The efficacy of sewage influent-isolated bacteriophages on *Pseudomonas aeruginosa* in a mixed-species biofilm

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ABSTRACT

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The growth of environmentally persistent biofilms in cooling towers causes several associated problems, including microbiologically-induced corrosion (MIC) and biofouling. Current chemical control methods are not only ineffective against biofilms and costly to procure, they also have downstream environmental impacts when released untreated, or incur additional treatment costs. Bacteriophages are alternative biofilm control agents that have the potential to be more effective, cheaper to produce and yet have a more benign effect on the environment. In this study, biofilms grown under conditions simulating seawater fed cooling towers were characterized and the differences in growth and community make-up across time and different substrates were assessed. An MIC associated bacterium common in cooling tower water, *P. aeruginosa*, was chosen. Seven bacteriophage strains found to be effective against the chosen bacterium were isolated from wastewater influent. The relative effectiveness of these strains was measured against *P. aeruginosa* across different salinities. Separate biofilms fed with *P. aeruginosa* enriched seawater were characterized and the effectiveness of the isolated strains, singly and in cocktails, against the enriched biofilms was measured.

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1. INTRODUCTION

Cooling tower water is a conducive growth medium for bacteria (Kusnetsov et al., 1993) to grow at a temperature ranging between 26 to 43 °C. The water is usually recirculated at least once, allowing for microbes, minerals and nutrients to accumulate and concentrate via evaporation. Some types of commonly used antiscalants may also contribute nutrients that support microbial growth (Sweity et al., 2013). As such, extensive biofilm growth is ubiquitous in cooling towers (Liu et al., 2009, Pagnier et al., 2009).

Biofilm growth creates a host of problems for cooling towers such as biofouling and MIC. MIC accounts for approximately 20% of industrial corrosion (Flemming et al., 2009) and costs $55 billion annually in the US alone (Koch et al., 2002). Aside from impeding flow and causing MIC, biofouling on cooling tower elements such as heat exchangers can lead to a reduction in cooling efficiency (Chien et al., 2012, Meesters et al., 2007). Biofilms composed of microbial consortiums can also serve as a colonization platform for pathogens which may be less inclined to form biofilms on their own or augment their environmental persistence (Abdel-Nour et al., 2013, Murga et al., 2001). One such pathogen would be *Legionella pneumophila* (Dondero et al., 1980), which can lead to outbreaks through both exposure to cooling tower water (Addiss et al., 1989) as well as aerosolization of pathogens (Ishimatshu et al., 2001, Pagnier et al., 2009, Declerck, 2009).

The typical means of dealing with biofilms in cooling towers are biocides and biodispersants (Simões et al., 2010, ASHRAE 2000). Chemical biocides are classified into either oxidizing biocides, such as sodium hypochlorite, or nonoxidizing biocides, such as carbamates, which kill microbes by inhibiting essential processes. Biodispersants, such as

sodium dodecyl sulphate, interfere with the biofilm's ability to adhere to the surface by breaking down components such as polysaccharides or lipids (Simões et al., 2006). Biofilm removal and viable cell number reduction are poorly correlated (Chen et al., 2000). A biocide alone may not be able to effectively penetrate layers of the biofilm (Liu et al., 2011, Simões et al., 2006). Not only is diffusion of the biocide through the biofilm hampered, chemical interactions with extracellular polymeric substances (EPS) also hinder delivery of the biocides (Stewart 2002, Flemming, Wingender 2010). Biodispersant application in isolation may simply dislodge cells. The dislodged planktonic bacteria can then form biofilms elsewhere or cause outbreaks if pathogenic. The effectiveness of biodispersants on biofilms is also dependent on how the biofilms were formed, with biofilms formed under turbulent flow having greater resistance to biodispersants than those formed under laminar flow (Simões et al., 2006). Furthermore, these are often broad spectrum controls, which make them either an environmental liability, should they be released with untreated water, or an additional treatment cost. Just as importantly, biofilms can develop resistance not only to antibiotics, but to chemical controls as well (Mah et al., 2001). Most chemical treatments are focused primarily on treating *L. pneumophila* as well (Pagnier et al., 2009).

Given the complications associated with biofilm chemical controls, it is worth investigating alternative solutions. One promising group of alternatives are bacteriophages. Bacteriophages, or phages for short, are viruses which infect bacteria. Phages are obligate parasites which depend on host cell machinery to propagate. Lytic phages are of interest in this study as they lyse and kill their hosts upon completion of their replication cycle. As discussed in the following section, phages offer a way to circumvent some of the shortcomings associated with conventional biofilm controls.

The objective of this thesis is to investigate the viability of using phages to remove MIC associated biofilms. This was done by characterizing the biofilms formed under industrial conditions as well as the planktonic community in source seawater, ascertaining the presence of MIC associated organisms and common pathogens in the biofilms, isolating phage strains effective against a target MIC associated bacterium and comparing the effectiveness of each strain against the target organism under varying conditions. The effects of the phage strains on a simulated MIC associated biofilm, singly and in a cocktail (Summer et al., 2009), were also studied. The biofilms were grown and studied under conditions designed to mimic as closely as reasonably possible those found in cooling tower pipes at KAUST. The cooling towers at KAUST source water from the Red Sea (3.5% salinity average, 28°C average). The insights provided by this study may eventually serve to better inform cooling tower disinfection protocols.

2. LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*: a common marine biofoulant

The MIC associated bacterium chosen for this study was *P. aeruginosa*, a known metal-reducing bacterium (MRB) (Beech et al., 1999, Abdolahi et al., 2014). *P. aeruginosa* is an environmental bacterium ubiquitous to seawater (Belila et al., 2016, Connon, Giovannoni, 2002). Its minimal survival requirements and unique adaptation mechanisms (Frimmersdorf et al., 2010) mean it can be found in a wide variety of environments apart from seawater including fresh water, brackish water, sewage, the mammalian gut and plants (Yoshpe-Purer, Golderman, 1987, Walker et al., 2004, Kimata et al., 2004). Elevated salinity and the relative lack of nutrients are not largely detrimental to the survival rates of *P. aeruginosa* (Cornax et al., 1990)

Kimata et al., 2004 showed that *P. aeruginosa* concentrations increased with salinity up to 30 psu and concluded that littoral seawater is most likely a natural reservoir for the bacterium. This was corroborated by the earlier findings of Mates, 1992, where *P. aeruginosa* was isolated from 14% of seawater samples from beaches in the Mediterranean. Furthermore, the presence of *P. aeruginosa* was not strongly correlated to that of fecal coliforms, suggesting that sewage discharge may not be a significant source of *P. aeruginosa* in marine environments as was previously believed.

Salinity has been shown to have little bearing on the ability to attach to stainless steel (Vanhaecke et al., 1990). This is a key attribute that would allow *P. aeruginosa* to form biofilms on structures in Red Sea, with its higher than average salinity seawater. Furthermore, Ca^{2+}, Fe^{2+} and Fe^{3+}, ions which are expected to be in high concentrations in

cooling tower water, stabilize alginate. Alginate, which is the primary EPS component of *P. aeruginosa*, serves to protect the bacteria, enhance biofilm integrity, as well as to enhance biofilm adhesion (Boyd et al., 1995). The more hydrophobic strains of *P. aeruginosa* can adhere to even electropolished SS 304 and SS 316 (Vanhaecke et al., 1990), increasing its persistence even in well designed and maintained cooling towers. The above would suggest an expected high representation of *P. aeruginosa* in cooling towers, or at least, the rapid establishment and dominance of *P. aeruginosa* once introduced into an existing biofilm.

To illustrate, *P. aeruginosa* outcompetes *Klebsiella pneumoniae* and *Flavobacterium* sp. (Liu et al., 2009), two other groups of opportunistically pathogenic or MIC inducing microbes found in seawater-based cooling towers (Beech et al, 1999) in batch experiments. In fact, the survival rate of *P. aeruginosa* was found to be independent of the presence of the other two bacteria. This was despite the relatively lower bioadhesion rate of *P. aeruginosa*. The results suggest that adhesion rate is less important than growth rate in long term persistence in cooling towers.

P. aeruginosa was chosen over other well-known MIC associated genera such as *Desulfovibrio* and *Desulfobacter* as 1) it was presumed that the latter 2 genera would be less well-represented in cooling tower pipes due to the large exposure to air as 2) as well as the relative ease involved in cultivating the former.

2.2 MIC causing mechanisms of *P. aeruginosa*

P. aeruginosa induces biocorrosion via several mechanisms, namely 1) H_2S production from thiosulfate reduction (Friedrich et al., 2001), 2) the removal of protective oxide layer

on metal surfaces (Obuekwe et al., 1981) and 3) the presence of acid polysaccharides in the EPS (Beech et al., 1999). *P aeruginosa* is capable of degrading alloys commonly used in the marine industry, such as SS 304 (Yuan, Pehkohnen, 2007, Hamzah et al., 2013), as well as 2707 Hyper-Duplex Stainless Steel, an alloy otherwise known to be highly corrosion resistant (Li et al., 2016).

Morales et al., 1993 and Yuan et al., 2008 demonstrated that the primary mechanisms through which *P. aeruginosa* corrodes SS 304 are pitting corrosion through depletion of the most electrochemically active metals (Beech et al, 2000) in the alloy (Yuan, Pehkohnen, 2007) and depletion of the protective chromium oxide layer. These mechanisms are more pronounced when a biofilm is formed (Hamzah et al., 2013) as a localized electrochemical cell is created between the anoxic biofilm and the aerated steel surface, or when the biofilm concentrates chloride ions which initiate the pitting process. Beech et al, 2000, describes the *P. aeruginosa*-induced, multistep electrochemical corrosion of stainless steel 316 (SS316) in detail: the anodic portion of the metal is oxidized and oxygen is reduced at the cathodic portions of the metal, creating H^+ ions. The resulting low pH around the anode hydrolyses the protective Cr and Mo from the stainless steel. Cl^- ions then aggregate around the anode to neutralize the charge, causing pitting through the formation of $FeCl_3$ and $MnCl_4$, both of which corrode stainless steel.

Considering this, any proposed treatment should prioritize effectiveness against adhered *P. aeruginosa* over planktonic *P. aeruginosa*. It should be noted, however, that some strains of *P. aeruginosa* may serve to inhibit biocorrosion by developing a protective layer (Wadood et al., 2015) with the extracellular polymeric substance (EPS). Rajasekar and Ting (2011) observed that *Pseudomonas* sp. biofilms accelerate corrosion in saline organic

media (in g/L: peptone, 5; beef extract, 5; yeast extract, 1.5; NaCl, 3.5; pH 7) but inhibit corrosion in saline inorganic media (in g/L: K_2HPO_4, 0.5; $NaNO_3$. 0.5; $CaCl_2$, 0.2; $MgSO_4.7H_2O$, 0.5; ammonium iron citrate, 0.6; NaCl, 3.5; pH 7). It was found that thick layers composed of Fe_2O_3 and Cr_2O_3 as well as deposits of insoluble iron phosphonates, were formed under the biofilms in inorganic media. These deposits are presumed to protect the steel from further corrosion. Rajasekar and Ting further suggested that the EPS bind selectively to certain metal ions and form complexes that eventually result in the deposition of protective oxides and that the preponderance of these processes is a function of ion concentration.

2.3 Impact of *P. aeruginosa* growth on seawater reverse osmosis (SWRO)

To gauge the potential impact of *P. aeruginosa* biofilm growth in seawater cooling towers, the literature on a related industry, seawater reverse osmosis (SWRO), was examined. Biofilms are more likely to form and persist in environments with high hydrodynamic shear (Donlan, Consterton, 2002). In fact, the structural integrity of a biofilm is proportional to the hydrodynamic shear (Liu et al., 2002). Elevated hydrodynamic shear induces greater EPS secretion, enhancing initial cell adhesion (Lopes et al., 2000) and increasing structural integrity (Chen et al., 1998). High hydrodynamic shear is expected in sections of both SWRO plants and cooling towers.

Biofouling is a major concern in SWRO plants, accounting for up to 50% of total costs (including membrane treatment and replacement) (Matin et al., 2011). Membrane biofouling can also have downstream effects on plant operating costs. To compensate for the decrease in flux, pump activity is usually increased, leading to an increase in energy consumption and pump maintenance costs. *P. aeruginosa* biofouling negatively affects RO

membrane performance via two distinct mechanisms: 1) the accumulation of cell bodies, which increases osmotic pressure on the membrane surface, and 2) the secretion of EPS, which decreases flux (Herzberg, Elimelech, 2007; Hong et al., 2016). Aside from causing MIC and biofouling, the *Pseudomonas* genus is known to mineralize organic matter (Palleroni, 1981). This could exacerbate scaling and fouling concerns.

The literature on the impact of *P. aeruginosa* on SWRO biofouling is divided. Ghayeni et al., 1998 showed that *P. aeruginosa* was dominant among a group of fast-adhering biofoulants. Furthermore, the number of *P. aeruginosa* which attached to the membrane was positively correlated to salinity. *Pseudomonas* spp. was found to be the major genera present in SWRO feed tank (Jamieson et al., 2016), although SWRO microbial communities can vary vastly depending on the plant location and the stage of treatment. *P. aeruginosa* is among the species involved in biofilm formation on RO membranes (Sinclair, 1982, Belgini et al., 2014, Bereschenko et al., 2008). Whilst *Sphingomonas* was the most dominant, Bereschenko et al., *Pseudomonas* comprised 19% of the total clones. Zhang et al. (2011) disputed the role of *P. aeruginosa* as an initiator, however, citing cultivation bias. Additionally, while *P. aeruginosa* is almost undoubtedly the primary biofilm initiator in wastewater RO membranes, Lee et al. (2010) posits *Bacillus* spp. playing the same role for SWRO instead. Although *Bacillus* sp. eventually outcompeted four other common strains (*P. aeruginosa*, *Rhodobacter* sp., *Flavobacterium* sp. and *Mycobacterium* sp.), *P. aeruginosa* was shown to be dominant at different phases of biofilm colonization, most notably within the initial four hours (Kim et al, 2014). The same study also noted that *P. aeruginosa* is more resistant to membrane pretreatment, with populations recovering the second fastest after exposure to NaOH pretreatment.

2.4 *P. aeruginosa* as a potential pathogen

Although primarily an environmental bacterium, the potential of *P. aeruginosa* to cause disease is well established (Defez et al., 2004, Bodey et al., 1983, Mena, Gerba, 2009, Kreger et al., 1980, Palleroni, 1981). It has been implicated in a variety of conditions not limited to malignant external otitis, endophtalmitis, endocarditis, meningitis, pneumonia, septicemia, gastrointestinal infections and cystic fibrosis complications. *P. aeruginosa* is the dominant infective organism in cystic fibrosis patients as well as the most common and most fatal pneumonia-causing organism. Kreger et al., 1980 found it to be the fourth most common etiologic agent isolated from septicemia patients. Although nosocomial infections are primarily associated with *P. aeruginosa* (it is the fifth most frequent cause of nosocomial infections), it has been known to infect non-immunocompromised individuals as well (van Asperen et al., 1995). *P. aeruginosa* was determined to be the cause of an outbreak of otitis externa contracted by 98 individuals swimming in a freshwater lake.

There are a few key traits that enhance *P. aeruginosa*'s effectiveness as a pathogen. Chief amongst them is *P. aeruginosa*'s hypermutability, which allows it to develop novel antibiotic resistance mechanisms (Webb et al., 2004, Oliver et al., 2000, Mah et al., 2003). This trait is not only observed in epidemic strains, but in environmental strains as well (Kenna et al., 2007). Moreover, the rate of horizontal gene transfer between *Pseudomonas* species is significantly greater within biofilms (Ehlers, Bouwers, 1999), allowing for resistance genes to be shared quickly.

The threat posed by *P. aeruginosa* in a non-hospital setting is undoubtedly lower than that posed by other environmental bacteria, such as *L. pneumophila*. Still, while *P. aeruginosa* cannot be aerosolized in a similar manner to *L. pneumophila*, it is still an opportunistic

pathogen which can cause infection through exposure to cooling tower water. Skin contact, as well as contact with the soft tissues of the eyes and ears are common exposure routes (Mena, Gerba, 2009). Though less common, ingestion is also a possible route.

2.5 Effectiveness of common disinfection strategies

Oxidizing biocides and biodispersants (Simões et al., 2006) are the most common means of dealing with biofilms in an industrial setting. Oxidizing biocides are broad spectrum, an advantage when removing multispecies biofilms, but disadvantageous regarding environmental sustainability. Biocides can also be pH dependent (Walker, Morales, 1997) Antibiotics are generally less broad spectrum, but discharging large volumes of antibiotics into an open environment is not feasible or even irresponsible from a medical standpoint. The release of antibiotics into water systems would raise several concerns: 1) alteration of aquatic ecology and the removal of ecologically significant bacteria (Corcoran et al., 2010), 2) contamination of water used for consumption or recreation, 3) the proliferation of bacterial antibiotic resistance (Costanzo et al., 2005, Zhang et al., 2009). UV disinfection avoids many of the problems associated with chemical controls, but is difficult to apply throughout a closed system and its efficacy is limited by turbidity.

These traditional means of disinfection are far less effective against biofilms than they are against planktonic bacteria. Biofilms protect bacteria from environmental stresses and, consequently, chemical controls (Donlan, Consterton, 2002). Chlorine penetration was measured at only 20% of the bulk mass in a mixed *P. aeruginosa* and *Klebsiella pneumoniae* biofilm (de Beer et al., 1994). *P. aeruginosa* is also prone to phase variation to deal with environmental change (Drenkard, Ausubel, 2002), allowing it to go into a sessile state to reduce disinfectant uptake. Biofilms in general, with their increased

metabolite buildup and lower metabolic rates as compared to planktonic cells, have a decreased disinfectant uptake rate (Tresse et al., 1995). The EPS is a major limiting factor on the effectiveness of any potential treatment option due to interaction of antimicrobial agents with EPS (Stewart, 1996). Biofilm penetration rates differ according to the antimicrobial agent used (Souli, Giamarellou, 1998).

Donlan et al., 2002, noted that the current industrial approach to preventing biofilms (exploiting oligodynamic metals and surface characteristics of structures) and dealing with biofilms (mechanical removal followed by oxidative biocides) are overall insufficient. Biofilms form even on oligodynamic metals such as silver and copper (Marrie, Costerton, 1983), albeit at a reduced rate. Kim et al., 2015 found that damaged *P. aeruginosa* cells can enhance biofilm formation rate. In the context of cooling tower biofouling, this can lead to enhanced biofilm regrowth in between chemical treatment phases.

2.6 Bacteriophages as a potential alternative

Phages may undergo either lytic or lysogenic cycles. After infection of a host cell, lytic phages utilize their host's machinery to synthesize viral components. After the components assemble into complete virions, the phages burst from the host cell, killing it. Lysogenic phages, on the other hand, integrate their genetic material (known as the prophage) into the host cell. The host cell, now known as a lysogen, may continue to function normally, passing on the prophage to daughter cells or the integrated prophage may enter the lytic cycle under certain environmental conditions. Lysogenic phages are ineffective as biofilm controlling agents and can possibly enhance biofilm persistence through horizontal gene transfer instead. Only lytic phages from environmental isolates were considered for use and all further mention of phages in this study refer to lytic phages. The propagative nature

of phages is a major advantage regarding the dosage required. Doolittle et al., 1996 reported the formation of plaques in a biofilm at the low multiplicity of infection ratio of 10^{-5} phages per cell. Furthermore, phages may be more cost effective than conventional treatments, requiring only host nutrient media, incubation and labor costs.

Biofilms offer limited protection against phages (Donlan, Consterton, 2002). Phages are less likely to be blocked by the EPS unlike commonly used biocides such as chlorine. A biofilm's maturity affects its susceptibility to antimicrobials (Stewart, 2004). This is due to increased layers of EPS, the development of deadzones and reduced metabolism. In contrast, Hanlon et al., 2001, demonstrated that a phage's effectiveness against a *P. aeruginosa* biofilm is not significantly affected by biofilm age. Phages, especially those that induce the production of depolymerases (Lu, Collins, 2007), can both penetrate biofilms and deactivate the component cells (Azeredo, Sutherland, 2008). The structural integrity of the biofilm can be compromised both through depolymerase activity and extensive cell lysis (Sutherland et al., 2004). The phage used in Hanlon et al., 2001, triggered depolymerase production in the host cell which in turn reduced alginate viscosity and facilitated phage transport through the biofilm. These depolymerases may or may not be integrated onto the virion. Depolymerase-associated phages have been shown to be effective even against biofilms composed of phage-resistant bacteria (Hughes et al., 1998). Additionally, a phage may express multiple tail fiber proteins, each capable of cleaving a different EPS polymer. This vastly increases its host range (Scholl et al., 2001). The ability of a phage to penetrate a biofilm's EPS depends not only on its ability to induce depolymerase production, but its surface characteristics. For example, a non-contractile tail may hinder the phage's penetration ability (Briandet et al., 2008).

It is unlikely that phages can completely supplant chemical controls. Instead, phages can be used as part of a multi-pronged solution to biofilm removal or to supplement existing chemical treatment protocols. Such an approach may have a lower chance of inducing resistance to any single treatment approach if the effects are additive or synergistic (Worthington et al., 2013, Bollenbach et al., 2015), reducing the required frequency of chemical treatment. Using the phages in a cocktail would further reduce the chance of developing resistance.

Still, as with any other antimicrobial control, the target host can develop resistance to phages. It does this mainly through alteration of the cell surface receptor structure, density or presence. Other resistance mechanisms include production of EPS constituents impermeable to a particular phage or production of restriction endonucleases that target the phage (Jensen et al., 1998). The transformation of the host cell into a lysogen by another phage can confer resistance to other phages via phage-coded repressors (Donlan, 2009). These repressors prevent the transcription of phage encoded genes and block cell surface receptors. Even within the same strain of *P. aeruginosa*, subpopulations with distinct phenotypes can exist (Fu et al., 2010, Drenkard, Ausubel, 2002) and possibly give rise to resistance. A greater phenotypic presence also translates to increased biofilm formation rates (Drenkard, Ausubel, 2002). The propagative nature of phage treatment can also work against it. Phages propagate radially throughout the biofilm (Doolittle et al., 1996). Propagation towards the center of the biofilm is also detrimental to the phage (Sutherland et al., 2004) as deadzones are created.

Unlike conventional microbial controls, however, circumventing bacteriophage resistance is far less arduous. Phages are necessarily more abundant (Azeredo, Sutherland, 2008) and

diverse than their hosts (Clokie et al., 2011) and possibly possess greater rates of mutation owing to the lack of proofreading during phage production. Isolating a phage effective against a newly resistant strain is therefore less complex. Furthermore, Lenski, 1988, surmises that phage-resistant bacteria have compromised ability to attach to surfaces due to alteration of the cell surface receptors. Phage resistance may also come at the cost of avirulence or loss of motility (Laanto et al., 2013), which in turn reduces the bacteria's ability to aggregate into a biofilm. The use of phage cocktails can partially alleviate this concern (Fu et al., 2010).

The literature for phage use in clinical settings against *P. aeruginosa* is relatively extensive. Most studies focus on phage therapy against *P. aeruginosa in vivo* (Wright et al., 2009, McVay et al., 2007, Soothill, 1994) A study examined phage use on biofilms formed on medical devices (Curtin, Donlan, 2006). Studies on phage use on *P. aeruginosa* or other biofilms in aquatic industrial settings are scarce by comparison, though not completely without precedent. One similar study by Bhattarcharjee et al., 2015, showed that *Delftia tsuruhatensis*-infecting phages could successfully reduce biofouling in a membrane bioreactor, restoring flux to 70% of pre-fouling levels. This was achieved by passing an influent spiked with phages at a concentration of 10^{12} PFU/ml over a period of 2 days.

The high specificity of phages to their hosts is both an advantage as there are less environmental implications should the water be released untreated, and a limitation as it means that there will be no significant effect on the biofilm if *P. aeruginosa* is not a keystone species. A few phages with broad spectrum bactericidal effects (Melo et al., 2014, Olsen et al., 1973, Jensen et al., 1998) have been identified, but these are the exception rather than the norm.

3. MATERIALS AND METHODS

This study was sectioned into two phases. Phase one consisted of 1) phage isolation, 2) the characterization of biofilms grown on different materials over a time and 3) the characterization of the microbial community in the source water. Phase two demonstrates the application of phages in reducing *P. aeruginosa* within biofilm matrix.

3.1 Bacterial strain and cultivation

P. aeruginosa DSM 1117 was used throughout this study. The growth media used was lysogeny broth (LB) with 2 mmol $CaCl_2$. Where required, the salinity of the LB was raised from the base level of 1.0% by mass to 3.5% or 6.0%. For the soft and hard agars used throughout the study, 7 g and 15 g of agar, respectively, was added for every 1 L of LB. *Pseudomonas* isolation agar

3.2 Biofilm reactor operation and coupon materials for biofilm attachment

The biofilms were grown in Communicable Disease Centre (CDC) bioreactors (Fig. 1) at 28 °C and a stirring rate of 200 rpm. Each bioreactor had eight coupon holders that could each be fitted with three coupons. The coupon materials tested were SS316, titanium, chlorinated polyvinyl chloride (cPVC), high density polyethylene (HDPE) and polycarbonate. The seawater used to feed the bioreactors was sourced from an inlet pipe carrying water from the Red Sea over 2 km to the LFO32 lab in the Water Desalination and Reuse Center, KAUST. The seawater was fed at a rate of 1.3 mL per min to each bioreactor from 20 L carboys. This resulted in a residency time of approximately 5 days for the water, not including the time spent in the inlet pipe. It should be noted that the water quality may have varied in seasonal blocks over the course of the study. Although the

physicochemical parameters of the feed water were not measured, changing turbidity gave an indication of this, with turbidity increasing in the later months. The bioreactors were covered with aluminum foil to limit exposure to light. Though not placed under strict anaerobic conditions, the bioreactors' exposure to air was similarly limited.

The coupon materials were chosen to be representative of cooling tower piping materials (Liu et al., 2009). Two types of stainless steel, SS304 and SS316, were available for use as coupons. Both are typically used industrial piping materials. SS316, with its inclusion of 2-3% molybdenum, has superior corrosion resistance to SS304 and was chosen over SS304. It should be noted, however, that SS316 is susceptible to the pitting corrosion described in the literature review (Scott et al., 1991). Titanium is highly resistant to corrosion in saline environments due to the formation of a protective TiO_2 layer (Shaba et al., 2011, Sousa, Barbosa, 1993), although it is less commonly found because of its prohibitive cost. Chlorinated poly vinyl chloride (cPVC), due to its resistance to crevice and pitting corrosion, is ubiquitous as a piping material in marine industries. High density polyethylene (HDPE) is commonly used as flooring in the marine industry due to its resistance to direct sunlight and saline corrosion, as well as easy machinability. Polycarbonate has similar properties and is used as a transparent alternative to glass and acrylic.

After the biofilm sampling for the characterization portion of this study was performed, the reactors were prepared for the second phase of the experiment. The reactors were cleaned and reconfigured with cPVC coupons in the coupon holders. Seawater was passed through the reactors under the same conditions as before. At the 1-month mark, visual confirmation of biofilm formation was obtained and the feed source was switched from seawater to a *P.*

aeruginosa-enriched solution and from an open circulation to recirculation. The enriched solution was replaced on an average of 5 days when turbidity dropped.

Each batch of enriched solution was prepared using seawater and *P. aeruginosa* overnight culture. Eight 50 mL centrifuge tubes, each containing 27.5 mL of 3.5% salinity, 2 mmol $CaCl_2$ LB were inoculated with single colonies of *P. aeruginosa*. The tubes were left to incubate at 37 °C overnight before being centrifuged at 5000 rpm for 30 minutes. For each tube, the supernatant was discarded and the cell pellet was resuspended with 1 mL of seawater. The resuspended culture was then emptied into a beaker containing seawater. Each tube was subsequently washed down with an additional 1 mL of seawater, which was emptied into the same beaker. This resulted in a feed solution with 1500 mL final volume.

3.3 Biofilm sampling

The biofilms from the coupons were sampled at regular intervals (i.e. 1, 2, 3 or 4 months after the commencement of the reactor) during the first phase of the study. In each sampling, four coupons for each material were used. The coupons were selected from varying depths to minimize any sampling bias that depth would have on biofilm growth. The sampled coupons were placed onto separate petri dishes for each material. Five mL of 1X phosphate-buffered saline (PBS) solution was added to each petri dish. Autoclaved cotton swabs were then used to scrape down as much of the biofilm as possible from the coupons into the solution. The resulting suspension for each material was transferred into clean 50 mL centrifuge tubes (tubes A). The used cotton swabs were cut and placed into separate 50 mL centrifuge tubes (tubes B) along with the corresponding coupons and 10 mL of 1X PBS solution. Subsequently, tubes B were vortexed at high speed for 10 minutes with the aim of dislodging any remaining biofilm. Following this, the supernatant from

each tube B was transferred to the corresponding tube A. The mixed supernatant was removed from tubes A, with 20 µl of the supernatant set aside for flow cytometry. The remaining residue was centrifuged at 10000 rpm for 10 minutes and the cell pellet was used for DNA extraction.

3.4 Quantitation of total cell numbers

For flow cytometry, 7 µL of the supernatant was diluted 100-fold with 1X PBS. 10 µL of 500 mM ethylenediaminetetraacetic acid (EDTA) was added to chelate metals and other inhibitors that can obscure flow cytometry readings. The mixture was briefly vortexed and incubated at 35 °C for 20 minutes. 7 µL of 100X SYBR Green I was added with vortexing at the 10-minute incubation mark. The number of cell bodies in the resultant mixture was then quantified using a BD Accuri C6 flow cytometer.

3.5 DNA extraction of biofilm and planktonic samples

The UltraClean® Soil DNA Isolation Kit by MO BIO Laboratories was used in the DNA extraction following a modified protocol. For each sample, approximately 300 µL of supernatant from the isolation kit's microbead solution was used to resuspend the residue. The resulting suspension was transferred to the microbead solution tube and 12 µL each of achromopeptidase and lysozyme of 1 mg/mL and 100 mg/mL respective concentrations were added. The tube was left to incubate at 35 °C for 1 h. 60 µL of S1 solution from the kit was added before the tube was vortexed briefly. 200 µL of IRS solution was added next and the tube was vortexed again for 10 minutes. Following this, the tube was centrifuged at 12,000 rpm for 30 s. The resulting supernatant was transferred to a new 2 mL tube and 250 µL of S2 solution was added. The tube was briefly vortexed and allowed to incubate

at 4 °C for 5 minutes before being centrifuged for 1 minute at 10,000 rpm. As much supernatant as possible was subsequently transferred to a new 2 mL tube along with 1 mL of mixed S3 solution. 650 μL of the supernatant was loaded onto a spin filter before being centrifuged at 10,000 rpm for 1 minute, with the filtrate discarded. This step was repeated using the same spin filter until no supernatant remained. 300 μL of S4 cleaning solution was added before centrifuging at 10,000 rpm for 1 minute. The spin filter was then transferred to a new 2 mL tube. 40 μL of molecular biology grade water was added to the center of the spin filter and allowed to stand for 1 minute before centrifuging at 10,000 rpm. The filtrate was retained as the DNA sample. The DNA concentration was then quantified using the Qubit Broad Range DNA Assay according to the manufacturer's protocol.

3.6 16S rRNA gene-based amplicon sequencing on Illumina MiSeq platform

16S rRNA gene-based high-throughput sequencing was carried out with primer pair 515F and 907R. The primers were modified with the Illumina library adaptor sequence at the 5'-end so as to facilitate downstream sequencing analysis on Illumina MiSeq platform. PCR reaction mixtures were prepared as described by Illumina. The first-step thermal cycling program included an initial denaturation stage at 95 °C for 3 min, 25 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30 s), and then a final extension stage at 72 °C for 5 min. The presence of PCR product was verified using Invitrogen SYBR® green nucleic acid gel stain (Thermo Fisher Scientific, Carlsbad, CA, USA) and gel electrophoresis. Controls for PCR reactions were negative for amplification. Amplicons were of the correct anticipated size of ~450 bp, and were purified with AgenCourt AmPure XP (Beckman Coulter). Individual samples were differentiated by

indexed PCR using Illumina Nextera XT index kit. The indexing thermal cycling program included an initial denaturation stage at 95 °C for 3 min, 8 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30 s), and then a final extension stage at 72 °C for 5 min. The indexed amplicons are purified again with AgenCourt AmPure XP (Beckman Coulter). The final concentration of each sample were then measured by Invitrogen Qubit 2.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Purified amplicons were submitted to KAUST Genomics Core lab for sequencing on Illumina MiSeq platform. Analysis was done based on procedures described in an earlier study (Al-Jassim et al., 2015).

3.7 Planktonic community characterization

Five liters of seawater were filtered through a 0.4 μm Whatman Nucleopore™ track-etched polycarbonate membrane filter. The filter membrane was subsequently cut into 5 mm strips and subjected to DNA extraction using the same modified UltraClean® Soil DNA Isolation Kit protocol as listed in the preceding section. The extracted DNA was then processed using the same MiSeq 16S rRNA library preparation workflow. Post-sequencing processing and analysis were likewise performed according to the protocol listed previously.

3.8 Phage isolation

An altered phage isolation protocol from that described in Summer et al., 2009 was used. 100 mL of 1X lysogeny broth (LB) with 2 mmol $CaCl_2$ was inoculated with a single colony of *P. aeruginosa* via an inoculation loop and left to incubate at 37 °C overnight. 60 mL of

municipal influent collected from KAUST wastewater treatment plant, adjusted to a salinity of 35 g/L, was centrifuged at 10,000 rpm for 15 minutes and the supernatant was passed through a 0.2 μm filter. 50 mL of the filtrate was then added to 50 mL of the 2X LB solution with 4 mmol $CaCl_2$, and 50 mL of the overnight grown *P. aeruginosa* culture. The mixture was incubated at 37 °C for 48 h. Following this, chloroform was added to a final concentration of 1% v/v. The resultant mixture was shaken and incubated at room temperature for 1 h before being centrifuged at 4 °C for 30 minutes. The supernatant was subsequently passed through a 0.2 μm filter and the filtrate was serially diluted with SM buffer to create lysate solutions of 10^{-1} to 10^{-5} dilution. For each dilution, 10 μL of lysate solution was added to 100 μL of overnight culture and 4 mL of soft LB agar with 2 mmol $CaCl_2$, shaken and poured onto hard LB agar plates. The plates were then incubated at 37 °C for 24 h and monitored for the presence of lysis zones. 10^{-5} was the dilution found to consistently yield discrete and countable lysis zones. Seven of the largest, discrete lysis zones were removed using inoculation loops. Each excised lysis zone was placed in a 2 mL microcentrifuge tube with 1 mL of the phage diluent, SM Buffer (50 mmol Tris-Cl pH 7.5, 100 mmol NaCl, 8 mmol $MgSO_4$) and masticated using a needle. The tubes were then mixed via inversion and the contents of each tube were passed through a 0.2 μm filter into a 15 mL centrifuge tube containing 9 mL of SM buffer for further purification. This yielded seven strains of lysate solution at 10^{-1} dilution, which were further diluted up to 10^{-5}. For each isolated strain, 10 μL of 10^{-5} lysate solution was added to 100 μL of overnight culture and 4 ml of soft LB agar with 2 mmol $CaCl_2$, shaken and poured onto hard LB agar plates. The lysis zone excision, filtering, dilution and plating steps were repeated for a total of seven purification cycles.

Once isolated, each strain was plated and reisolated every 2 weeks. This was done to keep a ready stock of viable lysate solution as phage counts in lysate solutions drop over time (Jończyk et al., 2011).

3.9 Colony forming units (CFU) count of overnight *P. aeruginosa* culture

With the exception of the cultures used in the phage isolation portion of the study, all overnight cultures were prepared by inoculating a single colony of *P. aeruginosa* into a 50 mL centrifuge tube containing 27.5 mL of 3.5% salinity and 2 mmol $CaCl_2$ LB. The tubes were incubated overnight (approximately 10 h on average) at 37 °C.

To enumerate the average cell count in an overnight culture, three separate CFU counts were performed. For each CFU count, the culture was first serially diluted with 1X PBS up to 10^{-7}. 200 μL of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were then pipetted and spread onto a 3.5% salinity, 2 mmol $CaCl_2$ LB agar plate. This was performed in triplicates, with the plates left to incubate overnight at 37 °C.

3.10 Effect of salinity on phage strain viability

The relative effectiveness of each isolated phage strain across different salinities (1%, 3.5%, 4% and 6% w/v) was measured via PFU counts. Because the PFU counts between the phage strains sometimes varied by an order of magnitude, the 10^{-4} and 10^{-5} dilutions of the lysate solution were plated for each strain. 10 μL of the chosen dilution was mixed with 100 μL of overnight culture and 4 mL of the chosen salinity, 2 mmol $CaCl_2$ soft LB agar. The resulting mixture was shaken and spread over a 2 mmol $CaCl_2$ LB agar plate with matching salinity. This was performed in triplicates for each salinity. The plates were incubated overnight at 37 °C.

Soft agar with salinities of 1%, 3.5%, 4% and 6% were used. LB soft agar has a base salinity of 1%. In addition, 3.5% and 4% salinity represent the average or upper limits of salinity in the Red Sea, respectively (Arz et al., 2003) and. The final 6% salinity was chosen to produce a smooth salinity gradient.

3.11 Demonstration of lytic effect on biofilms

Collectively referred to as Phase 2 of this study, the steps described in this section were repeated over two separate runs: trial 1 and trial 2.

After 1 month of feeding the CDC biofilm reactor with *P. aeruginosa*-enriched seawater, three cPVC coupons from each holder were then placed in individual 50 mL centrifuge tubes. Each tube was filled with a lysate solution (set 1) or SM buffer as a control solution (set 2). The lysate solutions tested were either undiluted solutions of the more effective phage strains or cocktail phage solutions. It should be noted that the performance of individual strains may vary by a magnitude from one experimental repeat to another due to the burst size of the lysis zone (Hadas et al., 1997). This is a function of host cell physiological variation. As such, the strains selected were those with the most consistent performances. For all cocktail solutions, equal quantities of constituents were used. The solutions tested can be found in Table 1.

The tubes were incubated at 37 °C for seven days, following which, DNA extraction, MiSeq sequencing and flow cytometry were then carried out on the coupons per the protocol described in preceding sections.

Another set of control tubes containing only phage lysate solution without coupons (set 3) were simultaneously incubated under the same conditions. After 7 days, the supernatant of set 1 tubes were filtered through a 0.2 μm filter. The filtered supernatant and the phage lysate solution of set 3 tubes were serially diluted up to 10^{-11}. Dilutions at 10^{-5} and 10^{-11} were chosen for plating. The same PFU enumeration protocol described in section 3.10 was used, with a fixed agar salinity of 3.5%.

Fig. 1: CDC biofilm reactor

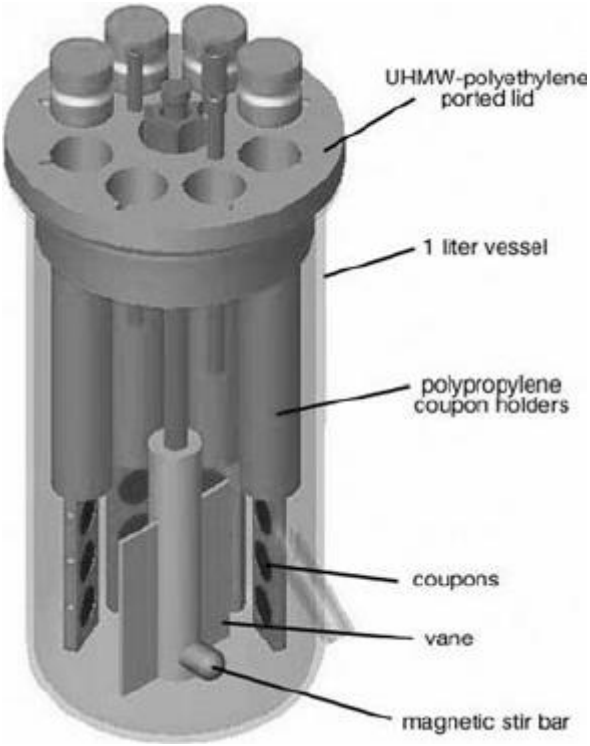


Table 1: Phage cocktail combinations used

Trial 1	Trial 2
A1+A2+B1+B2+C1+C2+D1 (ABCD)	A1+A2+B1+B2+C1+C2+D1 (ABCD)
A1+A2 (A)	B1+C1
B1+B2 (B)	C1+D1
	C1

4. RESULTS

4.1 Phage isolation

Phage isolation from seawater was initially attempted. Repeated attempts failed to procure any phages capable of triggering lysis in *P. aeruginosa*. Phage isolation from municipal wastewater proved successful, inducing lysis in *P. aeruginosa* at 1.0% salinity. However, these strains did not trigger lysis in *P. aeruginosa* at the target salinity (3.5%). An additional round of phage isolation was performed after the wastewater sample was enriched with NaCl to 3.5% salinity. In this repeat attempt, phage strains capable of lysing *P. aeruginosa* at that salinity were isolated.

4.2 Attached biofilm cell numbers per materials

Across all the materials tested, cPVC supported the greatest number of cells (Fig. 2). Taking the 1st month as reference, cPVC supported the greatest number of cells at 3.65 x 10^6 cells/mL, followed by HDPE at 2.16 x 10^6 cells/mL, polycarbonate at 1.9 x 10^6 cells/mL, titanium at 7.94 x10^5 cells/mL, and SS316 at 7.22 x 10^4 cells/mL. The difference in cell count between all the materials were significant (Table 2) at the 95% confidence level, except that between HDPE and polycarbonate.

The cell count numbers in the 2nd month increased significantly, except for HDPE and SS316. Despite the increases, cell counts remained within the same order of magnitude for all the materials, apart from titanium, which showed a greater than 3-fold increase. Cell count increases from the 2nd to the 3rd month across all the materials. Sharp increases in cell counts were observed for cPVC and titanium, by 3-fold and 6-fold respectively from the previous month. Titanium supported a significantly greater number of cells than cPVC

in the 3rd month. No SS316 samples were collected for the 3rd month due to sampling error. In the 4th month, the cPVC cell count almost doubled, to more than 3-fold the number of cells supported by the next highest material, polycarbonate. Titanium cell counts dropped by 56% from the 3rd to the 4th month. Overall, cPVC supported the greatest number of cells. Cell count numbers were most similar across all 4 months between HDPE and polycarbonate; both materials remained within the same order of magnitude throughout the study.

Upon removing the biofilm, pitting corrosion was observed on all SS316 coupons. Oxide grains were also present on the coupons and in the biofilms. Neither was observed in any of the other materials.

4.3 Biofilm microbial community

Metric multidimensional scaling (mMDS) plots were used to analyze the relative abundances of the bacterial genera present by material type (Fig. 3a) and time (Fig. 3b). Overall, the sample groupings indicate clear material effects. The microbial communities of HDPE and polycarbonate showed strong similarity both in average values and grouping. The microbial communities attached on cPVC, titanium and stainless steel was strongly dissimilar to those on polycarbonate and HDPE. Microbial communities varied the most amongst SS316 samples throughout the sampling period. The communities of the two metals were more similar to cPVC than to each other. The communities present in the seawater samples were tightly clustered and greatly dissimilar to those on any of the materials.

The microbial communities across the months showed some measure of sequentially, although there was an abrupt shift from the first to the second month. Outliers were present amongst all the months, with the second month possessing the most outliers. Grouping was most compact for first month samples and most diffuse for second month samples.

At the class level, Alphaproteobacteria was dominant (ranging from 8.2% for titanium to 18.2% for seawater samples) across the planktonic samples and all the materials except for SS316. Gammaproteobacteria made up the second most dominant class, ranging from 2.5% for HDPE to 16.1% for SS316. It should be noted that the dominance of Gammaproteobacteria amongst SS316 was primarily the result of an anomalously large amount of *Pseudomonas* on one sample.

Diatoms (*Bacillariophyta*) made up the bulk of the classifiable taxa amongst the materials. On average, *Bacillariophyta* made up 12.8% of cPVC biofilms, 31.5% of HDPE biofilms, 25.6% of polycarbonate biofilms, 11.7% of SS316 biofilms and 18.0% of titanium biofilms. By contrast, *Bacillariophyta* made up only 0.78% of the planktonic seawater community. Overall, the planktonic seawater community was more diverse than those on any of the materials, which tended to be dominated by a few taxa. Two hydrocarbon degrading genera, *Marinobacter* and *Cycloclasticus*, had large representations amongst the samples. *Marinobacter* comprised up to 8.69%, 1.71%, 0.22%, 0.18% and 0.38% of microbial community attached on cPVC, titanium, SS316, HDPE and in the seawater planktonic community, respectively. *Cycloclasticus* made up 0.28% and 0.25% of the microbial community attached on HDPE and polycarbonate, respectively. Four recently identified genera, *Kangiella*, *Lutaonella*, *Parvularcula* and *Maricurvus*, isolated from geographically distant sources (Korea, Taiwan, the Sargasso Sea and Japan) were present

in significant amounts as well. *Kangiella* (Arun et al., 2009), made up 1.66% and 4.57% of cPVC and titanium biofilm communities, respectively. *Lutaonella* (Han et al., 2009) composed 0.25% and 2.43% of cPVC and titanium biofilms, respectively. 0.23%, 0.23%, 0.16%, 1.85%, 2.09% and 0.51% of cPVC, SS316, titanium, HDPE, polycarbonate and seawater microbial communities were made up of *Parvularcula*(Cho, Giovannoni, 2003). *Maricurvus* (Iwaki et al., 2012) comprised 0.38% and 0.33% of HDPE and polycarbonate biofilms respectively. *Pelagibacter ubique,* a member of the *Pelagibacterales* order which is estimated to make up 1/3 of ocean surface bacteria (Morris et al., 2002), comprised 7.2% of seawater microbial communities. The species was still present on the materials, albeit in low amounts (0.025%, 0.019%, 0.04%, 0.013% and 0.073% of cPVC, SS316, titanium, HDPE and polycarbonate biofilms respectively.

4.4 Bacterial species of interest

Anaerobic sulfate-reducing bacteria (SRBs) were present on all materials (Tables 3a-f) as well as the seawater samples. Total SRB relative abundance showed a slight increase from the 1st month to the 4th month of 10.7%. *Desulfopila* was the most abundant genus amongst the SRBs and was present on all samples. Representation of *Desulfopila* averaged at 0.004%, was the highest in HDPE at 0.0065% and the lowest at 0.00091% on cPVC. The aerobic H_2S producing bacteria, *Thiobacillus*, was present on cPVC and HDPE. MIC associated bacteria not inclusive of the Pseudomonads comprised 0.0059%, 0.01%, 0.035%, 0.035%, 0.0048% and 0.053% of SS316, titanium, HDPE, polycarbonate, cPVC and seawater microbial communities respectively. The average representation of SRBs across all materials increased over time. *Comamonas*, a genus implicated in biofouling via the deposition of chromium (III) oxide from chromate reduction (Cooke et al., 1995), was

present on HDPE and polycarbonate at 4.2% and 4.8% of total microbial community respectively. *Comamonas* is also known to be an opportunistic pathogen under rare circumstances (Willemw, De Vos, 2006).

Amongst common pathogens not including the Pseudomonads, *Coxiella* was the most well represented at an average of 0.12% of microbial community, followed by *Acinetobacter* at 0.048%. Total relative abundance of these common pathogens increased by 2.6% from the 1st to the 4th month. These genera, along with *Mycobacterium*, were present on all samples. *Legionella*, the genus commonly associated with cooling towers, was present on all biofilm attached on materials, but not in any of the seawater samples.

Pseudomonas was present on all the samples. Representation of *Pseudomonas* ranged from 0.0008% on cPVC to 11.7% on SS316 on average. The large average representation on SS316 was primarily contributed by one sample, which had 70.2% of its community composed of *Pseudomonas*. *Aeromonas*, a pathogen also implicated in waterborne disease (Silvestry-Rodriguez et al., 2007) and which gives false positives on *Pseudomonas* isolation assays, was found on cPVC, HDPE and polycarbonate. Representation of *Aeromonas* was highest on titanium at 0.033% of total microbial community.

4.5 Salinity and lytic effect

Phage viability and infection rates depend on a variety of environmental factors (Silva et al., 2014, Jonczyk et al., 2011). Due to time constraints, only salinity was considered for this study.

At the reference salinity of 3.5%, A1 displayed the greatest lytic effect (Fig. 4). A1 had an average of 2.18 x 10^8 PFU/mL. A2 displayed similar lytic effect, with an average of 2.08

x 10^8 PFU/mL and was not significantly different at a 95% confidence level (p-value = 0.31). The strain with the weakest lytic effect, B1, was nevertheless within the same order of magnitude at 1.06 x 10^8 PFU/mL. D1, with a PFU count of 1.1 x 10^8 PFU/mL, was not significantly different from B1. Notably, there was no significant differences between A1 and D1, the strain with the second lowest lytic effect (p-value = 0.0699) B2 and C1 had similar PFU values of 1.33 x 10^8 PFU/mL and 1.35 x 10^8 PFU/mL, respectively, although the standard deviation between C1 repeats were an order of magnitude higher. The order of relative lytic strength (Table 4) was mostly unchanged across salinities.

A1, A2 and C2 remained the 3 most effective strains across all salinities. No significant difference was observed between the PFU counts at 1% and 3.5% (p-value = 0.67). The PFU counts at 6% were significantly lower than those of the other 2 salinities, with p-values of 0.0207 and 0.0259 when compared against 3.5% and 1% respectively. The lysis zones demonstrated a clear decrease in area with increasing salinity. At 6%, the lysis zones shrunk to a point that many of them were barely visible without magnification and enumeration was difficult (Fig. 5a and 5b).

4.6 Reduction in cell count

Except for when exposed to single phage B1 and cocktail phage C1D1, the cell counts in almost all control samples were higher than that of the samples exposed to phages (Fig. 6). However, there were only significant differences between controls and phage-treated coupons when exposed to A1, ABCD and C1D1, with p-values of 0.05, 0.0275 and 0.014 respectively. At a 90% confidence level, set B1 also showed a significant difference in cell count.

4.7 Change in seawater microbial community upon exposure to phage

Changes in bacterial taxa of interest, namely pathogens, MIC-associated bacteria and Pseudomonads were the focus of this portion of the study. *Pseudomonas* showed a 0.72-fold decreasefor set ABCD, though the change was not significant. The similar *Aeromonas* decreased from a 0.0019% representation on the coupon biofilm to 0%. The SRB, *Desulfocapsa* also decreased from a 0.0065% representation to 0%. *Chloroflexi* and *Chlamydiales*, two taxa with pathogenic members, showed 0.95-fold and 0.6-fold decrease respectively for set ABCD. *Chloroflexi* decreased 0.82-fold for set C1D1 and Chlamydiales decreased 0.41-fold for set B1.

4.8 Change in PFU count

PFU counts showed significant increment by 4 orders of magnitude for all phage cocktails, showing multiplication of the phages during the course of treatment (Fig. 7). All phage solutions showed a 10^4 increase in PFU count compared to their respective controls.

Fig. 2: Cell counts per material across time in log scale

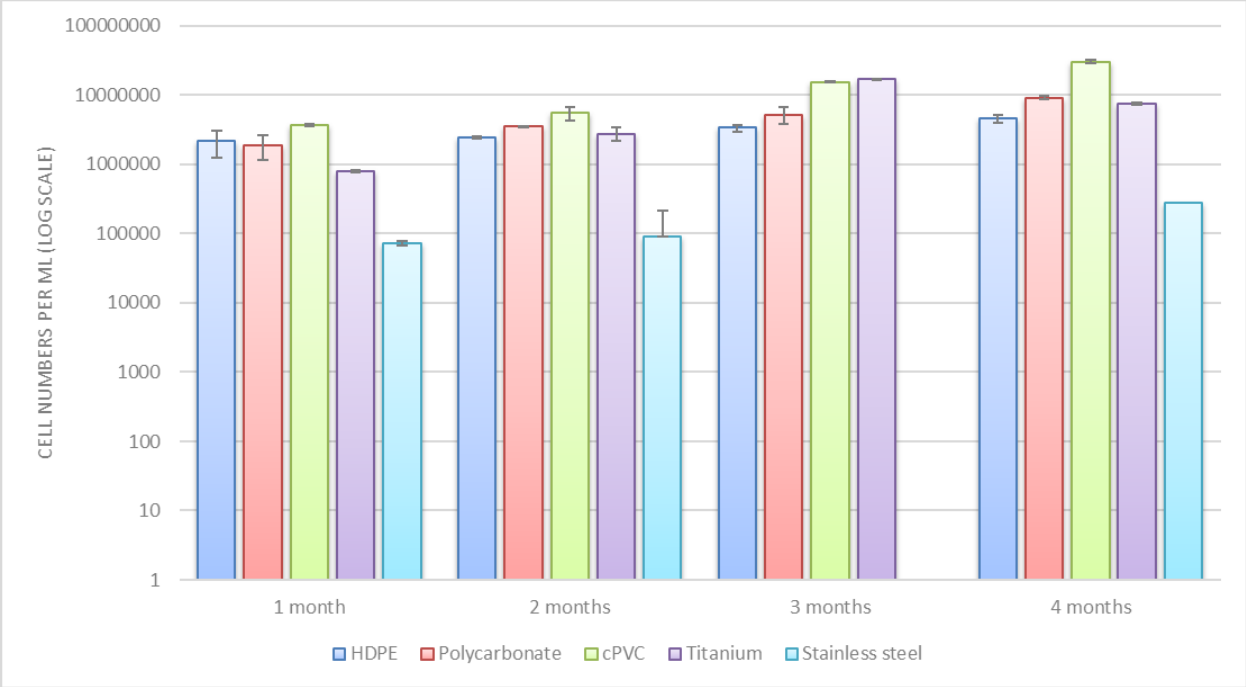


Fig. 3a: mMDS plot showing microbial community differences across materials

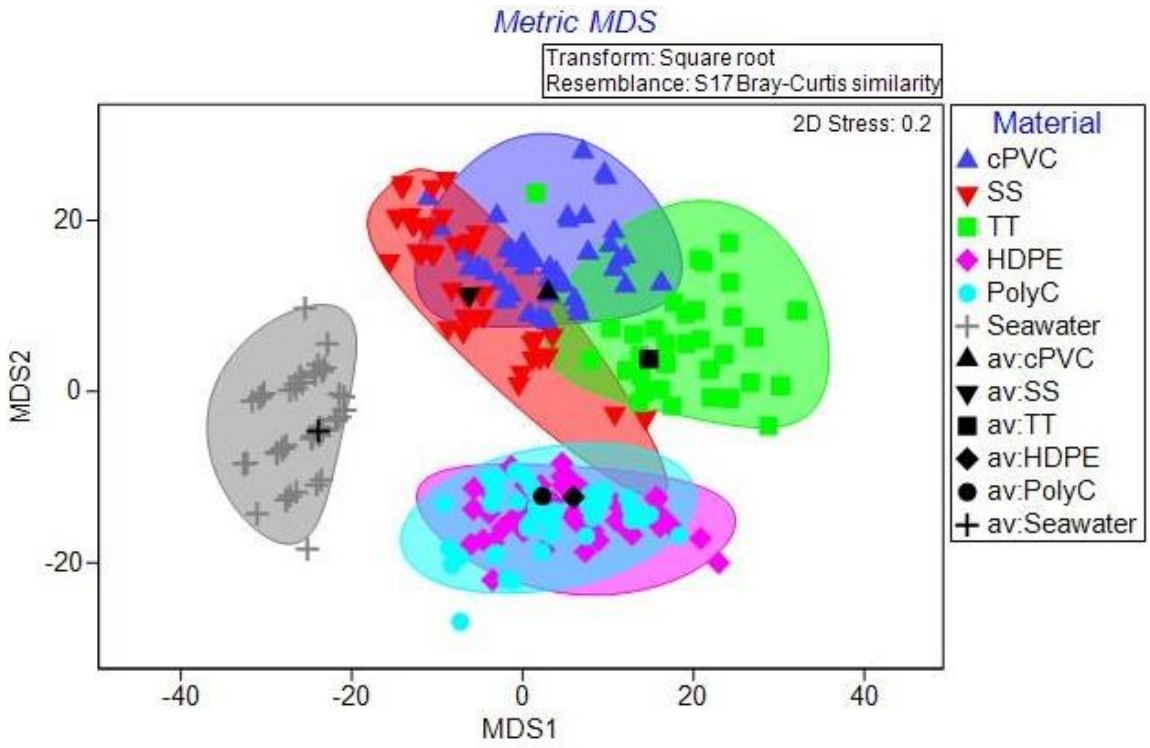


Fig. 3b: mMDS plot showing microbial community differences across time (months)

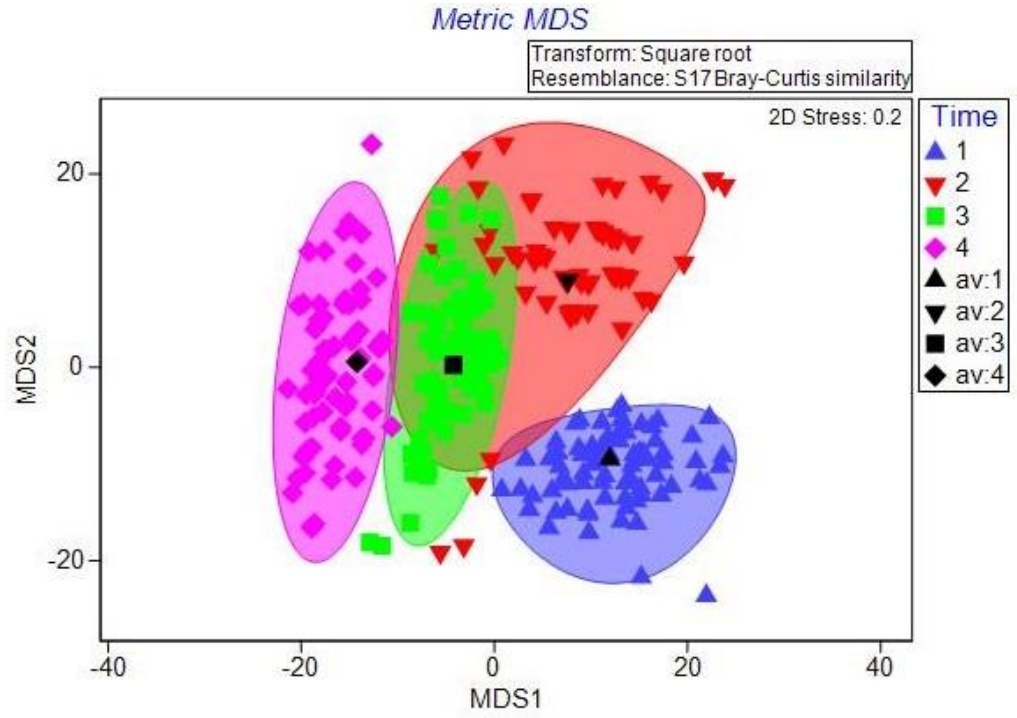


Fig. 4: Plague forming unit (PFU) count at each salinity

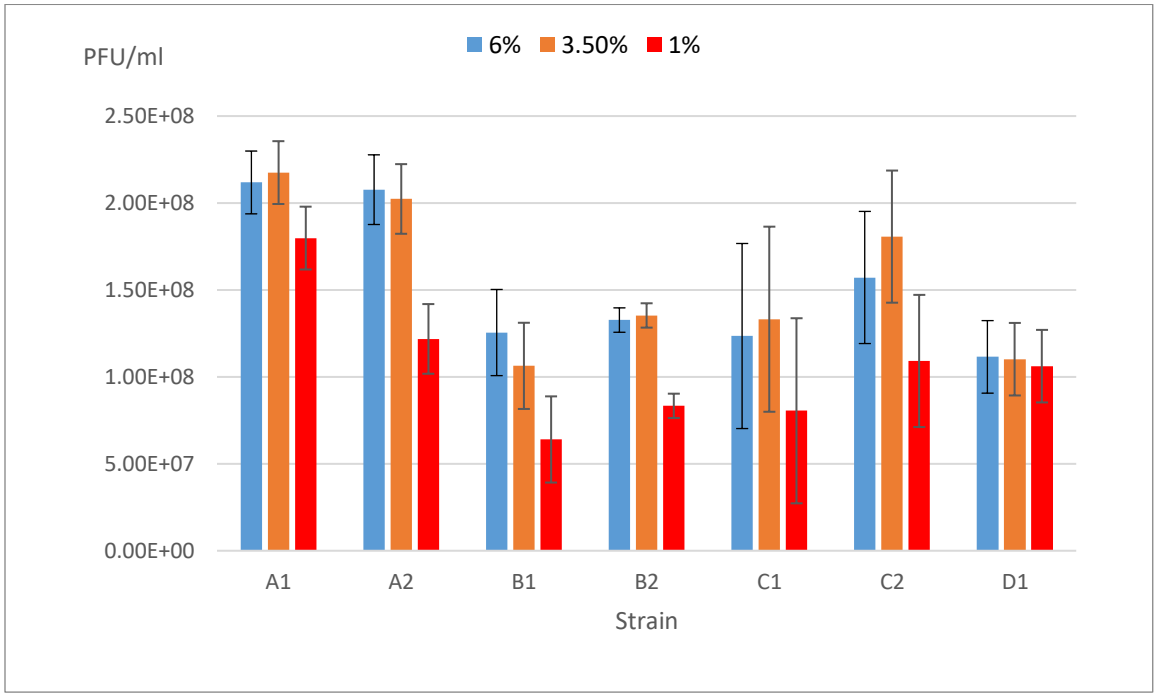


Fig. 5a: D1 strain phage lysis zones on 6% (left) and 1% (right) salinity double agar overlays

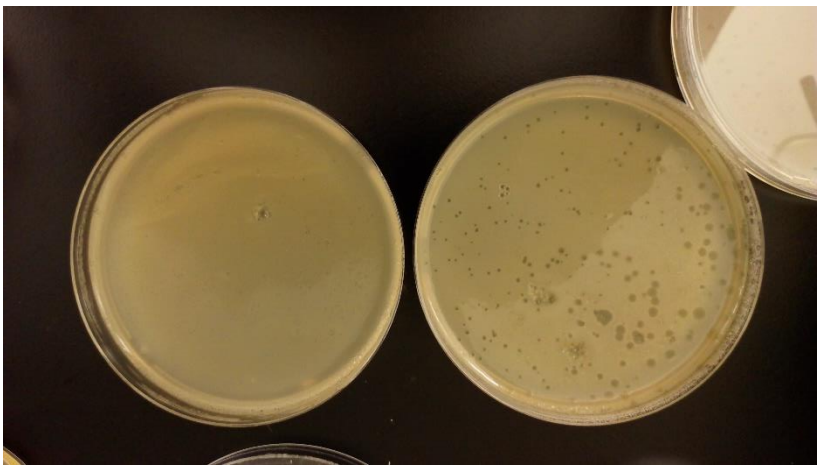


Fig. 5b: A2 strain phage lysis zones on 6% (left), 3.5% (middle) and 1% (right) salinity double agar overlays

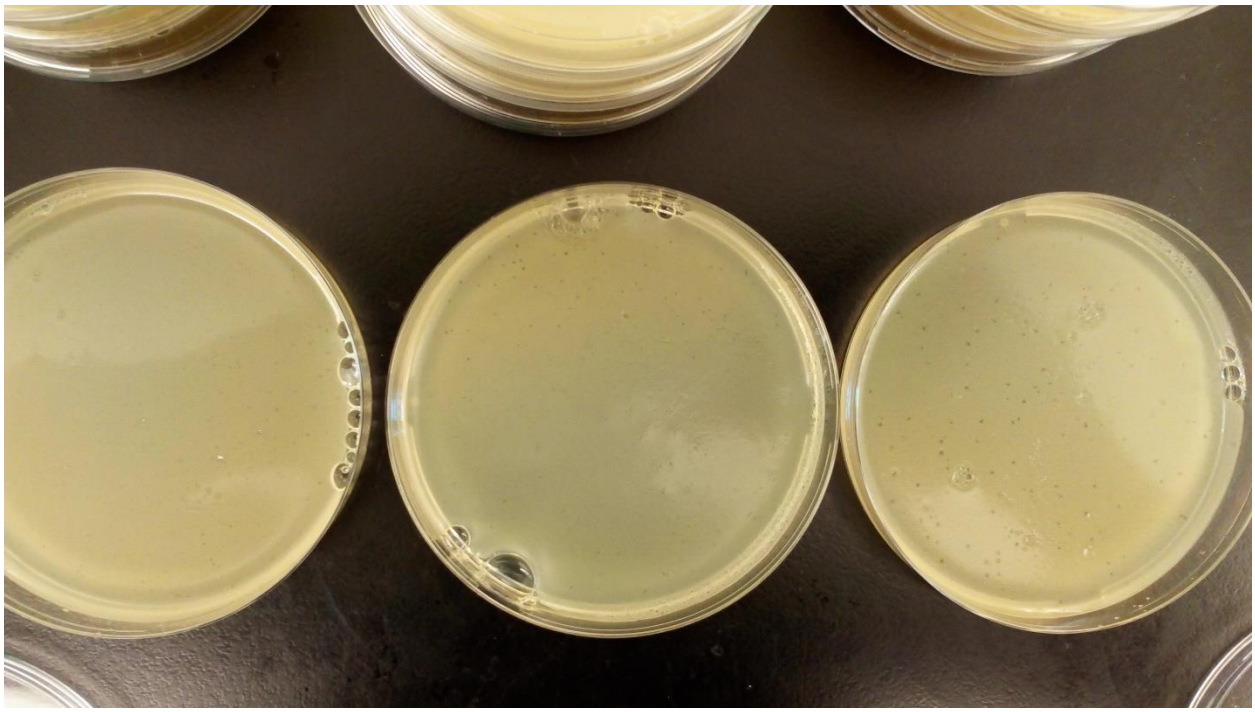


Fig. 6: Flow cytometry results for coupons exposed to phage lysate and control solutions

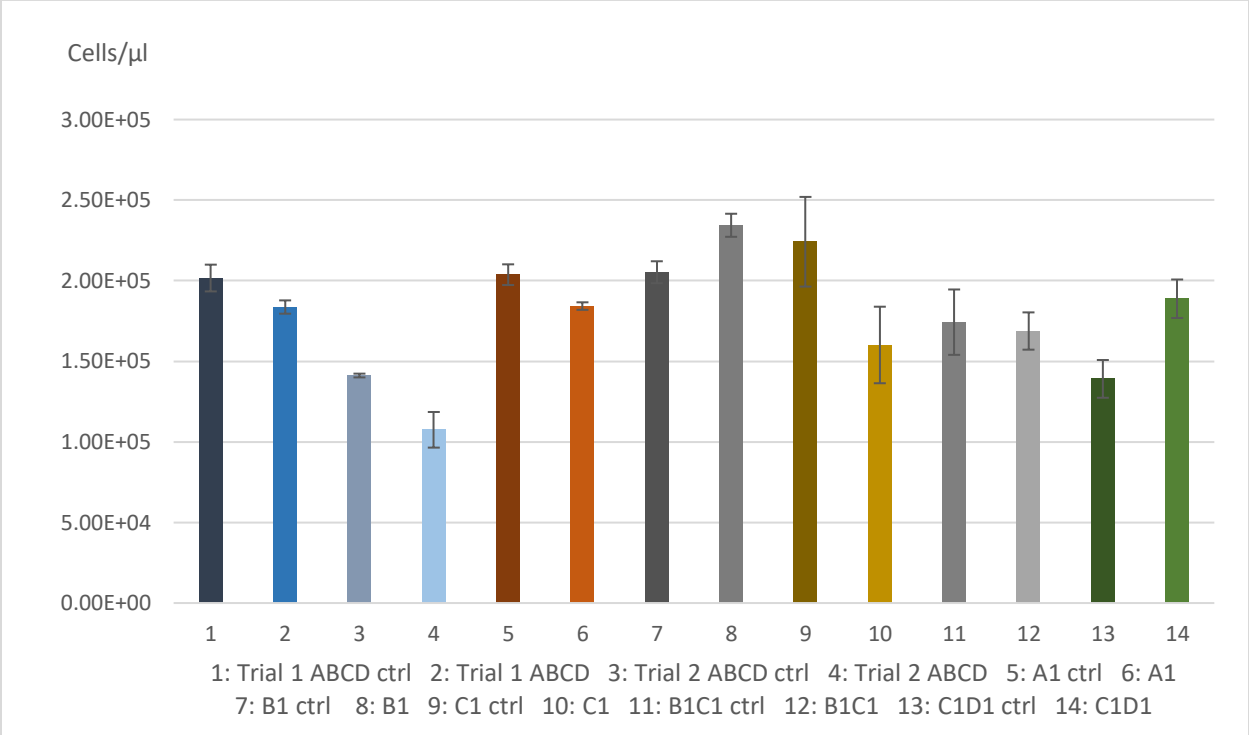


Fig. 7: Comparison of PFU counts between phage lysate solutions exposed to biofilms and controls

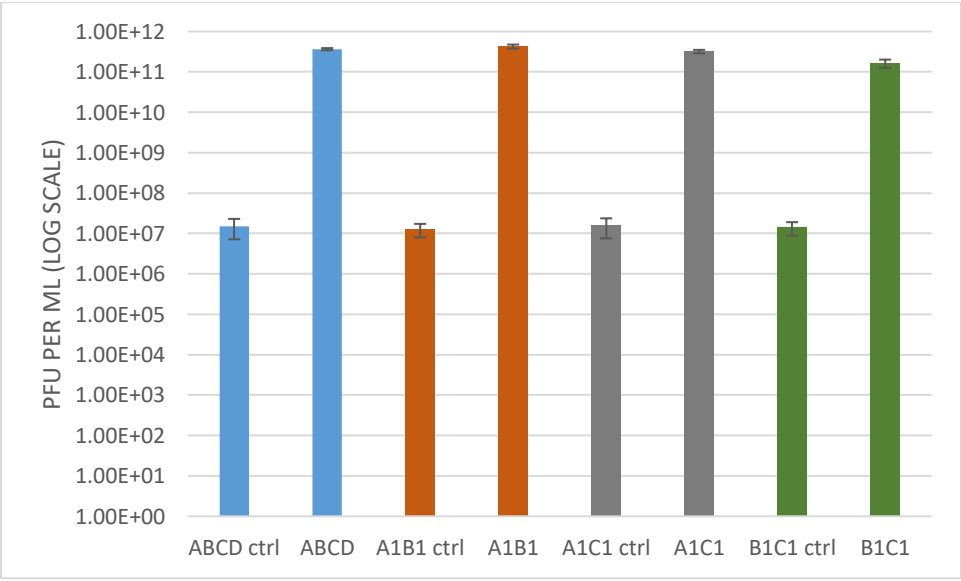


Table 2: p-values for differences in cell counts between materials. Only the value highlighted in red is not significantly different.

	HDPE	**Polycarbonate**	**SS316**	**Titanium**
cPVC	8.80292E-06	2.11991E-06	0.000513476	0.001270632
HDPE		0.636604279	0.007488969	0.01417695
Polycarbonate			0.006562713	0.004564836
SS316				2.52513E-05

Table 3a: Averaged relative abundance of bacterial species of interest for cPVC

Duration	1	2	3	4
MIC associated bacteria				
Desulfopila	0.001524	0.000666	0.000997	0
Desulforhopalus	0	0	0	0.004002
Thiobacillus	0	0	0	0.001168
Unclassified *Desulfobacteraceae*	0.002028	0	0	0.004086
Unclassified *Desulfobulbaceae*	0.00073	0.002666	0.001329	0
Pathogens				
Acinetobacter	0.013936	0.00785	0.007268	0.001123
Arcobacter	0.001916	0	0	0
Coxiella	0.223703	0.231021	0	0.859313
Desulfopila	0.001524	0.000666	0.000997	0
Escherichia/Shigella	0.000366	0.006607	0	0

Legionella	0.002142	0	0	0
Mycobacterium	0	0.000833	0	0.046702
Streptococcus	0	0	0.009462	0.002018
Vibrio	0.000171	0.000133	0	0.000266
Pseudomonads and *Aeromonas*				
Aeromonas	0.001851	0	0.000721	0
Pseudomonas	0.001698	0	0	0.002216
Unclassified *Pseudomonadaceae*	0.001435	0	0.007329	0.00049
Unclassified *Pseudomonadales*	0.000532	0	0	0

Table 3b: Averaged relative abundance of bacterial species of interest for HDPE

Duration	1	2	3	4
MIC associated bacteria				
Desulfobacter	0.000282	0.002782	0	0
Desulfobacterium	0.000376	0	0	0
Desulfofaba	0	0.000696	0	0
Desulfoluna	0	0.001391	0	0
Desulfopila	0.005588	0.003976	0.007371	0.007753
Desulforhopalus	0.000658	0	0	0
Desulfosarcina	0.00144	0	0.000298	0.00085
Desulfosporosinus	0.000432	0	0	0
Desulfovibrio	0	0.001672	0.000677	0

Thiobacillus	0.001152	0	0	0
Unclassified *Desulfobacteraceae*	0.008034	0.040343	0.006844	0.006788
Unclassified *Desulfobacterales*	0	0.000743	0	0
Unclassified *Desulfobulbaceae*	0.007233	0.011925	0.013245	0.005824
Unclassified *Desulfovibrionaceae*	0	0.002092	0	0
Unclassified *Desulfovibrionales*	0	0.001252	0	0
Pathogens				
Acinetobacter	0.292515	0.06599	0.051895	0.002897
Arcobacter	0.00517	0.00298	0	0
Coxiella	0.019513	0.058428	0.019656	0
Enterococcus	0.000493	0	0	0
Escherichia/Shigella	0	0	0.00017	0.000322
Legionella	0.002266	0	0	0
Mycobacterium	0.008195	0	0.000804	0.00081
Shewanella	0.00012	0	0	0
Streptococcus	0.001741	0	0	0
Vibrio	0.001306	0.000949	0.000136	0
Pseudomonads and *Aeromonas*				
Aeromonas	0.000944	0.047474	0.001716	0
Pseudomonas	0.0073	0.001057	0.001069	0.000215
Unclassified *Pseudomonadaceae*	0.015448	0	0.00167	0.000714
Unclassified *Pseudomonadales*	0.00024	0	0	0

Table 3c: Averaged relative abundance of bacterial species of interest for polycarbonate

Duration	1	2	3	4
MIC associated bacteria				
Desulfobacter	0	0.001387	0	0
Desulfocapsa	0	0	0.000397	0
Desulfomonile	0	0	0.002284	0
Desulfopila	0.003297	0.013186	0.013225	0.005197
Desulfosarcina	0	0.000604	0	0.001705
Desulfotomaculum	0	0	0.000163	0
Desulfovibrio	0	0.001665	0.000716	0
Unclassified *Desulfobacteraceae*	0.001154	0.025448	0.011609	0.011936
Unclassified *Desulfobulbaceae*	0.003297	0.015371	0.016999	0.004547
Unclassified *Desulfovibrionaceae*	0	0.002607	0	0
Unclassified *Desulfovibrionales*	0	0.003122	0	0
Pathogens				
Acinetobacter	0.059158	0.217254	0.049806	0.001457
Arcobacter	0.006917	0.001447	0.001787	0
Coxiella	0.020764	0.023882	0.045045	0
Legionella	0.00066	0	0	0
Mycobacterium	0.001647	0	0.000815	0.001623
Streptococcus	0	0.000299	0	0
Vibrio	0.00105	0.00057	0.000272	0

Pseudomonads and *Aeromonas*				
Aeromonas	0	0.01729	0.002835	0
Pseudomonas	0.009194	0	0.000217	0.000432
Unclassified *Pseudomonadaceae*	0.007754	0.001014	0.002681	0

Table 3d: Averaged relative abundance of bacterial species of interest for SS316

Duration	1	2	3	4
MIC associated bacteria				
Desulfopila	0.003378	0	0	0
Unclassified *Desulfobacteraceae*	0.000653	0	0.018002	0
Unclassified *Desulfobulbaceae*	0.001664	0	0	0
Pathogens				
Acinetobacter	0.004344	0.006278	0.023115	0
Arcobacter	0.001119	0	0	0
Coxiella	0.276528	0.166522	0.36005	0.315436
Enterococcus	0.000521	0	0	0
Escherichia/Shigella	0.000187	0	0	0
Legionella	0	0.000685	0	0
Mycobacterium	0.007335	0.00171	0	0.112663
Streptococcus	0	0.008992	0	0
Pseudomonads and *Aeromonas*				

Pseudomonas	0.001949	35.08383	0	0
Unclassified *Pseudomonadaceae*	0.003255	7.06585	0	0
Unclassified *Pseudomonadales*	0.000543	0.010952	0	0

Table 3e: Averaged relative abundance of bacterial species of interest for titanium

Duration	1	2	3	4
MIC associated bacteria				
Desulfobacter	0	0	0	0.001785
Desulfopila	0.005435	0.002637	0	0.003569
Desulforegula	0	0	0	0.001338
Desulforhopalus	0.00068	0	0	0.005099
Desulfosarcina	0.001189	0	0	0
Desulfotomaculum	0	0	0	0.000255
Desulfovibrio	0.000713	0	0	0
Unclassified *Desulfobacteraceae*	0.005349	0.000577	0	0.002677
Unclassified *Desulfobacterales*	0.000635	0	0	0
Unclassified *Desulfobulbaceae*	0.002717	0.003297	0.000951	0.00204
Pathogens				
Acinetobacter	0.217542	0.00111	0.083618	0.021163
Arcobacter	0	0	0	0.002293
Coxiella	0	0.004615	0	0.231405

Enterococcus	0.000509	0	0	0
Escherichia/Shigella	0.00034	0	0.000475	0
Legionella	0.008152	0.001318	0	0
Mycobacterium	0	0.001647	0.002373	0.010197
Shewanella	0	0	0	0.000189
Vibrio	0.001624	0.000263	0	0
Pseudomonads and *Aeromonas*				
Aeromonas	0.000737	0	0.161532	0.002953
Pseudomonas	0	0.000438	0.000631	0
Unclassified *Pseudomonadaceae*	0.000998	0.000485	0.829462	0.003749

Table 3f: Relative abundance of bacterial species of interest for seawater across batches

Batch	A	B	C	D	E
MIC associated bacteria					
Desulfobacter	0	0	0	0	0.003355
Desulfofaba	0	0	0.02011	0	0
Desulfomonile	0	0.065266	0	0	0
Desulfonispora	0	0	0	0	0
Desulfopila	0.013451	0	0	0	0

Desulfosarcina	0	0	0	0.003488	0.003355
Unclassified *Desulfobacteraceae*	0.004484	0.065266	0.040219	0.003488	0.010065
Unclassified *Desulfobulbaceae*	0.009608	0	0	0.019938	0
Unclassified *Desulfovibrionales*	0.001009	0	0	0	0
Pathogens					
Acinetobacter	0.001078	0	0	0	0
Coxiella	0	0	0	0.041856	0.201294
Escherichia/Shigella	0	0	0	0.015947	0.001919
Mycobacterium	0.003204	0	0	0	0
Shewanella	0.000238	0	0	0	0
Streptococcus	0	0	0	0	0.005797
Vibrio	0.018874	0	0	0.004758	0.006106

Pseudomonads and *Aeromonas*					
Pseudomonas	0	0	0	0	0.00255
Unclassified *Pseudomonadales*	0	0	0	0	0.00279 1

Table 4: Order of relative lytic strength across salinities

Salinity	**Order of relative lytic strength**
1%	A1 > A2 > C2 > B2 > C1 > D1 > B1
3.5%	A1 > A2 > C2 > B2 > B1 > C1 > D1
6%	A1 > A2 > C2 > D1 > B2 > C1 > B1

5. DISCUSSION

5.1 Attached biofilm cell count numbers per material

The cell count results across materials deviated sharply from predicted trends. Niquette et al., 2000 showed that polyvinyl chloride (PVC) and polyethylene (PE) supported the lowest amounts of biofilm growth when compared to several varieties of steel, cast iron and cement. cPVC, with a similar chemical structure to PVC, was expected to support lower amounts of biofilm growth. Instead, in this study, cPVC consistently had the highest cell counts, surpassed only by titanium in the 3rd month. This is especially unusual, considering the possibly repulsive interaction between the negatively charged lipopolysaccharides or teichoic acids on bacterial cell walls and the delta negative charge on the chloride groups of cPVC. The grouping of samples amongst the plastic materials in Fig. 3 shows that there are clear dissimilarity between the communities present on cPVC and the other two plastic materials. This could imply that the material characteristics of cPVC are different enough from the other two plastics to result in total biofilm growth differences as well.

Surface charge is only one component of cell adhesion. Cell adhesion is dependent on the relative strengths of different types of intermolecular bonding, the polarity of bacterial cell walls and EPS proportion of bacteria in the source water. Metal surfaces are hydrophilic and plastics are hydrophobic (Pedersen, 1990), unless subject to surface modification. These factors can also contribute towards the high cell count on cPVC as well as the greater cell count numbers of polycarbonate and HDPE when compared to SS316.

However, if hydrophobicity is a significant factor in cell adhesion (Ras et al., 2013), the relatively high cell count on titanium is not in concordance with the rest of the results.

Titanium was identified by Muller et al., 1985, as having antibacterial effects due its oligodynamic properties. This is mostly due to the formation of a TiO_2 layer (Kubacka et al., 2014, Shahba et all, 2011). Titanium was expected to have the lowest cell count amongst the materials. On the other hand, the metals were not electropolished and have a rougher surface than the 3 plastic materials. Chao et al., 2015, notes that the rougher surfaces of metals may allow for greater bacterial attachment. If this factor outweighs oligodynamic properties, SS316 should have similar biofilm growth to titanium. Furthermore, SS316 is not known to possess any oligodynamic properties, beyond those granted by the presence of molybdenum in the alloy. This does not corroborate with the cell count on SS316, which was consistently the lowest. Van der Kooij et al., 2005, reported that active biofilm mass (measured by ATP concentration) was similar in stainless steel and copper (a metal with oligodynamic properties (Yasuyuki et al., 2010)) pipe. The amounts in both were approximately half that in cross-linked polyethylene, which is chemically similar to HDPE. While it does not explain the anomalously high cell count on titanium when compared to SS316 and HDPE, it does support the lower cell count on SS316.

It should be noted that the samples were collected in different batches throughout the year due to limited equipment inventory and that seasonal changes in water parameters over the sampling period are likely. Variation in nutrient, mineral and cell concentration of the influent could have played a greater role than type of material on biofilm cell counts. Also, the seemingly conflicting results from literature (PE supporting lower biofilm growth than mild steel in Niquette et al., 2000 as opposed to van der Kooij et al., 2005 showing that stainless steel has lower active biofilm mass than cross-linked PE) imply that growth trends

may not be clearly predictable from similar materials. The inclusion of small amounts of another metal in an alloy or a change in functional groups in a plastic could drastically alter surface properties and hence attachment suitability.

5.2 Biofilm microbial community

SS316 showed the greatest dissimilarity between samples (Fig. 3a). This is likely the result of a batch effect, as SS316 was sampled over three sampling batches. Chao et al. 2015 showed that the difference in biodiversity at phylum level is approximately twice in stainless steel compared to plastics, with 47% of the biofilm being made up of *Proteobacteria* on stainless steel and 92-95% on plastics (Chao et al., 2015).

The greater similarity between the community on cPVC and those on the metals as opposed to those on the other two plastics is notable. It is possible that the presence of Cl functional groups (Fig. 8a) causes an inductive effect that confers upon cPVC greater net dipole moment (Shandong Tianchen Chemical Co., Ltd, 2013) relative to the other two plastics, whilst still remaining hydrophobic overall. HDPE, by contrast, is only composed of an unmodified C-H skeleton (Fig. 8b) and, while the polarity of polycarbonate can vary depending upon whether polar groups are introduced, its base composition (Fig. 8c) is hydrophobic (Caldwell, Jackson, 1968).

Chao et al., 2015 showed that the dominant genera changed with different phases of biofilm development. The time effect shown on sample similarity is consonant with this. It is interesting to note that the 1st month samples were more similar to each other than 2nd month samples. It is possible that the first month samples are dominated by similar groups of fast adhering, “pioneer” taxa. By the 2nd month, the importance of fast attachment fades

and material effects may start to dominate. By the 3rd and 4th months, communities stabilize. This is reflected in the similar distribution and close average values/groupings of the 3rd and 4th month samples.

A recent study (Belila et al., 2016) in a full-scale desalination plant utilizing seawater from the same location showed that the planktonic community in the inlet water consisted of approximately 66% *Proteobacteria*, 16% *Bacteroidetes*, 3% *Planctomycetes*, 3% *Cyanobacteria* and 2% *Firmicutes*. By contrast, the average seawater sample in this study was composed of 16% *Proteobacteria*, 7% *Bacteroidetes*, and 2% *Planctomycetes*. The representation of *Cyanobacteria* and *Firmicutes* were several orders of magnitude lower. 29% of taxa in this study were grouped under unclassified bacteria. This could account for the discrepancy between the two.

On the other hand, the planktonic seawater microbial community is vastly different from that associated with the biofilm matrix. Although measures were taken to shield the biofilm reactors from light, representation of *Bacillariophyta* was high on the coupons. On hindsight, the carboys used to store the feedwater should have been consistently kept opaque. Algal growth was found at the bottom of the translucent carboys after 5 days. Algal growth also accumulated around the inlet of the feed tubes, though these were changed on an average of two months. The high amounts of *Bacillariophyta* can be attributed to accumulation or commensal relationships between algae and the other constituents of the biofilm. Given that exposure to light was limited within the biofilm reactors, the second possibility is less likely. The relative absence of *Bacillariophyta* amongst seawater samples is more easily explained. The feed water travels over 2 km to the dispensing point in opaque pipes and may have a long residency time before being dispensed.

The hydrocarbon degrading genus, *Marinobacter*, was well-represented amongst the materials. Although seawater also displayed a high representation, it was vastly lower than that on some of the materials. The genus was present on almost all samples, but largely clustered around a few samples. The first month sample of cPVC, for example, was 58.9% *Marinobacter*, while the first month of titanium was 8.4%. This could indicate two possibilities: 1) that *Marinobacter* has favorable pioneer taxon characteristics for some materials, 2) that there was a spike in hydrocarbons, possibly from a nearby leak, in the feed water at the time of sampling (the two samples were from the same batch).

5.3 Bacterial species of interest

The increase in relative abundance of SRBs can be attributed in part to the increasing thickness of the biofilm layer. As the biofilm matures and thickens, the layers closer to the coupon become more anoxic and more favorable to SRB growth. Although common pathogen relative abundance increased from the 1st to the 4th month, the diversity dropped. This can be interpreted as the taxa with better growth rates becoming dominant. *Legionella* was present only on the material samples and not in the planktonic seawater communities. This is likely due to the genus' close association with biofilms (Murga et al., 2001, Declerck, 2010) and the parasitic relationship it has with biofilm-grazing protozoans. It is surprising to note that the relative abundance of *Legionella* decreased over time when an increase tied to biofilm growth was expected. The commonly found waterborne pathogen, *Klebsiella* (Duncan, 1988), was notably absent from all samples.

Given the tendency of *P. aeruginosa* to dominate in older biofilms (Liu et al., 2009), a higher representation of *P. aeruginosa* is expected as the biofilm matures. The anomalously large spike in relative abundance of *Pseudomonas* in the second month cannot definitively

support this notion. Furthermore, relative abundance of *Pseudomonas* dropped in the 3rd and 4th months.

5.4 Salinity and lytic effect

The shrinkage of lysis zones at with increasing salinity, together with the observation that no significant difference was observed between PFU counts at 1% and 3.5%, makes it highly possible that the drop in PFU count at 6% was the result of lysis zones shrinkage rather than an actual reduction in the number of plaques. This would mean that increased salinity either causes reduction in infected cell metabolism or inhibits phage replication in some other way, rather than virion inactivation or reduction in attachment efficiency, as the latter two would result in a reduction in lysis zone count. By contrast, Hidaka et al., 1971, showed that phages isolated from seawater had the highest inactivation rate in 0.5% NaCl solution as compared to distilled water, 3% NaCl solution, artificial seawater and seawater broth. A separate study showed that phage strains derived from the same environmental isolate could have optimum salinities as far apart as 0.25 and 1.65M (Zachary, 1976). No differences in the size of the lysis zones were mentioned in these studies. The literature cited phage inactivation and changes in phage receptor conformation as reasons for PFU count reduction. Whether through a reduction in plaque area or an actual decrease in the number of plaques, the result is a decrease in phage strain effectiveness against *P. aeruginosa* at higher salinities.

It should be noted that multiplicity of infection (MOI) was not kept constant throughout this study, although host cultures in the stationary phase were always used. This sometimes resulted in changes in magnitude in PFU counts between batches. However, MOI was constant within each batch of experiments and the relative strengths of the strains remained

mostly unchanged. It is also worthwhile to note that host physiology can vastly alter the size of phage bursts (You et al., 2002), sometimes by several orders of magnitude.

5.5 Reduction in cell count upon phage exposure

From the sequencing of the enriched bioreactor coupons, *P. aeruginosa* constitutes approximately 25% of the biofilm. Yet, there was an apparent lack of significant effect for most cocktails/strains tested. This can be attributed to a few reasons. Upon further review, the staining protocol was not optimal for measuring cell lysis. Sybr Green, being a nucleic acid dye, stains both viable and nonviable cells. Stiefel et al., 2015 reported stronger staining of nonviable cells by Sybr Green. A better alternative would have been to stain the cells with propidium iodide in conjunction with Sybr Green (Barbesti et al., 2000), allowing for better discerning of cell viability. Another less likely possibility is the development of bacteriophage resistance. Brockhurst et al., 2005, noted that phage exposure drove diversification of a population of *P. aeruginosa*. The *P. aeruginosa* used to enrich the seawater was prepared from monoculture plates of single colonies kept at -20°C, however, so it is unlikely that there are enough phenotypes for resistance to arise. Horizontal transfer of phage resistance traits between the introduced *P. aeruginosa* and other biofilm constituents is possible but unlikely as 1) the phages were not derived from the same environmental isolates and 2) such traits, unless they offered broad-spectrum protection, would confer no advantage.

Furthermore, Webb et al, 2004, observed that even with large dead zones created in the biofilms caused by phage lysis, the overall effect on the biofilm is not clear. The nutrients released by the lysed cells may be subsequently taken up by the surviving cells (Tolker-Nielsen et al., 2000, Sauer et al., 2002). Additionally, given that biofilms are only 15%

cells by volume (Donlan, Consterton, 2002) and are mostly made up of matrix material, the phages may not be reaching most of the cells, especially if they are protected by dead zones. A better approach may be to use biosurfactants in conjunction with phages, though the former needs to be evaluated for its effect on phage activity.

The biofilms used in phase 2 of the study were relatively young (2 months old with only 1 month of *P. aeruginosa* enrichment). The effects of age on biofilm susceptibility in the literature are not clear. Younger biofilms may be more susceptible to phage attack (Abedon et al., 2016). This is due to two primary factors. The higher metabolic rates of younger biofilm constituents make them more suited to virion production. The EPS surrounding younger biofilms is also naturally thinner and more vulnerable to phages (Chan, Abedon, 2015). On the other hand, when older cells die and break off in mature biofilms, more viral receptors are exposed (Sutherland et al., 2004).

5.6 Change in seawater microbial community upon phage exposure

The drop in relative abundance of *Pseudomonas* is an expected, direct consequence of phage exposure and presumably, lysis. Although the rarity of broad spectrum phages make it likely that the strains isolated are specific for the DSM 1117 strain of *P. aeruginosa*, drops in other taxa may be due to commensal relationships with *P. aeruginosa*. *P. aeruginosa* is known to be a prolific EPS producer (Boyd et al., 1995). The large amounts of alginate produced and the subsequent reduction post-phage exposure may induce die-offs or increases in dominance of other taxa.

The drop in *Marinobacter* casts doubt on the notion that drops in relative abundance is attributable to phage exposure. A drop in *Marinobacter* would be more likely due to the

coupons being cut off from a fresh feedwater and, presumably, from an exhaustion of available hydrocarbons in the media surrounding the biofilm. Likewise, the drops in relative abundance of most of the other taxa may simply be the result of the biofilms being cut off from feedwater nutrients. Furthermore, the salinity of SM buffer is only 0.58% as opposed to 3.5% in the feedwater. This could have caused die-offs due to osmotic shock. In review, the SM buffer should have been enriched to the same salinity.

Limitations to the DNA extraction and sequencing protocol should also be noted. As it stands, the protocol has no way of separating DNA from constituents that have died off from those remaining in the biofilm. The pelleting and supernatant removal steps prior to application of the DNA extraction kit does increase the relative DNA concentration of whole (if not viable) cells. It is not known if this is sufficient to accurately distinguish relative abundances of the taxa remaining.

5.7 Change in PFU count

The magnitudinal increase in PFU count across all cocktails show that the phages were still able to induce lysis under conditions more representative of industrial environments. The PFU counts also offer evidence of *P. aeruginosa* lysis in absence of confirmation via flow cytometry, for reasons highlighted in the preceding section. A1C1 and ABCD had the largest increases in PFU count. For these results to be translatable to phage cocktail effectiveness, however, average phage burst size must be assumed to be constant. The larger PFU count increases observed in ABCD, A1B1 and A1C1 cocktails may be attributable to the higher relative strength of the A1 strain demonstrated in section 4.5.

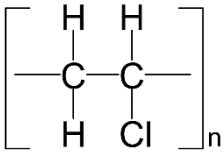
Fig. 8a: cPVC structure 

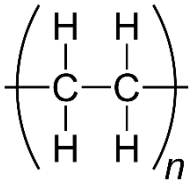
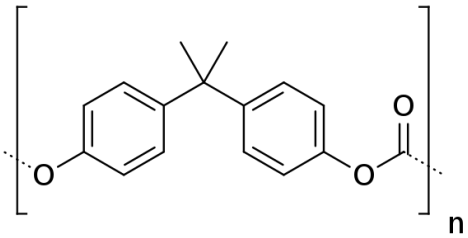
Fig. 8b: HDPE structure 

Fig. 8c: Polycarbonate structure 

6. CONCLUSION

This study concludes that phages are a potential alternative treatment for the removal of *P. aeruginosa* in seawater cooling towers. However, due to staining protocols used, the effect of phage treatment on the reduction of *P. aeruginosa* relative abundance could not be fully enumerated. The increase in phage concentration after incubation with coupon biofilms confirmed the effectiveness of the isolated strains against *P. aeruginosa* in a multi-species biofilm under simulated environmental conditions. Furthermore, the decrease in relative abundance of other bacterial species of interest possibly indicate other positive downstream effects of *P. aeruginosa* phage therapy.

The other objectives of this study were also met. The presence of MIC and other biofouling related microbes were confirmed on the entire selection of common cooling tower materials. The biofilms were also found to harbor common pathogens, further highlighting the importance of biofilm removal. Additionally, the isolated phage strains were proven effective in varying salinities.

This study raised a number of issues that warrant further investigation. While phages cannot completely supersede conventional treatments, they can be used to supplement or delay the time needed between conventional treatments. The effect of conventional disinfection protocols on the efficacy of the isolated phages should be ascertained to better inform the use of phages as part of a multi-pronged treatment approach. A modified flow cytometry staining protocol should be used henceforth to accurately distinguish viable cells from affected ones. The tendency of a host strain to develop phage resistance to phage cocktails as opposed to single strains should also be studied. Likewise, a multiple-host

enrichment protocol should be used to isolate broad range *P. aeruginosa* phages less likely to induce resistance in hosts. The experimental set-up for the second phase should also be improved, by passing the test solutions into the biofilm reactors rather than immersing the biofilm coupons in static test solutions. Phage coatings as preventative *P. aeruginosa* biofilm treatments are worth investigating.

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