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Pseudomonas aeruginosa Biofilms in Drinking Water and the Evolution of Antibiotic Resistance

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Pseudomonas aeruginosa Biofilms in Drinking Water and the Evolution of Antibiotic Resistance

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Thesis submitted to the Faculty of Graduate and Postgraduate Studies
in partial fulfillment of the requirements for the degree of

Master of Science
in
Microbiology and Immunology

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Ottawa, Ontario, Canada

September 2007

Your file Votre référence
ISBN: 978-0-494-41669-3
Our file Notre référence
ISBN: 978-0-494-41669-3

Abstract

In nature, bacteria grow attached to surfaces in communities called biofilms. Biofilm bacteria are phenotypically distinct from free-floating planktonic bacteria, and one of the phenotypic characteristics of biofilms is that they are highly resistant to antimicrobials. Because of this, biofilms growing inside water distribution systems are not killed by the residual water disinfectant. Bacteria that live inside drinking water distribution system biofilms are under stress due to the constant exposure to the residual disinfectant and the nutritional stress of living in an oligotrophic environment. These stresses can activate the bacterial stress response mechanisms, resulting in increased mutation rates. The mutations that occur are random, but the possibility exists for a mutation to arise that confers increased resistance to antibiotics.

The objective of this study was to determine if biofilm bacteria acquire antibiotic resistance when exposed to the residual water disinfectant chloramine. I grew biofilms of *Pseudomonas aeruginosa* in drinking water bioreactors and treated them with chloramine. The bacteria were sampled over time for isolates that were resistant to ciprofloxacin; isolates were obtained from both the chloramine treated and control biofilms. The ciprofloxacin-resistant isolates had some phenotypic differences from the wildtype *P. aeruginosa*, as well as being resistant to rifampicin and chloramphenicol, but not resistant to tobramycin and gentamicin.

Acknowledgments

I could not have accomplished everything that I have during these last 2 years without the help and support of many people. First, I would like to thank my supervisors: Dr. Thien-Fah Mah and Dr. Syed Sattar. I am grateful that they allowed me to work on this interesting project, and without their guidance I don't know if I could have overcome the difficulties that I have had during this project. Also, I would like to thank the members of my thesis advisory committee, Dr. Rees Kassen and Dr. Franco Pagotto, who have contributed greatly to my thesis by giving suggestions on different approaches that I could use to further this project.

I want to thank Susan Springthorpe, Justo Perez, and Reza Nokhbeh who were great resources on assembling and running the CREM bioreactors. The help I received from the staff at the Lemieux Island water purification facility was much appreciated: they were kind enough to give me a tour of the facility and answer any questions I had about the water purification process. I thank all of my labmates in both the Mah and Sattar labs for their assistance with various pieces of equipment, or procedures; with special thanks to Li Zhang and Fernando Matias. I also thank Dr. Karam Ramotar for performing the Vitek 2 identification of the ciprofloxacin resistant isolates. Funding for this project was provided by the Ontario Research Development Challenge Fund (ORDCF).

And last, but definitely not least, I need to thank my family for their support through all of my education, especially my husband Scott. Without his support and encouragement I could not have done this, and I will never forget it.

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List of Abbreviations

°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometer
13S	Ribosomal RNA
30S	Small Ribosomal Subunit
50S	Large Ribosomal Subunit
ABC	ATP-Binding Cassette
Alum	Aluminum Phosphate
AOC	Assimilable Organic Carbon
ATP	Adenosine triphosphate
BAC	Biological Activated Carbon
BDOC	Biodegradable Organic Carbon
Bp	Base Pair
CAT	Chloramphenicol Acetyltransferase
CF	Cystic Fibrosis
CLSI	Clinical Laboratory Standards Institute
Cm	Centimeter
DNA	Deoxy-Ribonucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
GAC	Granular Activated Carbon
Hr	Hour
L	Litre
LB	Luria-Bertani
LPS	Lipopolysaccharides
MATE	Multidrug And Toxic Compound Extrusion
MBC	Minimal Bactericidal Concentration
MFS	Major Facilitator Superfamily
Mg	Milligram
MIC	Minimum Inhibitory Concentration
mL	Milliliter
mm	millimeter
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone
Na^+	Sodium Ion

NCl_3	Monochloramine
NH_2Cl	Dichloramine
NH_2Cl_2	Nitrogen Trichloride
nm	nanometer
OD_{600}	Optical Density at 600nm
PBS	Phosphate Buffered Saline
pH	Power of Hydrogen
PIA	Pseudomonas Isolation Agar
Pol	Polymerase
RNA	Ribonucleic Acid
RND	Resistance-Nodulation-Division
rRNA	Ribosomal RNA
THMs	Trihalomethanes
TOC	Total Organic Carbon
tRNA	Transfer RNA
UV	Ultraviolet
VBNC	Viable But Non-Culturable

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INTRODUCTION

Introduction

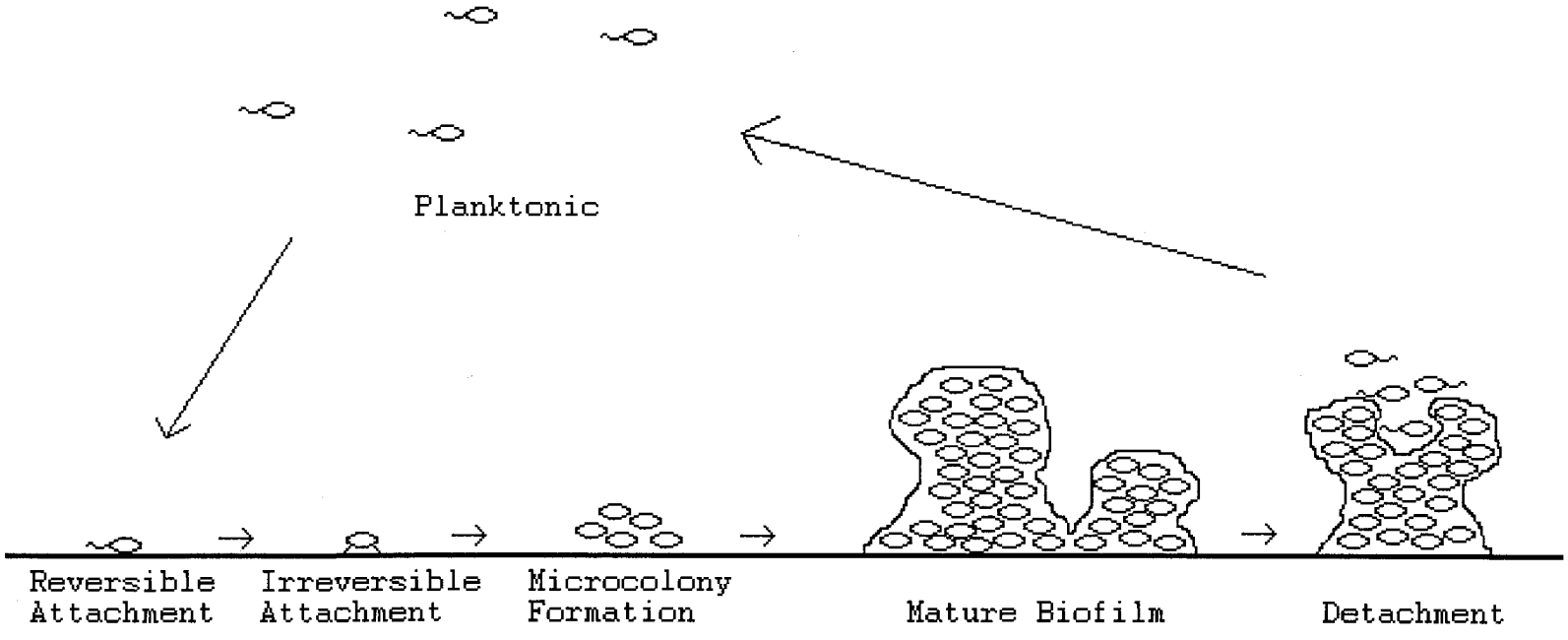
The most important resource on the planet is water, water that is clean and safe to drink. In Canada, we have an abundance of clean fresh water that once filtered and chemically treated is safe to drink. After the treatment process the water must travel from the water treatment facility to the consumer. During this transport, which takes place via pipes that form a water distribution system, it is difficult to maintain the water quality. Bacteria that are not killed during the water treatment process, or bacteria that enter the water distribution system after treatment, can attach to the walls of the pipes and form sessile bacterial communities called biofilms.

Biofilms

The definition of a biofilm can be as simple as 'a matrix-enclosed bacterial population adhering to other bacteria or a surface' (Costerton et al., 1995), or as specific as 'a sessile bacterial community made up of cells that attach to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric matrix, and exhibit an altered phenotype with respect to growth, gene expression, and protein production' (Shirtliff et al., 2002). The biofilm mode of growth is especially successful, as most bacteria are able to form biofilms and in many environments the vast majority of the bacteria present are part of a biofilm (Costerton et al., 1995).

The typical life cycle of a bacterial biofilm (Figure 1) has been well studied. Bacteria will come into contact with a surface either by chance or they will actively move towards a surface because of the presence of environmental signals such as osmolarity, pH, iron availability, oxygen tension and temperature (Davey and O'Toole, 2000). The types of environmental signals that initiate bacterial attachment are species specific. For example;

Figure 1. Model of biofilm development.
Planktonic bacteria come into contact with a surface and then become irreversibly attached. The attached bacteria migrate toward other bacteria and form microcolonies. The microcolonies grow to form a mature biofilm. Bacteria detach from the biofilm to become planktonic again.


Planktonic
Reversible Attachment
Irreversible Attachment
Microcolony Formation
Mature Biofilm
Detachment

when *Pseudomonas aeruginosa* is in a favorable environment, where there is a lot of nutrients, it will readily attach to surfaces to begin the process of biofilm formation (Costerton et al., 1995). Once the bacteria come into contact with a surface they attach to the surface in a reversible manner, allowing the bacteria the chance to detach from the surface if the conditions on that surface are unfavorable. During the reversible attachment phase the bacteria are able to move around the surface and many bacteria will come together and form a microcolony; *P. aeruginosa* use type IV pili to move over the surface and form microcolonies (O'Toole and Kolter, 1998; Davey and O'Toole, 2000; Stoodley et al., 2002). If the bacteria do not detach from the surface within a few hours, the attachment becomes irreversible because the bacteria begin to produce extracellular polymers (Stoodley et al., 2002). As the extracellular polymer accumulates, it forms a thick matrix. The polymers produced are different for each species. For instance, if the biofilm is composed mainly of *P. aeruginosa*, the matrix will be primarily alginate (Stoodley et al., 2002). A macrocolony begins to form as the bacteria within the microcolony multiply and more matrix is produced. Furthermore, planktonic bacteria can also colonize and become part of the growing macrocolony. A biofilm is composed of many macrocolonies that are separated by pores and channels through which water and nutrients can flow (Davey and O'Toole, 2000; Kreft, 2004).

Bacteria are able to detach from the mature biofilm in three different ways: as single bacteria sloughed from the biofilm, as whole clusters of bacteria breaking away from the biofilm or as a large planktonic group. Once a biofilm is mature the centre of a macrocolony can hollow out and the bacteria inside become planktonic, and will be released into the

surrounding media (Boles et al., 2005; Davey and O'Toole, 2000). These newly released bacteria can then move to a new location and begin the process of forming a new biofilm.

There are many advantages for bacteria that reside within biofilms. The first and most obvious benefit to forming biofilms is for the bacteria to remain in a favorable environment (Jefferson, 2004). Planktonic bacteria are carried along with the flow of the liquid that they are in. If there is an area with a high concentration of nutrients, the planktonic bacteria could not take advantage of that, while biofilm bacteria can. If the conditions once again are unfavorable, then the bacteria can detach from the biofilm and resume the planktonic mode of growth.

Another advantage of living in a biofilm is that being sessile and in close contact with neighboring bacteria allows for metabolic cooperation within a biofilm (Jefferson, 2004; Davey and O'Toole, 2000; Kreft, 2004). Each of the different species of bacteria inside a biofilm is able to produce and secrete different sets of enzymes. When the bacteria are in close association, as they are within a biofilm, the different enzymes are able to work together to break down complex substrates. Often a mixed-species biofilm is able to break down compounds that single species cannot. For example, *Streptoccocus oralis* and *Actinomyces naeslundii* are two oral bacteria that can only utilize saliva as a nutrient when both species are in close association (Kreft, 2004).

The final benefit to the biofilm mode of growth is protection. Bacteria residing within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, heavy metals toxicity, UV radiation, osmotic shock, the immune response, disinfectants and antibiotics better than planktonic bacteria (Jefferson, 2004; Davey and O'Toole, 2000). The most dramatic protective effect of growing in a biofilm is the resistance to antibiotics:

biofilm bacteria have been shown to be up to 1000 times more resistant to certain antibiotics compared to their planktonic counterparts (Nickel et al., 1985; Mah and O'Toole, 2001). The mechanisms of biofilm antibiotic resistance are different from planktonic resistance mechanisms. The increase in antibiotic resistance that is seen in biofilms is not due to the up-regulation of efflux pumps (DeKievit et al., 2001), there are biofilm-specific mechanisms that are known to contribute to the increased antibiotic resistance (Mah and O'Toole, 2001). The biofilm matrix is one of the mechanisms that increase the biofilms resistance to certain antibiotics as well as many of the other agents listed above. The matrix is able to physically restrict diffusion of positively-charged antibiotics, metals and toxins through the biofilm (Davey and O'Toole, 2000; Stewart, 2002); as well as resisting phagocytosis by macrophages (Leid et al., 2005). The biofilm matrix can play an additional role in antibiotic resistance by sequestering drug-inactivating enzymes such as beta-lactamase: in this way, the drug will be degraded by the enzymes as it diffuses through the biofilm (Gilbert et al., 2002). Another reason for the increased antibiotic resistance is the heterogeneity of biofilms. Even if the biofilm contains only one species of bacteria, there is great diversity within the biofilm with regards to nutrient and oxygen availability, and therefore growth rates (Wentland et al., 1996; Drenkard, 2003). Evans et al. (1990) demonstrated that the growth rate of biofilm bacteria greatly influences the effectiveness of tobramycin. Additionally, areas of limited oxygen will be less susceptible to the action of aminoglycosides, such as tobramycin and gentamycin (Stewart, 2002), even if the bacterial growth rate is high. There has also been recent evidence to support the idea of there being a biofilm-specific phenotype which is responsible for the increase in antibiotic resistance of biofilms that is unrelated to planktonic resistance mechanisms (Mah et al., 2003). This biofilm-specific phenotype is only seen when the

bacteria are part of a biofilm; if the biofilm is dispersed, the bacteria are no longer resistant to antibiotics because the resistance is not due to mutations, which is what causes planktonic antibiotic resistance (Mah and O'Toole, 2001). While all of the mechanisms described have a role in increasing the antibiotic resistance of biofilms they do not account for the total increase that is seen; therefore, there must be additional, as yet unknown, mechanisms that also contribute to biofilms' increased resistance to antibiotics.

Drinking Water Distribution System Biofilms

Biofilms are very common in drinking water distribution systems. In fact, more than 95% of the bacteria in the distribution system are found in biofilms (Hu et al., 2005), and any planktonic bacteria found inside the water distribution system are generally bacteria that have detached from an existing biofilm (van der Wende et al., 1989). Because the pipes of the water distribution system are often very old, there is a large biofilm population capable of producing planktonic bacteria that are able to colonize any new sections of pipes within a short period of time.

Inside the drinking water distribution system, the water is considered oligotrophic because it has a very small amount of organic carbon (LeChevallier et al., 1991); this is actually one of the reasons why most of the bacteria are found in biofilms. There is a phenomenon called 'the antenna effect' whereby the organic molecules in the flowing water accumulate at the pipe wall (Szewzyk et al., 2000). Since the concentration of nutrients is greatest along the wall of the pipe, the bacteria will form biofilms on the wall of the pipes in order to take advantage of the increased availability of nutrients. If the water is very oligotrophic, or under other stressful conditions, the bacteria may not get enough nutrients even when attached to the wall of the pipes, and the bacteria may employ survival strategies

such as decreasing respiration and metabolism or decreasing cell size (Roszak and Colwell, 1987). Bacteria that employ these survival strategies may enter a viable but non-culturable (VBNC) state, where the bacteria will grow when nutrients are available, but cannot be cultured by conventional means (Roszak and Colwell, 1987).

Drinking water distribution system biofilms are unwanted for a variety of different reasons: customer dissatisfaction due to taste, turbidity and odours (Momba et al., 1999; Gagnon et al., 2004), pipe corrosion (Costerton et al., 1987), mineral deposition (Szewzyk et al., 2000), and health concerns about exposure to pathogens (Momba et al., 1999). Taste, turbidity and odours are present in the drinking water when there is an increase in the number of planktonic bacteria or invertebrates in the water distribution system (Hargesheimer and Watson, 1996). Most of the larger pipes of the drinking water distribution system are made of metal, such as iron, and biofilms can cause corrosion of these pipes. Metal corrosion occurs when the biofilm contains sulfate-reducing bacteria (Costerton et al., 1987). The corrosion continues until a hole is formed in the wall of the pipe, leading to the need to replace the pipe. Biofilms in the drinking water distribution system can also deposit inorganic compounds onto the walls of the pipes (Szewzyk et al., 2000). The mineral deposits that are formed depend on the species of bacteria within the biofilm but over time the deposits can narrow the diameter of the pipes, thus restricting the water flow. The bacteria living inside drinking water biofilms are typically non-pathogenic (Wingender and Flemming, 2004). However, pathogenic bacteria such as *Legionella sp.* and *Mycobacterium sp.* can be routinely found in improperly treated drinking water (Pryor et al., 2004). It is for all of the above reasons that drinking water must be treated, and the pipes of the water distribution system should not develop too large a biofilm population.

Pseudomonas aeruginosa

P. aeruginosa, a Gram-negative motile rod, is one species of bacteria that is commonly found in drinking water distribution system biofilms (Szewzyk et al., 2000). While *P. aeruginosa* is not harmful for healthy individuals it is an opportunistic pathogen (Momba et al., 1999), and as such can cause serious infections in individuals with compromised immune systems or with underlying diseases such as diabetes or cystic fibrosis (Szewzyk et al., 2000). In individuals with cystic fibrosis, *P. aeruginosa* colonizes the lungs and forms biofilms that are highly resistant to treatment (Lambert, 2002). The strains of *P. aeruginosa* that were used in this study to form the drinking water biofilms were PA01 (Holloway et al., 1979) and PA14 (Rahme et al., 1995). The PA01 strain is the standard laboratory strain of *P. aeruginosa*, while the PA14 strain has been shown to be more pathogenic than PA01, in a variety of model systems (Rahme et al., 2000).

Drinking Water Purification

Disinfectants

There are many disinfectants that can be used for drinking water purification. The effectiveness of the different disinfectants depends on the water source, as the type and levels of pathogens found in ground water and surface water can be different. Some disinfectants are used for primary disinfection of the water, while others are added to the drinking water before it enters the distribution system as a residual disinfectant, to maintain the water quality in the drinking water distribution system.

Chlorine has been used for over 100 years as a water disinfectant. Chlorine was first used on a continuous basis to disinfect drinking water in Belgium in 1902 (American Water Works Association, 2006). Chlorine acts as a strong oxidant that can inactivate a wide range

of waterborne pathogens. There are three different forms of chlorine that can be used to disinfect drinking water: chlorine gas, sodium hypochlorite or calcium hypochlorite (HDR Engineering, 2001). Chlorine is used as a primary disinfectant to inactivate most bacteria, viruses and protozoans during the purification of drinking water, but it can also be used as a residual disinfectant within the drinking water distribution system (American Water Works Association, 2006). The effectiveness of chlorine as a drinking water disinfectant depends on the concentration of the chlorine in the water, the contact time, the pH of the water, the water temperature (American Water Works Association, 2006) and the turbidity, or level of total organic carbon (TOC) in the water (LeChevallier, 1981). Turbidity in drinking water is usually correlated with increased levels of TOC in the water (LeChevallier, 1981). Chlorine reacts with the organic carbon in the water and forms combined residuals, effectively reducing the chlorine concentration (American Water Works Association, 2006). The combined residuals that form from the reaction of chlorine with organic carbon in the water are called trihalomethanes (THMs), which have been shown to be carcinogenic (LeChevallier, 1981). In an effort to reduce the amount of THMs in drinking water, chloramine has become widely used as a drinking water disinfectant.

Chloramine is formed when ammonia and hypochlorous acid react, resulting in a mixture of three different chemical species; monochloramine (NH_2Cl), dichloramine (NH_2Cl_2) and nitrogen trichloride (NCl_3) (HDR Engineering, 2001). The most abundant species formed in water is monochloramine (American Water Works Association, 2006). Chloramine, like chlorine, is an oxidant (Kreft et al., 1985), so it disinfects in much the same manner as chlorine, though not as effectively. Chloramine has become widely used as a residual disinfectant because it is very stable through the drinking water distribution system.

Furthermore, chloramine produces very few THMs and adds very little taste and odour to the drinking water (American Water Works Association, 2006). Since chloramine is not as effective a disinfectant as chlorine, higher concentrations are required to achieve the same level of disinfection. However, the stability of chloramine in the drinking water distribution system allows for this higher concentration to be maintained throughout the system, making chloramine a viable alternative for residual disinfection. The main reason that many drinking water purification facilities have switched over to using chloramine as the residual disinfectant is to reduce the amount of THMs in the water. Mitcham et al. (1983) found that when chloramine was used to disinfect the drinking water, the levels of THMs in the drinking water was decreased by almost 80% from the levels found in drinking water disinfected with chlorine.

Drinking Water Purification Process

Drinking water is treated in much the same way as it has been for many decades. The widespread use of filtration and chlorination to treat drinking water began in the 1930s and 1940s throughout North America (Trussell, 2006). The Lemieux Island water purification facility was constructed in 1931 and began to purify drinking water for the city of Ottawa that same year (City of Ottawa, 2006). The water source is the Ottawa River, upstream of the city centre. The first treatment step is the addition of aluminum phosphate (Alum) and sulphuric acid to coagulate particulate matter in the water (City of Ottawa, 2006). The water passes through screens that remove all of the large debris and then silica is added. The addition of silica, together with the alum and sulphuric acid, allows the formation of flocs (City of Ottawa, 2006). The flocs are large agglomerations of particulates from the water that will settle to the bottom of the sedimentation tanks. After passing through the

sedimentation tanks, the water moves through the large granular activated carbon / biological activated carbon (GAC/BAC) filters (City of Ottawa, 2006). The GAC/BAC filters are comprised of many layers of sand and anthracite that is colonized by bacteria from the input water. The function of the filters is to remove AOC (Persson, 2006) and harmful disinfection by-products such as chlorite (Collivignarelli, 2006). The water leaving the filters goes into clear wells where the primary disinfectant, sodium hypochlorite is added, after which the pH of the water is adjusted using sodium hydroxide and carbon dioxide (City of Ottawa, 2006). Just before the water is pumped out to the water distribution system fluoride is added to the drinking water, as well as the residual disinfectant chloramine (City of Ottawa, 2006).

Stress Response and Mutations

Bacteria that live in the drinking water distribution system are subject to many stresses. The THMs that are formed as disinfection by-products have been shown to be mutagenic; one component of THMs, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) is one of the most potent bacterial mutagens known (DeMarini et al., 1995). In *Salmonella typhimurium* strains, MX predominantly induces GC→TA and GC→AT mutations in mutational hotspots (DeMarini et al., 1995). Drinking water that is treated with chloramine produces fewer THMs, and is less mutagenic than water treated with chlorine (Cozzie et al., 1993). However, mutagens are not the only way for mutations to arise in drinking water bacteria.

Exposure to the residual disinfectant is stressful for the bacteria living in the drinking water distribution system. Monochloramine, the main component of chloramine in water, damages bacterial proteins (Jacangelo et al., 1991), and protein damage is one of the inducers of the bacterial heat-shock response (Segal and Ron, 1998). In *Escherichia coli*, the heat-

shock response activates a cellular cascade that stabilizes the sigma factor RpoH (or σ^{32}) (Foster, 2005). RpoH can then upregulate the transcription of the RpoH-regulon that encodes chaperones, proteases and regulatory factors (Segal and Ron, 1998), one of which is the chaperone GroE that stabilizes the polymerase subunit of Pol V and prevents its degradation allowing PolV to accumulate (Foster, 2005). Pol V is an error-prone polymerase that can replicate damaged DNA, but causes frequent mutations (Foster, 2005). Thus, when the bacteria are exposed to chloramine, there could be an increase in the number of mutations in the genome.

Another stressful condition on the bacteria living in the drinking water distribution system is the oligotrophic environment. Starvation can induce the heat-shock response like protein damage can, as well as the induction of both the SOS response and the general stress response (Foster, 2005). When the bacteria do not have enough AOC in the water, the intracellular concentration of the repressor LexA decreases; resulting in the transcription of the SOS genes (Foster, 2005). Two of the products of SOS genes are Pol IV and Pol V, which are both error-prone polymerases (Foster, 2005). The alternative sigma factor, RpoS (or σ^{38}), is activated by stressful conditions that stop growth, starvation being one of them (Foster, 2005). Like the SOS response, the general stress response induces the production of the error-prone polymerases Pol IV and Pol V (Foster, 2005)

The transient increases in mutation rate that occur from activation of these stress responses can generate many different mutations (Figure 2) (Finkel and Kolter, 1999); however, most mutations are either deleterious or neutral (Kang et al., 2006). While most of the population will die, mutants that do not die can proliferate rapidly due to the sudden excess of nutrients generated by the death and lysis of the majority (Zinser and Kolter, 2004).

Very few of the mutations generated by the error-prone polymerases are advantageous, but advantageous mutants will proliferate in the population. One possibility for an advantageous mutation, is in genes that are important for biocide and antibiotic resistance, for example, efflux pumps (Lambert, 2002). If a mutation occurs causing the over-expression of efflux pumps; the bacteria will become resistant to antibiotics, as well as biocides, such as the water disinfectant.

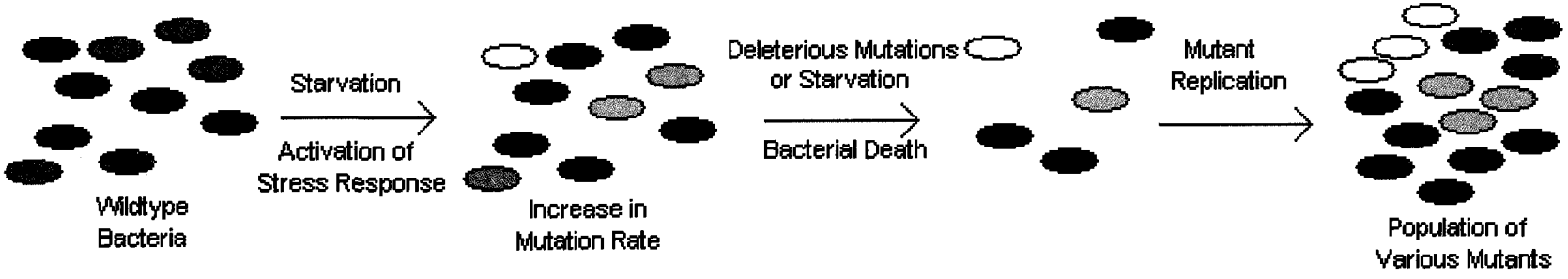
Antibiotic Resistance

Antibiotic Mode of Action

Antibiotics are classified according to their chemical structure and their mode of action. The most common mechanisms of action for antibiotics are: membrane damage, inhibition of cell wall synthesis, and inhibition of protein, RNA or DNA synthesis (Mascaretti, 2003). Fluoroquinolones, such as ciprofloxacin, target the bacterial type II topoisomerases; these enzymes alter the coiling of the DNA by passing an intact double helix through a transient double-stranded break in another section of the DNA (Anderson et al., 1998). Aminoglycosides, such as gentamicin and tobramycin, inhibit protein synthesis by interfering with the 30S subunit of the ribosome (Walsh, 2003). Rifampicin is one example of an ansamycin, it inhibits RNA synthesis by binding to the β-subunit of the bacterial DNA-dependent RNA polymerase (Mascaretti, 2003). Chloramphenicol is the only antibiotic in its class, and its target is the 50S subunit of the bacterial ribosome, thereby inhibiting protein synthesis. (Mascaretti, 2003).

Mechanisms of Antibiotic Resistance in Planktonic Bacteria

There are three basic mechanisms by which planktonic bacteria develop resistance to antibiotics: reduction of the intracellular concentration of the antibiotic, drug inactivation by


Starvation
Activation of
Stress Response
Deleterious Mutations
or Starvation
Bacterial Death
Mutant
Replication
Wildtype
Bacteria
Increase in
Mutation Rate
Population of
Various Mutants

enzymes, and alteration of the antibiotic target (Jana and Deb, 2006; Lambert, 2002). Most mechanisms of resistance are acquired either through mutations, or transferred from one bacterium to another on a plasmid or transposon (Mascaretti, 2003).

Bacteria decrease the intracellular concentration of an antibiotic by either altering the permeability of the membrane to the antibiotic, or by the expression of efflux pumps that pump the antibiotic out of the cell (Mascaretti, 2003). Furthermore, some bacteria can have innate resistance to antibiotics through the low permeability of their cell membrane. For example, in *P. aeruginosa* the outer membrane lipopolysaccharides (LPS) restrict the penetration of small hydrophilic molecules and exclude larger molecules (Lambert, 2002). Efflux pumps are a common mechanism for antibiotic resistance, particularly for multi-drug resistant pathogens (Jana and Deb, 2006). There are four different families of efflux pumps that are able to transport several antibacterial compounds out of the bacterial cell: the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxic compound extrusion (MATE) family, and the ATP-binding cassette (ABC) family (Mascaretti, 2003; Walsh, 2003). The efflux pumps require energy to remove antibiotics from the cell; the MFS and RND efflux pumps are driven by the proton motive force, the MATE efflux pump is driven by the counter-flow of Na^+ ions, and the ABC efflux pumps are powered by the hydrolysis of ATP (Walsh, 2003).

The MFS efflux pumps are the predominant efflux systems found in Gram-positive bacteria (Mascaretti, 2003). MFS efflux pumps do not have a very broad antibiotic range; they are primarily only able to export chloramphenicol and some fluoroquinolones (Walsh, 2003). The efflux pump QacA, which is found in *Staphylococcus aureus*, is a member of the MFS and is able to export quaternary ammonium compounds (Walsh, 2003).

The RND family of efflux pumps are found in Gram-negative bacteria, and because they must transport across an inner and an outer membrane, the pumps are composed of three different parts: the transporter, which is in the inner membrane, a periplasmic accessory protein and the outer membrane channel (Mascaretti, 2003; Lambert, 2002). The antibiotic range of RND efflux pumps is very broad; they are able to export β-lactams, tetracycline, erythromycin, chloramphenicol, and fluoroquinolones (Walsh, 2003). *P. aeruginosa* has many different RND efflux pumps; the one with the broadest antibiotic range is the mexAB-oprM efflux pump (Walsh, 2003).

The MATE family of efflux pumps is the least well known. In 1998, the first MATE family efflux pump, NorM, was first discovered in *Vibrio parahaemolyticus* (Morita et al., 1998). Since this discovery, homologs in other Gram-negative bacteria have been found. The NorM efflux pump is only able to export fluoroquinolones and some aminoglycosides (Morita et al., 1998).

The last family of transporters is the largest group. The ABC superfamily transport many different types of compounds and they are found in eukaryotes as well as in prokaryotes (Walsh, 2003). ABC transporters are found in both Gram-positive and Gram-negative bacteria, but there are actually very few that are able to pump antibiotics. LmrA is an ABC transporter that is found in *Lactococcus lactis*. It is a multi-drug efflux pump that is able to export aminoglycosides, some β-lactams, chloramphenicol, macrolides, fluoroquinolones, and tetracycline (Walsh, 2003).

Chloramphenicol, aminoglycosides and β-lactams are all susceptible to inactivation by bacterial enzymes (Mascaretti, 2003). The genes for the antibiotic inactivating enzymes are usually located on plasmids, so this type of resistance is easily transferred in the

environment. Chloramphenicol acetyltransferase (CAT) inactivates chloramphenicol by transferring an acetyl group from acetyl coenzyme A to chloramphenicol (Mascaretti, 2003).

There are three different enzymes that are capable of inactivating aminoglycosides (Jana and Deb, 2006, Mascaretti, 2003). Aminoglycoside acetyltransferases acetylate one of the amino groups on the antibiotic, and this acetylation reduces the antibiotics affinity for the target site by four orders of magnitude (Jana and Deb, 2006). Aminoglycoside nucleotidyltransferases transfer an adenyl group from Mg-ATP to the aminoglycoside to form O-adenylated aminoglycoside (Jana and Deb, 2006). Aminoglycoside phosphotransferases, or aminoglycoside kinases, phosphorylate the antibiotic dramatically decreasing the antibiotics ability to bind to the ribosome (Jana and Deb, 2006).

The most well known of the antibiotic inactivating enzymes are the β-lactamases, also known as penicillin-binding proteins (Mascaretti, 2003). These enzymes normally catalyze the transpeptidation reaction in cell wall biosynthesis, but when β-lactams are present the β-lactamases hydrolyze the β-lactam ring so the antibiotic can no longer inhibit its target (Walsh, 2003). Bacteria that are highly resistant to β-lactams usually have mutations in the regulatory gene ampR causing the over-expression of β-lactamases (Lambert, 2002).

Alterations to the antibiotic target are seen most frequently in bacteria that produce the antibiotics. Bacteria that produce aminoglycosides, such as *Streptomyces sp.*, express rRNA methylases that methylate amino acids in the 16srRNA A site so that the antibiotic it produces cannot bind and inhibit protein synthesis (Jana and Deb, 2006). In *P. aeruginosa* mutations in the gene *gyrA* are found that alter the binding site of fluoroquinolones on DNA gyrase (Lambert, 2002).

Experimental Rationale

The bacteria that are growing within the drinking water distribution system are found in biofilms, and these biofilms are exposed to the residual disinfectant at all times. However, the affect that exposure to the drinking water residual disinfectant or the oligotrophic environment has on the resident bacteria has not yet been studied. Because the bacteria are growing in biofilms they are not killed by the disinfectant; however, the exposure to the disinfectant may stress the bacteria and induce one or all of the bacterial stress response mechanisms. The induction of the stress response mechanisms can generate mutants, and one possibility is the generation of antibiotic resistance. I grew *P. aeruginosa* biofilms in drinking water and treated the biofilms with chloramine in order to determine if drinking water distribution system biofilms generate antibiotic resistant isolates because of chloramine exposure.

HYPOTHESES AND OBJECTIVES

Hypotheses

I propose that biofilms growing in the pipes of drinking water distribution systems are not eliminated by the concentration of chloramine that is present in the water, although this concentration of disinfectant does kill planktonic bacteria. While the concentration of chloramine maintained throughout the drinking water distribution system does not kill the biofilm bacteria, the chloramine exposure is a stress on the bacteria which can lead to mutations in the bacterial genome. The resultant mutations can give rise to bacteria that are cross-resistant to antibiotics.

Objectives

1. Demonstrate that planktonic *P. aeruginosa* are killed by chloramine at a concentration of 1 mg/L, while biofilm *P. aeruginosa* are not.
2. Establish persistent *P. aeruginosa* biofilms in drinking water bioreactors.
3. Treat *P. aeruginosa* drinking water biofilms with chloramine at two different concentrations, and for two different lengths of time, then assess for the generation of antibiotic-resistant isolates.

MATERIALS AND METHODS

Materials and Methods

Bacterial Strains

The strains of *P. aeruginosa* used were PA01 (Holloway et al., 1979) and PA14 (Rahme et al., 1995). Both PA01 and PA14 were used to determine chloramine susceptibility with the Minimum Bactericidal Concentration (MBC) assay for planktonic and biofilm cells (Mah et al., 2003). Drinking water biofilms of PA01 were grown in the CREM bioreactors. The two strains were compared for biofilm formation in drinking water using the Kadouri drip-fed reactor, but only PA14 was used to grow drinking water biofims in the Kadouri drip-fed reactor for the generation of ciprofloxacin-resistant isolates.

Chloramine Susceptibility

Minimum Bactericidal Concentration Assays

The MBC assays (Mah et al., 2003) can be performed for either planktonic or biofilm cells using 96-well plates, allowing a direct comparison of the antibiotic resistance of planktonic and biofilm cells. A stationary-phase culture of the two different strains of *P. aeruginosa* was diluted 1/1,000,000 in Luria-Bertani (LB) broth for the MBC assays. The chloramine was prepared by combining equal parts of 0.2 mg/mL sodium hypochlorite (Fisher Scientific) in phosphate buffered saline (PBS) (Fisher Scientific) with 0.8 mg/mL ammonium chloride (BDH) in PBS, and the resulting solution had a chloramine concentration of 200 mg/L.

For the planktonic assay a 96-well plate was inoculated with 10 μL of the diluted culture along with 90 μL of the chloramine solution diluted in LB broth to give the following final concentrations: 200 mg/L, 100 mg/L, 50 mg/L, 20 mg/L, 10 mg/L, 5 mg/L, 1 mg/L and 0 mg/L. The inoculated plate was then incubated at 37°C for 24 hours. After incubation

some of the bacteria inside the wells of the 96-well plate were transferred onto LB agar plates using a multi-pronged device. The LB agar plates were incubated at 37°C for 24 hours, and then examined to see at which concentration of chloramine the planktonic bacteria were unable to grow.

The biofilm MBC assay was similar to that for the planktonic bacteria with a few additional steps. A 96-well plate was inoculated with 10μL of the diluted culture into 90μL of LB broth and then incubated at 37°C for 24 hours to allow the bacteria to form biofilms inside the wells. After incubation the spent medium was removed from the wells and replaced with 100 μL of LB broth containing chloramine at the same final concentrations used for the planktonic MBC assay; then the plate was incubated again at 37°C for 24 hours. After incubation the medium containing chloramine was removed and replaced with 100 μL of fresh LB broth and the plate was incubated at 37°C for 24 hours, this allowed the biofilm bacteria to detach from the walls of the well. After incubation the detached biofilm cells were transferred from the 96-well plate onto LB agar plates using a multi-pronged device. The LB agar plates were incubated at 37°C for 24 hours and then examined to see at which concentration of chloramine the biofilm bacteria were unable to grow.

Bioreactors

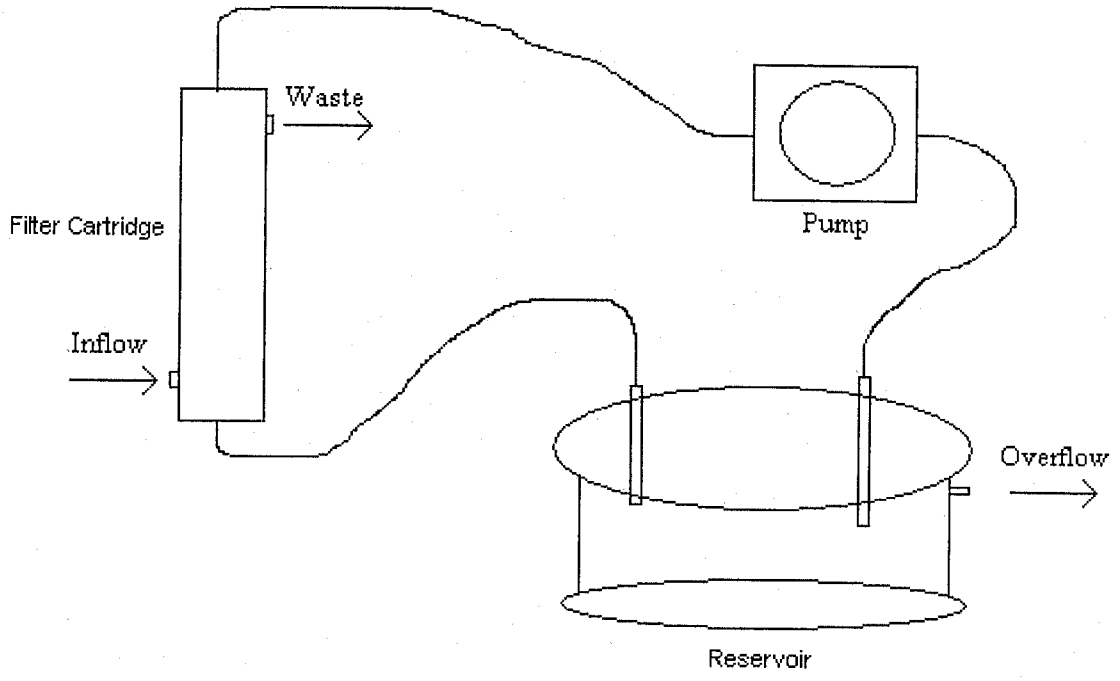
The CREM bioreactor (Springthorpe et al., 2001) and Kadouri drip-fed reactor (Merritt et al., 2005) were used to grow drinking water biofilms. The CREM bioreactor, used in initial tests, yielded inconsistent biofilm growth. For this reason the Kadouri drip-fed reactor was used in all subsequent experiments to form the biofilms that were treated with chloramine and to generate the ciprofloxacin-resistant isolates.

CREM Bioreactor

The CREM bioreactor was a large-scale device set up at the Lemieux Island Water Purification Facility in Ottawa, Ontario, Canada. A schematic of the bioreactor system is shown in Figure 3. Having the CREM bioreactor at the water treatment facility allowed us to use both chloraminated and unchloraminated water directly from the water treatment process without it having to travel through the distribution system. The unchloraminated water was diverted from the water treatment process immediately after filtration through the granular activated carbon/biologically activated carbon (GAC/BAC) filters. The chloraminated water was fully treated water that was diverted before entering the water distribution system.

The CREM bioreactor system has three main components; the reservoir where the biofilm forms, the pump that circulates the water, and a hollow-fiber cartridge. The cartridge, designed for use in kidney dialysis (F70NR; Fresenius), allows the flow of liquid and low molecular weight substances through the pores in the walls of the fibers while retaining any microorganisms present. The hollow-fiber cartridge was the junction between the internal and the external water flow of the CREM bioreactor system. The water in the internal flow started in the reservoir and was pumped out of the reservoir and into the cartridge, where it traveled inside the fibres. Once the water left the cartridge it was pumped back into the reservoir, which had overflow control to maintain the appropriate water level. The water in the external system started as inflow water which was pumped into the external chamber of the cartridge where the water could cross through the pores in the cartridge and enter the internal flow, or the water could remain in the external system. Once the water exited the cartridge it went to the waste.

Figure 3. Schematic of CREM bioreactor system.
The water circulates from the reservoir through the hollow fibre cartridge and back into the reservoir. Fresh water (inflow) can enter the system by passing through the walls of the hollow fibres inside the filter cartridge, if the water does not enter the system it leaves through the outflow. The water level inside the reservoir is regulated by the overflow.


Waste
Filter Cartridge
Pump
Inflow
Overflow
Reservoir

Bioreactor Construction

The CREM bioreactor reservoir (Figure 4) was constructed at the Carleton Science Technology Centre (Carleton University; Ottawa, Canada). The reservoir was a 190 x 100 mm Pyrex crystallization dish that was modified to have an overflow on the side to maintain the water volume at approximately 2 L. The crystallization dish was enclosed with a top made of lexan which had inflow and outflow tubes as well as an access port. There was a platform inside the reservoir with grooves in it which could hold up to 50 polycarbonate coupons measuring 2 cm by 5 cm vertically within the water. The bacteria formed biofilms on the polycarbonate coupons which could be removed through the access port in the reservoir's top allowing for repeated sampling of the biofilm.

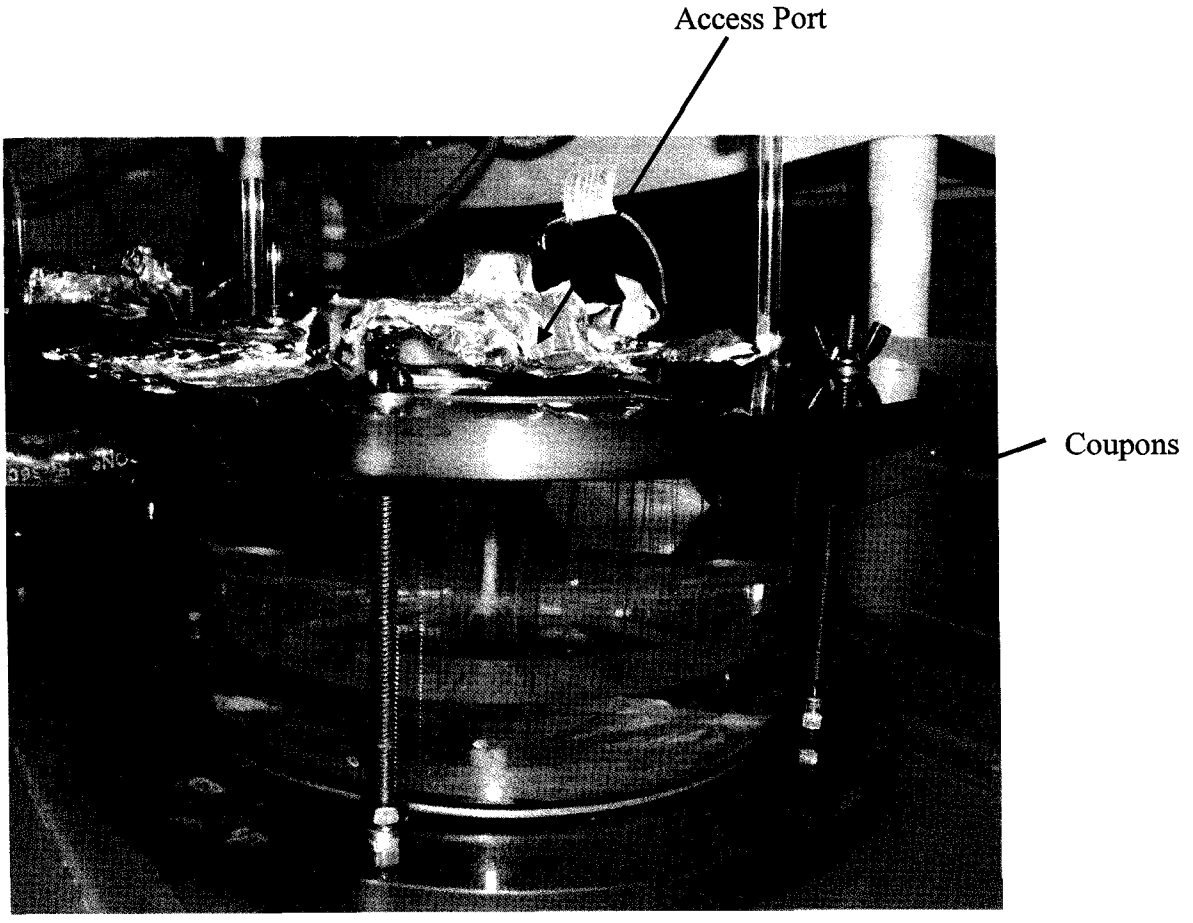
Inoculating and Running the CREM bioreactor

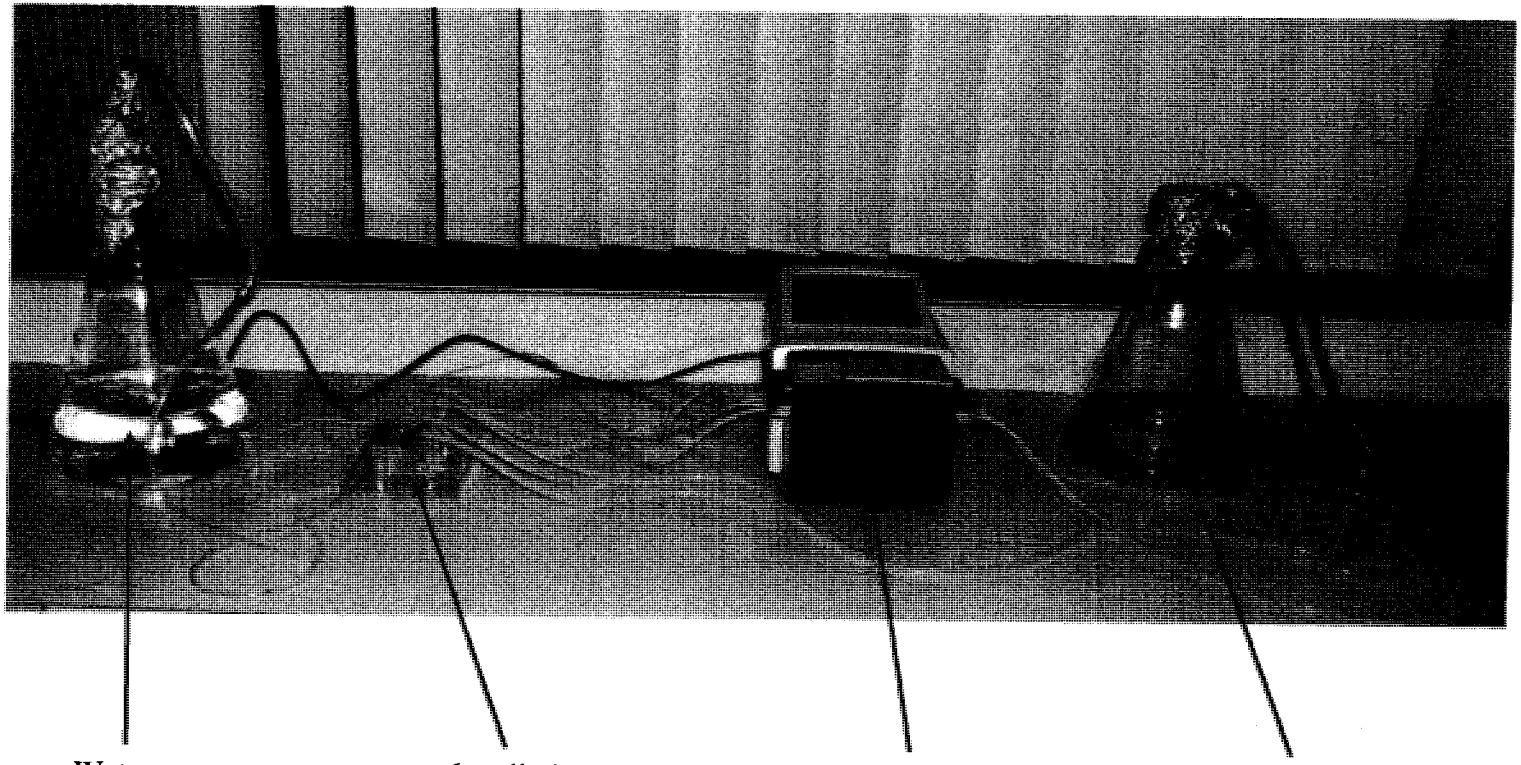
Initially, the reservoirs of the CREM bioreactors were loaded with coupons and sterilized by autoclaving prior to inoculation at the laboratory. Each reservoir was filled with 2 L of LB broth and then inoculated with 100 mL of stationary phase PA01. The inoculated reservoirs were then transported to the Lemieux Island water treatment facility and connected to the water system there. The water flow was not started for 24 hours to allow the bacteria to attach to the coupons. To allow the biofilm to mature, only unchloraminated water was used. The flow rate of the water in the internal flow was approximately 600 mL/hr, giving a water residence time of 3.3 hours, and the water temperature was 21°C. Two bioreactors were run in parallel under the same conditions.

Kadouri drip-fed Reactor

The Kadouri drip-fed reactor (Merritt et al., 2005) is a very simple device made from a 6-well plate (Figure 5). One well of the plate acts as the bioreactor chamber and 20-gauge

Figure 4. CREM bioreactor reservoir.
The reservoir contains polycarbonate coupons on which the *P. aeruginosa* biofilms are formed. The biofilm can be sampled by removing the coupons through an access port in the reservoir lid.


Access Port
Coupons


Water
6-well plate
(reactor chamber)
Pump
Waste Reservoir

needles inserted into polypropylene fittings act as the inflow and outflow ports. The volume of one reactor is approximately 5 mL. The water is pumped into the well through the inflow port and out to waste through the outflow. On the bottom of the reactor there are 8 pie-shaped polycarbonate coupons on which the biofilm will form, allowing for repeated sampling of the biofilm.

Inoculating and Running the Kadouri drip-fed Reactor

The tubing for the reactor system was sterilized between experiments by pumping through 600 mL of a 0.05% sodium hypochlorite solution, then rinsing with 1 L of sterile water. The flow rate for bleaching and rinsing was approximately 200 mL/hr. A new 6-well plate was used for every experiment. The plate containing sterile coupons was filled with 4 mL of LB broth then inoculated with 1mL of stationary phase PA14. The bacteria were allowed to attach to the coupons by incubating at 37°C for 24 hours; the reactor was then removed from the incubator as all the Kadouri drip-fed reactor experiments were run at room temperature.

For the first 24 hours of flow, only unchloraminated water was used and then treatment with chloraminated water was started on one reactor, while the control reactor received unchloraminated water. The water flow rate was 40 mL/hr. All of the water used for the experiments was brought fresh from the Lemieux Island water purification facility.

The unchloraminated water was sterilized by autoclaving at 121°C for 45 minutes, while the chloraminated water was filter sterilized using a 0.22μm membrane (Stericup), and stored at 4°C to maintain the concentration of chloramine. The concentration of chloramine in the water was determined using DPD total chlorine reagent for a 10 mL sample (HACH) and a DR/820 colorimeter (HACH). Unchloraminated water was mixed with chloraminated

water to produce the desired concentration of chloramine for the different chloramine treatments. There were three different chloramine treatment experiments: 1) treatment with 0.5 mg/L of chloramine for 15 days, 2) treatment with 1.0 mg/L of chloramine for 15 days, and 3) treatment with 0.5 mg/L of chloramine for 21 days.

Biofilm Sampling

CREM Bioreactor

The biofilms growing in the CREM bioreactor were sampled by removing two coupons from each reactor at each time point to examine biofilm development and to count the numbers of bacteria. The port at the top of the reservoir provided access to the water inside the system as well as the coupons. Each coupon was removed from the reservoir using flamed forceps and placed in a 50 mL Falcon tube containing 35 mL of reservoir water. Once transported back to the laboratory, the bacteria in the biofilm were removed from the coupon by scraping with a glass slide. The biofilm was sampled two weeks after inoculation, and once a week every week thereafter.

Kadouri drip-fed Reactor

The biofilm growing in the Kadouri drip-fed reactor was sampled by removing a coupon from each reactor at each time point to count the number of bacteria in the biofilm and obtain isolates. Each coupon was removed from the reactor using flamed forceps, and rinsed in 0.85% saline to remove any planktonic cells. The rinsed coupon was then placed in a 15 mL Falcon tube containing 5 mL of 0.85% saline. The bacteria were dispersed from the biofilm by vortexing the coupon vigorously. This bacterial suspension was used to determine the numbers of viable bacterial and to isolate antibiotic-resistant ones (see below). Some

coupons were viewed under the microscope (Leica CMI 6000B) before and after vortexing to confirm removal of the biofilm.

Bacterial Counts

The numbers of viable bacteria in the biofilms were determined by plate counts of serial dilutions as colony-forming units. The serial dilutions were made using 0.85% saline and the resulting dilutions were spread-plated on LB agar and incubated at 37°C for 24 hours. The bacteria grown in the CREM bioreactor were only culturable on LB agar for 2 weeks post inoculation. In an attempt to culture these bacteria, different culture media were used. These included R2A agar (Difco), agar plates made from CREM bioreactor reservoir water, and *Pseudomonas* Isolation Agar (PIA) (Difco). The biofilm bacteria from the CREM bioreactor were also cultured in LB broth, by scraping the bacteria from the coupon into 50 mL of LB broth and incubating the culture at 37°C for 24 hours while shaking.

Isolation of Antibiotic-Resistant Bacteria

The portion of the bacterial suspension that was not plated to determine bacterial counts was screened for the ability to grow on ciprofloxacin (1 μg/mL). The bacterial suspension was filtered by vacuum onto a filter with a pore size of 0.22 μm. The filter was then aseptically transferred to an LB agar plate containing 1 μg/mL ciprofloxacin (Sigma) and the plate was incubated at 37°C for 48 hours. Each colony that grew on the ciprofloxacin LB plates was then transferred to 5 mL of LB broth using a sterile swab and grown overnight at 37°C. A frozen stock of each ciprofloxacin-resistant isolate was prepared by adding 400 μL of 50% glycerol in water solution to 800 μL of the overnight culture and then frozen at -80°C.

Isolate Characterization

P. aeruginosa Confirmation

There were four different tests performed to confirm that the ciprofloxacin-resistant isolates were indeed *P. aeruginosa*: direct microscopic examination, Gram-staining, growth on PIA, and Vitek identification.

Microscopic Examination

A wet-mount slide of each isolate was made by placing a 10 µL drop from the overnight culture onto a clean microscope slide and covering the drop with a coverslip. The slide was viewed with the 63x objective of a Leica CMI 6000B microscope under phase contrast. A picture of the bacterial cells was taken and the isolate's cell morphology was compared to the wildtype PA14.

Gram-Staining

All of the isolates that had similar cell morphology to the wildtype PA14 were Gram-stained. A bacterial smear was made by spreading out a 10 µL drop of the overnight culture on a clean slide and leaving it to air dry; once the drop had completely dried the slide was passed through a flame three times to heat-fix the bacterial cells to the slide. The bacterial smear was completely covered with crystal violet for 30 seconds and then rinsed with distilled water. After rinsing the smear was covered with Gram's iodine for 60 seconds, and rinsed with water. The bacterial smear was then decolourized by rinsing with 75% ethanol until the runoff was no longer coloured. The smear was counter-stained with safranin for 60 seconds, rinsed with distilled water and blotted dry. The Gram-stained slide was viewed with the 63x objective of the Leica DMI 6000B microscope under bright field.

Growth on PIA

The isolates that had similar morphology to wildtype PA14 and were Gram-negative were checked for growth on Pseudomonas Isolation Agar (PIA). The overnight culture was streaked on a PIA plate using a sterile swab, and the plate was incubated at 37°C for 24 hours then examined for growth.

Vitek Identification

Some of the isolates (the first 32 obtained) that had similar morphology to wildtype PA14, were Gram-negative, and were able to grow on PIA were sent to Dr. Karamchand Ramotar at the Ottawa Hospital General Campus (Ottawa, Ontario, Canada) for identification using the Vitek 2 Identification System. The Vitek 2 system uses 47 colorimetric tests to identify fermenting and non-fermenting bacillus species, and as such it is able to positively identify *P. aeruginosa* with a very low error (Renaud et al., 2005). The test was performed as per manufacturer's recommendation using the GN card (software version WSVT2-R04.03, BioMerieux).

Growth Curves

Due to time constraints only the ciprofloxacin-resistant isolates obtained from the first replicate of all three experiments were used to determine growth rates. The growth rate of the ciprofloxacin-resistant isolates was determined and compared to the wildtype PA14 and a clinical ciprofloxacin resistant isolate (CipR#1) obtained from Wendy Ferris at the Children's Hospital of Eastern Ontario (Ottawa, Ontario, Canada). A flask containing 50 mL of LB broth was inoculated with an overnight culture to give an OD_{600} of approximately 0.07. The optical density was measured at a wavelength of 600 nm using a Spectronic 20D+ (Thermon Electron Corp.), using 3 mL of culture in a glass spec tube. The flask was shaken

at 37°C for 7 hours, and the optical density was measured every 30 minutes. Significance of the differences in growth rate were determined by performing a T-test on the growth rate of the cultures during exponential growth, only those with a p-value less than 0.05 were considered significant.

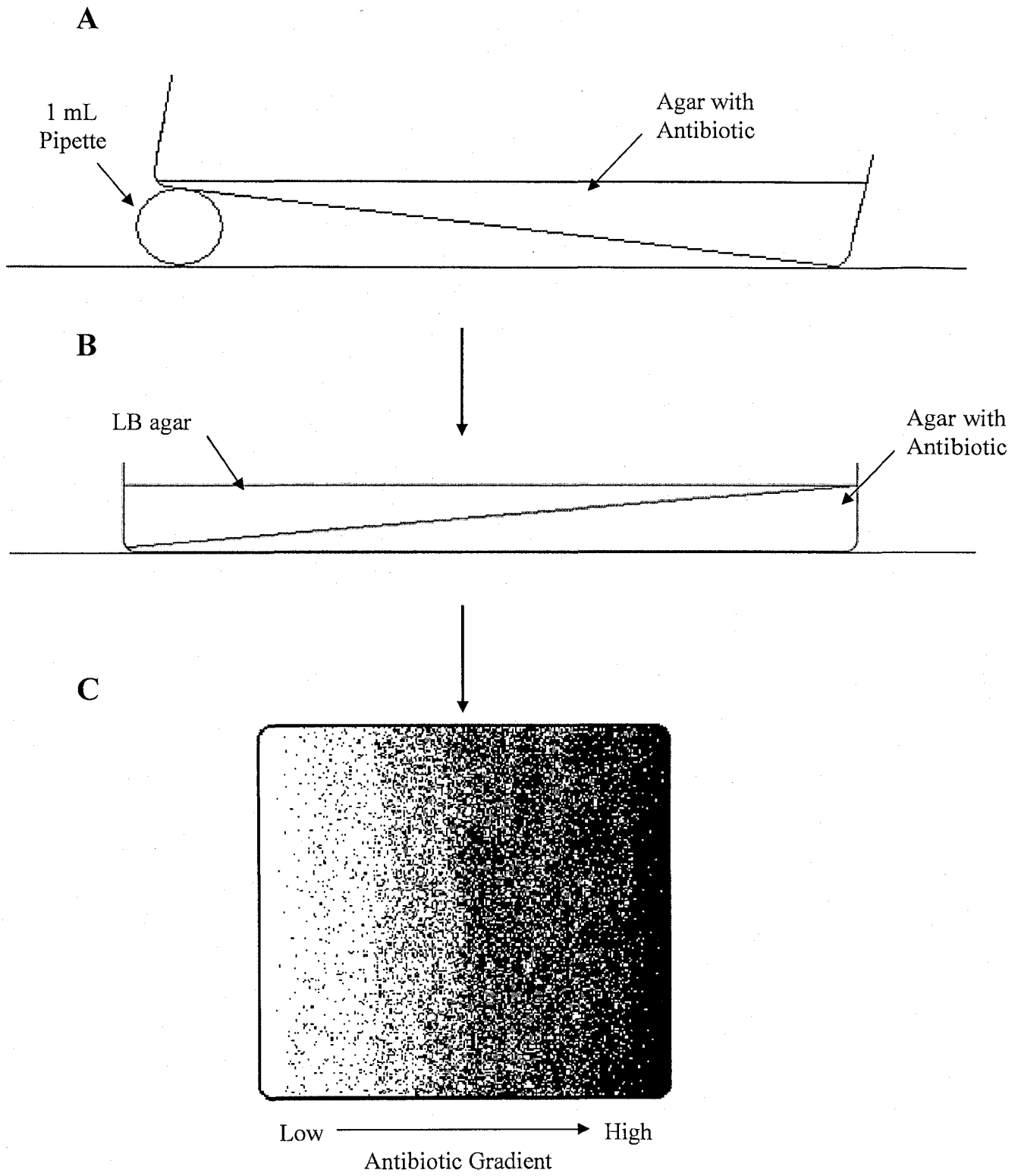
Determining Level of Antibiotic Resistance

Ciprofloxacin Gradient Plates

All confirmed *P. aeruginosa* isolates were assessed for the general level of ciprofloxacin resistance using ciprofloxacin gradient plates (Bryson and Szybalski, 1952). The gradient plates were prepared using two layers of LB agar, one without antibiotic and the other having 10 μg/mL of ciprofloxacin. The agar containing 10 μg/mL of ciprofloxacin was poured into a square Petri plate so the bottom of the plate was covered, and then one side of the plate was lifted to rest on a 1 mL pipette (Figure 6A). Having the plate on an angle allowed for an increasing depth of antibiotic-containing agar across the plate. Once the first layer of antibiotic had hardened the plate was once again set level on the bench-top and the second layer of agar was poured on top; just enough agar was added to completely cover the first layer (Figure 6B). The gradient plate was refrigerated for at least 24 hours to allow the antibiotic to migrate through the agar and establish the gradient, which was approximately 0 μg/mL at one side and approached 10 μg/mL at the opposite side (Figure 6C).

The gradient plate was inoculated with an overnight culture of the isolate as well as the wildtype PA14 as a negative control and CipR#1 as a positive control; the positive control has a minimum inhibitory concentration (MIC) for ciprofloxacin of 4 μg/mL (MIC predetermined by CHEO). The different strains were inoculated onto the gradient plate using a sterile swab that was soaked with the overnight culture and then streaking a single line

Figure 6. Making the antibiotic gradient plates.
Agar containing antibiotic is poured into a square petri dish to just cover the bottom of the plate. A-One side of the plate is elevated and placed on a 1 mL pipette until the agar hardens, then the pipette is removed. B-The antibiotic layer is overlaid with LB agar. C-The antibiotic forms a gradient across the plate.


A
1 mL
Pipette
Agar with
Antibiotic
B
LB agar
Agar with
Antibiotic
C
Low
High
Antibiotic Gradient

across the antibiotic gradient. The plate was incubated at 37°C for 24 hours and then a picture of the growth was taken using a gel documentation system under fluorescent lighting.

MIC Assays

The Minimum Inhibitory Concentration (MIC) assay is an assay strictly used to determine the concentration of an antibiotic required to inhibit the growth of planktonic bacteria. An MIC assay was performed for five different antibiotics: ciprofloxacin, tobramycin (Sigma), gentamicin (Sigma), rifampicin (Sigma) and chloramphenicol (Fisher Scientific). The MIC assays were determined according to the standard set by the Clinical Laboratory Standards Institute (CLSI) (2007). An overnight culture of the isolates as well as the controls described for the gradient plates were used for this assay; 8 μL of the overnight cultures were diluted into 16 mL of LB broth. Then 2x dilutions of the antibiotic listed above were prepared. The dilutions used for each antibiotic are listed in Table 1. In sterile culture tubes, 0.5 mL of the 2x antibiotic dilution or 0.5 mL of LB broth with no antibiotic was mixed with 0.5 mL of the diluted overnight culture. The tubes were incubated at 37°C for 24 hours and then examined for growth.

Table 1. Antibiotic concentrations for MIC assay.
The 2x concentrations are the working dilutions prepared, 500 μL of the working dilutions is mixed with 500 μL of the bacterial dilution to give the final concentrations.

Results

Results

Minimum Bactericidal Concentration

In order to confirm that the concentration of chloramine used as a drinking water residual disinfectant does not kill *P. aeruginosa* when growing in a biofilm, I performed MBC-B and MBC-P assays with both PA01 and PA14. Here in Ottawa, the concentration of chloramine that is added to the drinking water as a residual disinfectant is between 1.5 and 2.2 mg/L (City of Ottawa, 2006). The MBC assays allowed for a direct comparison of the concentration of an antimicrobial that is able to inhibit 99.99% of the bacteria when grown either planktonically or in a biofilm. When a 1/1,000,000 dilution of an overnight culture was used for the MBC-P assay, both the PA01 and PA14 bacteria were killed by 1 mg/L of chloramine (Figure 7). When a 1/1,000,000 dilution of an overnight culture was used for the MBC-B assay, both the PA01 and PA14 bacteria were killed by 100 mg/L of chloramine (Figure 7). This meant that the *P. aeruginosa* biofilms grown in one of the reactors could be treated with the same concentrations of chloramine found in the drinking water distribution system, and much higher concentrations without killing the biofilm bacteria.

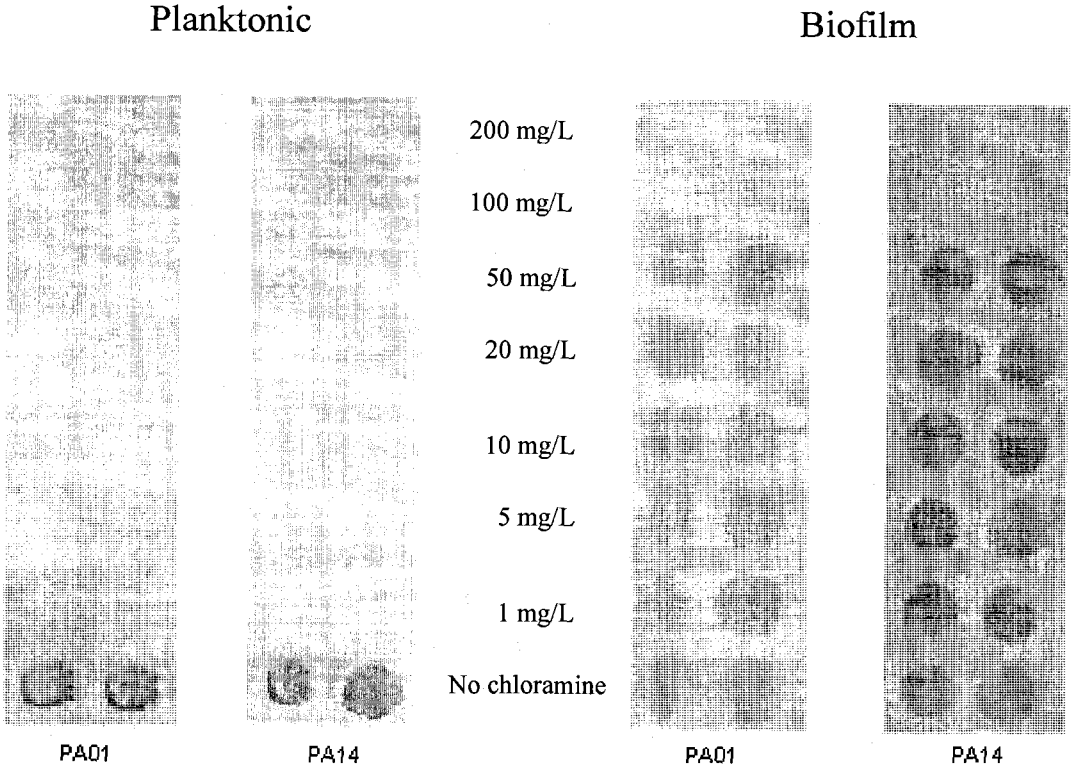
Growth of Biofilms in Drinking Water Systems

The main part of this project was setting up a system where biofilms of *P. aeruginosa* could be grown in drinking water. In doing this 3 experiments were performed with the CREM bioreactor, and 3 experiments were performed with the Kadouri drip-fed reactor.

CREM bioreactor experiments:

1) No chloramine, 105 days
2) No chloramine, 35 days
3) No chloramine, 64 days

Figure 7. Growth from biofilm and planktonic MBC assay for PA01 and PA14.
If the bacteria are not killed by the concentration of chloramine in the different wells of the plate, a spot of bacterial growth can be seen on LB agar. Using a 1/1,000,000 dilution of the saturated culture, the biofilm PA01 and PA14 were killed by 100 μg/mL of chloramine and the planktonic PA01 and PA14 were killed by 1 μg/mL of chloramine.


Planktonic
Biofilm
200 mg/L
100 mg/L
50 mg/L
20 mg/L
10 mg/L
5 mg/L
1 mg/L
No chloramine
PA01
PA14
PA01
PA14

Kadouri drip-fed reactor experiments: 1) 0.5 mg/L chloramine, 15 days

2) 1.0 mg/L chloramine, 15 days

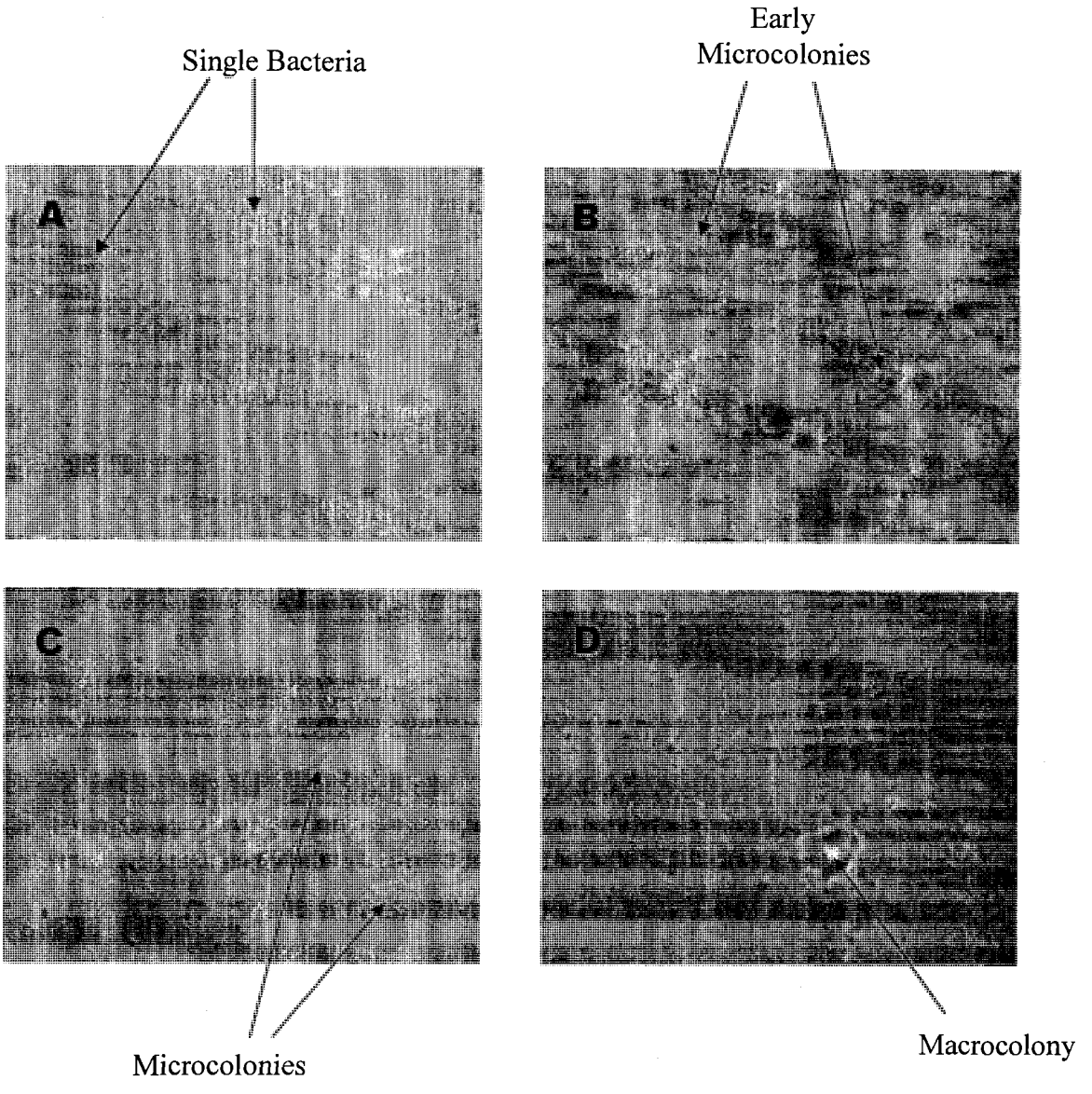
3) 0.5 mg/L chloramine, 21 days

CREM bioreactor

For the first attempt to grow the drinking water biofilms, 2 CREM bioreactors were set-up at the Lemieux Island water purification facility (please see Materials and Methods section for an overview; Figs. 3 and 4). Over a period of 12 weeks (Feb 2006 – June 2006), *P. aeruginosa* PA01 formed a biofilm on the coupons in the CREM bioreactors. *P. aeruginosa* PA01 was used because it is a commonly used laboratory strain that easily forms biofilms.

As can be seen in Figure 8, the bacteria attached to the coupons on day 1 and formed microcolonies that grew over time. After 2 weeks of growth in drinking water, the number of biofilm bacteria was greater than 10^7 CFU/coupon, based on plate counts (on LB agar) of the sampled coupons. After 3 weeks of growth in drinking water, bacterial isolates from the system did not grow on LB agar plates; however, when the bacteria were grown in liquid LB, the culture became turbid after 24 hours. In the subsequent weeks, I attempted to culture the biofilm bacteria on the nutrient-rich media, LB agar and the minimal media, R2A agar, as well as agar made from the CREM bioreactor overflow water, but there was no bacterial growth from any of these. In week 12, I was able to culture the biofilm bacteria by plating on PIA; however, the counts were only $1\text{-}3\text{x}10^2$ CFU/coupon, suggesting that not all of the biofilm bacteria were able to grow on PIA. PIA is selective for pseudomonads as it contains

Figure 8. Biofilm development in the CREM bioreactor.
A-bacteria have attached to the coupon (day 1), B-density of the attached bacteria increases and they begin to clump together (day 14), C-microcolonies have formed (day 42), D-microcolonies increase in height becoming a mature biofilm (day 56).


Single Bacteria
Early
Microcolonies
A
B
C
D
Microcolonies
Macrocolony

Irgasan, which inhibits other types of bacteria (Schweizer, 1998).

In the subsequent 2 experiments with the CREM bioreactor, the bacteria did not form mature biofilms (Figure 9), and the number of bacteria attached to the coupons steadily declined after inoculation. More specifically, after the initial incubation to allow the bacteria to attach to the coupons the bacterial counts on both LB agar and PIA were 10^8 CFU/coupon. However, after 7 days of growing in drinking water, the bacterial counts on both LB agar and PIA were 10^6 CFU/coupon, and after 35 days, the bacterial counts dropped to 10^3 CFU/coupon. At this point the experiment was terminated. Similar bacterial counts were obtained from the third experiment with the CREM bioreactor, and when *P. aeruginosa* PA14 was used. Therefore, this reactor was not used for subsequent experiments because the wildtype PA01 was unable to grow and form biofilms in the reactor. It is possible that there was a change in the characteristics of the water after the first experiment because the other parameters (bacterial strain, the number of bacteria inoculated, the time for their attachment) were not altered.

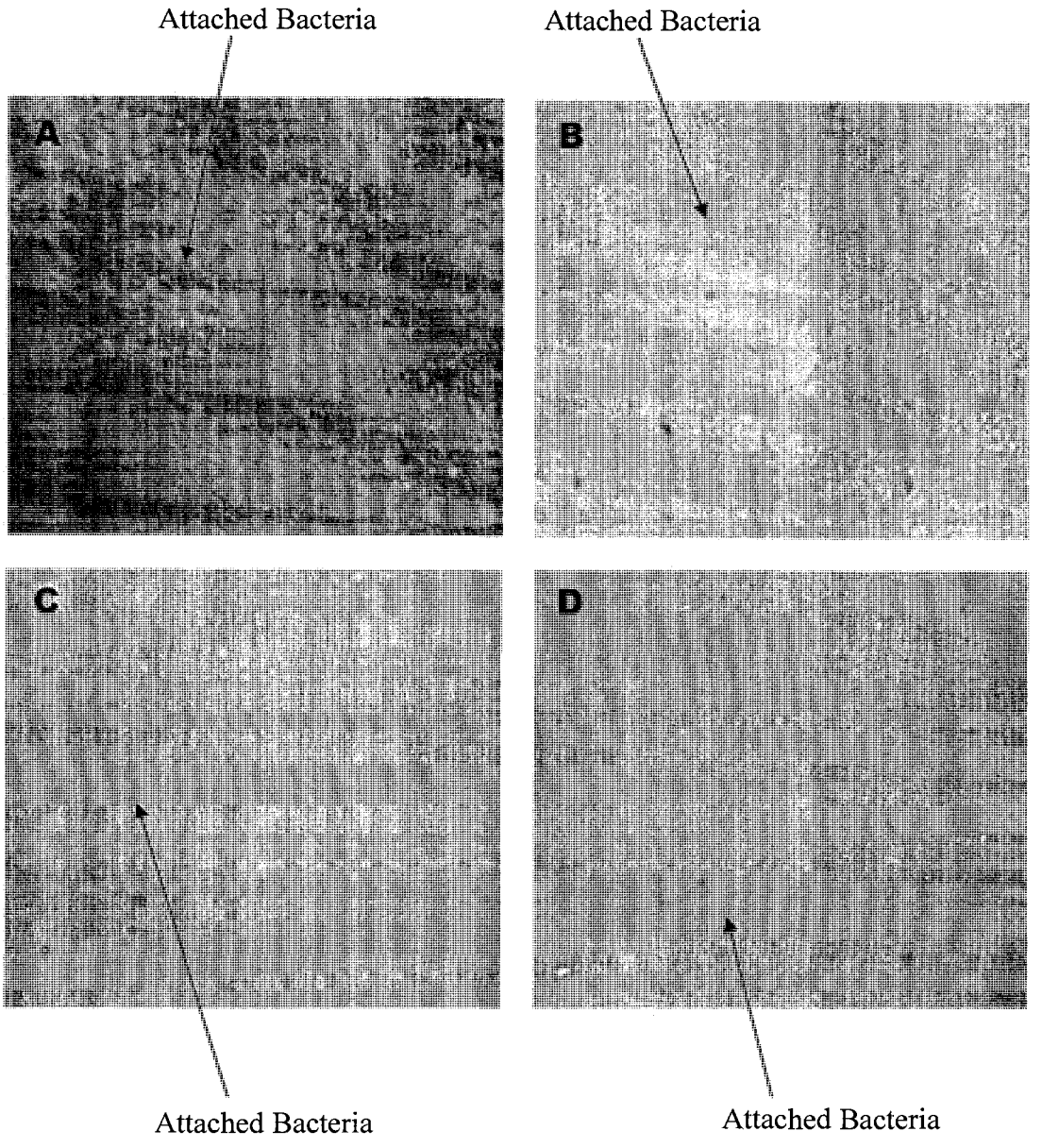
Kadouri drip-fed Reactor

This system was used because it is much smaller in scale; one well in a 6-well plate is used as a reactor (Figure 5), the total volume of a reactor being only 5 mL. Also, this system could be run in the laboratory, thus made monitoring the system and sample collection much easier.

Bacterial Persistence

Before the biofilms could be treated with chloramine, the ability of the wildtype *P. aeruginosa* to form biofilms in the Kadouri drip-fed reactor had to be established. If the bacteria could not form a biofilm in this reactor, then treating the bacteria with chloramine

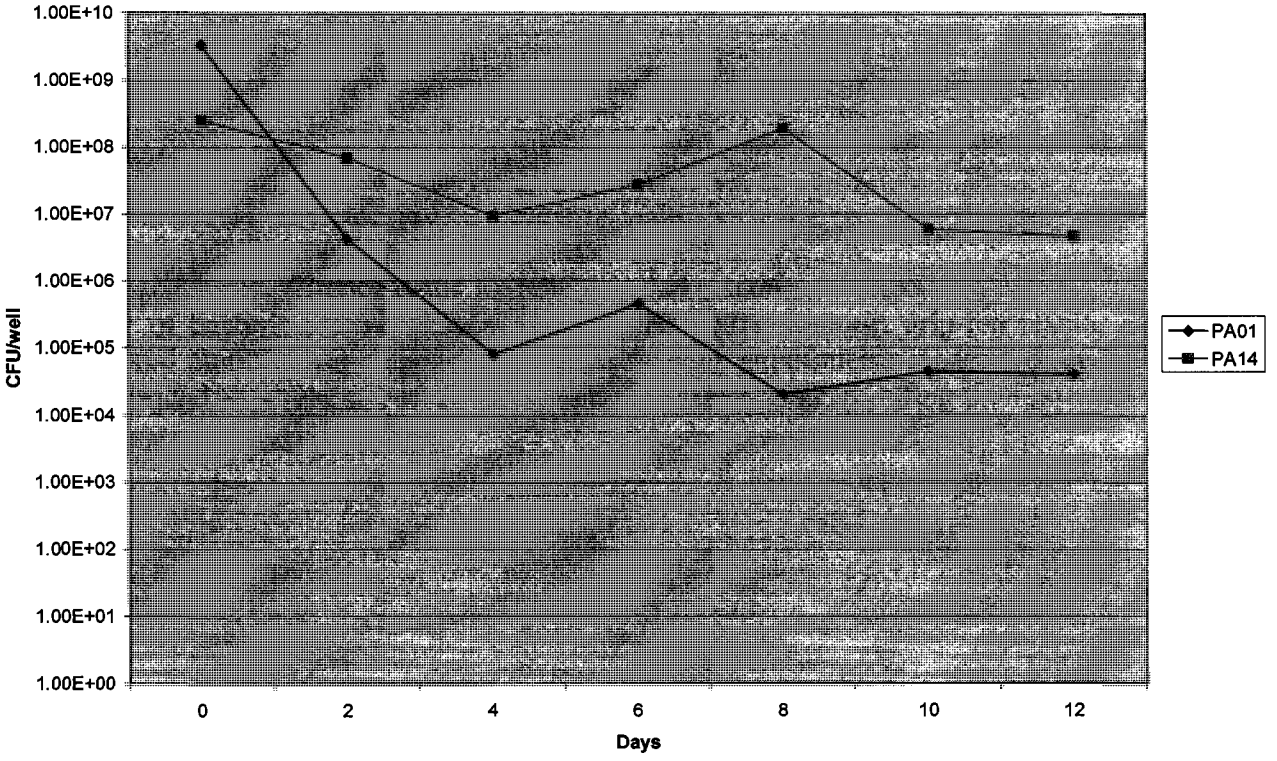
Figure 9. Failure to form biofilms in the CREM bioreactor.
A-bacteria have attached to the coupons (day 1). B-decrease in bacterial density. C and D-virtually no bacteria attached to coupons (day 10 and day 24 respectively).

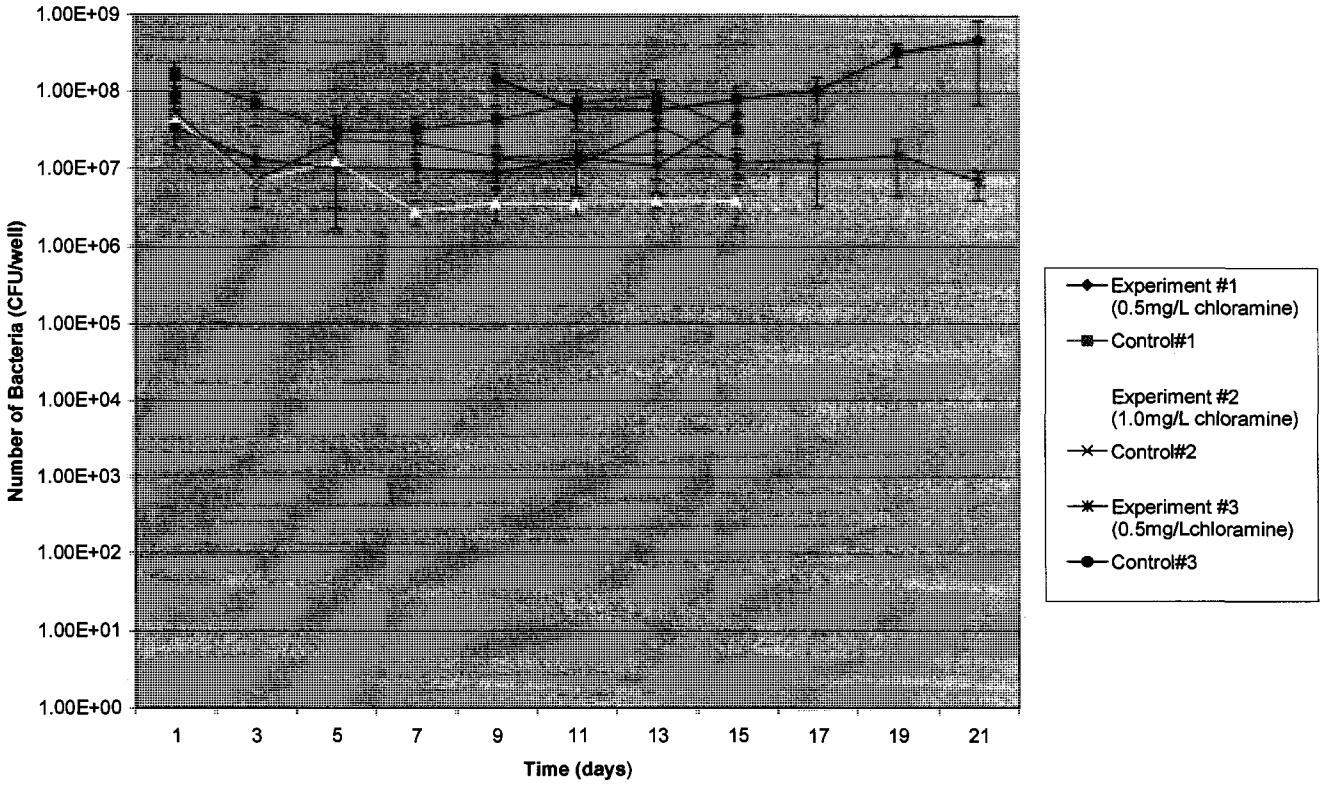

Attached Bacteria
Attached Bacteria
A
B
C
D
Attached Bacteria
Attached Bacteria

would kill all of the bacteria. Using the Kadouri drip-fed reactor the persistence of *P. aeruginosa* PA01 and *P. aeruginosa* PA14 as separate biofilms was compared. In a mature biofilm the bacterial numbers should remain stable; this was the objective. The bacteria in LB broth were allowed to attach (during a 24-hour incubation) to the polycarbonate coupons that were placed on the bottom of the well. The LB broth was replaced with water over 24hours and a coupon was then removed to sample the biofilm. The sampling took place once every 2 days, over a period of 14 days. The PA14 strain formed a biofilm that was able to persist over the course of the experiment, while the cell numbers in the PA01 biofilm dropped over the first 4 days of the experiment (Figure 10). Since PA14 is able to persist better in drinking water using the Kadouri drip-fed reactor, it was used to grow the biofilms for treatment with chloramine and the generation of ciprofloxacin-resistant isolates.

Three different experiments using PA14 biofilms in the Kadouri drip-fed reactor were performed. The experiments as outlined earlier were: 1) treatment with 0.5 mg/L of chloramine for 15 days, 2) treatment with 1.0 mg/L of chloramine for 15 days, and 3) treatment with 0.5 mg/L of chloramine for 21 days. The bacterial counts on day 1 for all of the experiments were between $3x10^7$ CFU/well and $2x10^8$ CFU/well. Over the course of experiment 1 (15 days), the biofilm bacterial counts for the treated biofilm, as well as the control biofilm, decreased by less than 1 log unit (Figure 11). Over the course of experiment 2 (15 days), the bacterial counts decreased by 1 log for the treated biofilm, and less than 1 log for the control biofilm. When the bacteria were treated with chloramine for a longer period of time (experiment 3, 21 days), the bacterial counts from the treated biofilm decreased by 1 log, while the control biofilm counts remained approximately the same. The samples taken

Figure 10. Persistence of *P. aeruginosa* strains PA01 and PA14 in drinking water using the Kadouri drip-fed reactor.


1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/well
0
2
4
6
8
10
12
Days
PA01
PA14


1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Number of Bacteria (CFU/well)
1
3
5
7
9
11
13
15
17
19
21
Time (days)
Experiment #1 (0.5mg/L chloramine)
Control#1
Experiment #2 (1.0mg/L chloramine)
Control#2
Experiment #3 (0.5mg/Lchloramine)
Control#3

to determine bacterial counts were also used to isolate bacteria that could grow in the presence of ciprofloxacin.

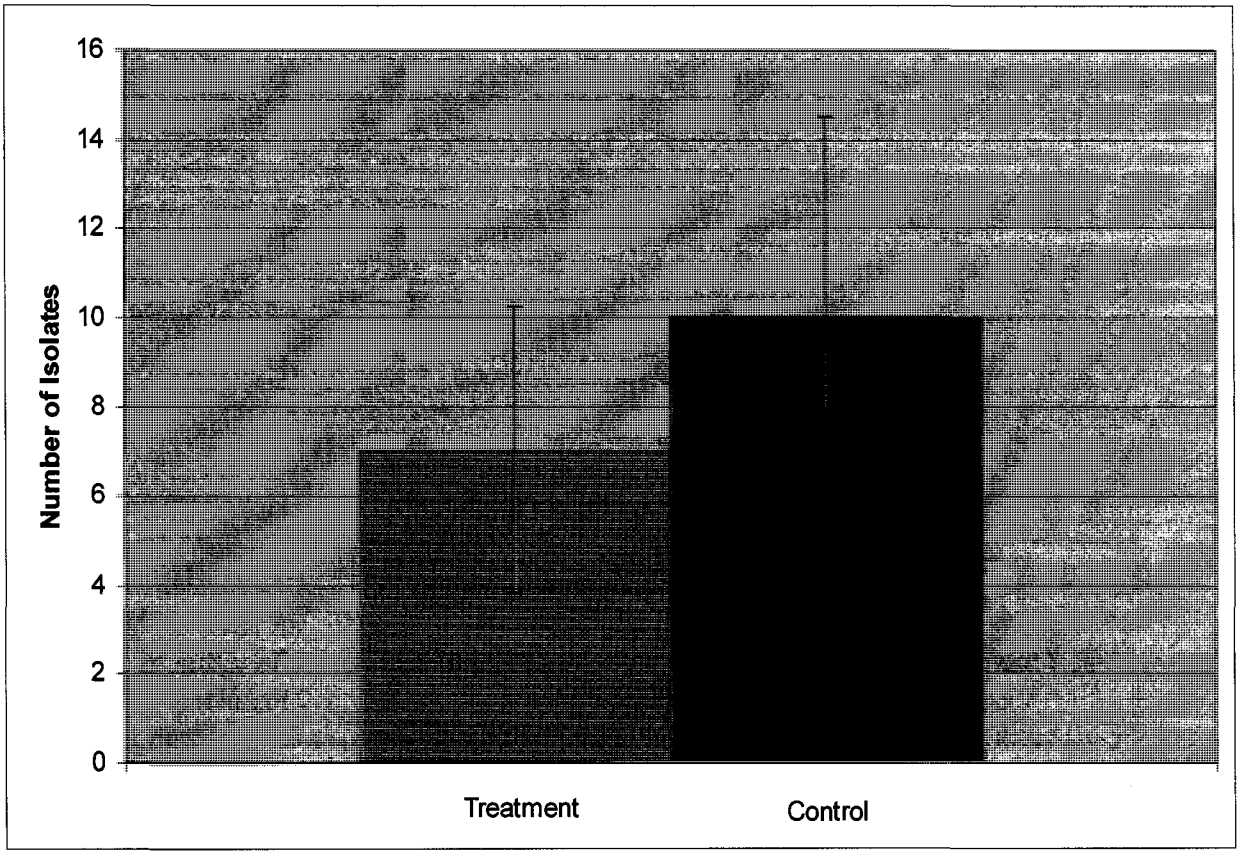
Ciprofloxacin-Resistant Isolates

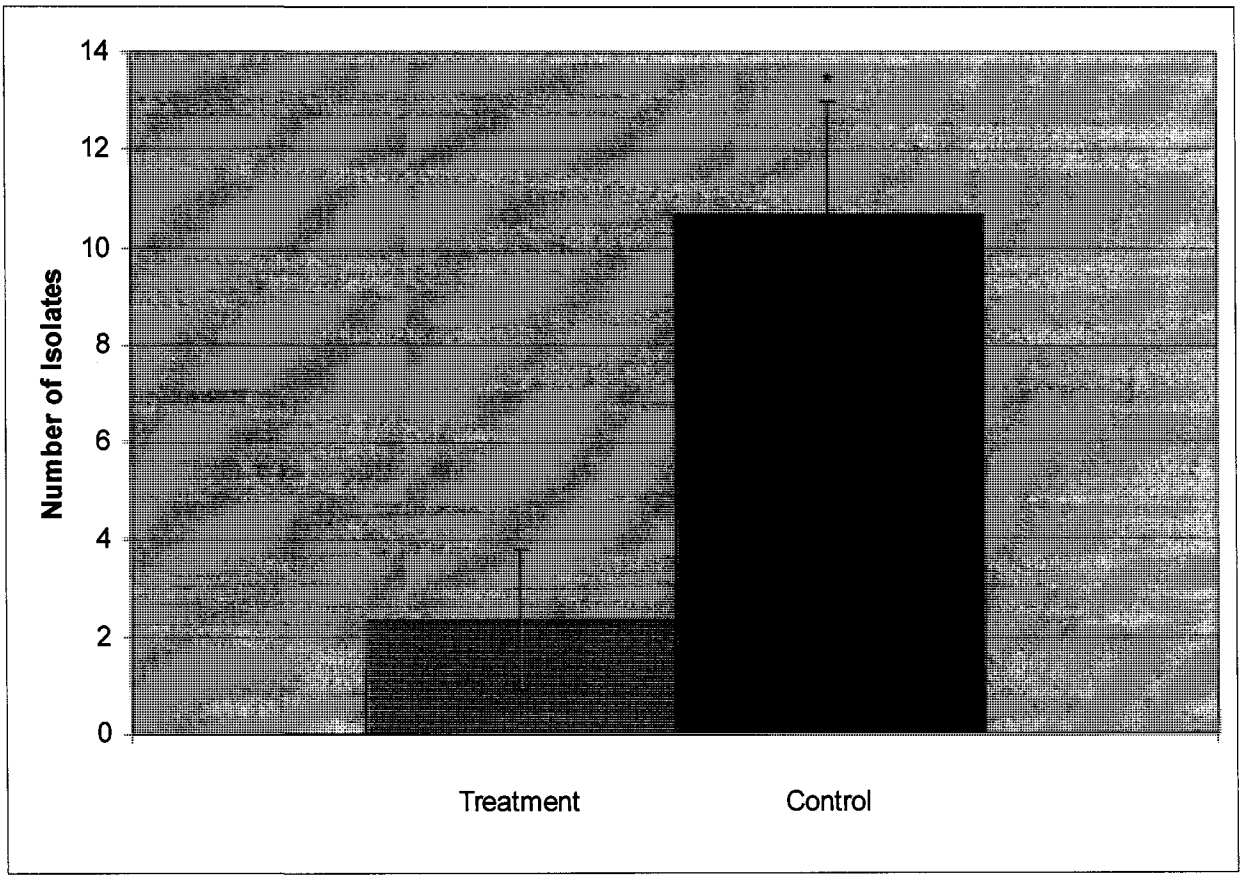
In order to determine if exposure to chloramine leads to antibiotic resistance, the bacteria isolated from the biofilms growing on the coupons from the Kadouri drip-fed reactor were plated on LB agar plates containing 1.0 μg/mL ciprofloxacin, a concentration that kills the planktonic form of wildtype *P. aeruginosa.* Isolates that grew on ciprofloxacin were obtained from both the treated and the control biofilms; in total 174 isolates were obtained from the 3 replicates of the different experiments that were run. When the biofilms were treated with 0.5 mg/L of chloramine for 15 days, the average number of isolates obtained from the treated biofilms was 7, and from the control biofilms was 10 (Figure 12); however, this is not statistically significant, based on a T-test.

When the biofilms were treated with 0.5 mg/L of chloramine for 21 days, the average number of isolates obtained from the treated biofilms was 2.3, and from the control biofilms was 25.7 (Figure 14). However, this is not a significant difference because there was a very large standard error with the number of isolates from the control biofilms, because one of the trials produced 66 isolates while another only produced 1. When the biofilms were treated with 1.0 mg/L of chloramine for 15 days, the average number of isolates obtained from the treated biofilms was 2.3, and from the control biofilms was 10.7 (Figure 13); in this case the control biofilms produced significantly more isolates able to grow on ciprofloxacin.

There was a lot of variation in the number of ciprofloxacin-resistant isolates obtained from the replicates of all the experiments. In one replicate of experiment #3, the control biofilm produced 66 ciprofloxacin-resistant isolates, while the chloramine treated biofilm

Figure 12. Number of ciprofloxacin-resistant isolates recovered from control and treated biofilms of experiment 1.
The treated biofilm was exposed to 0.5 mg/L of chloramine for 15 days. The experiment was performed three times. Error bars represent the standard error of the mean.


16
14
12
10
8
6
4
2
0
Number of Isolates
Treatment
Control


14
12
10
8
6
4
2
0
Number of Isolates
Treatment
Control

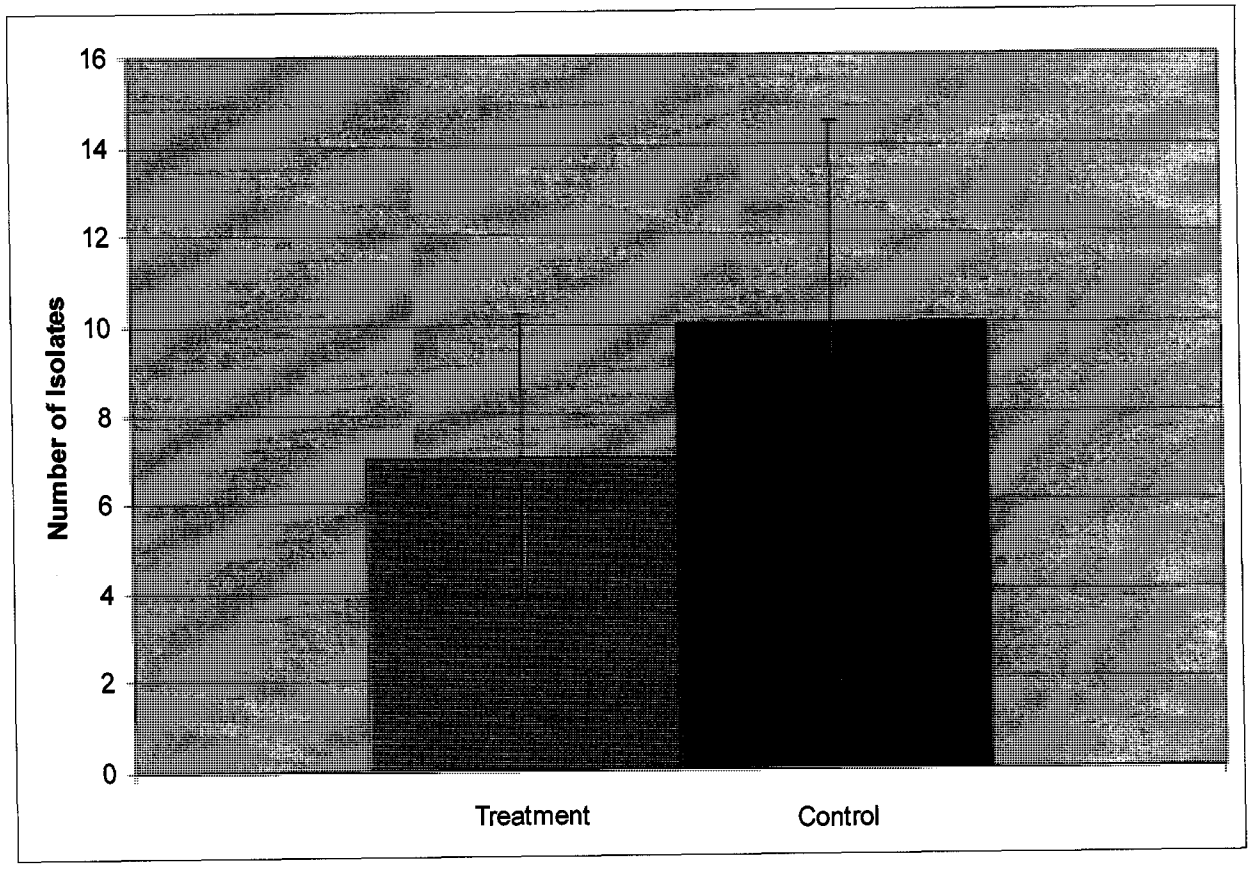

16
14
12
10
8
6
4
2
0
Number of Isolates
Treatment
Control

Figure 13. Number of ciprofloxacin resistant isolates recovered from control and treated biofilms of experiment 2.
The treated biofilm was exposed to 1.0 mg/L of chloramine for 15 days. The experiment was performed three times. Error bars represent the standard error of the mean. The control biofilms produced significantly more ciprofloxacin resistant isolates than the treated biofilms, as indicated by the star.

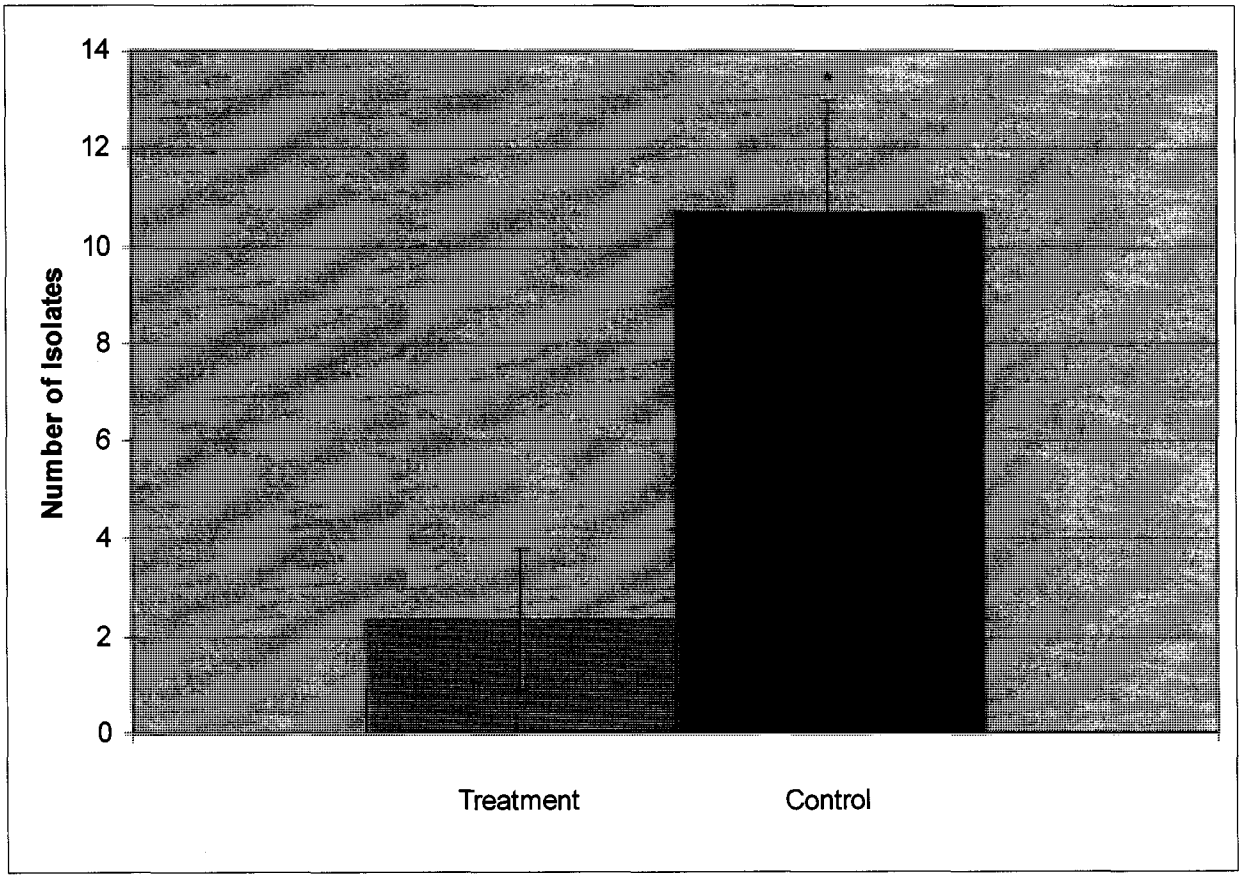
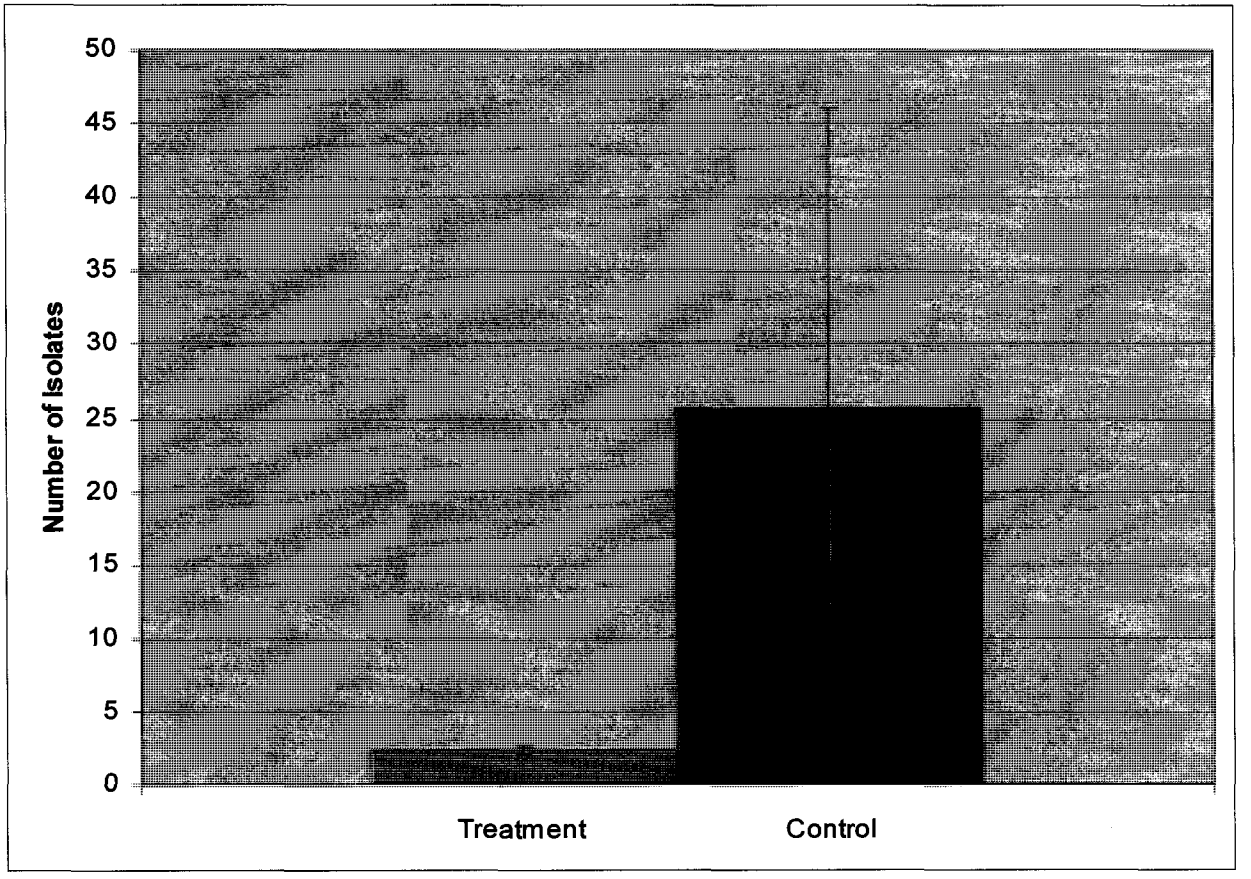

14
12
10
8
6
4
2
0
Number of Isolates
Treatment
Control

Figure 14. Number of ciprofloxacin resistant isolates recovered from control and treated biofilms of experiment 3.
The treated biofilm was exposed to 0.5 mg/L of chloramine for 21 days. The experiment was performed three times. Error bars represent the standard error of the mean.


50
45
40
35
30
25
20
15
10
5
0
Number of Isolates
Treatment
Control

only produced 3 ciprofloxacin-resistant isolates. In another replicate of the same experiment, the control biofilm only produced 1 ciprofloxacin-resistant isolate and the chloramine treated biofilm produced 2 ciprofloxacin-resistant isolates. The statistics are performed using the averages, so the great variability seen decreases the statistical significance, even though there were more ciprofloxacin-resistant isolates obtained from the control biofilms than the chloramine treated biofilms in all of the experiments. In fact, 139 of the ciprofloxacin-resistant isolates were obtained from the control biofilms, while only 35 were obtained from the chloramine treated biofilms. Even though both biofilms were growing in drinking water, the biofilms that were treated with chloramine produced fewer ciprofloxacin-resistant isolates, indicating that perhaps the chloramine was inhibiting ciprofloxacin resistance.

Characterization of Ciprofloxacin-Resistant Isolates

Since I inoculated the Kadouri drip-fed reactors with a pure culture of *P. aeruginosa*, I expected to recover only *P. aeruginosa*. However, in order to confirm that the ciprofloxacin resistant isolates were *P. aeruginosa*, I conducted a series of confirmatory experiments.

P. aeruginosa Confirmation

Of the 174 isolates that grew on the LB agar containing 1 μg/mL ciprofloxacin, 9 did not grow when subcultured into liquid LB to make a stock, so they were not further characterized. When the remaining isolates were viewed under the microscope, all but two had similar cell morphology to the inoculum (*P. aeruginosa* PA14). The morphology of these two bacteria was coccus and filamentous bacillus, and so these isolates were rejected as being *P. aeruginosa*. The remaining 163 isolates proved to be Gram-negative and were able to grow on PIA, indicating that they were *P. aeruginosa*. The first 32 isolates identified as *P.*

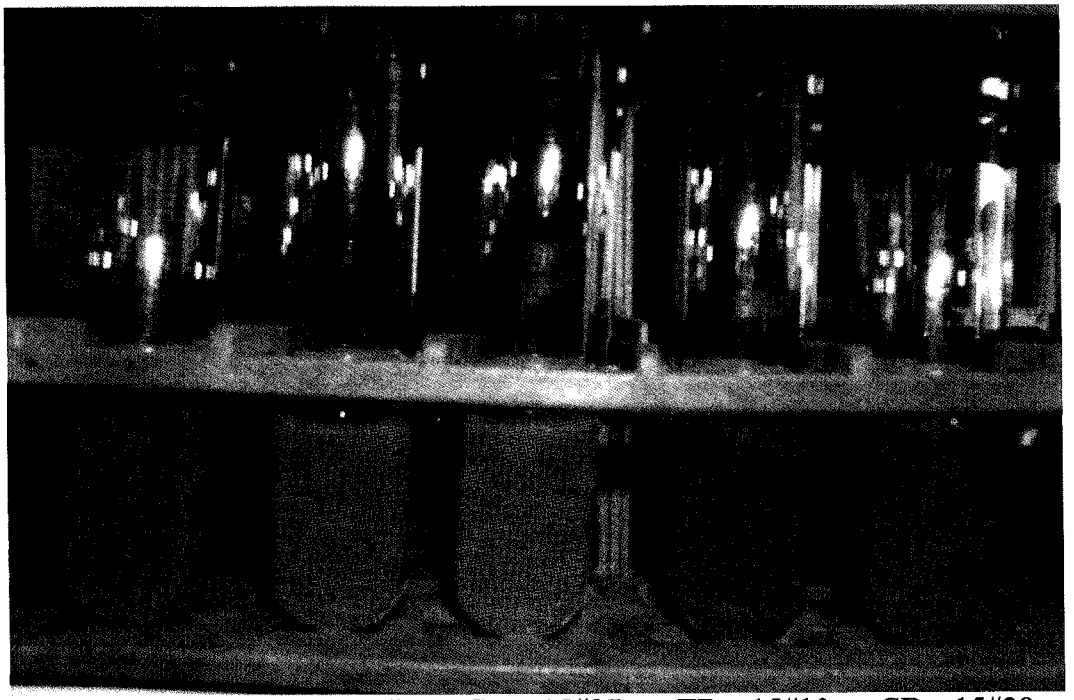
aeruginosa were also confirmed to be *P. aeruginosa* using the Vitek 2 system with a 99% probability (performed at the Ottawa General Hospital).

The ciprofloxacin resistant isolates had additional phenotypic differences from the wildtype PA14 aside from the resistance to ciprofloxacin. One example was the colour of the culture when the isolate was grown in LB broth (Figure 15). In stationary phase, the wildtype PA14 culture was bright green, while some of the isolates were also bright green during stationary phase (ex. isolate TDay15#10), other isolates were more yellow (ex. isolate CDay15#27), the colour of the culture was not determined by whether the isolate was obtained from a control or treated biofilm. Generally, liquid cultures of the wildtype PA14 in LB are green when they reach saturation because of the presence of pyocyanin. Pyocyanin is a blue secondary metabolite that is secreted by *P. aeruginosa* that is considered a virulence factor because it has been shown to be toxic to eukaryotic cells in-vitro (Lau et al., 2004). Therefore, the isolates may produce lower levels of pyocyanin.

Growth Rates

Growth curves were constructed to determine if the ciprofloxacin resistant isolates had any additional phenotypic differences from the wildtype PA14. The 3 ciprofloxacin-resistant isolates obtained from experiment 1 (treated with 0.5 mg/L of chloramine for 15 days) had a slower growth rate in LB broth compared to the wildtype PA14 during the exponential phase, but the difference was not significant (Figure 16). The exponential growth rate of the 2 ciprofloxacin-resistant isolates obtained from the biofilm treated with 1.0 mg/L of chloramine for 15 days (exp. 2) was significantly slower than the growth rate of the wildtype PA14 (Figure 17). The 14 ciprofloxacin resistant isolates obtained from experiment

Figure 15. Saturated liquid cultures of ciprofloxacin-resistant isolates.
The cultures were grown overnight in LB broth with agitation. From left to right they are: wildtype PA14, CDay15#26, CDay15#27, TDay15#10 and CDay15#28. The isolate identifier indicates if the isolate was obtained from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated.



PA14 CDay15#26 CDay15#27 TDay15#10 CDay15#28

Figure 16. Growth curve of ciprofloxacin-resistant isolates from experiment 1.
Only isolates obtained from the first replicate of experiment 1 were used. The wildtype PA14 and CipR#1 (a clinical ciprofloxacin resistant isolate) were used as controls, and the assays were performed three times. The isolate identifier indicates if the isolate was obtained from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated.

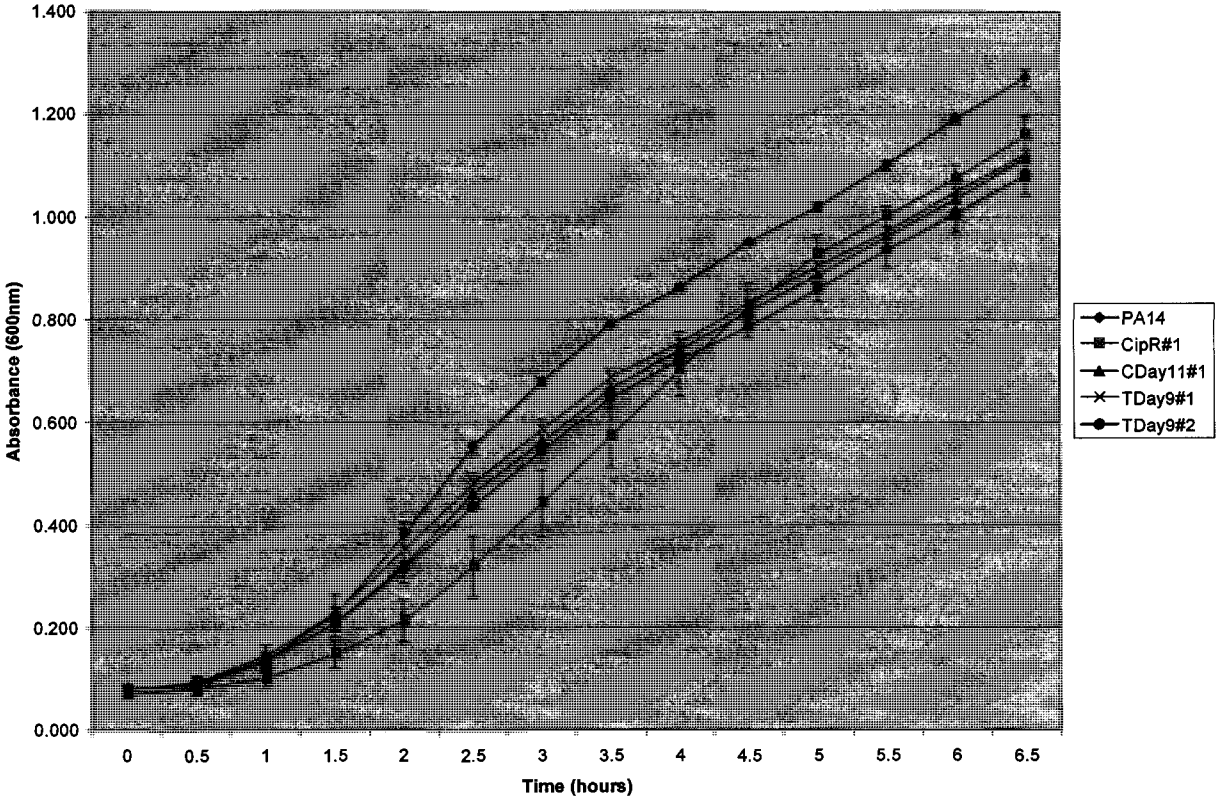
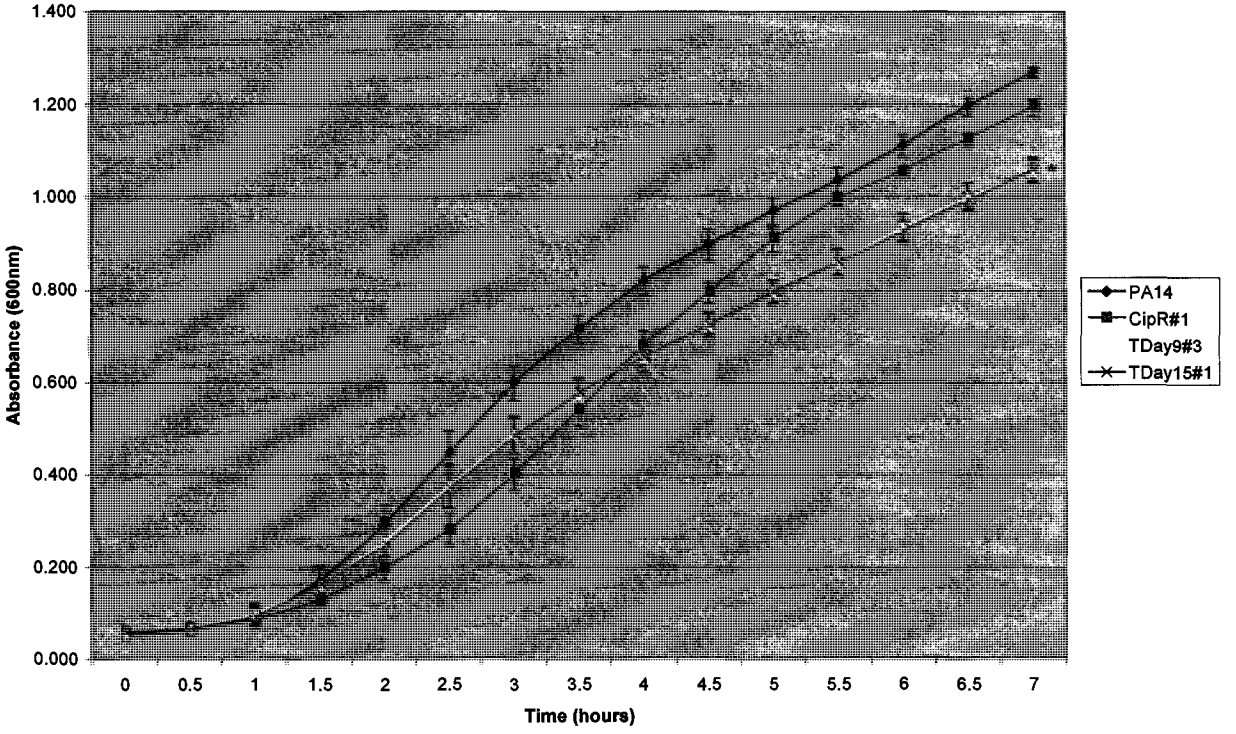

1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Absorbance (600nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
Time (hours)
PA14
CipR#1
CDay11#1
TDay9#1
TDay9#2

Figure 17. Growth curve of ciprofloxacin-resistant isolates from experiment 2.
Only isolates obtained from the first replicate of experiment 2 were used. The wildtype PA14 and CipR#1 (a clinical ciprofloxacin resistant isolate) were used as controls, and the assays were performed three times. The isolate identifier indicates if the isolate was obtained from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated. Both of the isolates had significantly slower growth during the exponential phase, as indicated by the star.


1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Absorbance (600nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
Time (hours)
PA14
CipR#1
TDay9#3
TDay15#1

3 (treated with 0.5 mg/L of chloramine for 21 days) had different rates of growth in LB broth; 7 of the isolates had the same growth rate as the wildtype PA14, while the other 7 had lower growth rates; however, the difference was not significant (Figure 18). None of the ciprofloxacin-resistant isolates tested achieved the same cell density as the wildtype after growing for 24 hours in LB broth (Table 2), with the exception of the 7 isolates from the biofilm treated with 0.5 mg/L of chloramine for 21 days (experiment 3) that also had similar growth rates to the wildtype. This indicates that most of the ciprofloxacin-resistant isolates have a growth impairment, even when growing in nutrient rich media.

Antibiotic Resistance

In order to examine more closely the antibiotic-resistance phenotype of the isolates, growth on ciprofloxacin gradient plates was assayed and MIC assays with different antibiotics were performed. The ciprofloxacin gradient plates allowed for a direct comparison between the resistance of CipR#1 (a ciprofloxacin resistant mutant obtained from Wendy Ferris at Children's Hospital of Eastern Ontario) and the ciprofloxacin resistant isolates. A single streak of a saturated culture was made across the antibiotic gradient of the gradient plates. Since there was a gradient of ciprofloxacin across the plates the length of the culture growth indicates how much antibiotic the bacteria can grow on. All of the isolates, as well as CipR#1 grew on the ciprofloxacin gradient plates, while the wildtype PA14 did not (Figure 19). Comparisons could not be made between plates, because the antibiotic gradients were not always the same on the different plates. In this case, the distance that the isolate grew on one plate had been compared to the distance that CipR#1 grew on the same plate. For the most part, the isolates grew between half as far as CipR#1 or a similar length (Figure 19), indicating that the ciprofloxacin resistant isolates had an MIC similar to that of CipR#1.

Figure 18. Growth curve of ciprofloxacin-resistant isolates from experiment 3.
Only isolates obtained from the first replicate of experiment 3 were used. The wildtype PA14 and CipR#1 (a clinical ciprofloxacin resistant isolate) were used as controls, and the assays were performed three times. The isolate identifier indicates if the isolate was obtained from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated.

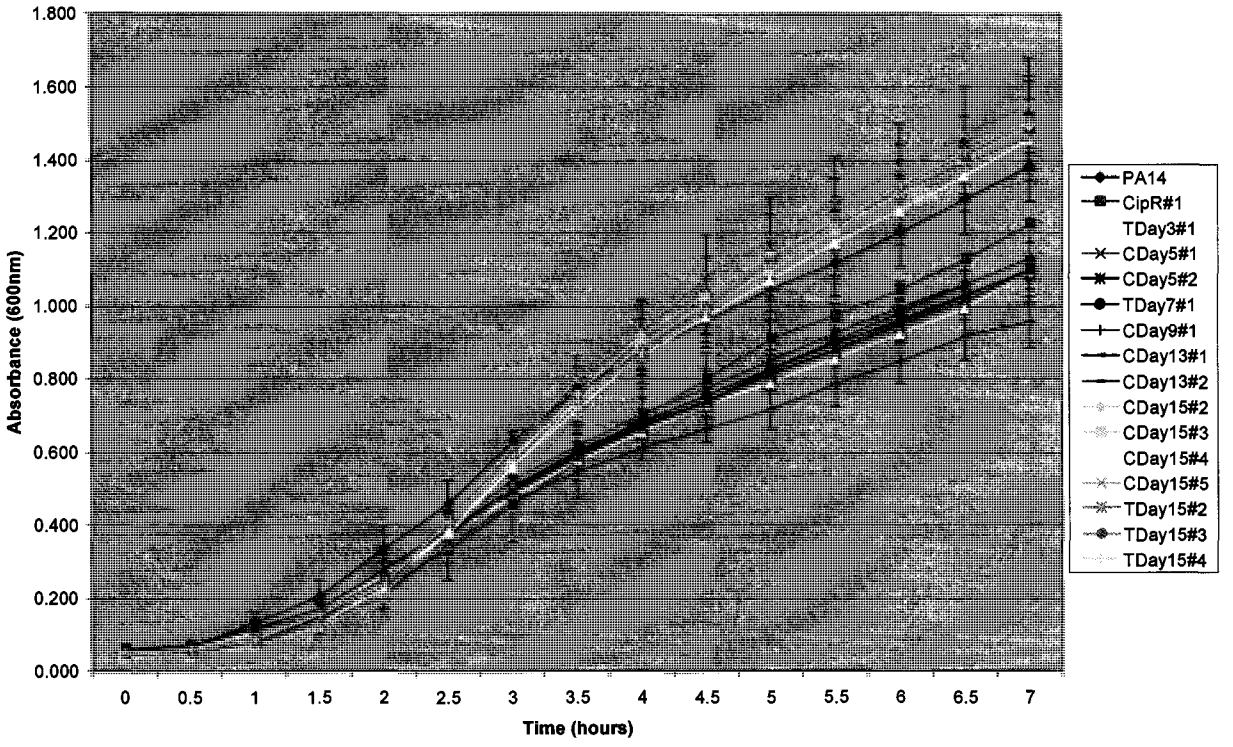

1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Absorbance (600nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
Time (hours)
PA14
CipR#1
TDay3#1
CDay5#1
CDay5#2
TDay7#1
CDay9#1
CDay13#1
CDay13#2
CDay15#2
CDay15#3
CDay15#4
CDay15#5
TDay15#2
TDay15#3
TDay15#4

Table 2. Optical density of 24 hour cultures.
The optical density of the ciprofloxacin resistant isolates from the first replicate of all three experiments grown in LB broth for 24 hours and diluted 10 times, was measured. Experiment 1 was treated with 0.5 mg/L chloramine for 15 days, experiment 2 was treated with 1.0 mg/L chloramine for 15 days, and experiment 3 was treated with 0.5 mg/L chloramine for 21 days. The isolate identifier indicates if the isolate was obtained from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated. The isolates that had similar optical densities as the wildtype PA14 are indicated with a star.

		OD_{600} (1/10 dilution)
	Wt	0.520
	CipR#1	0.390
Experiment 1	CDay11#1	0.462
	TDay9#1	0.404
	TDay9#2	0.382
Experiment 2	TDay9#3	0.348
	TDay15#1	0.335
Experiment 3	TDay3#1	0.331
	CDay5#1	0.329
	CDay5#2	0.362
	TDay7#1	0.444
	CDay9#1	0.424
	CDay13#1	0.428
	CDay13#2	0.424
	CDay15#2	0.518*
	CDay15#3	0.510*
	CDay15#4	0.500*
	CDay15#5	0.535*
	TDay15#2	0.488*
	TDay15#3	0.494*
	TDay15#4	0.496*

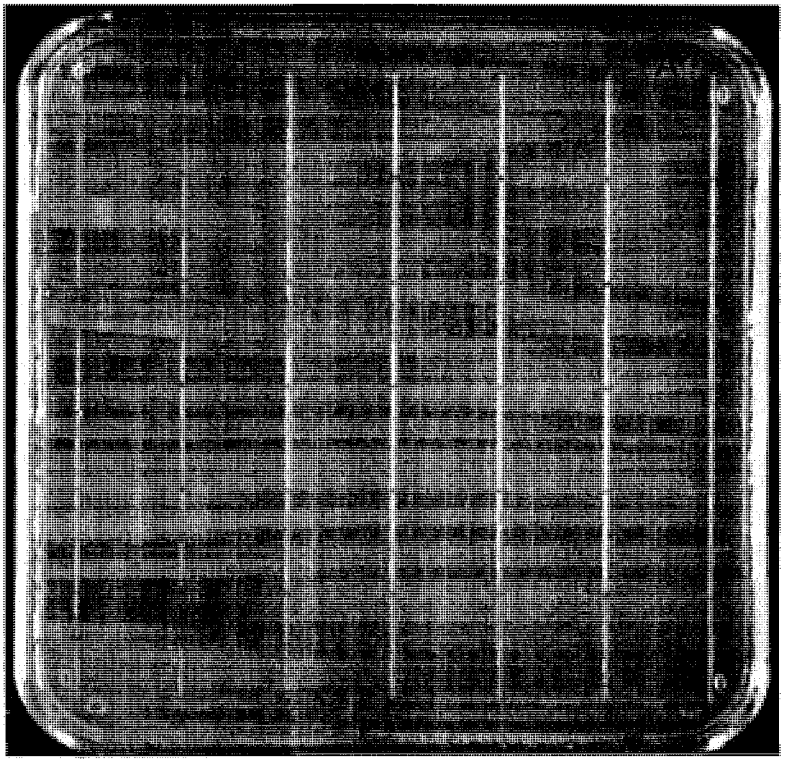
Figure 19. Growth on ciprofloxacin gradient plates.
Two examples of ciprofloxacin gradient plates: A-the isolates CDay11#8 and CDay11#9 grow approximately half the length of CipR#1(a clinical ciprofloxacin resistant isolate), B-the isolate CDay15#1 grows a similar length as CipR#1 (a clinical ciprofloxacin resistant isolate). The isolate identifier indicates if the isolate was obtained from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated.

CDay11#9

CipR#1

PA14 wt

CDay11#8



PA14 wt

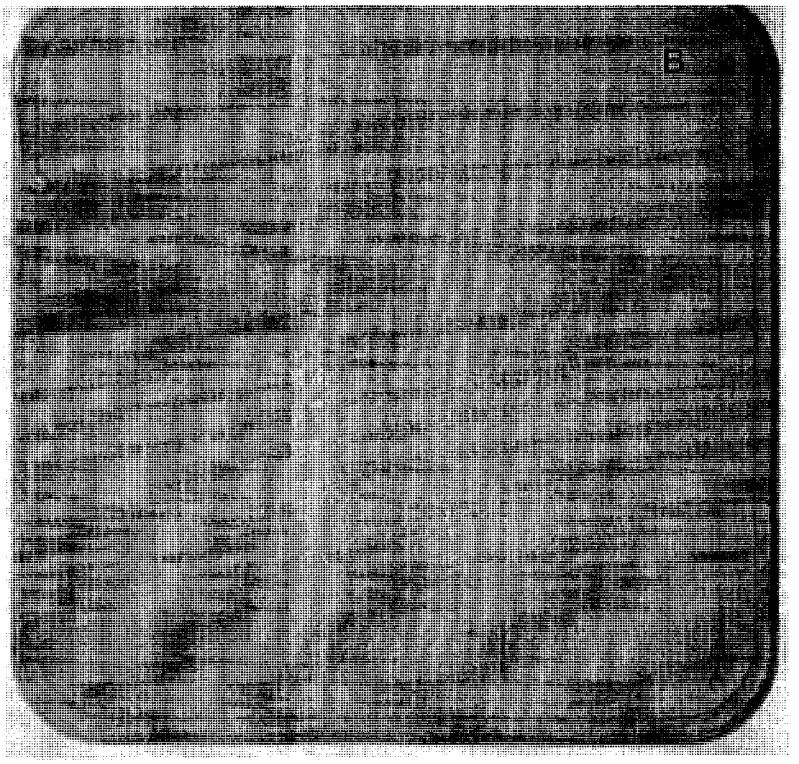
CDay15#1

CipR#1

PA14 wt

CDay15#1

CipR#1



If the isolates were only resistant to ciprofloxacin, the mechanism of resistance would likely be mutations to the antibiotic target, DNA gyrase. However, if the isolates were also resistant to antibiotics of other classes, the resistance mechanism would be a multi-drug resistance mechanism such as the hyper-expression of multi-resistance efflux pumps. In order to determine the isolates level of resistance for different antibiotics, MIC assays were performed. Most of the isolates were highly resistant to ciprofloxacin and had MIC values of 4 μg/mL, which is 8 times greater than the MIC for the wildtype PA14 (Table 3). Interestingly, the isolates were not resistant to tobramycin or gentamicin. In fact, the MIC values for the isolates were in most cases actually 2 or 4 times lower than the wildtype PA14 (Table 3). Most of the ciprofloxacin resistant isolates were also more resistant to rifampicin and chloramphenicol, with MIC values greater than 128 μg/mL for rifampicin and greater than 800 μg/mL for chloramphenicol (Table 3). These MIC values indicate that the increased antibiotic resistance of the ciprofloxacin resistance isolates is due to a multi-drug resistance mechanism.

Table 3. Multi-drug sensitivity of ciprofloxacin-resistant isolates.
MIC values of ciprofloxacin, tobramycin, gentamicin, rifampicin and chloramphenicol for the ciprofloxacin resistant isolates from the first replicate of all three experiments. Values are based on the assay being performed in duplicate. The mutant identifier indicates if the mutant was isolated from the control or treated biofilm (C or T), as well as the day of the experiment when it was isolated.

	Isolate	MIC				
		Ciprofloxacin	Tobramycin	Gentamicin	Rifampicin	Chloramphenicol
	PA14 wt	0.25µg/mL	4µg/mL	8µg/mL	32µg/mL	50µg/mL
	CipR#1	4µg/mL	>32µg/mL	>32µg/mL	32µg/mL	50µg/mL
Exp. #1	CDay11#1	4µg/mL	2µg/mL	4µg/mL	16µg/mL	>800µg/mL
	TDay9#1	4µg/mL	2µg/mL	4µg/mL	16µg/mL	>800µg/mL
	TDay9#2	4µg/mL	2µg/mL	4µg/mL	16µg/mL	>800µg/mL
Exp. #2	TDay9#3	4µg/mL	2µg/mL	2µg/mL	64µg/mL	>800µg/mL
	TDay15#1	4µg/mL	2µg/mL	1µg/mL	64µg/mL	>800µg/mL
Exp. #3	TDay3#1	2µg/mL	4µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay5#1	2µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay5#2	4µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	TDay7#1	4µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay9#1	4µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay13#1	4µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay13#2	4µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay15#2	2µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay15#3	1µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay15#4	1µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	CDay15#5	2µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	TDay15#2	2µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	TDay15#3	2µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL
	TDay15#4	2µg/mL	2µg/mL	2µg/mL	>128µg/mL	>800µg/mL

Discussion

Discussion

It is difficult to maintain the quality of drinking water inside the distribution system because biofilms form inside the pipes. Like all biofilms, these drinking water distribution system biofilms are highly resistant to antimicrobials (Frias et al., 2001; Cochran et al., 2000). In order to control the growth of bacteria in the drinking water distribution system, disinfectants such as chloramine are added to the drinking water before it enters the distribution system. However, due to the innate resistance of biofilm bacteria, drinking water biofilms are able to persist and grow inside the drinking water distribution system in the presence of the residual disinfectant.

Residual Chloramine Concentration

Here in Ottawa, the concentration of chloramine that is added to the drinking water as a residual disinfectant is between 1.5 and 2.2 mg/L (City of Ottawa, 2006), the concentration used is dependant on seasonal fluctuations in water quality. However, the concentration of chloramine in the water decreases as the water travels through the drinking water distribution system. While the concentration of chloramine in the system can range from 1.5 mg/L in areas closer to the water treatment facility, to as low as 0.3 mg/L in the outer limits of the system; the average concentration of chloramine throughout the system is approximately 1.0 mg/L (City of Ottawa, 2005). At these concentrations, planktonic bacteria are killed while biofilm bacteria are not (van der Wende et al., 1989). I used an MBC assay (Mah et al., 2003) to determine if this was also the case for the test organisms, *P. aeruginosa* PA14 and PA01. For either strain of *P. aeruginosa*, the planktonic bacteria were killed by the chloramine at the concentration typically found in the drinking water distribution system, while the biofilm bacteria could survive much higher concentrations of chloramine. This

means that these bacteria are able to survive the concentration of chloramine that is maintained throughout the drinking water distribution system, but only when growing in a biofilm.

Growing Drinking Water Biofilms

The CREM bioreactors were constructed to grow biofilms using drinking water at the water purification facility. Having the reactor at the water purification facility would allow for the use of drinking water that had already been purified before it travels through the drinking water distribution system, either with or without chloramine. As well, this reactor would allow for a more long-term experiment because each reactor is able to hold up to 50 polycarbonate coupons; so, even if 3 coupons are removed every 2 days for sampling, the experiment could run for 31 days.

The *P. aeruginosa* PA01 had no difficulty attaching to the polycarbonate coupons in the reactor, because after the 24 hour attachment period a large number of bacteria could be seen on the coupons (Figures 9A & 10A), and upon enumeration there was 10^8 CFU/coupon. During the first experiment using this reactor system, the biofilm was able to form and grow, but after 2 weeks I was unable to culture the biofilm bacteria on LB agar. These bacteria became viable but non-culturable (VBNC), likely due to the low nutrient conditions of the drinking water (Roszak and Colwell, 1987). In low nutrient conditions bacteria can become temporarily inactive and cannot be cultured on nutrient-rich media (Roszak and Colwell, 1987); however, these bacteria can sometimes be cultured on minimal media or selective media. The VBNC PA01 did not grow on agar made using the reactor water as a carbon source, or the minimal media R2A, but were able to grow on pseudomonas isolation agar (PIA). PIA contains the antimicrobial Irgasan (triclosan) which does not inhibit

pseudomonads, but does inhibit the growth of other bacteria (Schweizer, 1998). During the subsequent experiments using the CREM bioreactors the bacteria were enumerated using both LB agar and PIA.

During the subsequent 2 experiments with the CREM bioreactors, the bacteria again were able to attach to the polycarbonate coupons, but once the water flow was initiated and the medium was replaced with water, the number of bacteria attached to the coupons decreased, suggesting that they were either detaching from the coupon or that they were dying over the course of the experiment (Figure 10). These results were puzzling as there were no changes made to the protocol between the first and subsequent experiments; so, the difference between them was likely due to changes in the drinking water, a variable that I had no control over. In fact, upon investigation after the completion of the third experiment that I ran at the Lemieux Island facility, I learned that the GAC/BAC filters at the water treatment plant had been replaced over the period of time that the first experiment was running (Barry Duchene, City of Ottawa Water Quality Technologist, personal communication); so, by the time the second experiment was started, all of the water was passing through the new filters. GAC filters effectively remove AOC from the water. These filters are colonized by bacteria from the raw water, which in turn, increases the filters' ability to remove chlorine and chlorine by-products from the water (Uhl and Hartmann, 2005; Ellis et al., 1999). However, over time, the efficiency of the GAC/BAC filter decreases and they must be replaced. Therefore, when the filters were replaced at the Lemieux Island water purification facility, the concentration of AOC must have decreased, even though the total organic carbon (TOC) remained fairly steady before and after changing the filters (City of Ottawa, 2005). A large

decrease in the AOC has been shown to limit bacterial growth and biofilm formation in drinking water (Volk and LeChevallier, 1999).

With a lower concentration of AOC available in the water from the Lemieux Island facility, other parameters had to be changed in order to make conditions more favorable for the formation of drinking water biofilms. The Kadouri drip-fed reactor is a smaller-scale system, so there are fewer bacteria consuming the limited supply of AOC. The flow rate of the Kadouri drip-fed reactor is slower than that of the CREM bioreactor, but because the volume of the Kadouri drip-fed reactor is much smaller (only 5 mL), its volume exchange time is much greater. This means that the biofilm bacteria are getting more AOC when growing in the Kadouri drip-fed reactor and this may be why I was able to grow *P. aeruginosa* biofilms in drinking water using the Kadouri drip-fed reactor but not the CREM bioreactor.

Generation of Ciprofloxacin-Resistant Isolates

It is because biofilm bacteria are resistant to chloramine that there are resident bacteria in the drinking water system. I exploited the fact that there are resident bacteria in the system, and exposed them to chloramine while growing the biofilm in drinking water with low levels of AOC. However, I isolated planktonic cells from the biofilms looking for antibiotic resistant isolates.

The wildtype PA14 is susceptible to ciprofloxacin: its MIC is 0.25μg/mL. Using plates containing 1 μg/mL of ciprofloxacin as the selection medium ensures that any isolates obtained from the drinking water biofilm have a marked increase in ciprofloxacin resistance. All 174 of the ciprofloxacin resistant isolates were Gram-stained, examined for *P. aeruginosa* morphology and plated onto PIA to identify them as *P. aeruginosa*. Furthermore,

32 of these isolates were confirmed as *P. aeruginosa* using the Vitek 2 system. Not all of the identified *P. aeruginosa* isolates were sent for confirmation with the Vitek 2 system for two different reasons. Firstly, only the ciprofloxacin-resistant isolates obtained from the first replicate of all three experiments were further characterized, with growth curves and MICs, so only these isolates had to have their identification as *P. aeruginosa* confirmed. And secondly, because all of the isolates identified as *P. aeruginosa* using Gram-stain reaction morphology and growth on PIA were positively confirmed by the Vitek 2 system, so the additional confirmation seemed redundant for the remaining isolates. Only the ciprofloxacin-resistant isolates obtained from the first replicate of the experiments were further characterized mostly due to time constraints, and using isolates from the different experiments would be the best representatives for all of the isolates.

I chose the three different experiments used with the Kadouri drip-fed reactor to highlight any differences in the number of ciprofloxacin resistant isolates with higher concentrations of chloramine or longer growth in the drinking water. In the first experiment I used a low concentration of chloramine (0.5 mg/L), in order to ensure that the number of bacteria in the biofilms were maintained, while still being within the range of chloramine found in the city of Ottawa water distribution system. The time frame used was mainly dictated by only having 8 coupons to sample the biofilm; the experiment could be run for only 15 days while removing one coupon every 2 days. By increasing the chloramine concentration to 1.0 mg/L, I was looking to see if more ciprofloxacin-resistant isolates were obtained from the drinking water biofilms. If more ciprofloxacin-resistant isolates were obtained from the biofilms treated with 1.0 mg/L of chloramine than the biofilms treated with 0.5 mg/L of chloramine, this would support my hypothesis that the exposure to chloramine

stresses the bacteria, resulting in an increase in antibiotic-resistant bacteria. Finally, for the third experiment, the length was extended from 15 days to 21 days, to see if growing the biofilm in the drinking water for a longer period of time had a greater impact on the generation of antibiotic-resistant bacteria than an increase in chloramine concentration.

Overall, in the three different experiments performed using the Kadouri drip-fed reactor, treating the drinking water biofilm with chloramine did not produce more ciprofloxacin-resistant isolates than the control. In fact, when the biofilm was treated with chloramine, more ciprofloxacin resistant isolates were obtained from the control biofilms than the treated ones. This indicates that chloramine treatment does not cause ciprofloxacin resistance in drinking water biofilm bacteria, but perhaps growing in drinking water may cause antibiotic resistance. Chloramine disinfection decreases glucose metabolism (Chen et al., 1993), thus slowing the growth of biofilm bacteria, if the biofilm bacteria treated with chloramine were growing slower than the control biofilm bacteria fewer mutations would arise and fewer ciprofloxacin-resistant isolates would be generated.

Antibiotic Resistance

Ciprofloxacin was used as the selective factor to isolate antibiotic resistant bacteria because it is one of the antibiotics used to treat *P. aeruginosa* lung infections in those with cystic fibrosis (CF) (Canton et al., 2005). Ciprofloxacin is taken by CF patients with stable *P. aeruginosa* infections to prevent flare-ups, and also when there is a *P. aeruginosa* flare-up (Canton et al., 2005). However, *P. aeruginosa* is highly adaptable and can rapidly develop resistance to a variety of antibiotics including ciprofloxacin (Lambert, 2002).

Planktonic antibiotic resistance can be achieved in three different ways: restricted drug uptake or increased drug efflux, enzymatic inactivation and alteration of the target

(Lambert, 2002). The antibiotic resistant isolates obtained from the drinking water biofilms were resistant to ciprofloxacin, rifampicin and chloramphenicol, but not tobramycin and gentamicin suggesting that the mechanism of resistance is not specific for ciprofloxacin, but more general in nature. Such a mechanism could be increased efflux of the antibiotics due to the activation or increased activity of multi-drug efflux pumps.

As previously described, drinking water is an oligotrophic environment, where there is very little organic carbon available for the biofilm bacteria to utilize (LeChevallier et al., 1991). Also, the drinking water used to grow and treat the biofilms was especially low in AOC because the GAC/BAC filters were recently changed. Starvation can activate different stress responses: the general stress response, the heat-shock response and the SOS response (Foster, 2005). All of the stress responses lead to the activation of the Y-family of polymerases, Pol IV and Pol V, these polymerases are able to replicate damaged DNA, but they are error-prone and as such they cause frequent mutations (Foster, 2005). It is likely that one or all of these stress responses are induced in bacteria living in drinking water biofilms. These stress responses transiently increase the mutation rate in bacteria; however, the mutations occur randomly throughout the bacterial genome. Most mutations are either deleterious or neutral, but a small number of beneficial mutations will likely occur as well (Kang et al., 2006). Mutations increasing the antibiotic resistance of the bacteria would not be considered beneficial during the starvation state, but are beneficial if the bacteria come into contact with an antibiotic. While the bacteria were not assessed for the activation of stress molecules, the affect of the stress molecules was examined by looking for antibiotic resistance.

The increased antibiotic resistance that was seen in the isolates could also be due to phenotypic changes in the bacteria, rather than mutations. The one phenotypic change that was observed in the ciprofloxacin-resistant isolates was slower growth than the wildtype bacteria, and slow growth due to nutrient starvation has been shown to increase antibiotic resistance to some antibiotics (Tuomamen et al., 1986). When growing inside the drinking water biofilm the bacteria are nutrient deprived, but once the bacteria are isolated from the biofilm they are grown planktonically in nutrient rich media, so they are no longer nutrient deprived but still have slower growth. Slower growth would have the greatest impact on resistance to antibiotics that interfere with cell growth. Ciprofloxacin targets DNA gyrase, which is essential for proper DNA replication (Anderson et al., 1998). Gentamicin and tobramycin inhibits protein synthesis by targeting the 30S subunit of the ribosome (Walsh, 2003). Chloramphenicol also inhibits protein synthesis; however, its target is the 50S ribosomal subunit (Mascaretti, 2003). Rifampicin binds to the β-subunit of the DNA-dependent RNA polymerase and inhibits RNA synthesis (Mascaretti, 2003). Because all of these antibiotics inhibit cellular processes required for bacterial growth, if the slower growth was responsible for the increased antibiotic resistance, the isolates should have increased MIC values for all of these antibiotics. Since the isolates are not resistant to gentamicin and tobramycin, I believe that the isolates slower growth rate is not the cause of the increased antibiotic resistance. Other factors could also increase the antibiotic resistance of the isolates, such as differences in the outer membrane proteins. The loss of porin proteins contribute to increases in antibiotic resistance (Heinzel, 1998), but these mechanisms are not usually selective for different antibiotics. The most likely mechanism responsible for the

increased antibiotic resistance in the isolates is a mutation causing the over-expression of a multi-drug efflux pump.

Multi-Drug Efflux Pumps

If there is a mutation causing the over-expression of a multi-drug efflux pump, then by considering the different efflux pumps found in *P. aeruginosa* and their substrates I can identify the most likely candidate for the increased antibiotic resistance of the isolates. *P. aeruginosa* has a number of different characterized efflux pumps, mostly belonging to the RND family of pumps, the main ones being: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Poole, 2004). While all of these pumps have the same RND family structure, they have different substrate specificity and regulation. For example, MexAB-OprM and MexXY-OprM are constitutively expressed in wildtype *P. aeruginosa*, while the others are not (Poole, 2004), and the antibiotics and biocides that are substrates for each pump is different (Poole, 2005).

If the antibiotic resistance of the isolates from the drinking water biofilms is due to the mutation in an efflux pump, the most likely candidate is MexEF-OprN because it is the only pump that when over-expressed, the MIC for chloramphenicol as high as what was seen with these isolates, with an MIC value greater than 800μg/mL (Kohler et al., 1997). There are additional, uncharacterized efflux pumps (Stover et al., 2000); however, MexEF-OprN makes the most sense based on the information available on the characterized pumps.

MexEF-OprN has a variety of substrates; it is able to pump out fluoroquinolones, chloramphenicol, trimethoprim, as well as biocides such as triclosan and aromatic hydrocarbons (Poole, 2004). The genes for the MexEF-OprN efflux pump are located on the *P. aeruginosa* chromosome in the *mexEF-oprN* operon (Kohler et al., 1997). Unlike the

other RND efflux pumps MexEF-OprN is positively regulated, its regulator is a member of the LysR family of regulatory proteins, called MexT (Poole, 2005). The outer membrane porin OprD is inversely regulated with MexEF-OprN, and as such when the *oprD* gene is expressed the *mexEF-oprN* operon is not (Poole, 2005). In fact, it is because of the inverse regulation that bacteria hyper-expressing MexEF-OprN are resistant to the carbapenem class of antibiotics. Carbapenems are not a substrate for MexEF-OprN, but their route of entry is through the OprD porin, so when MexEF-OprN is hyper-expressed OprD is not, and the carbapenems cannot get into the cell (Sobel et al., 2005).

The increase in antibiotic resistance is not the only difference between the wildtype PA14 and the antibiotic resistant isolates. It was evident from the colour of the isolates cultured in liquid LB that they produced less pyocyanin than the wildtype PA14. And as previously mentioned, some of the antibiotic resistant isolates had a slower growth rate and did not reach the same final cell density as the wildtype PA14. These changes could also be attributed to the hyper-expression of MexEF-OprN and the down-regulation of OprD. MexEF-OprN is not specific for antibiotics and biocides, an intermediate in the pyocyanin biosynthesis pathway is also excreted by the MexEF-OprN efflux pump (Kohler et al., 1997), and because of this, pyocyanin production is reduced in mutants hyper-expressing MexEF-OprN. On the other hand, the isolates slower growth rate could be attributed to the down-regulation of OprD. The normal function of OprD is as an amino-acid porin (Tamber et al., 2006), which also happens to allow passage of carbapenems. So when its production is decreased due to the hyper-expression of MexEF-OprN the uptake of amino acids is impaired. This would not be detrimental to the bacteria while growing in drinking water because there are few nutrients available, but when growing in a rich media like LB, the

bacterial growth would be limited by the decreased up-take of amino acids. Coincidentally, the isolates that had growth rates the same as the wildtype PA14 (Figure 19), also had somewhat lower ciprofloxacin MICs (Table 3). This could indicate that these isolates may have a different mutation from the rest of the ciprofloxacin resistant isolates, so that the MexEF-OprN expression is not as high, and therefore there is also more expression of OprD. This type of expression could explain the difference in MIC and growth rates seen for these isolates.

Bacteria can acquire antibiotic resistance through hyper-expression of efflux pumps either by the acquisition, from another bacterium, of a plasmid or transposon capable of expressing the efflux pump, or by mutations in the bacterial chromosome (Mascaretti, 2003). Since only the inoculated strain of *P. aeruginosa* PA14 was present in the drinking water biofilms, there was no source for a plasmid or transposon containing antibiotic resistance genes, the antibiotic resistance must have been caused by mutations. These mutations were likely caused by the stress mechanisms described above. There are two genes that have been identified as having mutations which could cause the increased expression of MexEF-OprD and down-regulation of OprD: *mexT*, the MexEF-OprN regulator, or *mexS*, an oxidoreductase/dehydrogenase homologue (Poole, 2005). Many strains of *P. aeruginosa* have an inactive *mexT* gene, even though they are considered "wildtype", these inactive genes have an 8-bp insert in a direct repeat (Maseda et al., 2000). If our strain of PA14 has an inactive form of the *mexT* gene, then a reversion mutation that removes the 8-bp insert can occur resulting in production of a functional MexT protein and expression of the MexEF-OprN efflux pump. The gene *mexS* is important for detoxification of cellular metabolites, and as such it is constitutively expressed (Sobel et al., 2005). When there is a mutation in

mexS there is increased expression of the MexEF-OprN efflux pump (Sobel et al., 2005). The reason for this interaction is because MexS detoxifies cellular metabolites, so when there is enough MexS present in the cell MexEF-OprN is not required to export the toxic metabolites; however, if there is no MexS produced or if there is too much toxic metabolites being produced then the production of MexEF-OprN is induced (Poole, 2005).

After growing drinking water biofilms, which were exposed to starvation conditions and sublethal levels of chloramines, ciprofloxacin resistant bacteria were isolated from both the non-treated and treated biofilms. These isolates were found to be resistant to ciprofloxacin, rifampicin and chloramphenicol, but not resistant to gentamicin and tobramycin. There are different mechanisms that could contribute to the increase in resistance to antibiotics. I think that the most likely mechanism is a mutation causing the over-expression of the MexEF-OprN efflux pump. The other possibility is that there could be an alteration in the OM that restricts entry of antibiotics at a general level; however, this is less likely because the isolates were not resistant to all of the antibiotics tested.

Future Work

There are some additional tests that should be performed to obtain some missing information. All of the remaining isolates obtained from the second and third replicates of the 3 experiments run in the Kadouri drip-fed reactorcould be characterized, first confirming the *P. aeruginosa* identification with the Vitek 2 system, then performing growth assays and MIC assays. Also different antibiotics could be used for MIC assays in order to address whether MexEF-OprN is the efflux pump that is over-expressed. Another way to determine if the MexEF-OprN efflux pump is over-expressed would be to assay the expression of MexEF by real-time PCR.

To test the idea that growth in a low-nutrient environment is what generates ciprofloxacin-resistant bacteria, biofilms of wildtype PA14 should be grown in LB using the Kadouri-drip fed reactor for the same length of time used for the different drinking water experiments, and plated on the ciprofloxacin containing media to determine if any antibiotic resistant mutants are produced. If there are no or very few antibiotic resistant mutants isolated from the biofilms grown in LB, then this would support the theory that the antibiotic resistance was caused by growing in the drinking water. On the other hand, if there are resistant mutants isolated from the biofilms grown in LB, then this would indicate that spontaneous mutation within biofilms causes antibiotic resistance. To determine if the SOS or heat-shock responses are induced in the isolates, the expression of Pol IV or Pol V could be assessed.

In order to determine where the mutation has occurred in the antibiotic resistant bacteria isolated from the drinking water biofilm, as a start, the *mexT* and *mexS* genes of the isolatescould be sequenced, when the genes of the wildtype and the isolates are aligned, a mutation would be easily recognized. Alternatively, DGGE (discontinuous gradient gel electrophoresis), which can identify mutations in a genome sequence by comparing the banding pattern with the wildtype, could be used to find mutations in either in the *mexT* and *mexS* genes, or in any other genes.

Conclusions

Planktonic wildtype *P. aeruginosa* PA14 bacteria are killed by chloramine at a concentration of 1 mg/L, while 100 mg/L of chloramine is required to kill wildtype PA14 when growing in a biofilm. These bacteria were able to form biofilms in drinking water using the Kadouri drip-fed reactor.

The drinking water residual disinfectant chloramine did not cause antibiotic resistance in *P. aeruginosa* under the conditions tested here. However, under the growth conditions examined, the very low level of AOC in the drinking water seems to stress the bacteria, and activate the bacterial stress responses. The mutational rate of the bacteria likely increased transiently while growing in the drinking water biofilm, causing mutations that increased the antibiotic resistance of the bacteria. The increased antibiotic resistance could be due to mutations in either the *mexT* or *mexS* genes, causing hyper-expression of the MexEF-OprN efflux pump. However, this increase in resistance to ciprofloxacin could have also been due to alteration in outer membrane proteins or some additional genetic alteration. These possibilities need to be investigated in the future.

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Curriculum Vitae

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Objective:

I am looking for a position in a microbiology laboratory, where I can use my previous experience and education on interesting and important projects.

Education:

M.Sc. Microbiology/Immunology, University of Ottawa, 2007
Thesis Title - *Pseudomonas aeruginosa* Biofilms in Drinking Water and the Evolution of Antibiotic Resistance

B.Sc. Biology (Honours), Carleton University, 2004
Honours Thesis Title – Gamma-Glutamyltransferase Function Under Hypoxia.

Experience:

Graduate Student, 2005-2007
University of Ottawa, Dr. Thien-Fah Mah
Worked with *Pseudomonas aeruginosa* biofilms grown in drinking water reactors to determine if exposure to the drinking water disinfectant chloramine causes antibiotic resistance in drinking water biofilms. Performed characterization tests on *P. aeruginosa* mutants that are more sensitive to antibiotics when grown in biofilms than the wildtype.

Teaching Assistant, 2004(summer)
Carleton University, Joan Mallett
Assisted first year biology students during the laboratory session and graded assignments.

Summer NSERC and Honours Student, 2003-2004
Carleton University, Dr. William Willmore
Worked with mammalian cell lines examining the effect of oxygen depletion on the enzyme gamma-glutamyltransferase. Examined the effect of decreased oxygen on the expression of von Willebrand factor using PCR and Western blots.

Awards:

NSERC Summer Research Scholarship, 2003

Techniques:

- RNA/DNA isolation
- RT-PCR
- Western blot
- animal and bacterial cell culture
- growing bacterial biofilms
- bacterial transformation