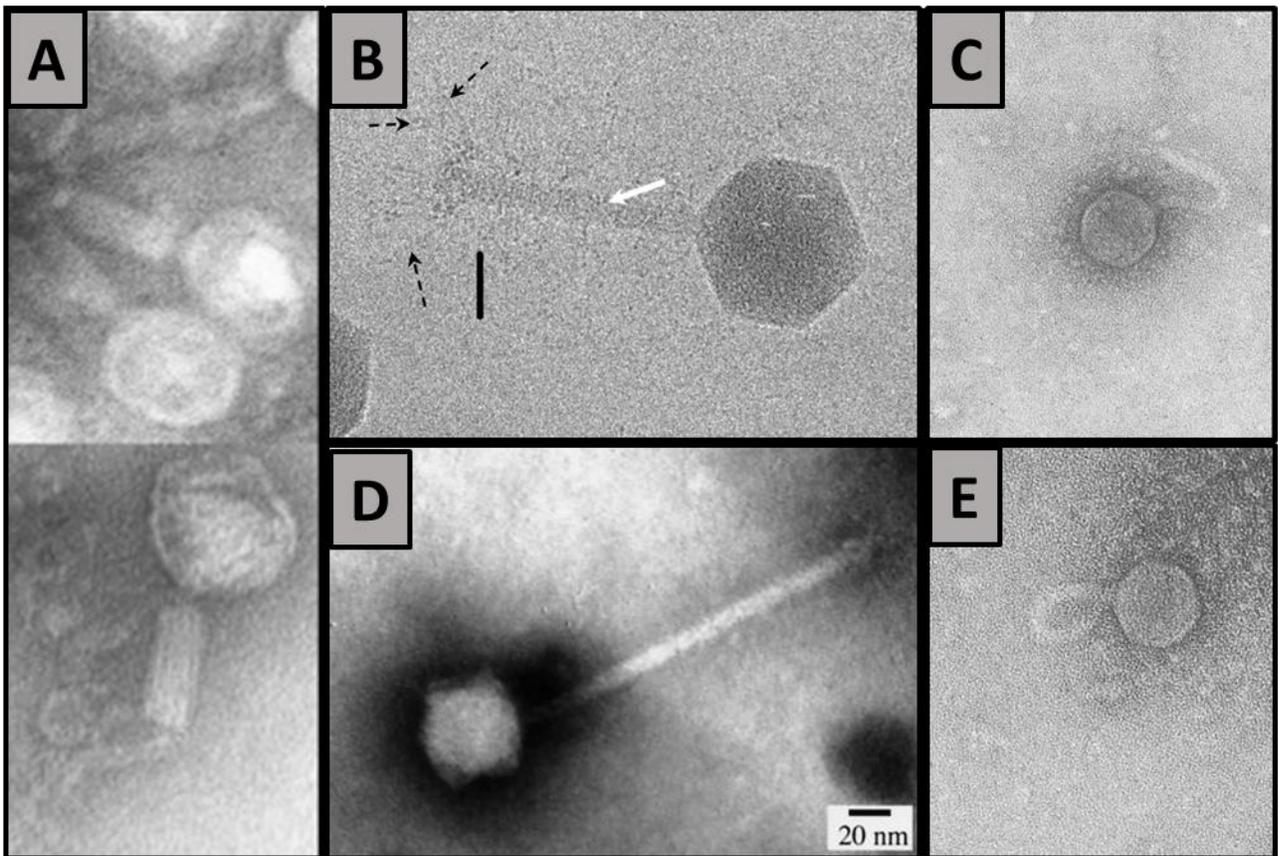


Figure 2. Images of the phages used in this study. (A) Habibi (Malki *et al.* 2015), (B) phiKZ (Fokine *et al.* 2007), (C) Greedy (K. Malki, unpublished data), (D) DMS3 (Budzik *et al.* 2004), and (E) Lust (K. Malki, unpublished data).

Growth Curves

Growth curves were conducted to determine the exponential growth phase of the bacteria. This knowledge is useful in deciding the opportune time to inoculate cultures with phage in isolation attempts. Furthermore, the growth curve enables us to estimate the number of bacterial colonies in a culture at a given time point, this is used to ascertain the appropriate phage concentration to use in treatments. Prior to this study our lab performed growth curves for *E. coli* and *P. aeruginosa* (unpublished data) which were referenced for those cultures.

To generate the growth curve for *P. mirabilis*, a single colony was used to inoculate a 50 mL flask of LB and glass beads, the culture was then grown for 21 hrs. 100 µl was used to subculture three flasks containing 300 mL of LB and glass beads. The glass beads were added to the culture to minimize biofilm formation. Three mL aliquots were taken from each replicate every 15 mins for that first 90 mins after inoculation. Samples were then taken every half-hour or hourly for 17.5 hrs post-inoculation. Additional samples were collected at 25 and 33.5 hrs to capture the stationary phase of growth. In total, 32 samples were collected. From the 3 mL sample collected, 2 mL was placed immediately on ice until plating/storage and 1 mL was assayed via the spectrophotometer. Readings were taken at 660 nm and 750 nm. For plating, 8-fold serial dilutions were performed; 100 µl of the aliquot was serially diluted into 1 mL of LB eight times, thus reducing the number of bacterial cells with each dilution by 1:10. For each dilution, 100 µl was spread on an LB plate using a sterile spreader. Plates were subsequently placed in a non-shaking 37°C incubator overnight. Colonies were counted the following day or replated if necessary. Figure 3 shows the growth curve produced, the first growth curve of *P. mirabilis* CDC PR 14 to our knowledge.

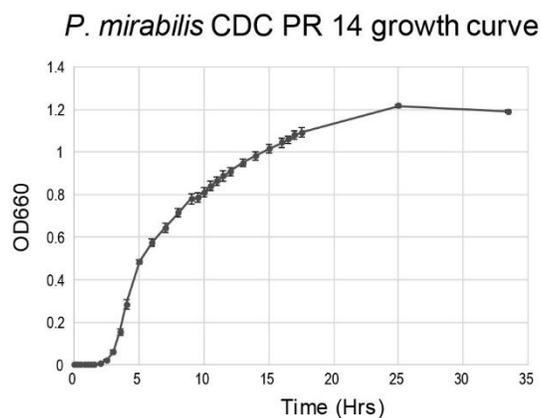


Figure 3. *P. mirabilis* growth curve. Standard deviation error bars are shown for the three replicates.

Biofilm Selection Experiment

A single colony of *P. mirabilis*, *E. coli*, or *P. aeruginosa* was added to 20 mL of LB within a 50 mL capped centrifuge tube containing a sterile microscope glass slide. After overnight growth at 37°C in a 200 rpm shaking incubator, a sterile loop was used to sample from the center of the biofilm formed on the slide. This sample was then used to inoculate 20 mL of LB, again within a 50 mL capped centrifuge tube containing a microscope glass slide. Samples from these biofilms were also streaked on an LB plate or low-salt LB (LSLB) (0.5 NaCl g/L) plate (LSLB plates reduce *P. mirabilis* swarming), to ensure contamination had not occurred. The following protocol was conducted for up to 19 passages. By selecting only bacteria which successfully biofilmed to propagate subsequent populations, this experimental protocol selected for strains that were efficient in forming biofilms. The passages were continued until a visible biofilm was established and could be reproduced from a single colony selected from the streaked bacterium. Images of representative biofilms are shown in Figure 4.

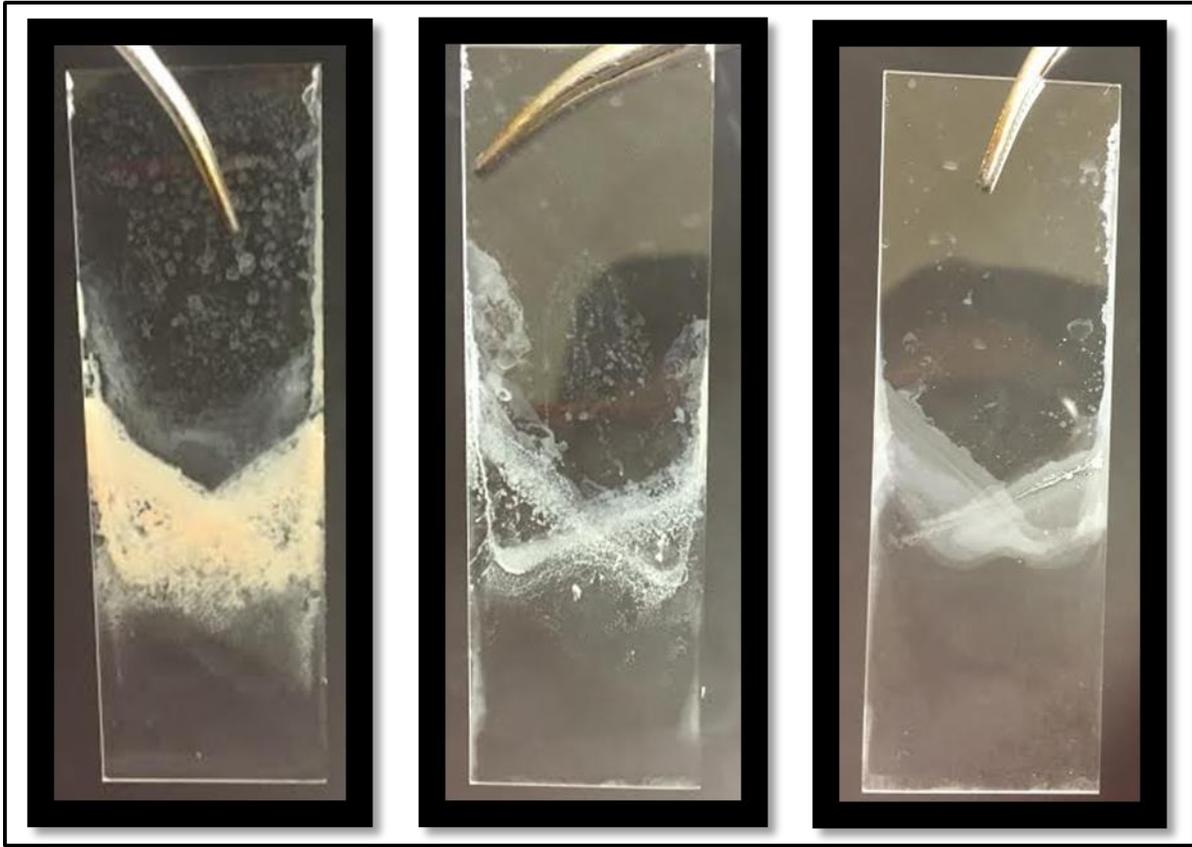


Figure 4. Biofilm formation on glass slides. Images are from left to right *P. aeruginosa*, *P. mirabilis* and *E. coli*.

Phage Growth and Titration

Each phage was plated from our lab's freezer stock and validated for uniform plaque morphology. A single plaque was harvested such that all subsequent work was derived from a single genotype. To harvest phage, a 1000 μ l pipette tip was used to puncture the agar around the plaque and then transferred to a microcentrifuge tube containing 1000 μ l of LB. The tubes were then vortexed for 10 mins using the Disruptor Genie vortex (Scientific Industries). Next, tubes were centrifuged at 10,000xg for 1 min and the lysate was transferred to a fresh microcentrifuge tube. Samples were chloroformed (1-2% by volume) and tubes were vortexed for 10 secs. Prior to transferring the lysate, samples were centrifuged again as described previously. Lysates were

then spotted on their respective bacterial hosts using the double layer agar method, in which a 1.7% agar LB plate was overlaid by 3 mL of 0.7% agar LB (LBSA) mixed with 1 mL of bacterial culture. These plates were then grown overnight at 37°C in a non-shaking incubator. Phage plaques were scraped off of the agar with a sterilized scoopula, transferred to a microcentrifuge tube containing 1000 µl of LB, chloroformed, vortexed and centrifuged.

An 8-fold dilution of the lysate was then performed and plated to determine phage titer. For dilutions: 100 µl of phage harvest was aliquoted into 1 mL of LB. A stepwise dilution was conducted, reducing the phage population with each transfer by 1:10. Lust, Greedy, phiKZ, and DMS3 were grown, harvested, and plated until plaques were visible at 10⁻⁸. The titers of Lust and Greedy were increased through growth on their host *E. coli* while the titers of phiKZ and DMS3 were increased through growth on their host *P. aeruginosa*. Habibi was propagated until plaques were visible at 10⁻⁷ dilution on *P. aeruginosa* and 10⁻⁵ dilution on *E. coli*. In order to retain Habibi's ability to successfully lyse both *P. aeruginosa* and *E. coli*, culture in both bacterial species was necessary. Titers for all five phages were confirmed via a pour plate in triplicate in which two consecutive dilutions were plated. Pour plates were produced as follows: 100 µl of the appropriate dilution was added to 1 mL of the phages host and placed on the benchtop for 10 mins before adding 3 mL of 50°C LBSA. This mixture was then poured over an 1.7% agar LB plate. After setting, plates were placed in a 37°C non-shaking incubator and allowed to grow overnight. Plaques were then counted.

Biofilm Experimental Design

The experimental design is depicted in Figure 5. Briefly, 20 mL of LB was added to a 50 mL capped centrifuge tube in addition to a sterile microscope glass slide. Glass slides were sterilized by submerging them in 70% EtOH after which they were placed in a sterile hood overnight for

EtOH evaporation. Each tube was inoculated with a single colony of the bacterial species. (When more than one bacterial species was used to form a biofilm, the culture was inoculated simultaneously with a single colony from each species.) The tube was incubated at 37°C, shaking at 200 rpm. After a 24-hr incubation, the glass slide was removed with sterile forceps. Excess liquid was removed from the bottom of the slides with a Kimwipe, and the slides were placed into new 50 mL tubes with 20 mL of fresh LB. Each slide was then treated with phage (or no phage in the case of the control). The 24 hr time point was chosen because after a day organization of bacterial species and the matrix has taken place and is truly considered a biofilm (Chandki *et al.* 2011). Following treatment, tubes were returned to the incubator and allowed to grow for an additional 24 hrs at 37°C, shaking at 200 rpm. Next the glass slides and a portion of the planktonic fraction were removed for further analysis.

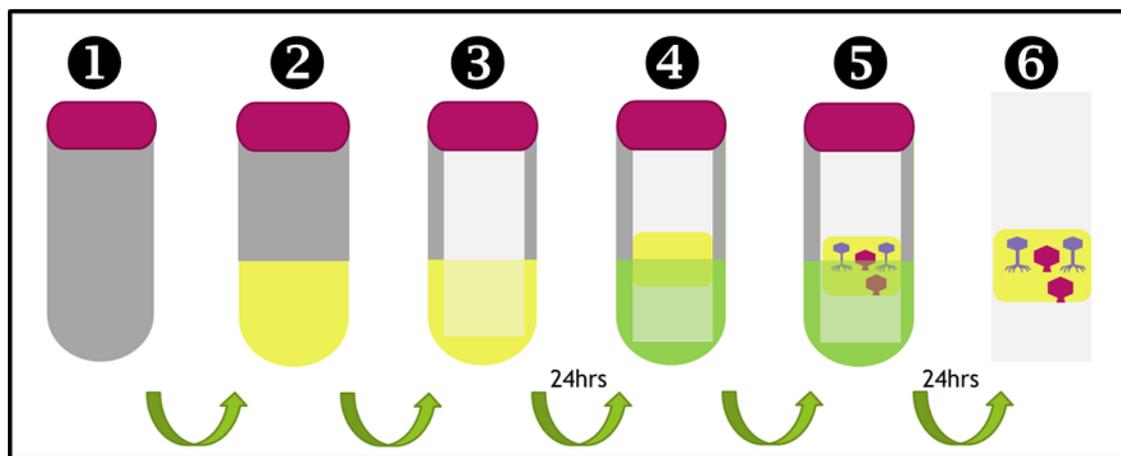


Figure 5. A visualization of the experimental design including biofilm growth and treatment. (1) Empty sterile 50 mL centrifuge tube. (2) 20 mL of LB and (3) a sterile glass slide was placed in the tube. A single bacterial colony per species is added and allowed to grow for 24 hrs resulting in biofilm formation (4). (5) The glass slide was removed and placed into a centrifuge tube containing fresh LB. Treatment was then applied to the slide and placed in the incubator for an additional 24 hours. (6) The slide and planktonic fraction from the tube is separated for subsequent analyses.

For Greedy, Lust, phiKZ, and DMS3 treatments, 100 μl of phage ($\sim 10^6$ phage/ μl) was added to 200 μl of phosphate buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 , and 0.24 g/L KH_2PO_4 , adjusted to pH=7). For Habibi treatments, 100 μl of phage ($\sim 10^5$ phage/ μl in *P. aeruginosa* and $\sim 10^3$ phage/ μl in *E. coli*) was added to 200 μl of PBS. The decreased titer of Habibi was used to remain consistent in treatment volumes and because of Habibi's unique ability to infect and lyse two potential members of the biofilm.

All combinations of the three bacteria received each individual phage treatment in addition to no treatment (PBS) in triplicate. *E. coli*, *P. aeruginosa*, and *E. coli* + *P. aeruginosa* (EP) biofilms also received a phage cocktail treatment consisting of phiKZ and Greedy. The cocktail treatments included 50 μl of each phage ($\sim 10^6$ phage/ μl) in 200 μl of PBS. Alongside each set of biofilm samples, a fourth biofilm was also grown to check for contamination of slides, LB, and/or PBS. Serving as a negative control, these samples were not inoculated with any bacterial species and were treated with 300 μl PBS at the 24 hr mark. Figure 6 lists the treatments conducted.

Mass

Biofilm formation and effects of phage treatment were evaluated based upon the mass of the biofilm. Dry weights of 1.5 mL microcentrifuge tubes were recorded using a scale. One mL of sterile LB broth was added and weight was again recorded. For biofilms: glass slides were removed from 50 mL centrifuge tubes using sterile forceps, the bottom of the slide was touched a Kimwipe to remove excess liquid, and the established biofilm was scraped off of both sides of the slide using inoculating loops and placed into the LB containing microcentrifuge tubes. All tubes were subsequently vortexed and weighed. For the planktonic fraction: tubes containing LB were centrifuged and LB was removed and replaced by 1 mL of the planktonic fraction from

biofilm samples. Dry weights and LB weights were subtracted from final weights to obtain the mass of the biofilm and planktonic fraction.

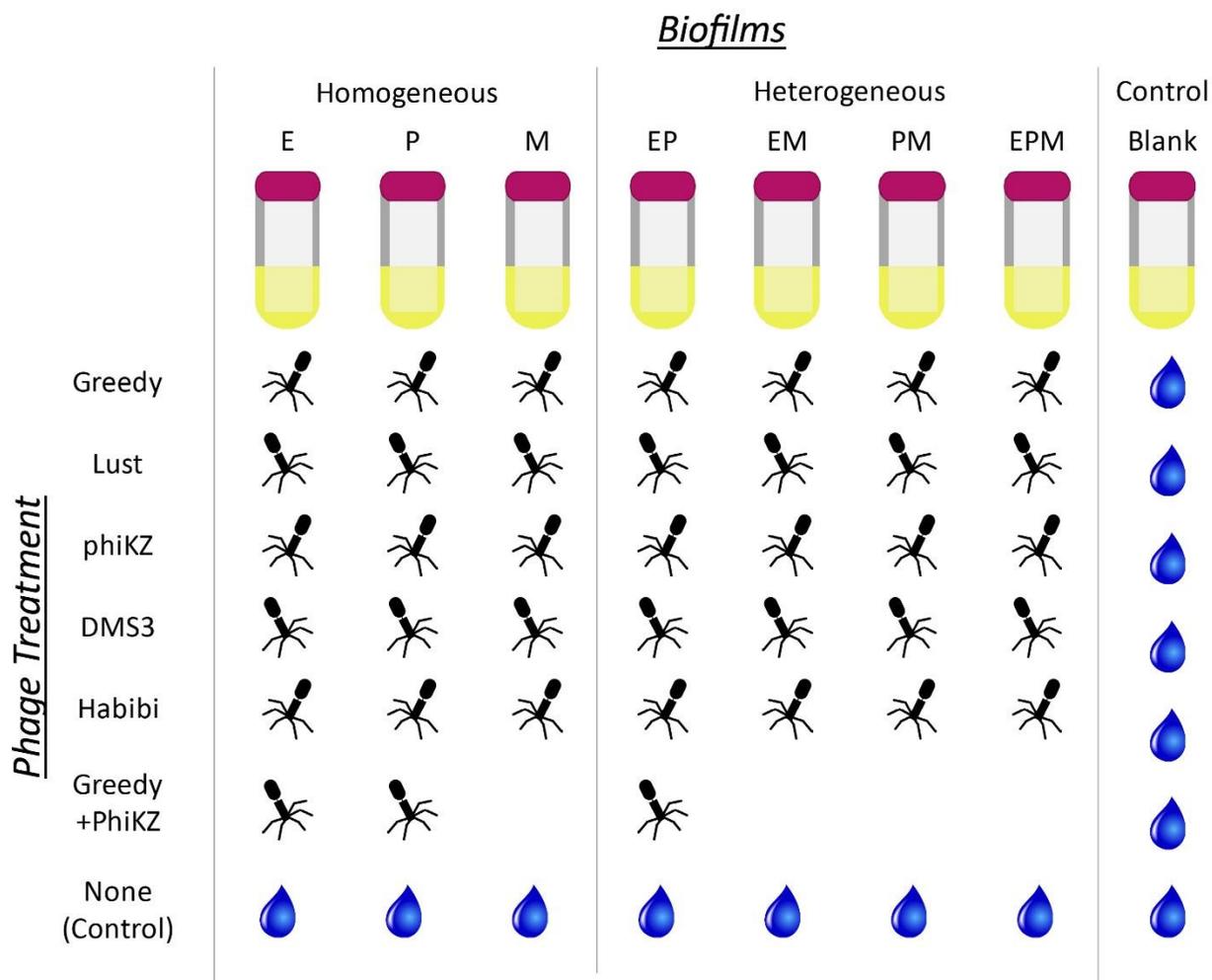


Figure 6. An image depicting biofilms generated and the treatments which were applied to them. The phage cartoon indicates that phage treatment was applied and a water droplet represents PBS treatment (control). All treatments for biofilms were conducted in triplicate. Biofilm cultures are abbreviated according to the bacteria used in the culture as follows. E=*E. coli*; P=*P. aeruginosa*; M=*P. mirabilis*; EP=*E. coli* and *P. aeruginosa*; EM=*E. coli* and *P. mirabilis*; PM=*P. aeruginosa* and *P. mirabilis*; and EPM=*E. coli*, *P. aeruginosa*, and *P. mirabilis*. Biofilms and the planktonic fraction were analyzed via absorbance, colony counting and host range.

Spectrometry and Colony Counting

All spectrometry readings were done at 660 nm; blanks consisted of 1 mL LB broth. For the planktonic fraction, 1 mL was removed from the sample following 48 hrs of growth, vortexed for 10 secs, aliquoted into a cuvette, and the absorbance was recorded. Resuspended biofilms from the mass reading were vortexed for 10 secs, removed from the microcentrifuge tube, and aliquoted into a cuvette and the absorbance was recorded. After recording the absorbance, each sample was returned to its original microcentrifuge tube for further analysis. For colony counts, 100 μ l of the resuspended biofilm or planktonic fraction was used to perform an 8-fold serial dilution as described previously. Two dilutions were plated for each sample; 100 μ l of the dilution was aliquoted onto an LB plate or a low-salt LB (LSLB) (0.5 NaCl g/L) plate, spread using a sterilized spreader, and allowed to dry. The plate was then incubated at 37°C overnight. Colonies were counted for each plate and recorded. For samples inoculated by more than one bacteria, counts were determined for each individual species as identified visually via colony morphology and color. Plates were stored at 4°C.

Bacterial Resistance Assay

LSLB and LB plates were prepared for assessing the phage sensitivity of bacterial isolates from untreated and treated biofilms; LSLB plates were used to test *P. mirabilis* isolates and LB plates were used to test *E. coli* and *P. aeruginosa* isolates. Regardless of the bacterial species or phage being examined, plates were prepared as follows: 50 μ l of naïve phage lysate was spread down the center of the plate and allowed to dry. From the plates produced for colony counting, three random colonies were picked for each species and streaked across the prepared plate, across the region containing the phage lysate. The plates were then incubated overnight at 37°C. Figure 7 presents a representation of the results observed. If the bacterial growth was consistent through

the phage treated portion of the plate, it was considered resistant (Figure 7A). Bacterial growth that stopped at the beginning of the phage treated portion of the plate was considered sensitive (Figure 7B). Variation between resistant and sensitive growth was considered partially resistant (Figure 7C). As a control, an additional colony was streaked through an area of the plate containing no phage treatment (Figure 7D).

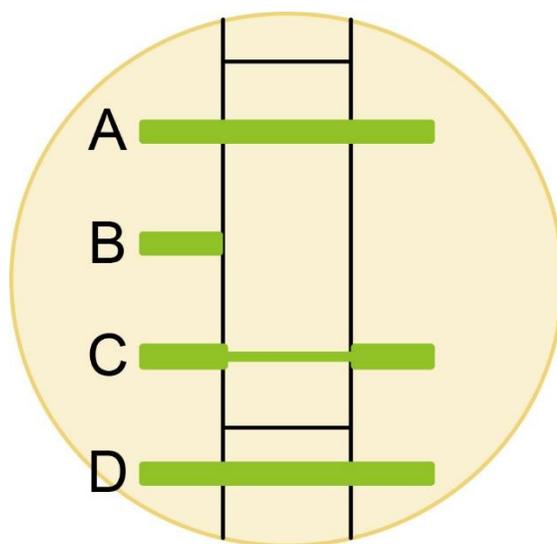


Figure 7. Generalized visualization of method for determining resistance categories: (A) resistance to phage infection (B) susceptibility, (C) partial resistance, and (D) negative control. Green lines represent bacterial growth. Phage treatment was applied in the black box containing A-C. No phage was applied to the black box containing streak (D).

Phage Identification

Phage specific primers were designed using primer3 (Untergasser *et al.* 2012) for all phages as well as the documented prophage within the *P. aeruginosa* strain used in this study. For primer design, the genomes of each of the five phages were retrieved from NCBI. To determine the prophage sequence, we used PHASTER (Arndt *et al.* 2016, Zhou *et al.* 2011). The primers designed are listed in Table 2. All primer pairs were checked for self-priming and dimerization using the ThermoFisher Scientific Multiple Primer Analyzer (ThermoFisher Scientific 2017).

Additionally, all primer pairs were checked for specific amplification computationally by querying each sequence via BLASTX against the nr/nt collection, and at the bench via PCR against DNA from each other phage and each bacterial host.

Phage	Primers	Amplicon Size	Targeted Region
Lust (KX534338)	GATGCGCAAGGAGTTAGAGC ACGTGACACTCGCTTCACAC	873 bp	DNA methylase
Greedy (KX534337)	TGCTAGTGGCCAGCATAGTG TTGTAGAAGTGCAGCCGATG	758 bp	hypothetical protein
Habibi (KT254132)	ACCGACTCACGACGATGG CGGCAAGGTGTTCGCTTA	904 bp	putative structural protein
DMS3 (NC_008717)	AACCGAACCTGAAAATGAC ATCCAAGTCAGAAAGCTGGT	625bp	putative C repressor
phiKZ (NC_004629)	CACGCGTGTAATCAAGACC CCTACTCGTTGGCCAAGTC	964 bp	structural head protein
<i>P. aeruginosa</i> prophage (NZ_CP017149)	GTGGAGCCAGAGTTTTACTG ATAGCGTAGGGAAAGGAATC	527 bp	annotated prophage

Table 2. Primers used for the amplification of phage species. Accession numbers are listed for the genome sequences used to design the primers.

The PCR primers designed here were tested against genomic DNA extracted for each of the phages used in this study and for the *P. aeruginosa* genome (for the detection of the prophage sequence). PCR set up was as follows: 25 μ l Ready PCR mix 2x (Amresco LLC), 0.5-1 μ l forward primer, 0.5-1 μ l reverse primer, 0.5-3 μ l template, 20-23.5 μ l nuclease free water. Thermocycler conditions for each phage tested are listed in Table 3. It is worth noting that this table does not include thermalcycling conditions for the identification of phiKZ. Attempts at phiKZ-specific PCR were unsuccessful due to insufficient phage concentration. Other phiKZ primers in our collection were also tested (results not shown) but were not successful in producing visible amplicons either. To identify phiKZ, plaques were tested against the prophage primers, in the event of no amplification, phiKZ was assumed.

Phage	Initial Denature	Denature	Annealing	Extension	# cycles	Final extension
Greedy and Lust	94°C 10 mins	94°C 30 secs	55.5°C 45 secs	72°C 1 min	30	72°C 5 mins
Habibi	94°C 15 mins	94°C 30 secs	52°C 45 secs	72°C 45 secs	45	72°C 5 mins
DMS3	94°C 15 mins	94°C 30 secs	54°C 45 secs	72°C 45 secs	30	72°C 5 minutes
<i>P. aeruginosa</i> prophage	94°C 10 mins	94°C 30 secs	55°C 30 secs	68°C 30 secs	30	68°C 5 mins

Table 3. Thermocycler conditions for phage identity testing.

Planktonic and biofilm samples were chloroformed (2% by volume), vortexed for 10 secs, and lysate was separated from the pellet. Subsequently, an 8-fold dilution was performed using 100 μ l of serially diluted lysate. Dilutions were spotted onto naïve lawns of each bacterial species present in the biofilm. If lysis was observed after overnight growth, pour plates at the corresponding dilutions were performed. Ten random plaques were harvested from each plate as previously described (section “Phage Growth and Titration”). Plaque harvests were stored at 4°C.

To identify the phage(s) producing the observed plaques, 50 μ l of phage lysate was used to inoculate 1 mL of a naïve culture of the bacterial host, the bacterium on which the phage was harvested. Following overnight growth each sample was chloroformed (20 μ l), vortexed, centrifuged (16,100xg rpm for 2 mins) twice, and the lysate was transferred to a sterile microcentrifuge tube. PCR reactions were immediately performed following the phage-specific protocol listed in Table 3. For positive controls 1 μ l DNA was used. For PCR reactions of the samples, 3 μ l of lysate was used. Amplicons were visualized using a 1.2% agarose gel.

If phage lysate PCR was unsuccessful, 10 μ l of the phage lysate was plated using the double layer agar method previously described. Following overnight growth at 37°C, spots were

harvested and suspended in 300 μ l nuclease-free H₂O. This suspension was vortexed for 10 mins, chloroformed (20 μ l), vortexed 10 secs, and centrifuged at 16.1 rpm for 2 mins. Each sample was chloroform treated three times to ensure no viable host cells remained. Lysate was removed, separated from the pellet, and used as the sample input for PCR. If this PCR was unsuccessful, lysate was regrown as previously described and phage DNA was extracted using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories). The manufacturer's protocol was followed with the following exceptions: rather than pelleting down the sample for bacterial extraction, 300 μ l of viral lysate was used with 250 μ l of bead solution. Furthermore, a 70°C heat step was added to increase DNA yield.

Phage Host Range Assay

Phage host range was tested in triplicate before experimental treatment. To test, 10 μ l of pure phage cultures were spotted on bacterial lawns, allowed to dry, and incubated overnight at 37°C. To reduce the risk of contamination, spotting was limited to a single phage per plate. For phages that were able to infect multiple hosts, dilutions were plated so that individual plaques could be harvested. Single plaque harvests were subsequently used to confirm that plaques on multiple species were in fact the same phage. Post experimental host range was recorded using phage identification results.

Statistical Analysis

Statistical analyses were performed in R (R core Team 2013). Data for each biofilm was considered separately. For example, data collected for all of the 18 *E. coli* biofilm absorbance data points were considered and checked for normality using the shapiro.test function before and after any necessary transformations. Abnormally distributed data was log transformed, if the transformation failed to normalize the data a box-cox transformation was performed using the

bctransform function in the MASS (Venables and Ripley, 2002) package. If normalization was successful, a one way ANOVA was performed using the aov. function in the Rcmdr package (Fox and Bouchet-Valat, 2017; Fox 2017; Fox 2005). If the ANOVA resulted in a significant difference between treatments, a Tukey's multiple comparison test was performed using the TukeyHSD function to identify which treatments were significantly different from each other. If data could not be normalized the Kruskal.test function was used.

CHAPTER THREE

RESULTS AND DISCUSSION

Five phage species were selected for this study and are described in detail in the Methods and Table 1. Briefly, bacteriophage Greedy and Lust, both isolated from the microbiota of the human bladder (Malki *et al.* 2016), can effectively infect and lyse several *E. coli* strains including *E. coli* C. The bacteriophage phiKZ, isolated from sewage (Krylov *et al.* 1978), and DMS3, induced from *P. aeruginosa* clinical isolates (Budzik *et al.* 2004), are both capable of infecting and lysing *P. aeruginosa* including several human pathogenic strains of *P. aeruginosa* (Mesyanzhinov *et al.* 2002). The fifth phage selected for this study is bacteriophage Habibi, which was isolated from Lake Michigan nearshore waters and is capable of infecting both *E. coli* and *P. aeruginosa* strains, amongst other bacterial taxa (Malki *et al.* 2015). Habibi is considered a broad host range phage, as it is capable of infecting across genera, while the other four phages used in this study are narrow host range phage, capable of infecting only strains of the same species.

Thus, our study includes phage species capable of infecting two of the bacterial species frequently associated with biofilms within catheters and CAUTIs. None of the phages, however, can infect the CAUTI-associated bacterium *P. mirabilis*. Although significant efforts were made to isolate a phage capable of reliably infecting and lysing *P. mirabilis*, we were unable to isolate a phage that could work consistently under standard laboratory conditions (results not shown). Furthermore, our attempt to purchase a *P. mirabilis*-infecting phage from the Felix d'Herelle Reference Center for Bacterial Viruses (Quebec, Canada) was also unsuccessful; the curators

were unable to propagate the one *P. mirabilis* phage in their collection.

Effects of Phage Treatment on Homogeneous Biofilms

Biofilms of *E. coli*, *P. aeruginosa*, and *P. mirabilis* were first treated by each of the five phage strains. As detailed within the Methods chapter, phage treatment was administered after the biofilm was established. Thus, our evaluation is focused on the effectiveness of phage treatment in reducing/eradicating biofilms. Furthermore, each of the bacterial strains used in this study were the result of a selection experiment in which bacterial colonies that were efficient in their ability to form biofilms were passaged serially (see Methods). Each treatment was conducted in triplicate along with a control (no treatment). After treatment of each of the three homogeneous biofilms, the efficacy of phage treatment was measured based on the biofilm's absorbance and individual species colony counts. While the former captures the presence of both viable and non-viable bacteria, the latter more closely represents the viable cells within the biofilm post-treatment. We also measured the mass of the biofilm before and after treatment, but found these measurements to be unreliable as often biomass differences were not detected due to the lack of precision available by our nanoscale. We thus do not include these measurements in our discussions here. All boxplots shown represent untransformed data.

To ascertain the effectiveness of the five phages under investigation, we first examined the treatment of monoculture biofilms of *E. coli* and *P. aeruginosa*. Statistically significant effects were evaluated through the comparison of the biofilm density (absorbance or colony counts) of untreated biofilms (control) and the treated biofilms. Figure 8 depicts boxplots of absorbance and colony count data for *E. coli* and *P. aeruginosa* biofilms. Figures 8A and 8B show the absorbance and *E. coli* colony counts, respectively, for the control lines (untreated) as well as the treatments using Lust, Greedy, and Habibi. *E. coli* absorbance data was log

transformed and an ANOVA was performed ($F^{3,8}=9.792$ $P=0.0047$). We found that treatments with the phages Lust ($P=0.02597$) and Greedy ($P=0.00427$) were significantly different from the control treatment when considering the absorbance (Figure 8A). However, treatment with Lust was not found to be significant when considering the colony count data. As shown in Figure 8B, the number of viable *E. coli* colonies (log-transformed) retrieved from the biofilm after treatment with Lust was highly variable. Nevertheless, Greedy ($P=0.00919$) was significantly different from control treatments for colony count data ($F^{3,8}=7.279$ $P=0.0113$) (Figure 8B).

Figure 8C shows the measured absorbance from the untreated (control) *P. aeruginosa* biofilms as well as those treated by the phages phiKZ, DMS3, and Habibi. Statistical methods similar to that described for the absorbance data for the *E. coli* biofilms were conducted. No significant differences between the control and the treatments were identified (Figure 8C). We observed, however, that both phiKZ ($P=0.00254$) and DMS3 ($P=0.04907$) treatments resulted in statistically significant differences from the untreated control biofilms (Figure 8D). This was determined by performing box-cox normalization ($\lambda=-0.34$) for the colony count data and an ANOVA ($F^{3,8}=10.1$ $P=0.00427$).

From the results of these assays, we found that Greedy and phiKZ are the most effective at reducing biofilms of *E. coli* and *P. aeruginosa*, respectively (Figure 8). Quantifying the effects of biofilm treatment via both absorbance and colony counts provides a means of distinguishing between viable and inviable biofilms. For instance, the success of phiKZ treatment on the *P. aeruginosa* biofilm was not significant for the absorbance data (Figure 8C); it was, however, significant for the colony count data (Figure 8D). Thus, we believe that the colony count data provides a more robust measure of the effect of phage treatment of biofilms.

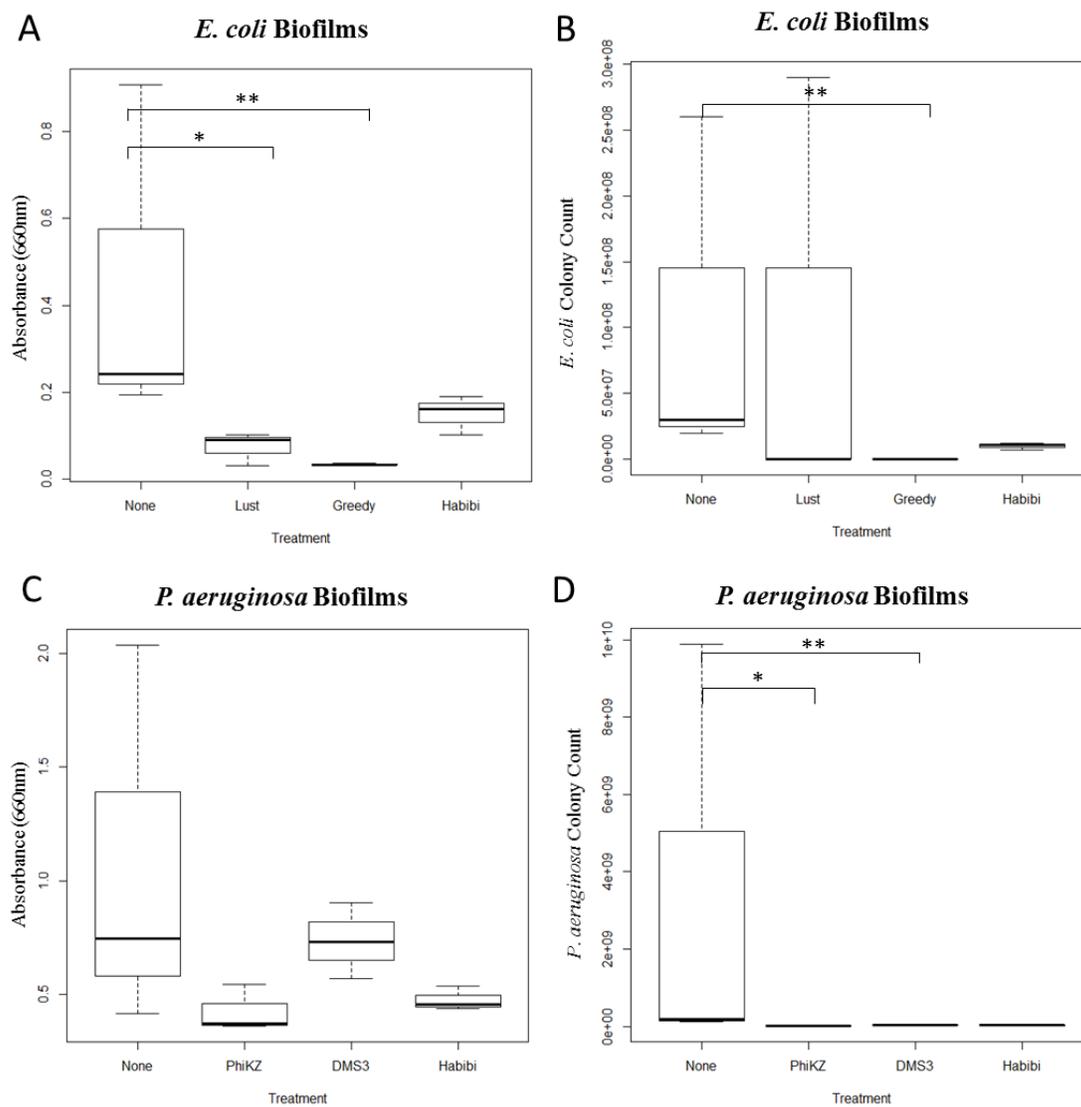


Figure 8. Effects of coliphage and *P. aeruginosa* phage treatments on homogenous *E. coli* and *P. aeruginosa* biofilms measured by absorbance (panels **A** and **C**) and colony counts (panels **B** and **D**). An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where * indicates a P-value < 0.05 and ** denotes a P-value < 0.01.

To date, the method by which Greedy and Lust infect the *E. coli* C cell is unknown.

While a handful of coliphages exhibiting similar genomic sequences (Carstens *et al.* 2015, Doan *et al.* 2015, Smith *et al.* 2015) have recently been published, they have not been characterized in the laboratory; their growth parameters and mechanisms of infection are unknown. Within the

Putonti lab both Greedy and Lust have been tested against several *E. coli* laboratory strains and clinical *E. coli* isolates from the bladder, including strains from patients suffering from UTIs, cystitis, and pyelonephritis as well as asymptomatic “healthy” individuals. While both Lust and Greedy can lyse some *E. coli* strains from UTI patients, they were unable to lyse all UTI-associated *E. coli* strains tested. This is not surprising as the differences in genotype and phenotype between the *E. coli* strains themselves are unknown and the same symptoms may have different etiologies. Against this subset of bacteria tested, however, it is worth noting that Greedy exhibited a broader host range than Lust as it was successful in infecting and lysing more *E. coli* strains (in particular clinical strains) than Lust (Putonti *et al.*, in preparation). The observed difference between Greedy and Lust’s ability to reduce the *E. coli* biofilm is an interesting area for further investigation.

In contrast to the coliphages, the *Pseudomonas*-infecting phages phiKZ and DMS3 have been extensively characterized in the laboratory. In a study using the same strain of *P. aeruginosa* used here, they found that phage KTN4 (a phiKZ-like-virus) disturbed biofilms and reduced the production of pyocyanin and siderophores (Danis-Wlodarczyk *et al.* 2004). Pyocyanin is a *P. aeruginosa* produced toxin that gives it its characteristic green color and plays a role in biofilm formation (Lao *et al.* 2004). PhiKZ has also been used in studies that aerosolized phage to test the feasibility of using inhalers to treat *P. aeruginosa* lung infections (Golshahi *et al.* 2011, Matinkhoo *et al.* 2011). PhiKZ was found to have a dose dependent effect on *P. aeruginosa* infections in mice lungs (Henry *et al.* 2013). Another study used phiKZ, along with other *Pseudomonas* phages, to test the resilience of clinical isolates from chronic *P. aeruginosa* infections; they found that these isolates had an increased susceptibility to phage infection (Friman *et al.* 2013). DMS3 has been studied with particular relevance to its effect on

biofilms. It has been shown to reduce swarming motility in *P. aeruginosa* PA14 via the CRISPR-Cas system (Zegans *et al.* 2009, Chung *et al.* 2012). Furthermore, DMS3 lysogeny blocks biofilm formation in *P. aeruginosa* PA14 with a 50% reduction of attachment in the first 30 mins (Zegans *et al.* 2009). Given this prior work, it is thus not surprising that DMS3 was effective in disrupting the viability of the *P. aeruginosa* biofilm (Figure 8D).

Phage treatments for biofilms of non-host species included the treatment of *P. mirabilis* biofilms with all five phages, coliphage treatment of *P. aeruginosa* biofilms, and *Pseudomonas* phage treatment of *E. coli* biofilms. Figure 9 shows the absorbance measurements of these treatments while Figure 10 shows the colony counts for these treatments. Looking first at the absorbance results, no statistically significant effect in the *P. mirabilis* biofilms was observed (Figure 9A). While visually it appears that the Habibi treatment did lead to a decrease in *P. mirabilis* biofilms (per absorbance measurements), this was not statistically significant ($P=0.281$). In contrast, treatment of *E. coli* biofilms by both *Pseudomonas*-infecting phages had an effect (Figure 9B). *E. coli* absorbance data was normalized (box-cox transformation, $\lambda=-0.94$) and an ANOVA was performed ($F^{2,6}=45.63$ $P=0.000235$). The effect of phiKZ (relative to the untreated control biofilms) was statistically significant ($P<0.001$) as was the effect of DMS3 ($P<0.001$). No statistically significant effect was identified for coliphage treatments of *P. aeruginosa* biofilms (Figure 9C).

To ascertain if the treatments had an effect on the viable bacterial community of the biofilm, colony count data was considered (Figure 10). Paralleling that observed for the absorbance data (Figure 9), no significant effect was observed for the *P. mirabilis* (Figure 10A) and *P. aeruginosa* (Figure 10C) biofilms. However, the *Pseudomonas* phages phiKZ and DMS3 treatment of the *E. coli* biofilms had an effect on the biofilm community; *E. coli* colony count

data was normalized (box-cox transformation, lambda= -0.69) and an ANOVA was performed ($F^{2,6}=20.64$ $P=0.00204$). PhiKZ ($P=0.00546$) and Greedy (0.00245) were found to be significantly different from the control treatment.

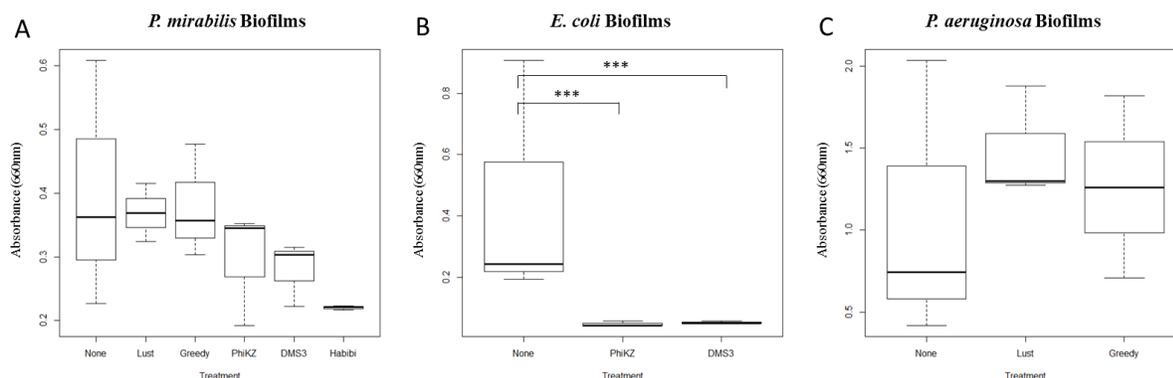


Figure 9. Effects on the biofilms, as measured by absorbance, for phage treatments of non-host phage species. (A) Treatments for all five phages on *P. mirabilis* biofilms. (B) Treatments for phiKZ and DMS3 phages on *E. coli* biofilms. (C) Treatments of coliphages Lust and Greedy on *P. aeruginosa* biofilms. An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where *** indicates a P-value < 0.001.

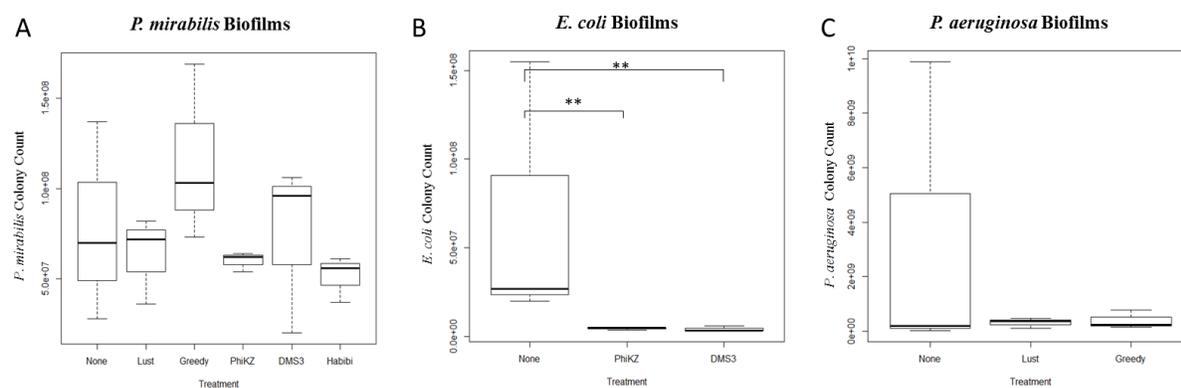


Figure 10. Effects on the biofilms, as measured by colony counts, for phage treatments of non-host phage species. (A) Treatments for all five phages on *P. mirabilis* biofilms. (B) Treatments for phiKZ and DMS3 phages on *E. coli* biofilms. (C) Treatments of coliphages Lust and Greedy on *P. aeruginosa* biofilms. An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where ** indicates a P-value < 0.01.

It is interesting that *P. aeruginosa* phages had a significant effect on *E. coli* biofilms despite their inability to lyse *E. coli*. Lysis is not the only way that a phage can inhibit bacterial

growth. For example, as mentioned previously, when DMS3 switches to lysogeny it inhibits *P. aeruginosa* biofilm formation (Zegans *et al.* 2009). Adsorption induced lysis as a result of high concentrations of phage is another possibility (Abdeon 2011, Turner and Chao 1998).

Additionally, phages may produce toxins or enzymes that inhibit bacterial growth, or perhaps the phages are attaching to the cell wall but are unable to degrade and enter the cell. There is still much to learn about bacteriophages and the ways they interact with bacteria.

Effects of Phage Treatment on Heterogenous Biofilms

In addition to the phage treatments applied to homogenous biofilms of *E. coli*, *P. aeruginosa*, and *P. mirabilis*, complex communities of biofilms were created. Biofilms consisting of each pair as well as all three of these CAUTIs-associated bacteria were established in the same manner as the homogeneous biofilms (see Methods). Thus, four heterogeneous biofilms were considered in this study: *E. coli* and *P. aeruginosa* (EP), *E. coli* and *P. mirabilis* (EM), *P. aeruginosa* and *P. mirabilis* (PM), and all three bacteria (EPM). Again, each treatment was conducted in triplicate in addition to a control (no treatment).

While absorbance measures revealed that Lust and Greedy phage treatments had a statistically significant effect on homogeneous *E. coli* biofilms (Figure 8A), the same cannot be said for the heterogeneous biofilms. As shown in Figure 11, no statistically significant effect was detected by means of absorbance for any of the heterogenous biofilms treated with any of the phage. Total colony count data, however, show a different trend; several of the treatments of the PM (Figure 12C) and EPM (Figure 12D) biofilms reveal significant effects. The total colony count represents the number of viable bacteria, regardless of species, in the heterogenous biofilm. The PM total colony count data was normalized (log-transformation) and an ANOVA was performed ($F^{5,12}=3.466$ $P=0.0361$). A significant effect to the biofilm was only found when

considering the untreated (control) and Lust treatments ($P=0.0294$) (Figure 12C). The EPM total colony count data was also log-transformed and an ANOVA was performed ($F^{5,12}=5.096$ $P=0.00977$). As Figure 12D shows, a significant difference was found between the untreated control and four of the treatment regimens: Greedy ($P=0.03056$), Lust ($P=0.00998$), phiKZ ($P=0.02728$), and Habibi ($P=0.01750$).

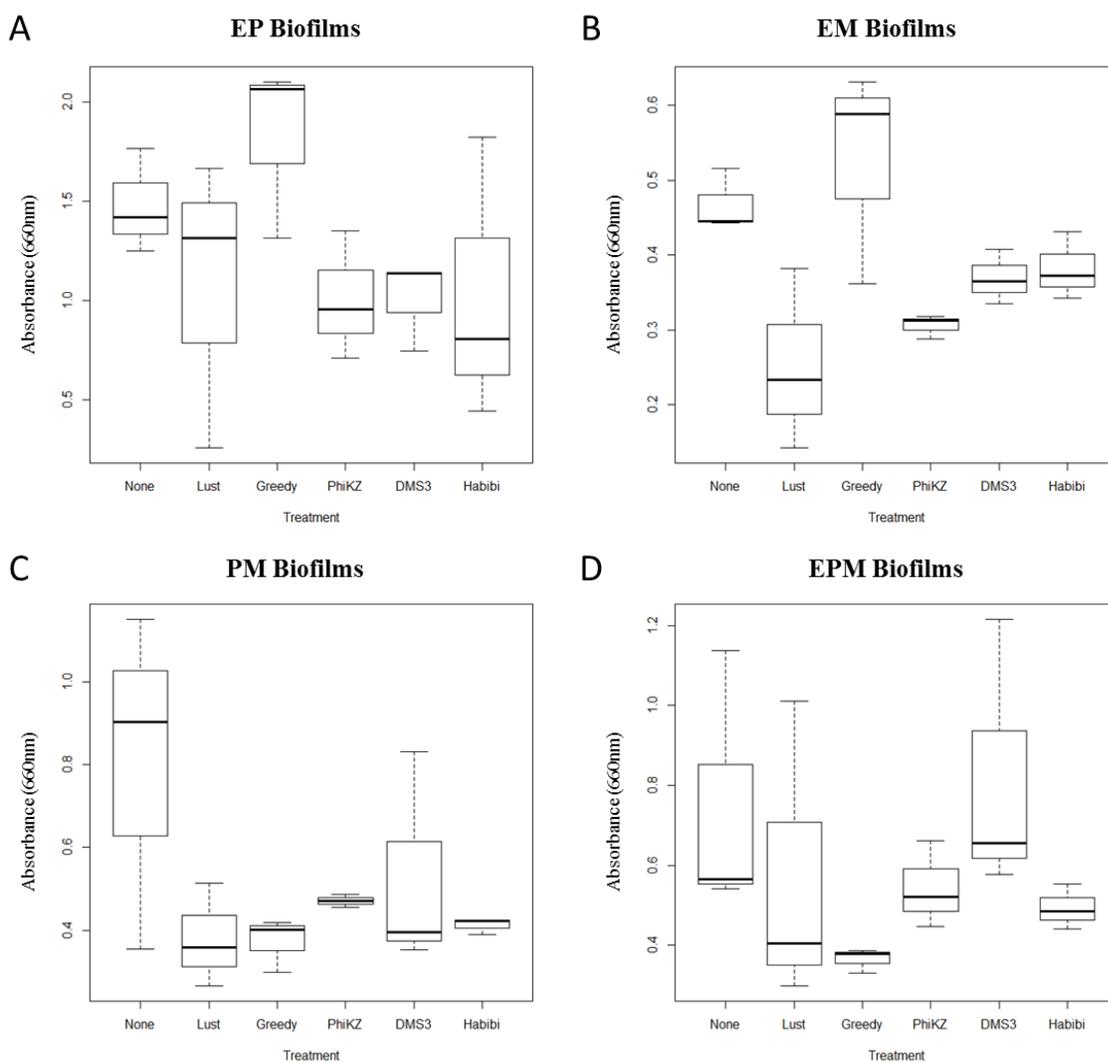


Figure 11. Effects of phage treatments, quantified by absorbance, on heterogenous biofilms of: (A) *E. coli* and *P. aeruginosa*, (B) *E. coli* and *P. mirabilis*, (C) *P. aeruginosa* and *P. mirabilis*, and (D) *E. coli*, *P. aeruginosa*, and *P. mirabilis*.

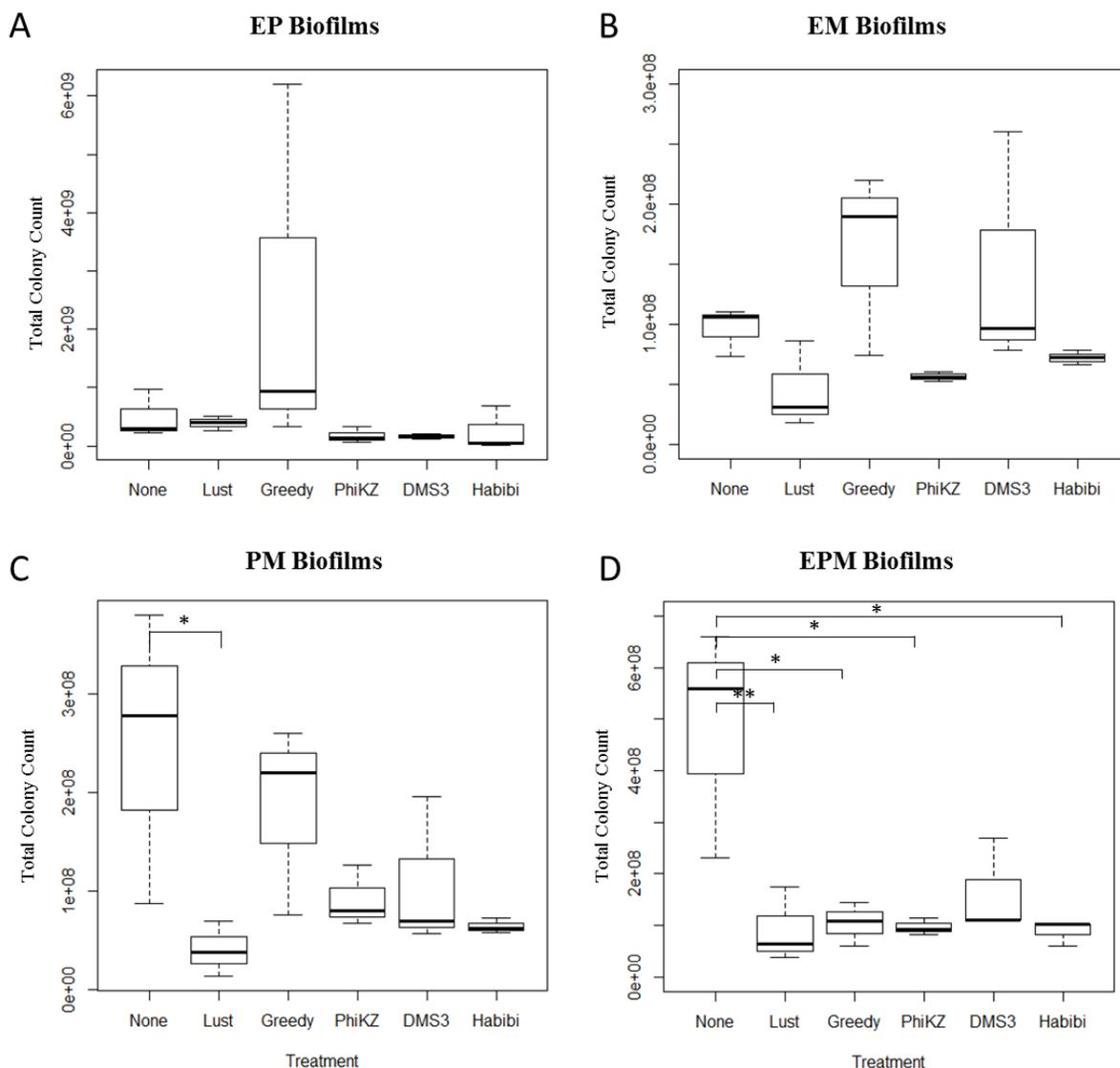


Figure 12. Effects of phage treatments, quantified by total colony count, on heterogenous biofilms of: **(A)** *E. coli* and *P. aeruginosa*, **(B)** *E. coli* and *P. mirabilis*, **(C)** *P. aeruginosa* and *P. mirabilis*, and **(D)** *E. coli*, *P. aeruginosa*, and *P. mirabilis*. An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where * indicates a P-value < 0.05 and ** indicates a P-value < 0.01.

While Figure 12 captures the overall viability of the biofilm community, we were interested in investigating specifically how each of the phage treatments impacted the individual constituents of the complex community. Thus, for each plate, individual colonies were identified

by species based upon their morphological features. Figures 13 and 14 present a breakdown of the colony counts by species for the two-species biofilms and three-species biofilms, respectively.

Where we saw four of the five phage treatments were significantly different from the control in EPM biofilms in regards to total colony counts (Figure 12), we only see a significant effect in *P. aeruginosa* individual colony counts (Figure 14). *P. aeruginosa* colony counts were normalized (log-transformation) and ANOVA was performed ($F^{5,12}=4.403$ $P=0.0165$). PhiKZ was found to have a significant effect on EPM biofilms ($P=0.0397$). Furthermore, for the two-species biofilms we see significant effects with respect to *P. aeruginosa* colony counts in PM biofilms (Figure 13F). After log-transformation, an ANOVA was performed ($F^{5,12}=5.311$ $P=0.00838$), and effects were found for both phiKZ ($P=0.0124$) and DMS3 ($P=0.0397$). We also identified a significant difference between treatments for *E. coli* colony counts in the EM biofilms when a Kruskal-Wallis was performed ($\chi^2=15.739$, $df=5$, $P=0.007631$). *P. mirabilis* colony counts (log-transformed) also showed a significant ANOVA in EM biofilms ($F^{5,12}=3.548$ $P=0.0336$); there was a significant difference between Greedy and Lust treatments ($P=0.0403$). For *E. coli* colony counts treated with Greedy, no colonies were observed at the dilutions plated. To perform a box-cox transformation ($\lambda=0.23$) $1.0e+04$ was used in place of zero, this number was chosen because it is the dilution *E. coli* colonies plated at when homogenous *E. coli* biofilms were treated with Greedy. Then an ANOVA was performed ($F^{5,12}=4.146$ $P=0.0203$); Greedy ($P=0.0213$) and Habibi ($P=0.0250$) were significantly different from control treatments.

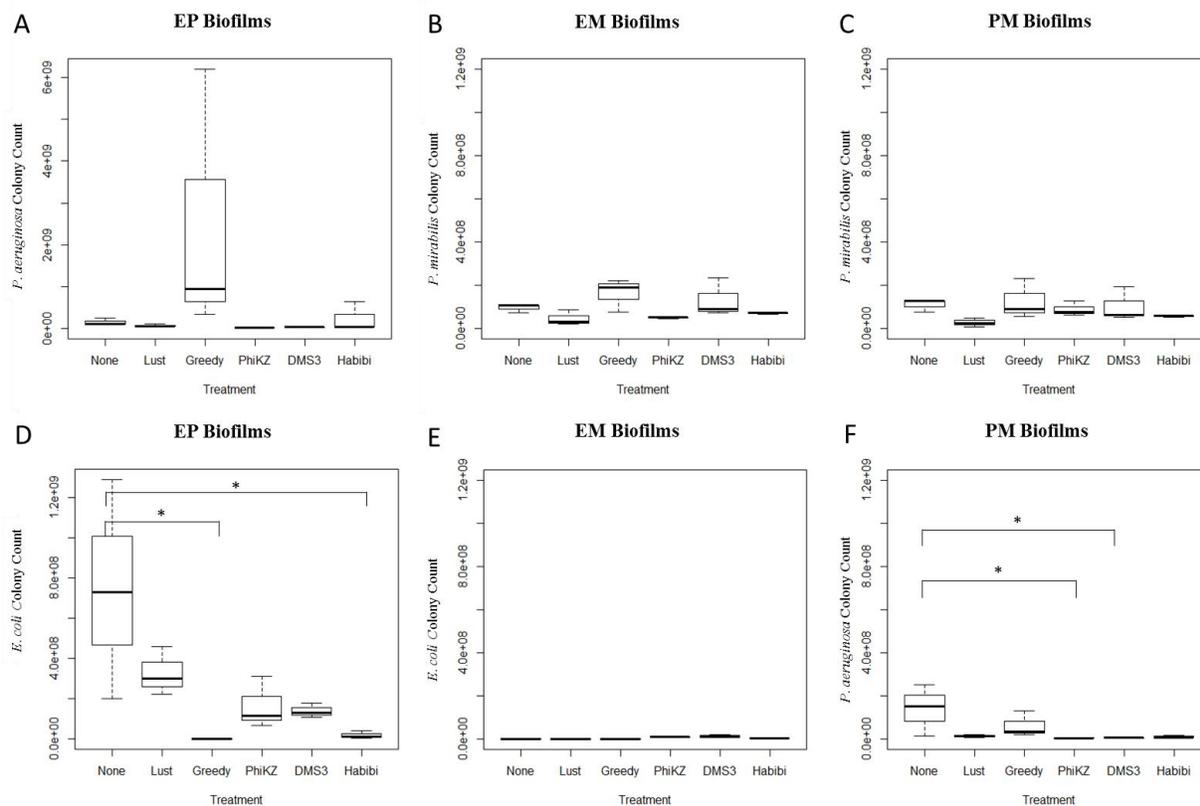


Figure 13. The effects of phage treatments, quantified by species colony counts, on heterogenous biofilms. **(A)** *P. aeruginosa* colony counts in *E. coli* and *P. aeruginosa* biofilms, **(B)** *P. mirabilis* colony counts in *E. coli* and *P. mirabilis* biofilms, **(C)** *P. mirabilis* colony counts in *P. aeruginosa* and *P. mirabilis* biofilms, **(D)** *E. coli* colony counts in *E. coli* and *P. aeruginosa* biofilms, **(E)** *E. coli* colony counts in *E. coli* and *P. mirabilis* biofilms, and **(F)** *P. aeruginosa* colony counts in *P. aeruginosa* and *P. mirabilis* biofilms. An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where * indicates $p < 0.05$.

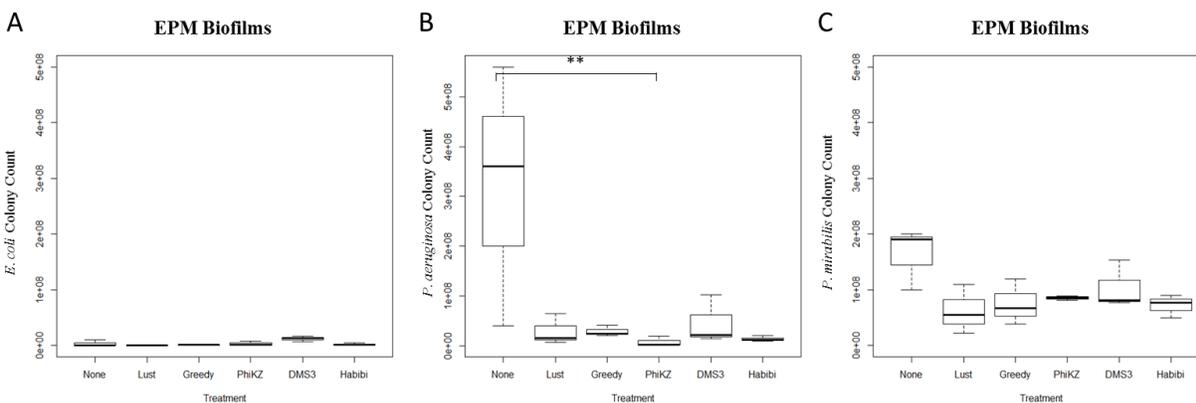


Figure 14. The effect of phage treatments, quantified by species colony counts, on heterogeneous *E. coli*, *P. aeruginosa*, and *P. mirabilis* biofilms. **(A)** *E. coli* colony counts, **(B)** *P. aeruginosa* colony counts, and **(C)** *P. mirabilis* colony counts.

Biofilm Response to Treatment with Broad Host Range Phage Habibi

Given Habibi's ability to infect both *E. coli* and *P. aeruginosa*, we originally hypothesized that it would be an effective phage treatment for both homogenous biofilms of *E. coli* and *P.*

aeruginosa as well as a heterogeneous biofilm containing the two bacteria species. While presented in prior figures, the direct comparisons for no treatment and Habibi treatments are shown here. While absorbance data indicated no significant effects (Figure 15), a statistically significant effect was detected using colony counts data for the *P. aeruginosa* biofilms (Figure 16C) as well as for the *E. coli* population of the EP biofilms (Figure 16B).

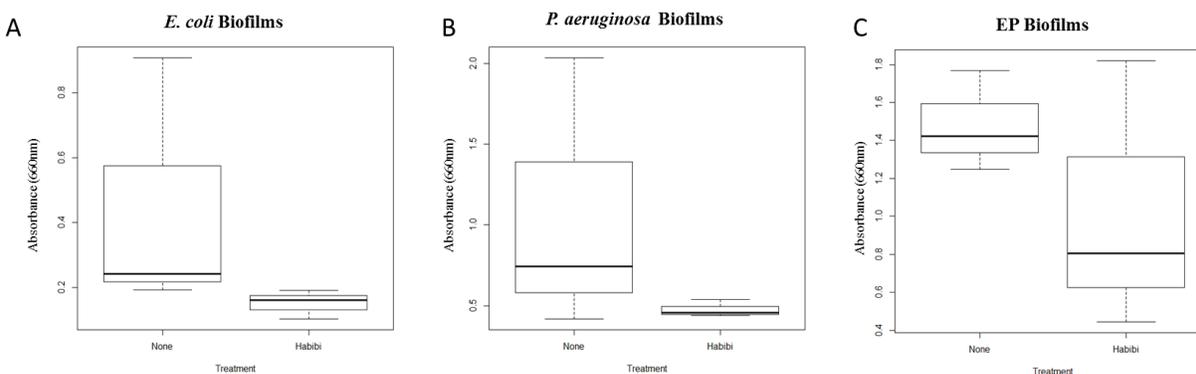


Figure 15. Effects of Habibi phage treatments on (A) *E. coli*, (B) *P. aeruginosa*, and (C) *E. coli* and *P. aeruginosa* biofilms, assessed via absorbance.

When considering the viable bacteria within the heterogeneous EP biofilm, we anticipated that Habibi treatment would have a greater effect on *Pseudomonas* as we have previously observed a higher plating efficiency of Habibi on *P. aeruginosa* relative to *E. coli*. The homogenous *P. aeruginosa* biofilms supported this conjecture (Figure 16C). *P. aeruginosa* colony count data for *P. aeruginosa* biofilms was normalized (box-cox transformation, $\lambda = -0.38$) and an independent sample t-test was performed ($t = 4.4422$, $df = 2.4384$, $p = 0.03235$). Despite this observation for the homogenous biofilm, the *P. aeruginosa* colony count from the EP biofilm Habibi treatments did not show a decrease in the number of viable cells. From a purely visual inspection of the log-transformed data in Figure 16D, Habibi had a varied effect on the *P. aeruginosa* cells; one of the replicates had 10x more *P. aeruginosa* colonies than the other two replicates. A statistically significant effect was observed for the *E. coli* colonies within these same treatments of the heterogeneous EP biofilms. *E. coli* colony counts for EP biofilms were normalized (log-transformation) and an independent samples t-test was performed ($t = 4.508$, $df = 3.8486$, $P = 0.01175$). *E. coli* densities between the three replicates were consistent.

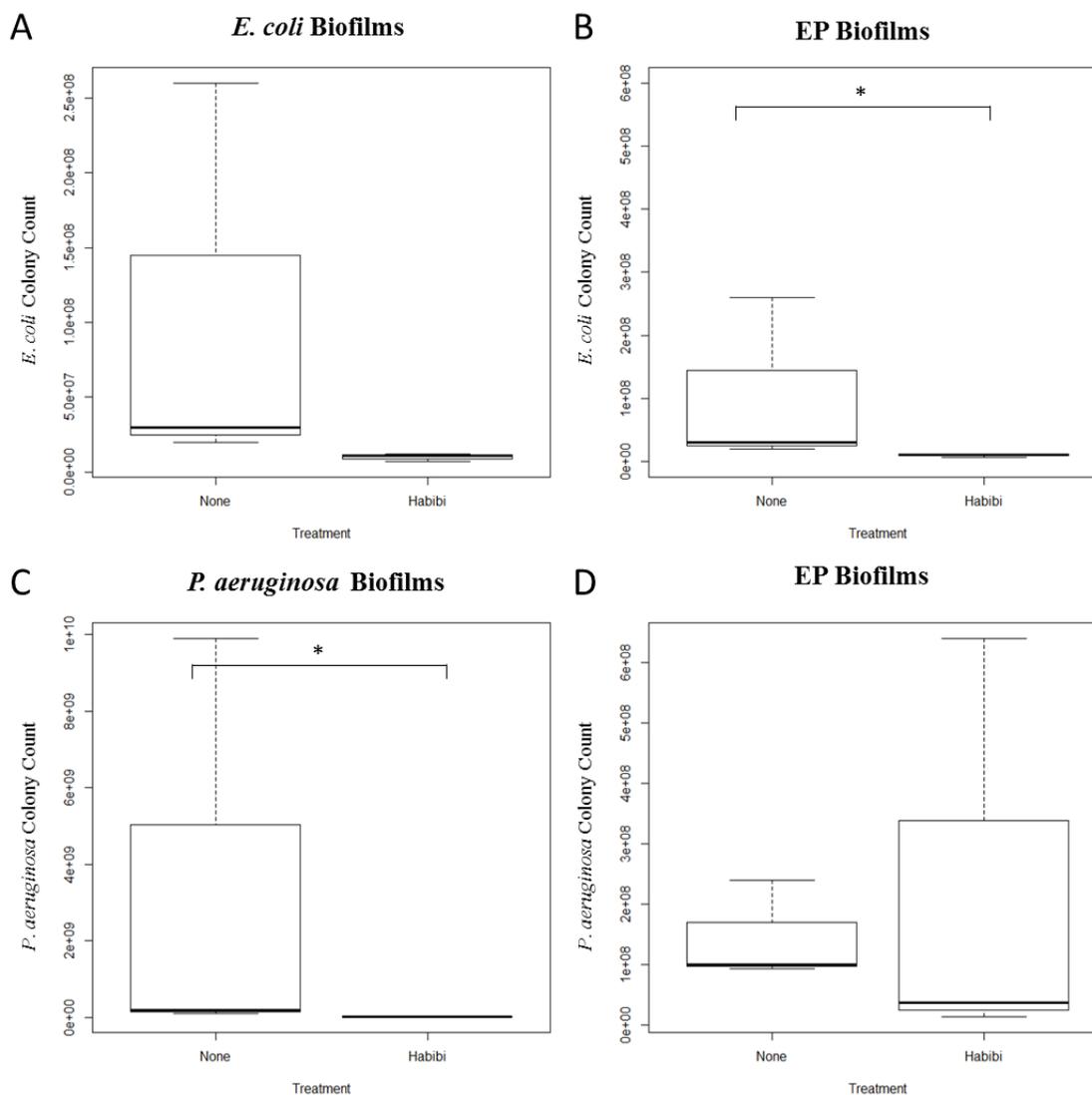


Figure 16. Effects of Habibi phage treatments, quantified by colony counts, on (A) *E. coli* in homogeneous *E. coli* biofilms, (B) the *E. coli* population in EP biofilms, (C) *P. aeruginosa* in homogeneous *P. aeruginosa* biofilms, and (D) the *P. aeruginosa* population in EP biofilms. An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where * indicates a P-value < 0.05.

Biofilm Response to Treatment with Phage Cocktails

Due to the fact that the broad host range phage of Habibi was not effective in reducing both the *P. aeruginosa* and *E. coli* populations of the EP biofilm, we decided to try and target the two bacteria by means of a phage cocktail. From the individual phage treatments of both

homogeneous and heterogeneous biofilms, the coliphage Greedy and the *Pseudomonas* phage phiKZ were selected for the phage cocktail. As with Habibi, we assessed the differences between Greedy, phiKZ, and Greedy+phiKZ (henceforth denoted as “G+phi”) treatments by both the recorded absorbance (Figure 17) and single species colony count data (Figure 18).

The *E. coli* biofilm absorbance data was normalized using the box-cox transformation ($\lambda = -0.94$). As shown in Figure 17A, we see that every phage treatment was significantly different from the untreated control (ANOVA, $F^{3,8} = 18.2$ $P = 0.000622$). Tukey’s multiple comparison test was performed finding statistical significance between: untreated and G+Phi treatment ($P = 0.00575$), untreated and Greedy ($P < 0.001$), and untreated and phiKZ ($P = 0.00434$). The absorbance data collected for the *P. aeruginosa* biofilms was log transformed and an ANOVA was performed. In contrast to the results observed for the *E. coli* biofilms, none of the phage treatments had a significant effect on the *P. aeruginosa* biofilm per the absorbance measure (Figure 17B). In the heterogeneous EP biofilms (Figure 17C), only the G+Phi treatment was statistically significant in relation to the untreated biofilms (ANOVA, $F^{3,8} = 17.39$ $P = 0.00726$). Tukey’s multiple comparison test between untreated and G+Phi treatment resulted in $P = 0.00285$.

Statistically significant effects due to phage treatment of the *E. coli* biofilms were also observed when considering colony counts. In Figures 18A and 18B we see a significant effect in the *E. coli* populations after treatment of both the *E. coli* and EP biofilms. Colony counts from the *E. coli* biofilms were log-transformed and an ANOVA was performed ($F^{3,8} = 33.74$ $P = 6.87 \times 10^{-5}$). Tukey’s multiple comparison test was performed finding statistical significance between: no treatment and G+Phi treatment ($P < 0.001$) and no treatment and Greedy ($P < 0.001$). Phage treatment of the *E. coli* population in EP biofilms was also significant (box-cox transformed,

lambda=0.07; ANOVA, $F^{3,8}=103.4$ $P=9.74e-07$). Tukey's multiple comparison test was next performed identifying statistical significance between the control (no treatment) and the G+Phi treatment ($P<0.001$) and between the control (no treatment) and the Greedy treatment ($P<0.001$).

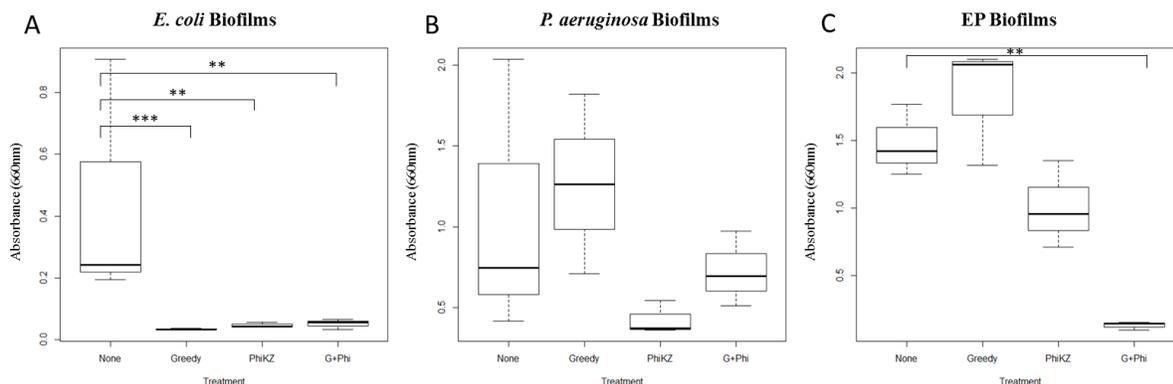


Figure 17. Effect of phage treatment of Greedy, phiKZ, and Greedy+phiKZ cocktail on (A) *E. coli*, (B) *P. aeruginosa*, and (C) EP biofilms based upon absorbance (660 nm). An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where *** indicates a P-value < 0.001 and ** indicates a P-value < 0.01.

While the absorbance assays indicated no significant effect in the concentration of *P. aeruginosa* bacterial cells (or cell matter) within the homogenous biofilm, colony counts tell a different story. In Figures 18C and 18D, a significant effect is observed for phage treatment of both the *P. aeruginosa* and EP biofilms. *P. aeruginosa* biofilms' (log transformed) ANOVA was significant ($F^{3,8}=7.405$ $P=0.0107$); Tukey's multiple comparison test between no treatment and phiKZ treatment is statistically significant ($P=0.0166$). Similar statistics were performed for the *P. aeruginosa* colony count data from the EP biofilms (log-transformed ANOVA, $F^{3,8}=18.22$ $P=0.000619$; Tukey's multiple comparison test between no treatment and G+Phi treatment ($P=0.00908$).

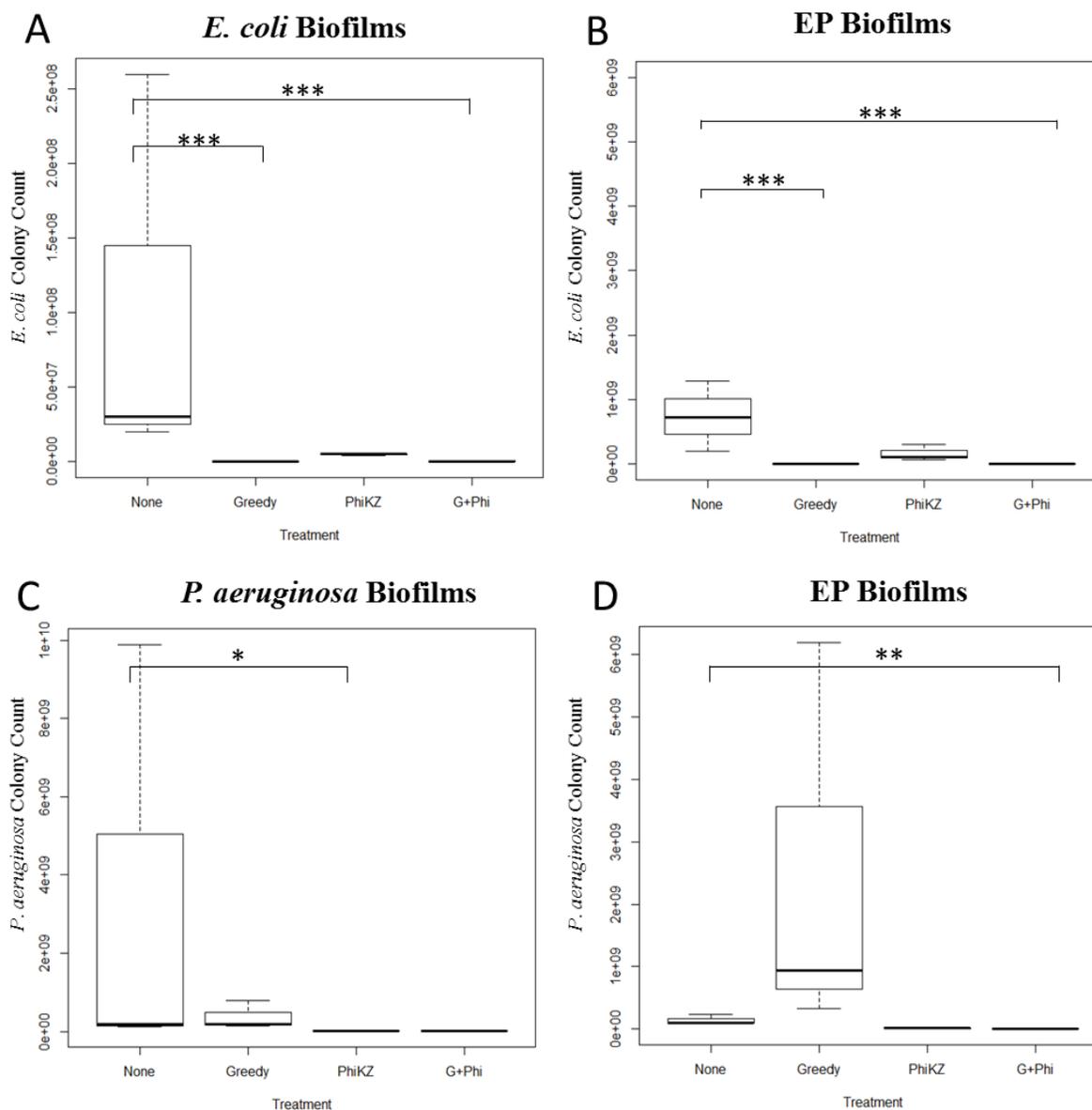


Figure 18. Effects of phage treatments of Greedy, phiKZ, and Greedy+phiKZ (G+Phi) cocktail on (A) *E. coli* in homogeneous *E. coli* biofilms, (B) the *E. coli* population in EP biofilms, (C) *P. aeruginosa* in homogeneous *P. aeruginosa* biofilms, and (D) the *P. aeruginosa* population in EP biofilms. An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where * indicates a P-value < 0.05, ** a P-value < 0.01, and *** a P-value < 0.001.

From the results of the cocktail treatments, in particular the colony counts presented in Figure 18, three observations can be made. First, Greedy was highly efficient in reducing the number of viable cells within the homogeneous *E. coli* biofilm. This efficiency in killing *E. coli*

cells was also apparent when it was used to treat the EP biofilm (Figure 18B). While it had no substantial effect on the *P. aeruginosa* biofilm (Figure 18C) it produced a dramatic effect on the *P. aeruginosa* population within the EP biofilm. While Greedy is highly unlikely to have “promoted” *P. aeruginosa* growth, this is likely a residual effect of its efficacy in lysing *E. coli* cells within the biofilm. Thus, we speculate that the decline in *E. coli* within the treated EP biofilms increased the resources available for *P. aeruginosa* growth. Based on *P. aeruginosa*’s ability to persist and even proliferate in the absence of *E. coli* within these treated biofilms, we hypothesize that the two bacteria species are not as cooperative as has been observed within other multispecies biofilms (Nozhevnikova *et al.* 2015) and may even be antagonistic, fighting for resources. Secondly, phiKZ was only found to produce a significant effect on the homogeneous *P. aeruginosa* population. The cocktail, however, was not found to be significant in the *P. aeruginosa* biofilm, perhaps because of the reduced titer of phiKZ. Lastly, the phage cocktail was effective on both bacteria species within the heterogenous EP biofilm.

Planktonic Fraction

The planktonic fraction, or the free-living independent bacteria outside of the biofilm, have substantially different metabolic characteristics (Davey and O’Toole 2000). They are, nevertheless, a vital part of the ecosystem representative of bacterium which can be recruited and integrated during biofilm development or dispersed members of a biofilm (Figure 1). Thus, in addition to examination of the biofilm bacterial communities, the planktonic fraction was also evaluated post-treatment. The same metrics were used to evaluate the planktonic fraction as were used for evaluation of the biofilms. The absorbance data and colony counts from the planktonic fraction of the homogeneous biofilm treatments and the heterogeneous biofilm treatments are shown in Figures 19 and 20, respectively.

For the *E. coli* biofilm's planktonic fraction, we detected a significant difference in absorbance between the treatments (Figure 19A); a Kruskal-Wallis test was found to be significant ($\chi^2 = 14.099$, $df = 5$, $P = 0.01499$). Similarly, the five treatments had different effects on the bacterial community within the planktonic fraction of the *P. aeruginosa* biofilms (Figure 19B). The Kruskal-Wallis χ^2 statistic = 14.17, $df = 5$, p -value = 0.01457. As expected, no significant effect was observed for the *P. mirabilis* community.

In addition to measuring the absorbance, aliquots from the planktonic fraction were plated and colonies counted (see Methods). For the *E. coli* biofilm treatments, an ANOVA was performed ($F^{5,12}=18.4$ $p=2.96e-05$). As Figure 19D shows, only the Greedy treatment was found to have a statistically significant relative to the untreated control ($P=0.00603$). To assess the putative effects on the *P. aeruginosa* population within the planktonic fraction, the colony count data was normalized (log-transformation) and an ANOVA was performed ($F^{5,12}= 20.41$ $p=3.15e-05$). PhiKZ ($P=0.01726$), DMS3 ($P<0.001$) and Habibi ($P=0.00386$) were found to have a significant effect relative to the untreated (control) biofilm planktonic fraction (Figure 19E). Again as expected, no significant effect was observed for the planktonic fraction's *P. mirabilis* community.

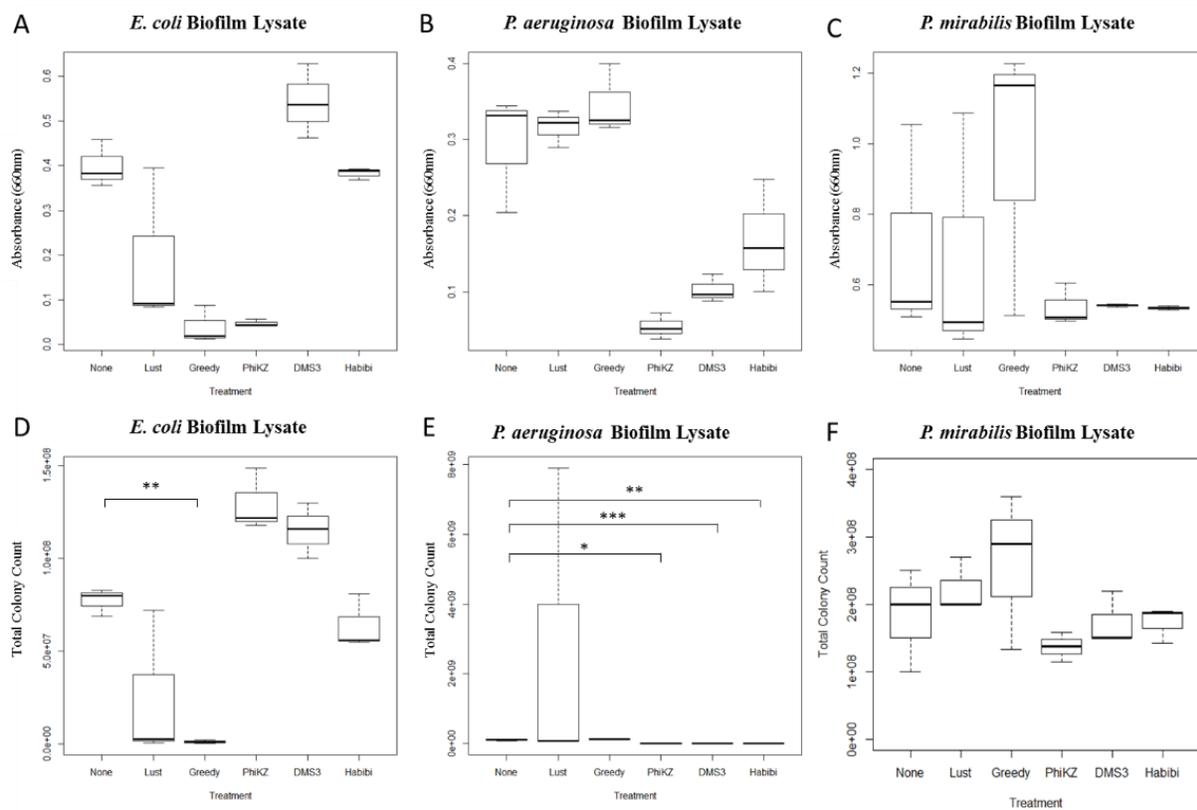


Figure 19. Effects of phage treatments on the planktonic fraction of homogenous *E. coli*, *P. aeruginosa*, and *P. mirabilis* biofilms measured by absorbance (panels A through C) and colony counts (panels D through F). An asterisk denotes a statistically significant difference by Tukey's multiple comparison test where * indicates a P-value < 0.05, ** denotes a P-value < 0.01, and *** indicates a P-value < 0.001.

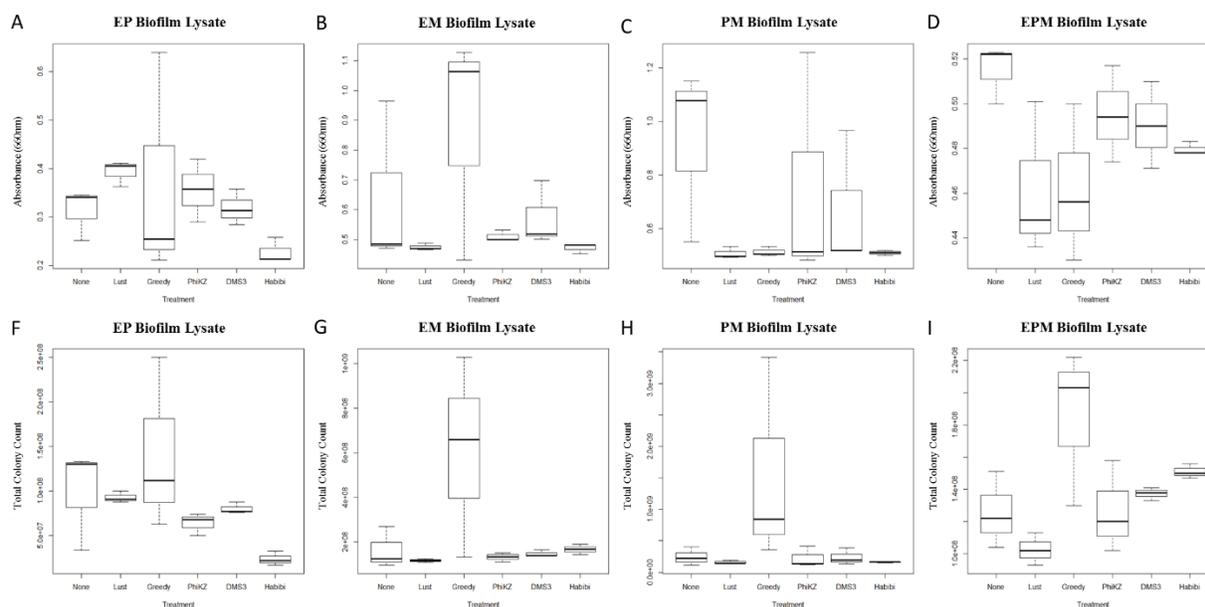


Figure 20. Effects of phage treatments on the planktonic fraction of heterogeneous biofilms measured by absorbance (panels **A** through **D**) and colony counts (panels **F** through **I**).

Evaluation of the effects of phage treatment within the planktonic fraction was next evaluated for the heterogeneous biofilms, again using absorbance (Figure 20, panels A through D) and total colony counts (Figure 20, panels F through I). From these total colony counts, the only statistically significant result was from an ANOVA of the EPM treatments ($F^{5,12}=3.629$ $p=0.0313$). Given this observed difference, Tukey's multiple comparison test was performed. While no statistically significant difference between the control and individual treatments was found, the Lust treatment was significantly different from Greedy treatment ($P=0.018$); this difference between Lust and Greedy was the reason the ANOVA produced a significant result.

To ascertain if the changes in total colony counts were due to an individual species, we next investigated these colony counts by species (Table 4). The most intriguing result found was the increase in *P. mirabilis* in the EM and PM biofilm planktonic fraction in response to treatment by Greedy. While one could hypothesize that this increase in the EM was due to the

effectiveness of Greedy in lysing *E. coli* cells, and thus reducing resource constraints for *P. mirabilis*, it is confounding to see a similar – and more profound – effect in the PM biofilm’s planktonic fraction. This warrants further investigation of *P. mirabilis* and *P. aeruginosa* interactions within liquid culture.

Biofilm	Bacteria	Treatment					
		None	Lust	Greedy	PhiKZ	DMS3	Habibi
EP	<i>E. coli</i>	6.73E+07	7.07E+07	N/A	6.40E+07	8.03E+07	2.27E+07
	<i>P. aeruginosa</i>	3.17E+07	2.23E+07	1.42E+08	N/A	N/A	1.33E+06
EM	<i>E. coli</i>	2.00E+06	N/A	N/A	2.50E+07	0.00E+00	0.00E+00
	<i>P. mirabilis</i>	1.72E+08	1.15E+08	6.08E+08	1.06E+08	1.27E+08	1.53E+08
PM	<i>P. aeruginosa</i>	9.00E+06	6.67E+06	9.00E+06	N/A	N/A	6.67E+05
	<i>P. mirabilis</i>	2.93E+08	1.50E+08	1.51E+09	2.25E+08	2.39E+08	1.59E+08
EPM	<i>E. coli</i>	1.10E+07	N/A	6.67E+05	7.00E+06	2.60E+07	1.67E+06
	<i>P. aeruginosa</i>	7.00E+06	4.00E+06	2.33E+06	N/A	3.33E+05	1.33E+06
	<i>P. mirabilis</i>	1.19E+08	9.87E+07	1.82E+08	1.20E+08	1.21E+08	1.48E+08

Table 4. Colony counts within the planktonic fraction for heterogeneous biofilm treatments by species. N/A means that no colonies grew at the dilutions plated.

Host Resistance to Phage Infection

Because no single phage or the phage cocktail completely destroyed the biofilms, although Greedy was close to successful for the *E. coli* biofilm treatments (Figure 8A and B), we next determined if the resilience of the biofilm was the result of bacterial resistance to the phage. Randomly selected bacterial colonies were thus selected and challenged by the phage used in the treatment (see Methods Figure 7). As expected, *P. mirabilis* remained resistant to all five phages, regardless if it was a homogeneous or heterogenous biofilm. Figure 21 provides a graphical representation of the resistance observed for *E. coli* colonies from the five treatment regimens of *E. coli* biofilms (Figure 21A) and the planktonic fraction (Figure 21C). Figure 21 panel B and D show the results for the *P. aeruginosa* colonies in the biofilm and planktonic fraction, respectively.

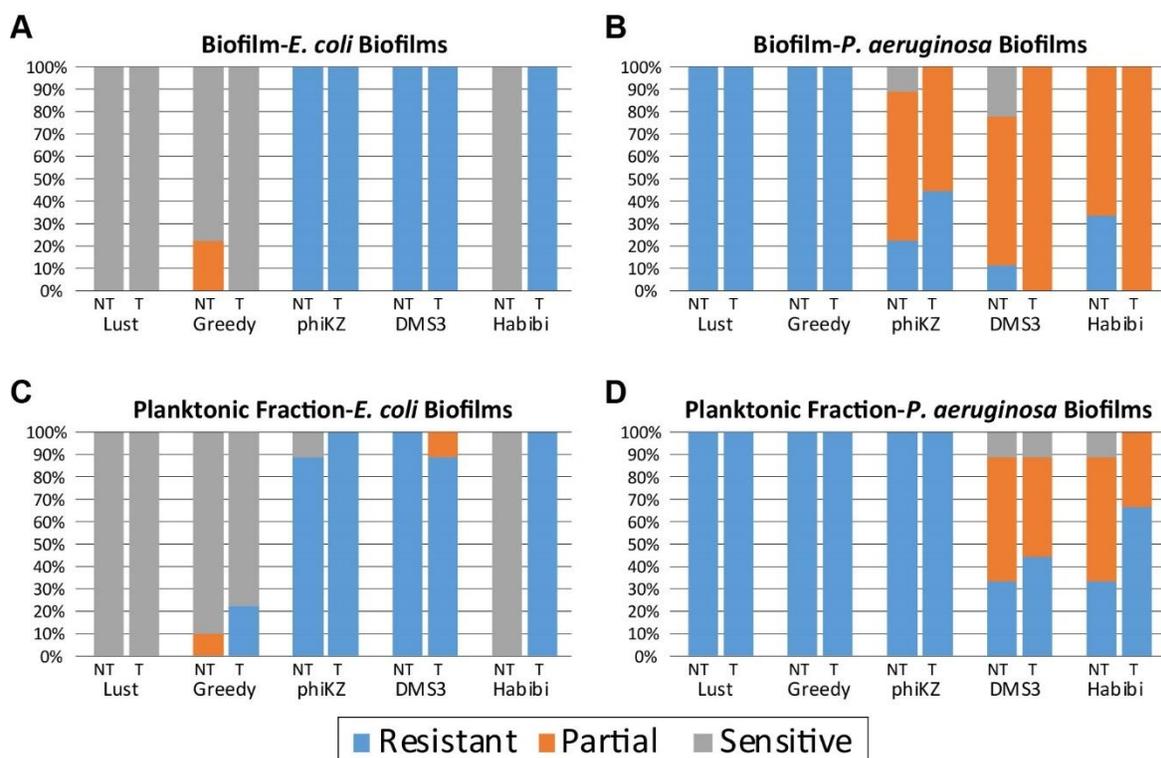


Figure 21. Bacterial resistance for bacterial isolates from within homogeneous biofilms. NT signifies isolates collected from the untreated (control) biofilms, and T represents those collected for the phage treatment samples. Panels **A** and **B** present the percentage of colonies resistant, partially resistant, or phage-sensitive from the *E. coli* and *P. aeruginosa* biofilms, respectively. Panels **C** and **D** include the percentages for colonies from the planktonic fraction of *E. coli* and *P. aeruginosa* biofilms, respectively.

Colonies from both the untreated (NT) and treated (T) samples are included in the graph. The NT bar charts represent the likelihood of resistance occurring *a priori*. It is important to note that phage sensitivity is not an absolute in, for example, *E. coli*. Colonies resistant to Greedy were observed both in the biofilm as well as the planktonic fraction (21A and C). Also of note is the occurrence of *E. coli* colonies which were sensitive to either phiKZ or DMS3 which is not believed to be infective of bacteria outside of the *Pseudomonas* genus. While these innate resistances and sensitivities may have arisen as a result of the selection experiment deriving the strains used for our experiment, the fact that they are seen in such small numbers suggests this is

not the case. Similar unexpected results were observed for the NT colonies tested from the *P. aeruginosa* experiments. In fact, the majority of the biofilm colonies were resistant or partially resistant to phiKZ and DMS3 and all of the biofilm colonies were resistant or partially resistant to Habibi. This may be a contributing factor to the minimal effect observed for the Habibi treatments (Figures 15 and 16). Furthermore, the ability for a phage to infect and successfully lyse a bacteria cell is traditionally assayed either via liquid culture or on a plate, as we have done here. The ability for a phage to lyse within the unique spatial organization of a biofilm is unknown.

For the four complex biofilms, resistance was tested for colonies of each (in the case of EP, EM, and PM) or all three (EPM) bacterial species. In complex biofilms with *E. coli* present treatment with Greedy and Lust resulted in few if any *E. coli* colonies growing at the dilutions plated. As such, we were unable to test for host resistance. Similarly, in multispecies biofilms with *P. aeruginosa* present treatment with PhiKZ, DMS3 and Habibi resulted in few if any *P. aeruginosa* colonies growing at the dilutions plated for the planktonic fraction. Thus, only *P. aeruginosa* from the biofilm was able to be tested for host resistance. Partial susceptibility to phage infection was more common in *P. aeruginosa* than *E. coli*. Table 5 summarizes the results of these resistance assays for the heterogenous biofilms for colonies isolated from the biofilms themselves, while Table 6 summarizes the results from the planktonic fraction. The results in Table 6 show an intriguing trend in which the *P. aeruginosa* colonies from the phage treatments were more sensitive than the untreated cultures. This contradicts our assumptions that resistance arises in response to phage predation and thus warrants further investigation. The results of the resistance assays for the G-phi treatments are shown in Figure 22.

		Category	Phage				
			Lust	Greedy	phiKZ	DMS3	Habibi
<i>E. coli</i>	NT	Resistant	0	0	24	26	0
		Partial	0	0	3	0	7
		Sensitive	27	27	0	1	20
	T	Resistant	0	0	25	27	10
		Partial	0	0	1	0	8
		Sensitive	9	2	0	0	3
		N/A	18	25	1	0	6
<i>P. aeruginosa</i>	NT	Resistant	27	27	0	0	0
		Partial	0	0	24	26	25
		Sensitive	0	0	3	1	2
	T	Resistant	27	27	3	1	0
		Partial	0	0	19	15	22
		Sensitive	0	0	1	11	5
		N/A	0	0	4	0	0

Table 5. Bacterial resistance for isolates from within heterogeneous biofilms.

		Category	Phage				
			Lust	Greedy	phiKZ	DMS3	Habibi
<i>E. coli</i>	NT	Resistant	0	0	26	25	0
		Partial	0	0	1	2	11
		Sensitive	27	27	0	0	16
	T	Resistant	0	0	22	27	20
		Partial	0	0	5	0	2
		Sensitive	9	1	0	0	0
		N/A	18	26	0	0	5
<i>P. aeruginosa</i>	NT	Resistant	27	27	3	3	3
		Partial	0	0	20	20	22
		Sensitive	0	0	4	4	2
	T	Resistant	27	27	0	0	0
		Partial	0	0	0	1	11
		Sensitive	0	0	0	0	1
		N/A	0	0	27	26	14

Table 6. Bacterial resistance for isolates from within planktonic fraction of heterogeneous biofilms.

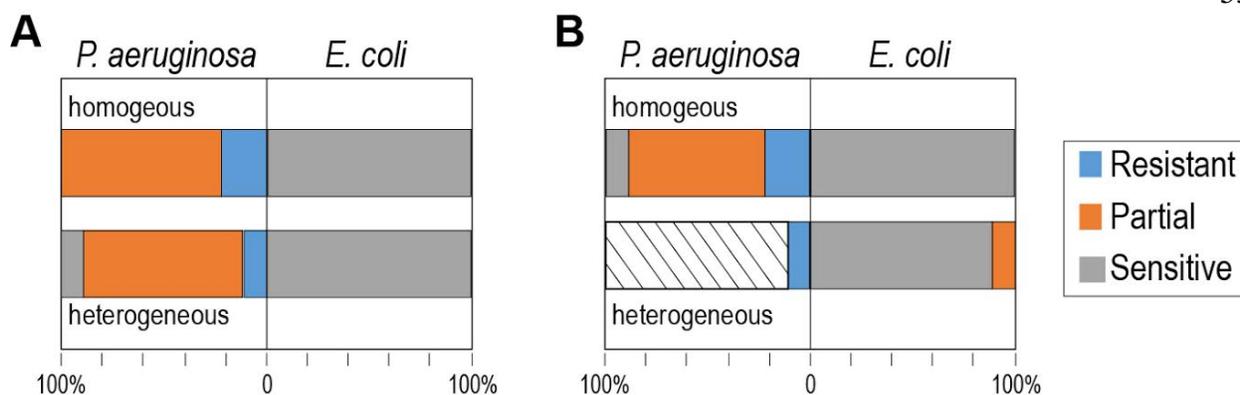


Figure 22. Bacterial resistance for isolates treated with phage cocktail within the (A) biofilm and (B) planktonic fraction. Areas denoted by the hashed bar indicate samples for which the effectiveness of the phage treatment resulted in no retrievable colonies of the particular bacteria.

Phenotypic Changes in Bacterial Species

In addition to determining the relative number of viable colonies for each species present in a biofilm, plating also allowed us to observe multiple morphological changes in colony phenotype. In six of the samples containing *P. aeruginosa*, a red colony morphology was observed (Figure 23). These samples included isolates from both the biofilm and planktonic fraction of the same replicate of the untreated *Pseudomonas* control. Two of the replicates from the *Pseudomonas* biofilm treated with phiKZ also contained a “Red *Pseudomonas*.” Other samples include one replicate of the untreated PM planktonic fraction, one replicate of *Pseudomonas* treated with Habibi, and two replicates of *Pseudomonas* treated with G+phi. These colonies appeared as normal, however once streaked on an LB plate, a red pigment was diffused through the agar. Pseudomonad species are known to diffuse colored pigments throughout their media. The *P. aeruginosa* 1C strain, used routinely in our lab, typically produces pyocyanin which is responsible for its characteristic green color and pyomelanin (brown) when the culture is old (Ogunnariwo and Hamilton-Miller 1974). This was the first time we observed the red-brown pigment pyorubin in our laboratory strain.

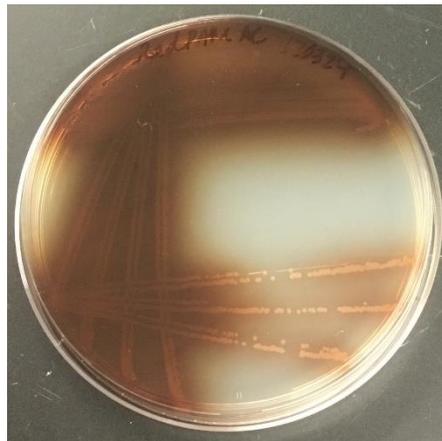


Figure 23. *P. aeruginosa* red colony phenotype.

P. aeruginosa biofilms treated with DMS3, phiKZ, or Habibi resulted in numerous colonies presenting a morphology quite different from those observed in untreated biofilms. Altered phenotypes consisted of *P. aeruginosa* colonies which maintained their color but were usually smaller in size and appeared as though pieces of their colonies were missing. These colonies had jagged edges rather than their normal circular shape. Restreaking these colonies resulted in their return to normal colony morphology; thus, the change in morphology was not likely due to a genetic source as it was not inheritable. These phenotypes had higher frequencies in the planktonic fraction than the biofilm portion of the samples. This is consistent with previous findings that show *P. aeruginosa* phages are effective at reducing additional biofilm formation by preventing the aggregation of dispersed members of the biofilm in the planktonic fraction (Darch *et al.* 2017).

Novel colony morphologies were also observed in *E. coli* biofilms treated with Lust and Greedy. *E. coli* colonies were smaller and jagged-edged in contrast to smooth-edged. Despite the fact that these two phages were isolated in our lab (Malki *et al.* 2015) and have been plated with

E. coli C numerous times prior, this was the first time this *E. coli* C morphology has been observed. As a precaution, the *E. coli* C biofilm treatment with Greedy was repeated, again in triplicate, and produced similar *E. coli* C cell morphologies. Colonies from these plates were selected and restreaked producing a second generation of aberrant colonies. Furthermore, in contrast with the *E. coli* C lab strain in our collection (and the ancestor of the inoculant of the biofilms), the restreaked *E. coli* grew slower. It is important to note that not all colonies on a plate showed these altered morphologies, and similar to phenotypic responses observed in *P. aeruginosa*, the altered colonies were more frequently observed in the planktonic fraction rather than in the biofilm community. This observation is consistent with the protective nature of biofilms.

An additional change to colony morphology was observed in biofilms containing both *E. coli* and *P. aeruginosa* treated with DMS3. Here a few colonies from the biofilm portion of the samples appeared to have swarming abilities. While frequently observed for *P. aeruginosa* and *P. mirabilis* colonies from all biofilms, *E. coli* swarming colonies were only observed in EPD biofilms. Perhaps this morphology was a result of horizontal gene transfer which occurs in higher rates in biofilms (see review Madsen *et al.* 2012).

Phage Populations within Treatments

While spotting phage to test host range and isolate individual plaques for identification, the last dilution at which plaquing occurred was recorded, though specific titers were not. From this we noticed a few interesting trends. As expected the concentration of phage was higher in the planktonic fraction than the biofilm fraction of samples for every phage. Additionally, all phages except Habibi had their lowest concentrations in EPM biofilms, for Habibi it was *E. coli* biofilms. Another interesting observation about Habibi was that it had a higher concentration in

EM biofilms than *E. coli* biofilms despite no observed plaques on *P. mirabilis* lawns. Something similar occurred with phage DMS3 which had equal concentration of phage in *P. aeruginosa* and EP biofilms despite a lack of plaques on *E. coli* lawns. This is probably because when treated with DMS3 EP biofilms did better than *P. aeruginosa* biofilms. Furthermore, this is intriguing when you consider the fact that while DMS3 and Habibi had no plaques on *E. coli* or *P. mirabilis* lawns; after treatment of EM, PM, and EPM biofilms, both phages resulted in visibly less turbid lawns when spotted on *E. coli* and *P. mirabilis*. This was not observed in *E. coli* or *P. mirabilis* biofilms treated with the phages individually. Lust had its highest concentrations in EP and EM biofilms. Greedy did the best in *E. coli* biofilms. PhiKZ and DMS3 were most successful in *P. aeruginosa* biofilms. Habibi saw its highest concentrations in EP biofilms, which was expected considering its ability to infect both members of the biofilm.

***Pseudomonas aeruginosa* Prophage**

Despite previously unsuccessful attempts from members of our lab trying to manipulate *P. aeruginosa* to release its prophage, throughout this experiment we observed the prophage multiple times. A prophage is a bacteriophage genome inserted into the bacterial chromosome or plasmid that is only released upon activation by things such as host stress (Figure 24). It was released in untreated *P. aeruginosa* biofilms and also in untreated biofilms composed of both *P. aeruginosa* and *E. coli*. Interestingly, it was not released in untreated biofilms containing *P. mirabilis* (PM or EPM biofilms). Additionally, the prophage was not released in *P. aeruginosa* biofilms treated with either Lust or Greedy. However, in EPM biofilms treated with Lust, one replicate showed the appearance of the prophage. In EPM biofilms treated with Greedy, the prophage appeared in all three replicates. Plaque morphology was observed as pin point plaques with crisp edges and a very clear center. Upon spotting the prophage on both *E. coli* and *P.*

mirabilis lawns, no lysis was observed. In a few instances, spotting the prophage on *P.*

aeruginosa resulted in plaquing that was not confined to the spots but rather was observed throughout the plate.

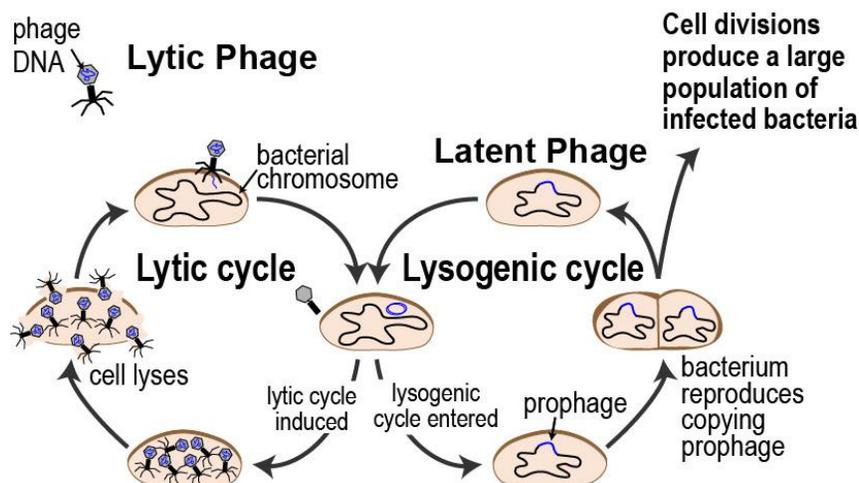


Figure 24. Bacteriophage life cycle.

Phage concentrations ranged from 10^2 - 10^8 with planktonic concentrations about 10-fold higher than their biofilm counterparts. In all cases except *P. aeruginosa* untreated biofilms, the concentration of prophage released was higher in replicates with higher absorbance values and thus thicker biofilms. Combined, these observations suggest that there is a relationship between prophage release and increased biofilm production in our strain of *P. aeruginosa*. This hypothesis is supported by other studies observing a similar correlation. For instance, *Streptococcus pneumoniae* biofilm production is aided by prophage mediated lysis which releases DNA into the environment which aides in biofilm formation as extracellular DNA is an important component of the matrix (Carrolo *et al.* 2010). Enhanced biofilm formation due to phage mediated release of DNA was also observed in *Shewanella oneidensis* (Gödeke, *et al.* 2011), and *E. faecalis* (Rossman *et al.* 2015).

CHAPTER FOUR

CONCLUSION

Our examination of the literature found phage to be more effective at biofilm inhibition than eradication, as such we did not initially expect any of our phages to substantially reduce biofilm density. In this aspect the effects of Greedy treatments exceeded our expectations. We saw that Greedy produced statistically significant reductions in homogeneous biofilms, when compared to the control, quantified by both absorbance and colony counts. We also found Greedy's effect spread into the *E. coli* colony counts in the planktonic fraction. Furthermore, Greedy had a significant effect in EPM biofilms, with regards to the total colony count data, and in EP biofilms (for *E. coli* colony counts). When combined with phiKZ, the cocktail treatment of EP biofilms resulted in a significant effect, per the absorbance data. This is particularly intriguing as absorbance data represents both the viable and non-viable portions of the biofilm, thus giving a more accurate representation of biofilm density. Greedy treatments also produced altered colony morphologies; bacterial colonies were both smaller and had a slower growth rate relative to their ancestor. As on-going research in our lab characterizing Greedy has found, it is able to infect and lyse several clinical *E. coli* strains isolated from patients presenting with UTIs. For these reasons, we believe that Greedy is a strong candidate for future research in biofilm reduction, and more so in phage treated catheter studies for biofilm prevention. Follow up studies, investigating the effectiveness of Greedy in preventing biofilm formation as well as the efficacy of Greedy treatments over time, would provide greater insight into its potential for phage therapy.

This study was the first, to our knowledge, to examine three species biofilms composed of CAUTI-associated bacteria. Despite catheter biofilms, and biofilms in general, being composed of multiple species of bacteria, much of the current research focuses on homogenous biofilms. Prior studies have found catheter biofilms containing up to five different bacterial taxa (Saint and Chenoweth 2003). As we have shown here, an effect on a single species biofilm is not necessarily reflected in more complex, heterogeneous biofilms. For example, while Lust had a significant effect on *E. coli* biofilm absorbance, it loses that effect in EP, EM and EPM biofilm absorbance. We did not observe any significant effects of any phage treatment on heterogeneous biofilms quantified by absorbance. Furthermore, we saw no significant effects, based on total colony counts, in EP biofilms despite all five of our phages infecting both species within it. We urge future studies to consider the more naturally occurring complex, multispecies biofilms.

Among the many interesting results of this study, perhaps the most alarming observation was the effect of phage treatment on non-host bacteria. While *P. mirabilis* was not significantly affected by any of the phage treatments, we did see an inflation of *P. mirabilis* colonies counts in the planktonic fraction of EM and PM biofilms in response to treatment with Greedy. Furthermore, *P. aeruginosa* phage treatments with phiKZ and DMS3 also had a significant effect on *E. coli* biofilms; within the literature, neither phage has been identified as capable of lysing *E. coli* cells. Additionally, we showed that phages Lust, Greedy, phiKZ, and DMS3 can reduce *E. coli* C populations in the multispecies EPM biofilm community. Our study clearly shows that phage treatment of heterogeneous biofilms does not only affect the species in which it is capable of lysing. This provides direct evidence that phages can inhibit bacterial growth or induce lysis without successful infection.

Future work in phage remediation should consider the entire microbial population at stake rather than focusing solely on the most problematic bacterial species. This is especially true for phage therapy of human infections, as maintaining a “normal” microflora is critical for human health. Phage treatment has the potential to disrupt beneficial microbes that could lead to unintended consequences. Nevertheless, phage therapy has significant potential to improve human health and combat infections including CAUTIs, particularly given the increase in antibiotic-resistant bacteria.

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VITA

Alexandria F. Cooper was born and raised in the suburbs of Chicago. She attended Loyola University Chicago for her undergraduate degree, a Bachelor's of Science; she majored in Biology.