Effect of Bacteriophage Infection on the Viability of *Escherichia coli* and *Pseudomonas aeruginosa* in Mixed Species Biofilms

THESIS

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ABSTRACT

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SUPERVISING PROFESSOR: GARY M. ARON

There has been renewed interest in the use of bacteriophage for the treatment of disease. Recent studies have focused on phage infection in single and mixed species planktonic culture. In chronic bacterial disease, however, pathogenic bacteria form biofilms, which facilitates colonization and the development of chronic infection. This

study investigated the effect of phage infection on cell survival in *Escherichia coli* and *Pseudomonas aeruginosa* mixed species biofilms. In the absence of phages, *E. coli* and *P. aeruginosa* were stably maintained during daily serial passage. *P. aeruginosa* maintained high cell densities in both single-species planktonic and biofilm cultures when infected with its phage, PB-1. Monoculture planktonic and biofilm populations of *E. coli* were also stable in the presence of its phage, λW60. In contrast, phage λW60 infection of two-species cultures resulted in the extinction of *E. coli* in planktonic but not in biofilm populations. In mixed species planktonic and biofilm cultures *P. aeruginosa* maintained high cell densities when infected with phage PB-1. The increased susceptibility of *E. coli* to its phage in planktonic culture may be due in part to competition with *P. aeruginosa*. This is the first report on the effect of bacteriophage infection in a mixed species biofilm and demonstrates the advantage bacterial populations gain by forming biofilm communities.

INTRODUCTION

The effect of bacteriophage infection on host cells has been studied almost exclusively in pure culture (10, 18, 24). Only recently has the study of multi-species bacterial populations gained interest in the scientific community (4, 9, 28). Studies have shown that growing in a mixed species culture can have profound effects on the genotypes and phenotypes of the bacteria involved (7, 8, 11, 14). These changes can affect cell growth and resistance to environmental stressors (7). Biofilm density can also be influenced in multi-species cultures (4).

Bacteriophage, viruses which infect bacteria, have been studied for nearly a hundred years, yet only recently has their role in the ecosystem been evaluated (8). Bacteriophage have a genome consisting of either DNA or RNA and, as with all viruses, they are obligate intracellular parasites requiring host cell machinery to replicate their genome (19). Phage are known to play an important role in bacterial ecosystems by mitigating the transfer of genetic material between populations and by causing phenotypic variation within populations of bacteria (43). Webb et al. demonstrated that *Pseudomonas aeruginosa* cells infected with bacteriophage Pf4 produced smaller colonies than uninfected cells (43). Bacteria often inherit phenotypic traits from invading bacteriophage through generalized and specialized transduction (4). In specialized transduction only a small part of the bacterial genome is transferred by the infecting

bacteriophage, unlike generalized transduction where all, or most, of the bacterial genome is transferred (48).

Phage infection plays an important role in all known food webs, most notably in marine systems (4). Aquatic environments allow phage a major role in controlling the biomass found in a system (19). A study by Harcombe et al. found significant changes in bacterial susceptibility to phage attack in a mixed species system as opposed to a single-species system (13). In this study, *Escherichia coli* lost its ability to establish a phage-resistant population when grown in a two-species community with *Salmonella typhimurium* (11). *S. typhimurium,* however, was able to maintain a phage resistant population when present in a two-species community (11). Bacteriophages have also come to the forefront of medicine due to their ability both to control bacterial infections, and spread resistance and virulence genes among populations of pathogenic organisms (12, 47). Industry has also developed a great interest in bacteriophage as means of controlling infection (17).

The phenomenon of host-induced modification has been observed throughout phage research (40). Bacteria of the same species often have different DNA restriction and modification systems (40). In host-induced modification, phage stocks prepared on laboratory stocks of *E. coli* K12 will have its genome modified at sites specified by a bacterial K-specific modification system. When these phage infect cells of *E. coli* B, with a B-specific modification system, the phage DNA are degraded upon entry into the new host cell (40). This is a challenge for phage as therapeutic agents because it means phage have to be specific to the strain of bacteria and not just the species. Another hurdle for phage therapy is the development of anti-phage antibodies following prolonged treatment

with bacteriophage (26, 36). However, since immune recognition is primarily based on epitopes on the viral capsid, phage variants can be isolated which can not be recognized by the immune system, making possible phage therapy as a viable treatment (26).

Bacteria have been studied in planktonic culture for nearly one hundred years and until recently it was believed that bacteria lived independent and unicellular lives (45). Bacteria are now known to take part in a variety of complex systems of cooperation, communication, and synchronization (45). Cells in biofilm communities exhibit quorum sensing. Quorum sensing allows bacterium living in close communities to regulate gene expression in response to cell-population density (27). In a study by McClean et al., it was demonstrated that N-acyl homoserine lactones (AHLs) act as signal molecules in *Chromobacterium violaceum* cultures leading to the production of violacein (25). Violacein is an antimicrobial pigment, produced by *C. violaceum,* which also has been shown have anti-viral activity (32). Bacteria also release chemical signals called autoinducers, which increase in concentration as a function of cell density (27). Quorum sensing molecules and other bacterial products are often referred to as common goods (45). Although the production of these common goods is metabolically costly to the individual, it can benefit the biofilm community as a whole (45). The production of common goods can have direct and indirect effects on every cell in the community (45).Within a biofilm, metabolic activities are integrated and developmental sequences can be detected (24). Genes within biofilm cells are expressed which result in structural interrelationships and phenotypes that are very different from those seen in a planktonic culture of the same species (39). One hypothetical cell state proposed to exist in a biofilm is the "persister" cell (38). A cell, in this so-called persister state, is thought to be

protected from most types of antimicrobial assaults. These cells would be in a "spore-like" state and have essentially no metabolic function (38). Cells of this nature could also be protected against phage infection due to the dependence viruses have for living and actively multiplying host cells.

Biofilms comprise highly structured, matrix-enclosed communities which make up the majority of bacteria in a natural ecosystem (39). Biofilms, both in pure culture and in natural ecosystems, show a basic structure which consists of cells growing in matrix-enclosed microcolonies separated by networks of water channels (39). The matrix is composed primarily of microbially produced exopolysaccharide, EPS (18). This matrix provides protection from the environment as well as antimicrobial substances (18, 40). There has been increased interest in the study of biofilms due to the high tolerance towards antibiotics, often exceeding the highest deliverable dose, as well as host immune systems (14, 31). Resistance has been noted from antimicrobials ranging from oxidants, such as chlorine, to antibiotics with very specific cellular targets (38). However, when biofilms are dispersed the antimicrobial susceptibility of the released cells is quickly restored (38). The addition of abiotic particles, such as silt, dead cells, and fibers, into the biofilm system has also been shown to increase antimicrobial resistance (38). These particles reduce diffusive transport and increase sorptive capacity (38). Ramage et al. found that genes encoding efflux pumps were upregulated during *Candida albicans* biofilm formation (30). These efflux pumps play a major role in antimicrobial resistance by removing antimicrobial substance from the cell (30). *C. albicans* strains with double deletions in genes responsible for efflux pumps exhibited hypersusceptability to

fluconizole when planktonic, but still maintained some level of resistance when in biofilms (31).

Only inside a laboratory can one truly find pure cultures of bacteria. In the natural environment bacterial communities are very complex and diverse and interactions between these different species can greatly influence factors such as biofilm development as well as the impact stressors, such as bacteriophage, have on the bacterial communities (7, 13). In a study by Burmølle et al. it was shown that biofilm formation can be greatly enhanced in mixed species cultures of common saprophytic bacteria (7). Biofilms formed by a mixed culture of four strains were found to increase in biomass by more than 167% over the single species biofilms (7). The same study also showed that when in a mixed culture, cells showed enhanced resistance, up to 90%, to tetracycline and hydrogen peroxide (7). In another study, Harcombe and Bull demonstrated a direct influence on phage susceptibility in single species planktonic culture as opposed to mixed species planktonic cultures (13).

Biofilms have been shown to provide a relative amount of protection for the bacteria involved. Bacteria in a biofilm show enhanced resistance to antimicrobials and antibiotics (10). Dispersed bacterial cells are approximately 15 times more susceptible to tobramycin than the same species of bacteria growing in a biofilm (16). It has been observed that when bacteria were introduced into a mixed species consortia the level of protection and biofilm biomass increased (10). Initially it was believed that the biofilm matrix simply limited diffusion of antimicrobials and acts as a physical barrier. However, it is now known that a biofilm only limits diffusion when the diffusing molecule actually

reacts with the biofilm matrix (10, 37). This is an important aspect of biofilm behavior because of the relative scarcity of pure culture biofilms in nature.

Phage release differs in planktonic cultures versus biofilms and has been shown to be related to the differentiation and maturation of a biofilm (31). Resch et al. demonstrated that phage expression in planktonic cultures of *Staphylococcus aureus* declined rapidly after 8 hours, however biofilms of the same species yielded infectious phage particles for as long as 72 hours (31). It is known that phage carry their own polysaccharide depolymerase enzymes which can aid in the degradation of bacterial capsular material as well as the biofilm matrix (18). In planktonic culture, phage-born polysaccharide depolymerases are able to degrade bacterial capsular material in a very defined sequence. The phage depolymerse binds to the capsular material and degrades it until the phage is able to bind to the outer-membrane receptors and infect the bacterium (18). However, very little is known about the relationship between phage and host in a biofilm (9). Since most bacteria exist in nature in biofilms, the question of phage-biofilm interaction is of great importance (18). In a recent study by Sillankorva et al. it was demonstrated that biofilms of *Pseudomonas fluorescens* experienced a 3-5 log reduction in biomass after a 4 hour exposure to the lytic bacteriophage ϕIBB-PF7A (35). However, this study was limited to single species systems.

Cells in biofilm and in planktonic culture spontaneously release phage into the surrounding environment (18). While both settings allow for phage release, bacteriophage are detected over a much longer period of time in biofilms and it is thought that phage release in biofilms is a frequent event (31). In both planktonic cultures and biofilms, phage release has been stimulated by addition of UV radiation and mitomycin-C (3, 31).

A study by Corbin et al. found that the presence of a bacteriophage in an *E. coli* biofilm leads to a reduction in biofilm density (9). However, in a more recent study, it was demonstrated that bacteriophage BA3 is effective in reducing *Thalassomonas loyana,* the bacterium responsible for white coral plague-like disease. If BA3 was added within one day bacterial density was lowered substantially. However, if phage was added after two days of bacterial infection *T. loyana* density was not affected (11). This led researchers to conclude that the use of phage may be more useful to prevent transmission of white coral plague-like disease rather than to cure infected corals (9).

Study of bacteria in pure cultures has been the standard since Koch first began using media solidified with agar to isolate bacterial colonies. While studying bacteria in a single species system does provide important information about the behavior of the bacteria being studied, a new approach to microbiology has begun to play an important role. Bacteria are rarely found in pure culture in nature and study of multiple species cultures is turning up new aspects of bacterial interactions (4, 9).

Little research has been done which compares phage infection in single species biofilms to mixed species biofilms. It has been found that planktonic cultures of *S. aureus* release phage for 8-16 hours whereas biofilms of the same organism release phage for as long as 48-72 hours (31). At higher cell densities it has been noted that phage release is elevated (31). In a study conducted by Webb et al., phage release was shown to increase in mature biofilms (43). Cell death inside biofilms plays an important role in the dispersal and differentiation of the surviving subpopulation of biofilm cells (43). This could give an advantage to remaining cells in a biofilm, or planktonic culture, by making nutrients available and limiting competition through microbial antagonism (31).

E. coli is a gram-negative bacilli, 2μm - 6μm in length, occurring singly or in pairs (5, 20). They are facultative anaerobes exhibiting both respiratory and fermentative types of metabolism. Colonies on nutrient agar appear smooth, low, convex, moist, and grey with a shiny surface and entire edge (5, 20). Most strains have numerous fimbriae and some produce enterotoxins. *E. coli* is a clinically relevant organism usually causing diarrhea, cramping, and nausea associated with food born illness and contaminated drinking water. The bacterium is also a common microflora of the intestinal tracts of most mammals (5, 20). Recently the appearance of strain O157:H7 has caused an increase in interest by both research and public communities (34). An outbreak in 1982 in the northern United States infected more than 10,000 people, killing 12 (34).

P. aeruginosa is a gram-negative bacillus sometimes, 1.5μm – 3μm in length, exhibiting a slight curved shape and is motile by a single polar flagella (5, 20). *P. aeruginosa* is aerobic having only one metabolic pathway with oxygen as the terminal electron acceptor. Colonies on nutrient agar are smooth, shiny, round, and exhibit a greenish pigment (5, 20). *P. aeruginosa* is a common noscomial infection showing up commonly in patients in burn wards and those with immune suppression and respiratory syndromes such as Chronic Obstructive Pulmonary Disease (C.O.P.D.) and Cystic Fibrosis Pneumonia (21, 24).

This study investigates the effect of bacteriophage infection on the survival of bacteria growing in single-species versus mixed-species cultures. *E. coli* and *P. aeruginosa*, both common clinical opportunistic pathogens, were grown in one and two-species planktonic and biofilm communities. These cultures were challenged with bacteriophage specific for one species in the community. PB1, a lytic phage of *P.*

aeruginosa, or λW60, a lytic phage of *E. coli,* was introduced at the same time that the culture was inoculated and the viable cell density was assayed over five days and compared with the cell densities of uninfected cultures. This is the first investigation of the effects of phage infection on mixed species biofilm communities.

MATERIALS AND METHODS

Cultures and conditions. *Escherichia coli* MG1655 (MG1655) (2), and *Pseudomonas aeruginosa* PA01 were maintained on LB (Luria-Bertani, Becton Dickinson, Sparks, MD) agar at 37°C. High titer stocks of bacteriophage λW60 (W60) (ATCC 97537) and bacteriophage PB-1 (ATCC 15692-B3) were grown by the agar overlay technique as described by Adams (1). λW60 is a lytic bacteriophage of *E. coli* that has been proposed for treatment of sepsis (26), and PB-1 is a lytic bacteriophage of *P. aeruginosa* (15).

Effect of bacteriophage infection on planktonic cultures. The method of culture infection and passaging used was described by Harcombe and Bull (13). Briefly, 125 ml flasks containing 10 ml of LB broth were inoculated with 0.1 ml of an overnight culture of either a monoculture of one bacterium or a two-species culture of both bacteria at a final cell density of 10^6 cells ml^{-1}. For phage infection cultures, virus was added at a multiplicity of infection (MOI) of ~10. Infected and uninfected cultures were grown at 37°C in a reciprocating shaker bath (Forma Scientific model 2564, Marietta, Ohio). At 24 h 0.1 ml of culture was serially transferred into 10 ml of fresh LB medium. Bacterial densities were determined at 24 h intervals by dilution plating on either antibiotic LB medium plates with 50 μg ml^{-1} ampicillin selective for *P. aeruginosa,* or 20 μg ml^{-1}

cefsulodin selective for *E. coli.* Plates were incubated at 37°C for 24 h and colony forming units (CFU) determined.

Biofilm growth. Biofilms were grown on autoclaved 7mm diameter silicon-rubber disks, prepared from 1-mm thick sheets of silicon rubber (Dapro Rubber Inc., Tulsa OK). Disks were placed into 50 ml LB broth inoculated with 0.1 ml of an overnight culture of either *P. aeruginosa, E. coli,* or equal volumes of both organisms. Cultures were then incubated at 37°C on an orbital shaker (Thermo Scientific Max Q 2000, Waltham, MA) for 48 h at 150 rpm. Disks were then removed, rinsed with 0.1M PBS to dislodge planktonic cells, sonicated for 5 min, and vortexed for 30 s to disassociate the biofilm. Suspended biofilm cells were measured by dilution plating using the antibiotic media described above.

Effect of bacteriophage on biofilm density. Biofilms were grown on silicon disks as previously described. After 48 h of incubation, biofilm-colonized disks were removed from the broth, gently rinsed to dislodge planktonic cells, then placed into a phage suspension at an MOI of ~10 for 15 min. Individual disks were then placed into 10 ml of LB broth and incubated at 37°C in a shaker bath (Forma Scientific model 2564, Marietta, Ohio). At 24 h intervals, 9 ml of spent medium was removed and replaced with 9ml of fresh LB. Three disks with biofilms were randomly selected at 24 h intervals and biofilms assayed for CFUs as previously described.

RESULTS

Without phage (planktonic). In monoculture the bacteria maintained densities of 10^9 to 10^{10} cells per ml for each day of the trial (FIG 1). The mean $\log_{10}$ density of *E. coli* reached a mean $\log_{10}$ density of 9.6 +/- 0.32 cfu per ml. The grand mean $\log_{10}$ density of *P. aeruginosa* reached 10.2 +/- 0.26 cfu per ml. When in a mixed species system both *E. coli* and *P. aeruginosa* maintained relatively high and stable cell mean $\log_{10}$ densities of 9.8 +/- 0.68 cfu per ml and 9.9 +/- 0.7 cfu per ml respectively.

***E. coli* with W60 (planktonic).** In monoculture, phage W60 had little long-term effect on cell density and *E. coli* cell numbers reached similar densities as the challenge without phage (FIG 2). The mean $\log_{10}$ density of *E. coli* with phage W60 reached 9.6 +/- 0.3 cfu per ml, mirroring the trial without phage. When mixed species cultures were subjected to phage, *E. coli* exhibited a rapid decrease in viable cell density, going to extinction after day three.

***P. aeruginosa* with PB-1 (planktonic).** In monoculture phage PB-1 did seem to affect *P. aeruginosa* initially, lowering the cell density by a little less than a factor of 10 from the culture without phage (FIG 3). A mean $\log_{10}$ cell density of 9.8 +/- 0.2 cfu per ml was

reached and maintained over the course of the experiment. In a mixed species population *P. aeruginosa* showed low initial (day1) cell densities as compared to the single species culture, but still reached high cell densities by day three. At day 5, both cultures with and without phage, had reached equivalent cell densities.

Without phage (biofilm). In monoculture both bacteria reached biofilm densities of 10^5 to 10^6 cells per mm^2 (FIG 4) *E. coli* reached a mean $\log_{10}$ density of 6.3 +/- 0.2 cells per mm^2. *P. aeruginosa* reached a mean $\log_{10}$ density of 6.0 +/- 0.4 cells per mm^2. In a two species community, both species reached and maintained high biofilm density, similar to the single species biofilms.

***E. coli* with W60 (biofilm).** When *E. coli* grew in a monoculture biofilm and was challenged with phage W60 viable cell density dropped by a factor of ten by day three of the trial (FIG 5). However, cell density recovered to levels equivalent to that of the biofilm without phage. A mean $\log_{10}$ density of 6.1 +/- 0.8 cells per mm^2 was maintained over the course of the trail.

***P. aeruginosa* with PB1 (biofilm).** When *P. aeruginosa* was grown in a monoculture biofilm and was challenged with phage PB1 viable cell density dropped slightly by day three of the trial (FIG 6). After day three, viable cell density also recovered to levels equivalent to that of the biofilm without phage. A mean $\log_{10}$ density of 5.8 +/- 0.4 cells per mm^2 was maintained over the course of the trail.

DISCUSSION

Research involving bacteriophage as therapeutic agents in the treatment of chronic bacterial infections ceased in the United States after WWII, primarily due to the widespread success of antibiotics (40). Phage research did continue, until quite recently, in eastern countries including (41). In a study by the World Health Organization, bacteriophage therapy was used to treat cholera patients in Pakistan during the 1960s. Results of this study showed that at a very high MOI, 100-200, bacteriophage were as effective as tetracycline in treatment of the disease (23, 28). This is a very important observation as antibiotic resistance is becoming a major problem in the treatment of bacterial diseases. In another study, over 65% of *Pseudomonas aeruginosa* strains isolated from septicemic neonates were found to be multidrug-resistant (MDR) (42). When mice were infected with a fatal dose of MDR *P. aeruginosa* followed by dose of bacteriophage CSV-31 within 5 hours of infection 100% of the test mice survived (42). Even when the septicemic mice were allowed to become moribund, the phage treatment proved 50% successful in rescuing the sick mice.

This study examined the effect of bacteriophage infection on mixed species *E. coli* and *P. aeruginosa* planktonic and biofilm communities. The data revealed *P. aeruginosa* maintained a high cell population when challenged with phage PB-1 in mixed species planktonic culture. On the other hand, E. *coli* was unable to establish a resistant

population when grown in a two-species planktonic community with *P. aeruginosa.* When *E. coli* and *P. aeruginosa* were grown in a two-species biofilm the bacteriophage had little effect on either bacterial community, suggesting that bacteria gain a protective advantage when in biofilms to phage. This can create a major obstacle for the use of phage in treatment of chronic, biofilm associated bacterial infections.

Bacteriophage are known to play an important role in ecosystems through the transfer of genetic material between populations and causing phenotypic variation within populations of bacteria (43). Phage also influence all known food webs, especially in marine systems (4). Aquatic environments allow phage to play a major role in controlling the biomass found in a system (19). Bacteriophage have also come to the forefront of medicine not only due to their ability to control bacterial infection but also to spread resistance and virulence genes through populations of pathogenic organisms (12, 47).

Many studies have shown that phage infection does not have a long-term effect on bacterial populations in pure culture (6, 13, 22). In a planktonic community high cell numbers are maintained though the development of resistant populations (8, 13, 22, 44). Even though there is initially a rapid decline in viable cell numbers the establishment of a resistant community occurs quickly (8, 14, 44). More recently, Harcombe et al. showed that *E. coli*, growing in a mixed species community with *Salmonella typhimurium,* became extinct when challenged with phage T7 (13). However, when *E. coli* was grown in monoculture it was able to establish a resistant cell population and was largely unaffected by its phage (14). This was encouraging for the use of phage in the treatment of chronic bacterial infections. However, many chronic bacterial infections exist as biofilms (10, 21, 24, 29).

In this study, bacteriophage were more effective in reducing the number of viable *E. coli* cells compared to the number of *P. aeruginosa* cells when grown in a two-species planktonic culture. The extinction of *E. coli* in mixed species planktonic cultures with *P. aeruginosa* agrees with the findings of Harcombe et al. which showed increased susceptibility of *E. coli* to phage when in the presence of *S. typhimurium* (13). It is possible that in mixed species cultures *E. coli* experiences competition and is therefore more susceptible to bacteriophage infection. *P. aeruginosa*, on the other hand, is also known to produce antimicrobial compounds called phenazines, the most notable being pyocyanin (5). These compounds could act synergistically with phage and eliminate the population. It is noteworthy that *E. coli* did not experience the same increase in susceptibility when grown in a mixed species biofilm with *P. aeruginosa.* This clearly illustrates the protective advantages that a biofilm offers its inhabitants and the challenge biofilm development can be for the use of phage as therapeutic agents.

Extra-cellular polymeric substance (EPS), which make up a large part of bacterial biofilms, can be a physical barrier against phage infection. This could account for the observation that mixed-species biofilms proved less sensitive than the mixed-species planktonic cultures to phage infection. Some bacteriophage carry enzymes capable of degrading capsular material of bacteria and EPS of biofilms in order to gain access to susceptible host cells (18, 41). The phage depolymerase binds to the capsular material and degrades it until the phage is able to bind to the outer-membrane receptors and infect the bacterium (18). These enzymes could play an important role in phage infection in biofilms.

In this study, *E. coli* gained a protective advantage from phage infection in mixed species biofilms compared to planktonic culture. Biofilms comprise highly structured, matrix-enclosed communities which make up the majority of bacteria in natural ecosystems (39). A biofilm, both in pure culture and in natural ecosystems, is a basic structure which consists of cells growing in matrix-enclosed microcolonies separated by a network of water channels (39). The matrix is composed primarily of microbially produced EPS (18). The development of phage resistance in combination with the inaccessibility of the host cell could account for the increased survival of *E. coli* when grown in a mixed species biofilm as compared to a mixed species planktonic community. EPS has been shown to allow bacteria to survive phage infection by making the bacterial cells inaccessible to the attacking phage (18, 41). Phage must be able to recognize receptors on the bacterial surface in order to attach to the host cell (41, 44). Infection can not take place if these receptors are covered by EPS (41). Indeed, Doolittle et al. showed that *E. coli* biofilms were more easily infected than *P. aeruginosa* biofilms due to the thickness of the EPS (11). Bacteria have also been shown to form denser biofilms when in a multi-species community (7). Reduced metabolic activity of host cells in the biofilm community can also affect bacterial sensitivity to phage infection (39). Many cells in a biofilm have greatly reduced metabolic functions, due to decreased oxygen and carbon resources, which do not support phage replication (39). Even poor and non-biofilm forming bacteria could benefit by growing in community with a strong biofilm-forming organism.

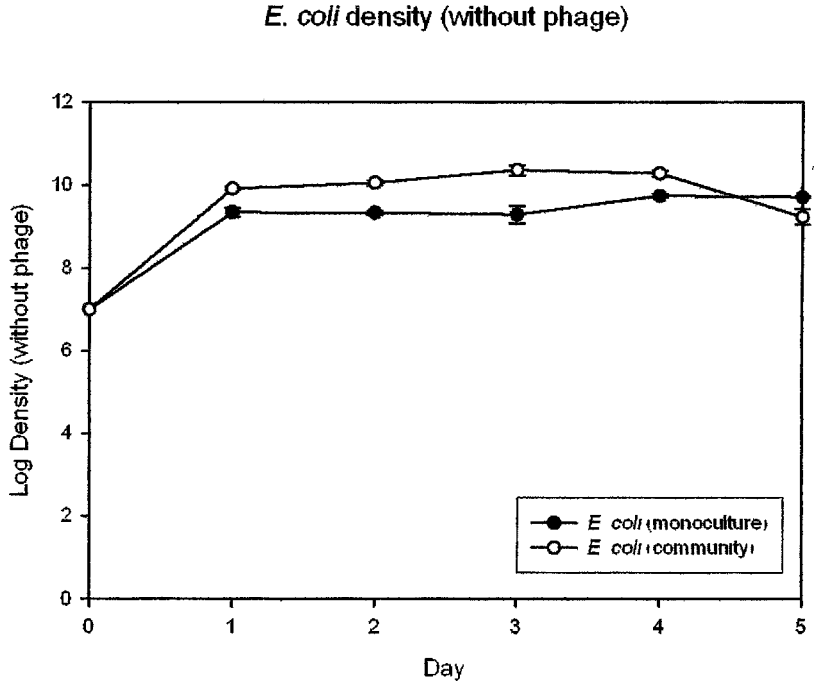
Although earlier studies have suggested that phage therapy has the potential to reduce bacterial cell densities in the environment (13), this study demonstrates that if the

bacteria form a biofilm, phage will have a very limited effect on the bacterial population. In aquatic environments, bacterial communities must reach a certain density before a phage infection can be supported (33). If the bacterial density drops below that level phage multiplication will cease (8, 46). In vitro, this will allow resistant populations to dominate and the infection to persist. However, in vivo, at low cells densities surviving bacteria could possibly be cleared by the host's immune system.

The results of this study demonstrate the efficacy of bacteriophage in the treatment of bacterial infections. However, the state in which the bacteria are growing needs to be taken into account in order for phage therapy to be effective. Bacteriophage infections of complex bacterial communities should be investigated further to determine the effect phage infection in other mixed species interactions. These studies would not only improve our understanding of microbial ecology, but also bacteriophage evolution. In addition, the effect of multiple phage, or "phage-cocktails", on mixed species biofilms should be investigated. The "killing the winner population" hypothesis states that phage expand on the fastest growing host population in a given ecological setting (33). This could be exploited through the use of sequential phage infections to reduce one cell population, making the other population the "winner" and now more susceptible to phage infection.

Figure 1.

A.



B.

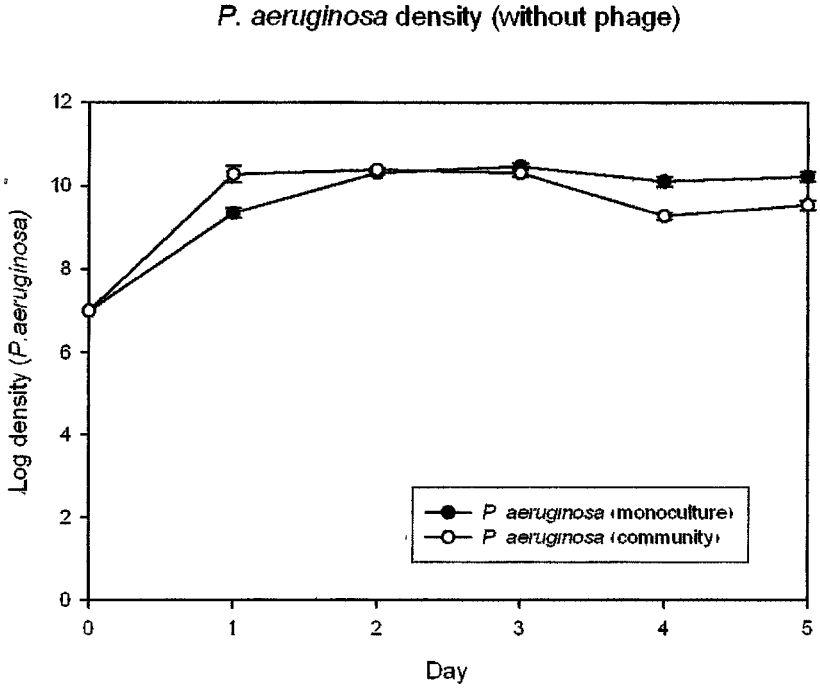
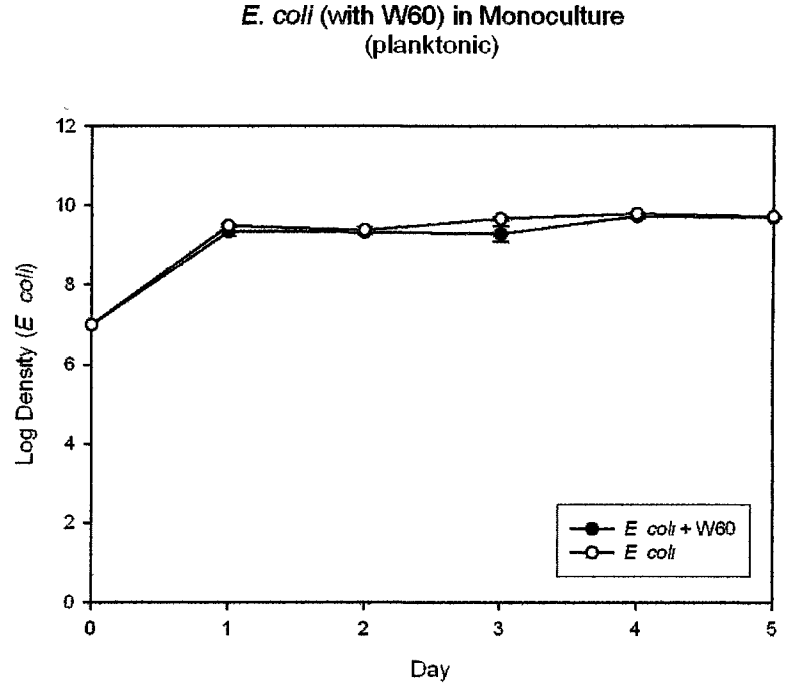


Figure 1. Mean planktonic bacterial density in the absence of bacteriophage. The data represent the log densities reached at 24h intervals. (A) *E. coli* densities in monoculture (□) and in two-species community (■). (B) *P. aeruginosa* densities in monoculture (□) and in two-species community (■).

Figure 2.
A.



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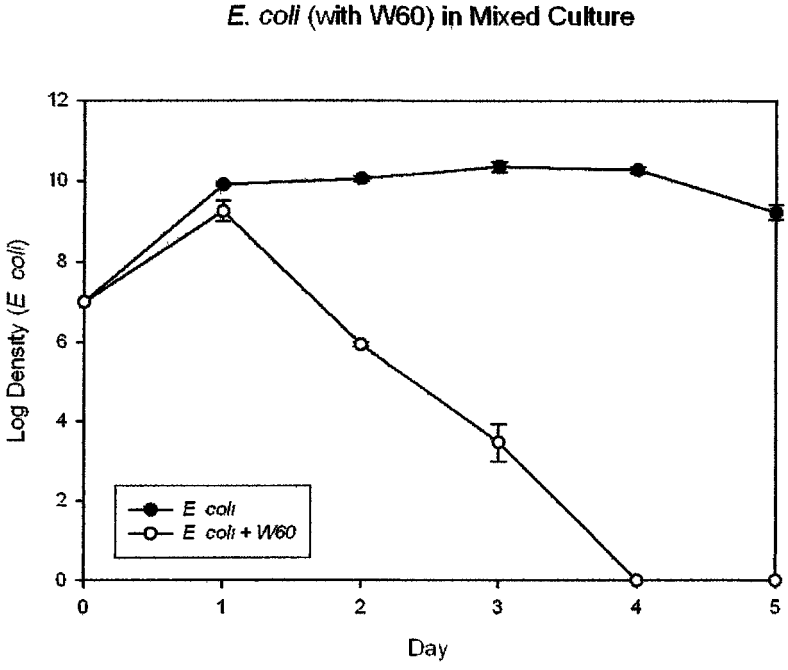
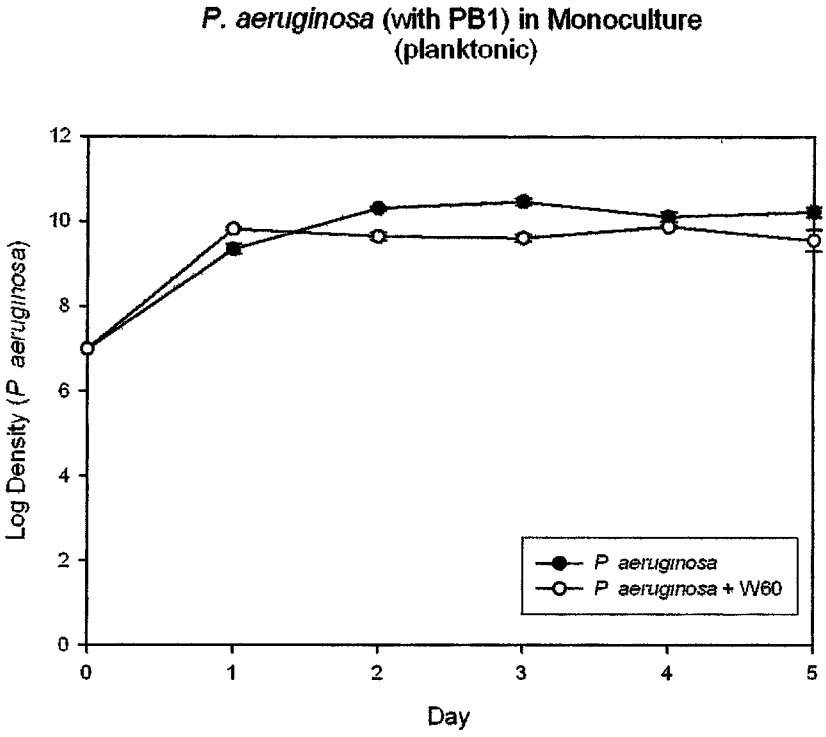


Figure 2. *E. coli* mean planktonic density when in the presence of phage W60. The data represent the log densities reached at 24h intervals. (A) *E. coli* in monoculture in the absence of W60 (○) and in the presence of W60 (●). (B) *E. coli* in a two-species community in the absence of W60 (□) and in the presence of W60 (■).

Figure 3.
A.



B.

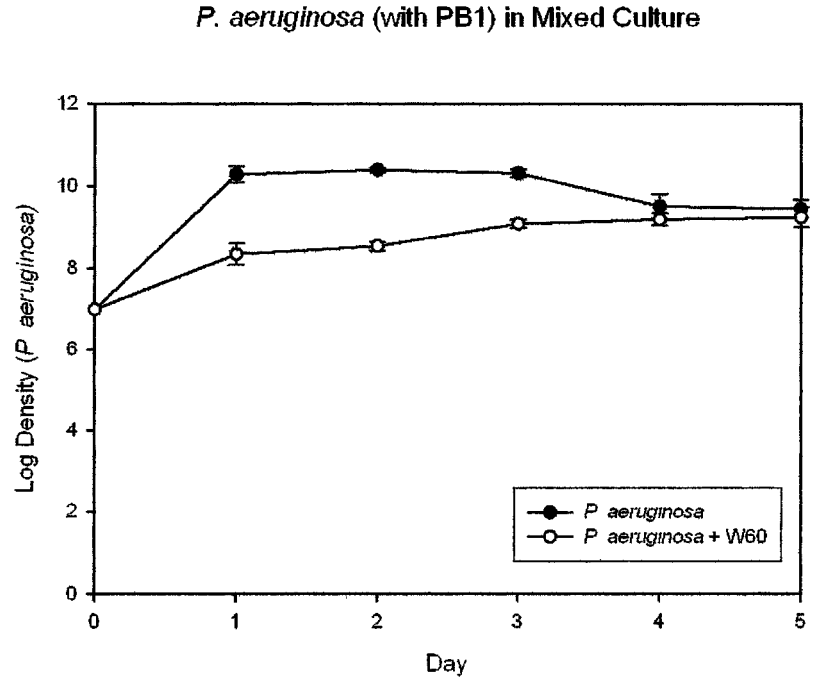
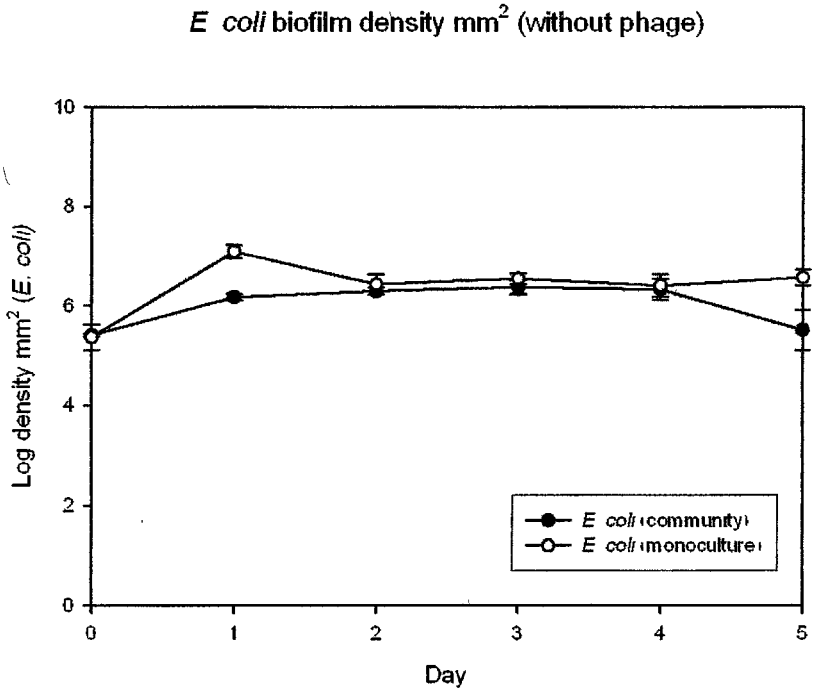


Figure 3. *P. aeruginosa* planktonic density when in the presence of phage PB-1. The data represent the log densities reached at 24h intervals. (A) *P. aeruginosa* in monoculture in the absence of PB-1 (○) and in the presence of PB-1 (●). (B) *P. aeruginosa* in two-species community in the absence of PB-1 (□) and in the presence of PB-1 (■).

Figure 4.
A.



B.

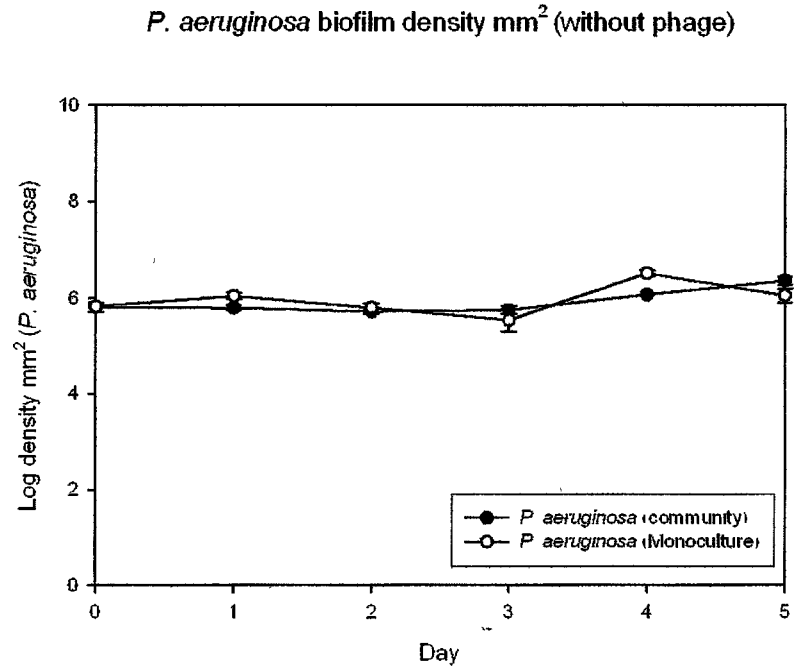
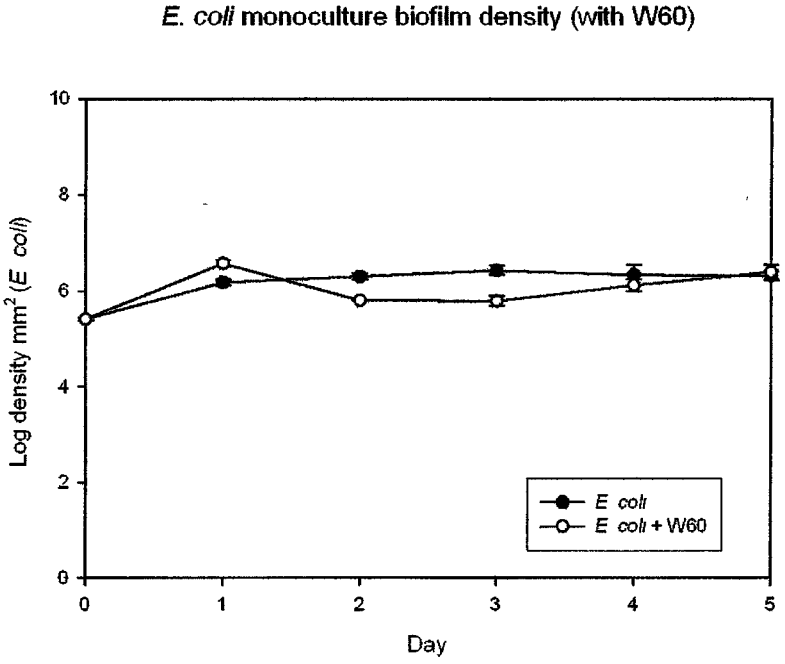


Figure 4. Mean biofilm density in the absence of phage. The data represent the log biofilm densities reached at 24h intervals. (A) *E. coli* densities in monoculture (□) and in two-species community (■). (B) *P. aeruginosa* densities in monoculture (□) and in two-species community (■).

Figure 5.
A.



B.

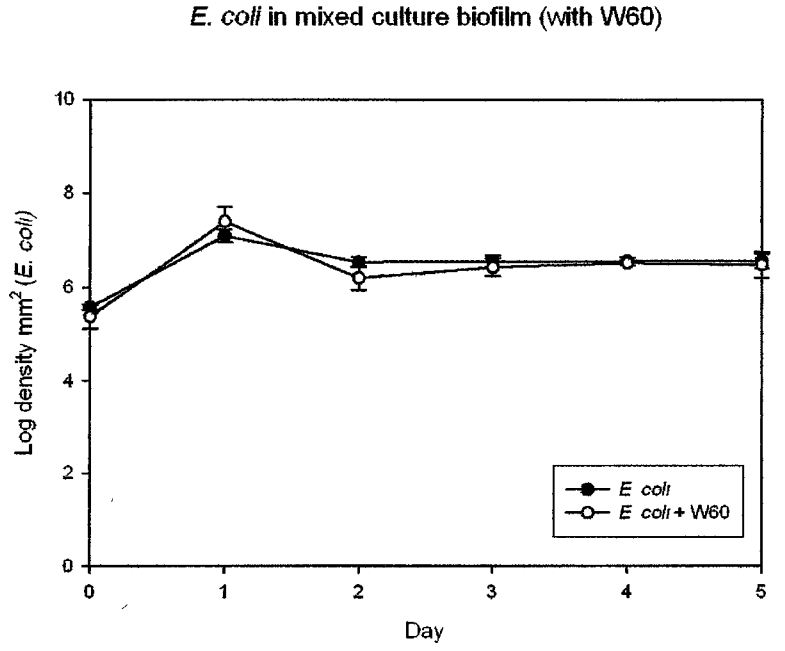
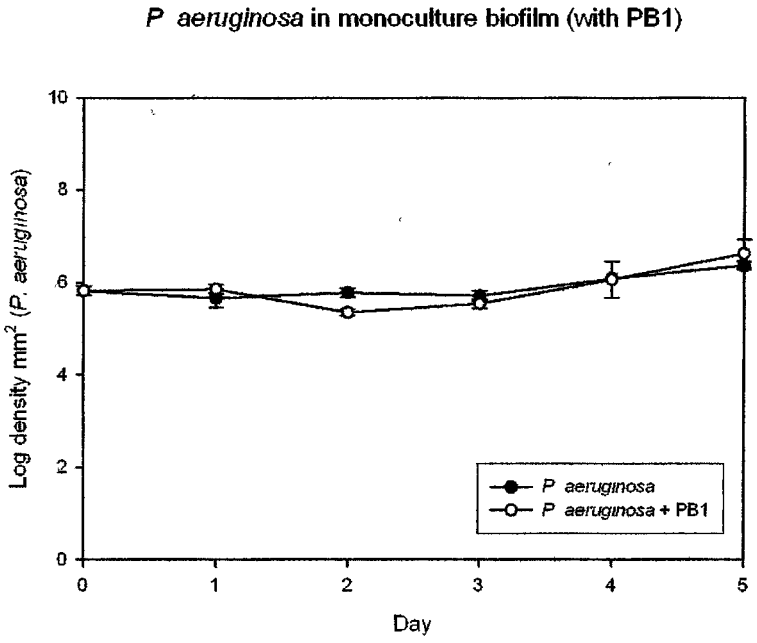


Figure 5. *E. coli* mean biofilm density when in the presence of phage W60. The data represent the log biofilm densities reached at 24h intervals. (A) *E. coli* densities in monoculture in the absence of W60 (○) and in the presence of W60 (●). (B) *E. coli* densities in two-species community in the absence of W60 (□) and in the presence of W60 (■).

Figure 6.
A.



B.

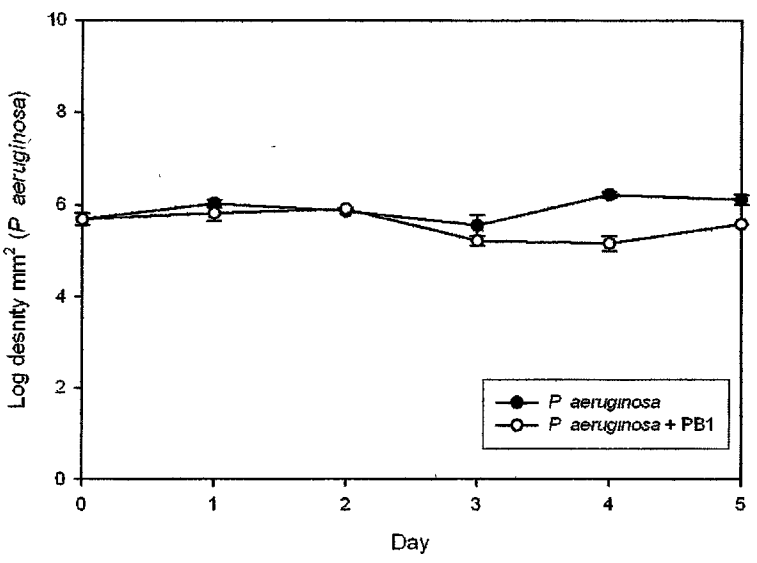


Figure 6. *P. aeruginosa* biofilm density when in the presence of phage PB-1. The data represent the log biofilm densities reached at 24h intervals. (A) *P. aeruginosa* biofilm densities in monoculture in the absence of PB-1 (○) and in the presence of PB-1 (●). (B) *P. aeruginosa* densities in two-species community in the absence of PB-1 (□) and in the presence of PB-1 (■).

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