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MUTABILITY AND SURVIVAL OF *PSEUDOMONAS AERUGINOSA* IN MULTI-SPECIES DRINKING WATER BIOFILM COMMUNITIES

by

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Thesis for the degree of Doctor of Philosophy

February 2012

General abstract

Pseudomonas aeruginosa is an important opportunistic pathogen of humans and also has the ability to form biofilms in drinking water. However, the survival, persistence and the control of this pathogen within mixed-species bacterial communities in drinking water distribution systems remains poorly understood.

Strains of *P. aeruginosa* obtained from natural and pathogenic biofilms are often hypermutable due to defective DNA error repair systems. However, the role of mutation in determining survival and fitness of *P. aeruginosa* within the environment has not been explored. This work, investigated the mutability, persistence and survival of hypermutable *mutS*, wild type and environmental strains of *P. aeruginosa* within mixed species drinking water consortia and the effects of oxidative stress and water treatment practices including chlorination and UV irradiation on their mutability and persistence within these biofilms using a flow cell continuous culture system. Our results show that *P. aeruginosa* hypermutator strains integrated and persisted within the biofilm more readily than the wild type and environmental strains. Moreover, growth of *P. aeruginosa* within a multi-species biofilm led to a 5-fold increase in the mutation frequency (resistance to rifampicin, RifR) of the wild type strain compared to monospecies *P. aeruginosa* biofilms, suggesting that interactions within polymicrobial communities may promote genetic diversification. Our results also show that antioxidants (L-proline and N-acetyl-cysteine) had an average of 4-fold reduction effect in the mutation frequency of the wild type *P. aeruginosa* within the mixed species biofilms. However, the mutation frequency exhibited by the *mutS* strain within the biofilms is independent of oxidative stress. UV irradiation of *P. aeruginosa* cells, but not exposure to chlorine, led to increases in *P. aeruginosa* RifR mutation frequency and enhanced the persistence of surviving *P. aeruginosa* cells within drinking water biofilms. These findings therefore have provided new insights into mechanisms by which drinking water biofilms may harbour important pathogenic micro-organisms and how these interactions within the multispecies biofilms can enhance genetic adaptation and evolution of microbial pathogens.

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Declaration of authorship

I, ENYIOHA KENNEDY IHEANYICHUKWU

declare that the thesis entitled

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and the works presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that

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Acknowledgements

My deepest gratitude goes to my supervisor Dr Jeremy Webb for all his guidance and direction throughout the period of this research. He has been a mentor, teacher as well as a friend and he believed in me even when I didn't believe myself. I feel honoured to have had this opportunity to work with him. I would also use this opportunity to thank my second supervisor Prof. Bill Keevil for all his constructive criticisms and his professorial ideas offered throughout the course of this work. I would also like to thank Dr Tim Conibear for his help, ideas and thoughts that contributed immensely to the success of this work. My thanks go to Dr Hans Schuppe of the imaging unit of the School of biological Sciences for many hours spent in advising and directing me on confocal imaging.

I would especially like to thank my course mate Miss Susanna Sherwin for her kindness and tolerance she accorded to me as an international student. She never for once got tired of me when pestering her for one problem or the other in our laboratory and she is always ready to render help whenever the need arises and to all postdoctoral researchers and postgraduate students in my office and also to all postgraduate students of the School of biological Sciences University of Southampton.

I am highly indebted to my wife and daughter Agnes and Jennifer respectively for their support especially my wife and best friend for being a hand to hold and a source of encouragement throughout the course of this study.

I would like to offer a special thank you to my family for their love and financial support for this programme. Without whom I would not be able to complete this study. I would say I am so lucky to have such amazing people in my life. I would also like to thank my special Nigerian friend Mr Wole Solanke whom we started this PhD programme together for his companionship and love throughout our moments at the University of Southampton and finally I thank the Almighty God for his protection and guidance throughout this programme.

Abbreviations

ANOVA	Analysis of variance
AOC	Assimilable organic carbon
ATP	Adenosine triphosphate
AWWA	American Water Works Association
BDOC	Biodegradable dissolved organic carbon
CF	Cystic fibrosis
CFU	Colony forming unit
CTC	5-cyano-2,3ditolyl tetrazolium chloride
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DVC	Direct viable count
DWB	Drinking water bacteria
ENVPA	Environmental strain of *P. aeruginosa*
EPS	Extracellular polysaccharides
FC	Fluorescing colonies
FISH	Fluorescence in situ hybridization
GAC	Granular activated carbon
GFP	Green florescent protein
HPC	Heterotrophic plate count
LB	Luria Bertani
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MMR	Mismatch repair
M-PCR	Multiplex polymerase chain reaction
NF	Nano-filtration
NFC	Non-fluorescing colonies
PAC	Powdered activated carbon
PNA	Peptide nucleic acid
RNA	Ribonucleic acid

RND	Resistance nodulation and cell division
RO	Reverse osmosis
USEPA	United states environmental protection agency
UV	Ultra violet
VBNC	Viable but not culturable
WHO	World Health Organisation
WT	Wild- type

Chapter One

1.0 General introduction

Biofilms are complex communities of micro-organisms attached to surfaces and play a major role in the sorption of planktonic micro-organisms from the bulk water onto surfaces including microbial pathogens (Block *et al.*, 1993; Szewzyk *et al.*, 2000; Keevil, 2002) (figure 1.1). Drinking water distribution pipe biofilms are implicated in reducing the aesthetic (taste, colour, and odour) and microbiological quality of water through continual detachment of biomass into the bulk water (Penna *et al.*, 2002) (figure 1.2). Biofilms are also important reservoirs for pathogenic bacteria. *Pseudomonas aeruginosa* is an important opportunistic pathogen of humans and also has the ability to form biofilms in drinking water (LeChevallier *et al.*, 1987). Much research has been carried out on *P. aeruginosa* single species biofilm formation and its role in human infections, but not much is known about its biofilm formation and interactions from an ecological perspective. A recent finding is that strains of *P. aeruginosa* obtained from natural biofilms are often hypermutable due to defective DNA error repair systems (Oliver *et al.*, 2000). However, the role of mutation frequency in determining survival and fitness of *P. aeruginosa* within the environment has not been explored. The research presented in this thesis aims to study how *P. aeruginosa* interacts and persist within multi-species natural biofilms from drinking water systems and the effect of biofilm micro-environmental conditions such as oxidative stress and drinking water disinfection practices (chlorination and UV treatments) on its mutation frequency, persistence and survival within this system. This will provide new insights into mechanisms by which drinking water biofilms may harbour important pathogenic micro-organisms and how these interactions within the multispecies biofilms can enhance genetic adaptation and evolution of microbial pathogens.

1.1 Introduction to biofilms

Claude Zobell (1943) was the first to recognise that aquatic bacteria are more numerous on solid surfaces in aqueous environments than as single suspended cells. However, this concept of preferential bacterial growth on surfaces did not include an understanding of the complexity of biofilm structure, until as late as 1987, when biofilms were still perceived as simple "slab" of matrix material in which sessile bacterial cells were randomly embedded (Costerton *et al.,* 1994). The modern biofilm era began when the first confocal scanning laser microscopic images of living biofilm (Lawrence *et al.,* 1991), and episcopic differential interference light microscopy (Rogers and Keevil 1992) showed that sessile bacteria grow in matrix-enclosed micro colonies interspersed between open water channels. Since then, the combination of high resolution three-dimensional imaging techniques, specific molecular fluorescent stains, molecular reporter technology and biofilm-culturing apparatus has shown that biofilms are not simply massive assemblages of cells that are stuck to surfaces, but are structurally and dynamically complex biological systems (Doyle 2001). This has led to various biofilm studies in industrial, ecological and environmental settings more relevant for public health. Biofilms can be defined as matrix-enclosed microbial accretions that adhere to biological or non-biological surfaces (Hall-Stoodley and Stoodley 2002) whose cells express genes in a pattern that differs profoundly from that of their planktonic counterparts (Sauer *et al.,* 2002).

The plaque formed on the teeth and the slippery slime on river stones are examples of biofilms. Noncellular materials such as mineral crystals, corrosion particles, clay or silk particles or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix.

Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water systems, piping, and in many natural aquatic systems (Donlan and Costerton 2002). Biofilms can be composed of single-species, or multi-species communities of bacteria, depending on the environmental parameters under which they are found.

1.1.1 Why do bacteria form biofilms?

The association of bacteria with a surface and the development of a biofilm can be viewed as a survival mechanism. Surfaces provide a niche that can be colonized and occupied by bacteria and this gives them a degree of stability (Hall-stoodley *et al.,* 2004). Potable water, especially high-purity water systems are nutrient-limited environments but nutrient concentrations too low to measure are sufficient to permit microbial growth and reproduction (Dreeszen, 2003). Bacteria have evolved the means to find and attach to surfaces in order to increase chances of encountering nutrients. The advantages offered by adhesion to surfaces and development of biofilms include, concentration of trace organics on the surfaces and further concentration of trace elements from the bulk water by means of extracellular polymers. Secondary colonisers utilize the waste products from their neighbours and by pooling their biochemical resources, several species of bacteria each armed with different enzymes can break down food supplies that no single species could digest alone (Dreeszen, 2003).

Protection against environmental stress is another reason why bacteria form biofilms. Once microorganisms have attached to surfaces they often become capable of withstanding normal disinfection processes. Biofilm bacteria display a dramatic tolerance towards biocides. LeChevallier demonstrated that biofilm associated bacteria may be 150-3000 times more tolerant of free chlorine and 2-100 times more resistant to free monochloramine than free-floating bacteria (LeChevallier, 1988). A germicide flushed through the water distribution system typically kills the majority of planktonic microorganisms but it often, cannot kill bacteria embedded in biofilms. Biofilms can often exhibit enhanced growth and exopolymer production after biocide treatment (Borenstein, 1994). Biofilm formation typically affords protection from a wide range of environmental stresses such as UV exposure (Espeland *et al*., 2001), metal toxicity (Teitzel *et al*., 2003), acid exposure (McNeill *et al.,* 2003), dehydration and salinity (LeMagrex- Debar *et al.,* 2002), Phagocytosis (Leid *et al*., 2002) and several antibiotic and antimicrobial agents (Gilbert *et al.,* 2002; Mah and Stewart *et al.,* 2001). In biofilms bacteria do not differentiate, rather they respond to environmental surroundings by adapting their gene expression to suit their own needs for survival. Biofilms are thus interactive communities rather than multicellular organisms. In addition to the advantage of resistance to environmental changes, the

biofilm may benefit from a number of properties of communal existence including division of metabolic burden, gene transfer, and selfless behaviour. The frequency of gene transfer in planktonic cells is probably lower than that seen in cells found within biofilms (Roberts *et al.,* 2001). A number of studies indicate that transfer of genes is a common phenomenon in biofilms (Lebaron *et al.,* 1997; Li *et al*., 2001; Licht *et al.,* 1999). In single species biofilms on glass beads, a strain of donor *Escherichia coli* harbouring three different plasmids transferred these plasmids to an *E. coli* strain present as a biofilm (Lebaron *et al.,* 1997). Horizontal gene transfer by transformation was also demonstrated in strains of *Streptococcus mutans* by Li *et al*., (2001). A rifampicin-resistant strain of *E. coli* (recipient) was allowed to form biofilms on glass, and on day eight, a donor strain of *E. coli* carrying the plasmid R1drd19 (which confers resistance to chloramphenicol and ampicillin) was added to the biofilm. Within 24 hours, rifampicin-resistant transconjugants with resistance to chloramphenicol and ampicillin were isolated (Licht *et al.,* 1999).

1.1.2 Impacts of biofilms

Microbial biofilms on surfaces cause major economic losses through equipment damage, product contamination, energy losses and medical infections. Conversely, microbial processes at surfaces also offer opportunities for positive industrial and environmental effects.

1.1.2.1 Medical effects:

Medically, biofilm formation can be thought of as a virulence factor i.e. a bacterial strategy that contributes to its ability to cause infection. Intravenous catheters, prosthetic heart valves, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts and endotracheal tubes save millions of lives, but all have an intrinsic risk of surface-associated infections. (Peters *et al.,* 1981; Christensen *et al.,* 1982; Marrie *et al.,* 1982). The microorganisms that are most frequently associated with medical devices are the *staphylococci* particularly *Staphylococcus epidermidis* and *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* and a plethora of other environmental bacteria that opportunistically infect a host who is compromised by invasive medical intervention (Stoodley *et al.,* 2002). Biofilm formation on medical implants, has led to the characterization of a new infectious disease called chronic polymer-associated infection (von Eiff *et al.,* 1999;

Gotz, 2002). *S. epidermidis* was not considered an opportunistic pathogen until the widespread use of medical devices (Stoodley *et al.,* 2002). Biofilm infections within the body which are characterized by adherent bacteria on tissues, might also include host cells and molecules as part of the surface-associated infection: bacterial endocarditis being a typical example (Donlan and Costerton 2002). Another common disease associated with biofilms is cystic fibrosis pneumonia (CF). CF is an autosomal recessive disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene that results in dysfunctional electrolyte secretion and absorption (Stoodley *et al.,* 2002). Pulmonary colonisation of the lower respiratory tract of CF patients begins during infancy or early childhood, most commonly by S. *aureus* and *Haemophilus influenza.* However, by adolescence and early adulthood, most CF patients have become colonized with *P. aeruginosa* (Koch and Hoiby 1993; Lyczak *et al.,* 2002). The shift from colonisation with other bacteria to chronic infections with *P. aeruginosa* seems to be the result of the peculiar environment of the CF lung which includes asialylated receptors on epithelial cells that facilitates pseudomonal attachment in addition to impaired mucociliary clearance (Koch and Hoiby 1993; Lyczak *et al.,* 2002).

Other diseases caused by biofilm-associated micro-organisms include periodontitis, otitis media, and chronic prostatitis.

Antibiotic resistance is another vital area where biofilms exhibit great medical impact. Bacteria within biofilms are protected against antibiotics. *P aeruginosa* for instance is extremely difficult, if not impossible, to eradicate using conventional antibiotic treatments in the large part due to its propensity to form biofilms (Costerton *et al.,* 1998). Resistance to antibiotics in biofilms can involve multiple physiological and genetic factors, including a protective effect of polymeric matrix which contributes to persistence in infections even in the face of vigorous chemotherapy. *In vitro* experiments suggest that bacteria encased in biofilms may be 50 to 500 times more resistant to chemotherapy than planktonic bacteria of the same strain (Poole *et al.,* 1993).

1.1.2.2 Environmental/industrial effects:

Conversely, biofilms serve as a reservoir of bacteria which can affect animal health. Any microorganism (including some pathogens) present in water may attach, or become enmeshed in the biofilm. Primary pathogens, which cause disease in

healthy humans, may survive for a time in the biofilms (Camper *et al.,* 1986; LeChevallier *et al.,* 2003).

A wide range of primary and opportunistic pathogens have demonstrated the ability to survive, if not grow, in drinking water biofilms. These pathogens are of both faecal and non-faecal origin, and have a multitude of pathways through which they can enter the distribution system. Some of the pathogens identified as growing or potentially surviving in biofilms include *Legionella spp., Mycobacterium avium* complex, *P. aeruginosa,* Poliovirus 1, Coxsackie *virusB* and several species of fungi (Hall-Stoodley *et al.,* 2004). Infection can occur by ingestion of contaminated water, inhalation of aerosols containing pathogens or contact of skin, mucous membranes, eyes and ears (WHO, 2006), Metabolic products such as hydrogen sulphide and nitrite or endotoxins also belong to the impact of biofilms to the hygienic quality of water.

Besides being a reservoir of bacteria which can affect animal health, biofilms can also cause corrosion in stainless steel or copper piping systems (Pedersen, 1990; Giao *et al.,* 2008). Products of their metabolic activities including enzymes, exopolymers, organic and inorganic acids as well as volatile compounds such as ammonia or hydrogen sulphide can affect cathodic and or anodic reactions, thus altering electrochemistry at the biofilm/metal interface, a phenomenon often referred to as "biocorrosion" or "microbial influenced corrosion" (figure 1.2) (Burlingame and Anselme 1995). Microbiologically influenced corrosion has been documented for metals exposed to sea water, fresh water, demineralised water, processed chemicals, food stuffs, soil, aircraft fuels, human plasma, and sewage (Costerton 1994). Biofouling is another area where biofilms create a significant environmental and industrial impacts. This refers to the undesirable accumulation of biotic matter on a surface (Geesey *et al.,* 1994; Percival and Walker 1999; Flemming *et al.,* 2000). It represents a significant expense to operators of oil and water pipelines, power plants (heat exchanger), desalination plants; container ship fleets (ship and paper mills) (Mittelman 1991; Bachmann and Edyvean 2006). It may cause blockage of drains in domestic and public buildings and contributes to the bacterial contamination of drinking and ultra-pure water systems. A better understanding of the factors which promote biofouling as well as the development of environmentally friendly biological and physicochemical control methods are therefore vital for a successful strategy in combating biofouling.

Bioremediation is an emerging in situ technology for the clean-up of environmental pollutants using microorganisms. The biological processes for treating toxic effluents are better than chemical and physical methods in terms of their efficiency and economy (Paul *et al*., 2005) and the potential of biofilm communities for bioremediation processes has been realised. Biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation with planktonic microorganisms because cells in a biofilm have a better chance of adaptation and survival during stress as they are protected within the matrix (Decho, 2000; Singh *et al.,* 2006). The use of biofilms for water and wastewater treatments was reported in the early 1980s (Akinson, 1981). They are employed for biological purification of drinking water in biofilters (Gimbel *et a*l., 2006; Vinarov *et al.,* 2002). However, biofilm reactors have become a focus of interest for researchers in the field of bioremediation.

1.1.3 Biofilm formation in potable water

The degradation of water quality due to bacterial growth in the drinking water distribution systems is a major concern for consumers as well as drinking water producers. Biofilms attached to the surfaces of drinking water distribution pipelines form an ecosystem which is both stable and difficult to remove (figures 1.1 and 1.2). It has been shown that bacterial growth in the liquid phase, even in the absence of chlorine, is negligible (LeChevallier *et al.,* 1987; van der Wende and Characklis 1990).

Essentially, only the bacteria in the biofilm attached to the walls of the distribution pipeline are multiplying and due to shear loss, constitute one of the main causes of the deterioration of microbiological quality of water distribution systems (Geldreich, 1996). Micro-organisms can enter distribution systems through a wide range of avenues, including treatment processes or through deficiencies of the distribution system infrastructure. Microbial presence in the distribution system can result in colonisation of the distribution system infrastructure. Once biofilm development begins, subsequent organisms and contamination introduced to the distribution system can become entrapped in the biofilm. The biofilm can protect microbes from disinfection and allow microbes injured by environmental stress and disinfectants to

recover and grow (Characklis and Marshall 1990). This project aims to study how *P. aeruginosa* interacts within drinking water biofilms. This will provide insight into mechanism by which drinking water biofilms may harbour important pathogenic microorganisms.

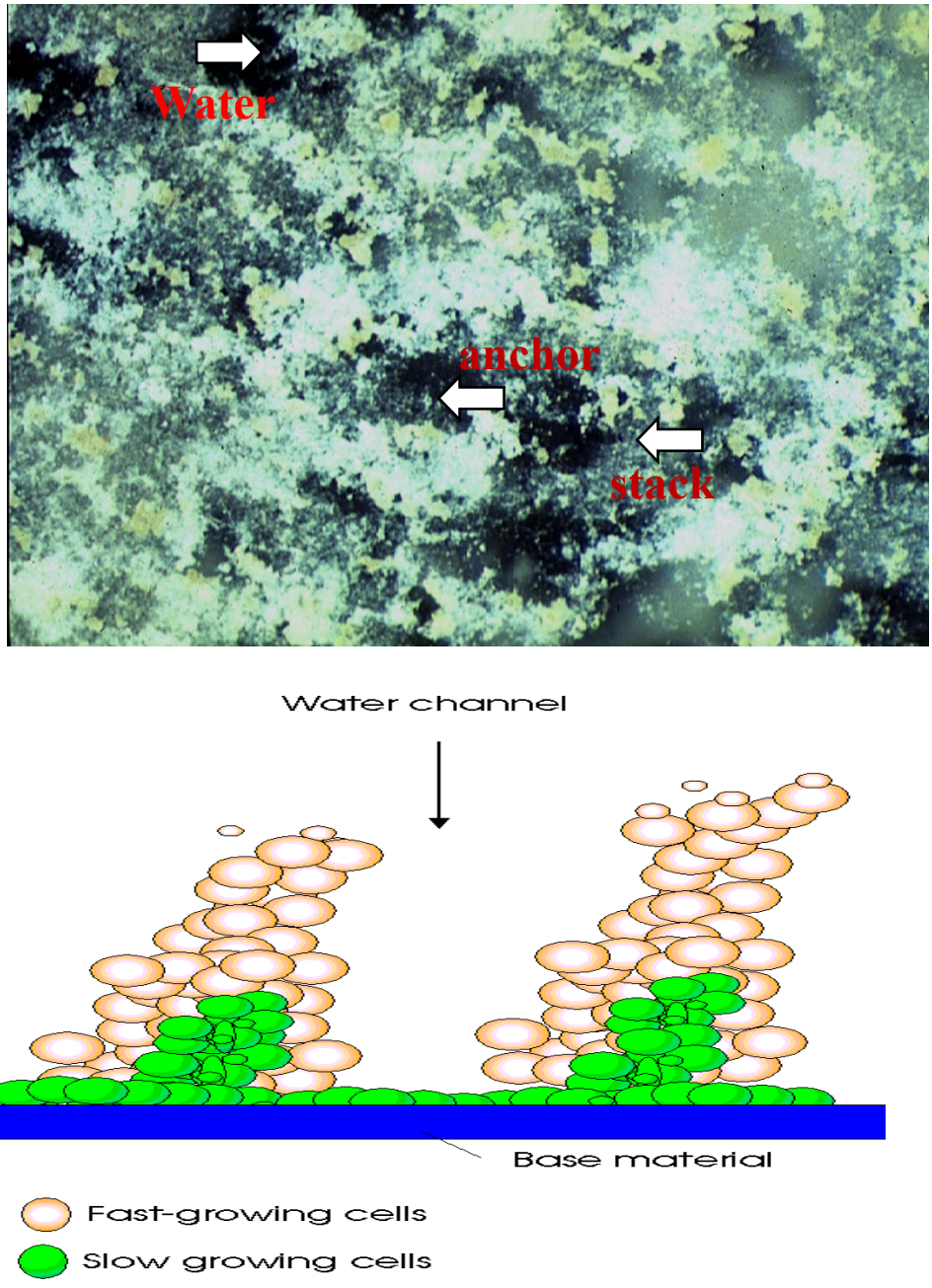


Figure 2.1 Heterogeneous drinking water biofilm and representative microcolony model (Keevil *et al.*2002*)*

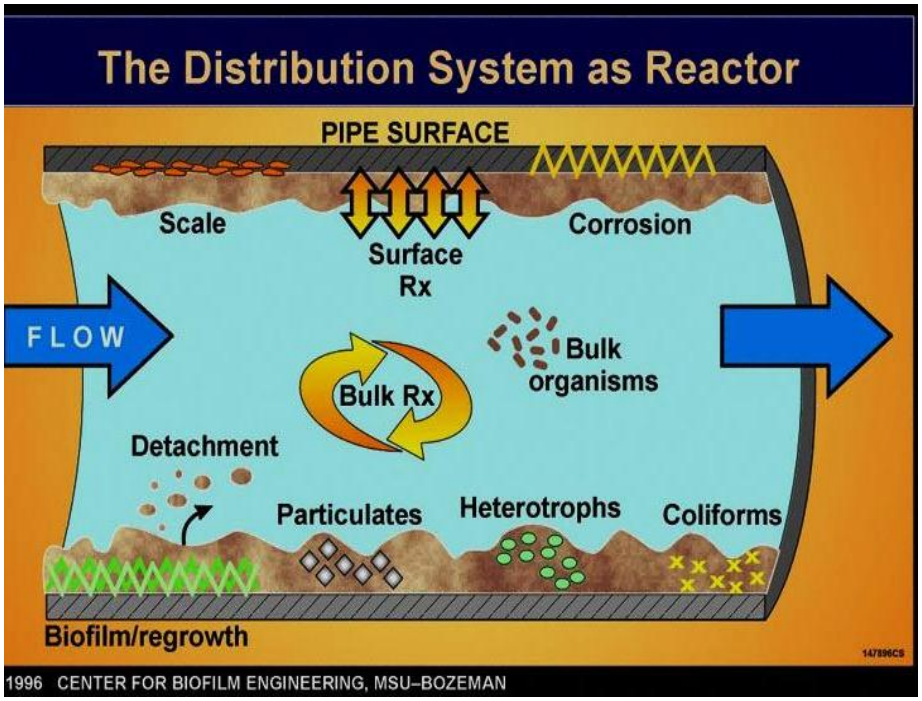


Figure 1.2 A typical drinking water distribution system showing the formation and detachment of microcolonies from biofilms formed by drinking water bacteria and the corrosion of the water distribution pipeline due to the metabolic activities of biofilm bacteria. (Centre for biofilm engineering Bozeman).

1.1.4 Physical factors that influence biofilm formation and structure in drinking water systems

A variety of physical factors affect pipeline biofilm development. Some of these factors vary often, making predictions difficult. Most of the data on the physical factors that influence biofilm formation are based upon changes in the total viable counts for example heterotrophic plate counts or on changes in the growth of specific indicator coliforms.

1.1.4.1 Mass transfer and velocity effect. A variety of hydraulic conditions such as long residence time due to low flow rates or dead ends, high flow rates or fluctuating flow rates can influence the formation and structure of biofilms. A simple relationship between the hydraulic effects and microbial growth in biofilms does not exist. Among the many factors that can influence flow rates are pipe layout, condition and size, demand, pump operation and elevation. High water velocities may increase the level of nutrients and disinfectants in contact with the biofilm and cause greater shearing of biofilms. Interrupted or pounding water flows, or reversal of water flows may shear biofilms (LeChevallier, 1990). High turbulence improves the opportunity for cells to arrive at the pipe surface and initiate biofilm formation. Under high unidirectional flows, shear becomes increased and biofilm cell clusters become elongated in the downstream direction to form filamentous streamers (Stoodley *et al.*, 1999). Filamentous biofilms commonly occur in both archeal and bacterial biofilms growing in fast-flowing environments such as hot springs (Rasmussen, 2000) or acid mine drainage runoff (Edward *et al.*, 2000). Filamentous biofilm streamers can also be formed by non-sheathed species such as *P. aeruginosa* (Reysenbach *et al.*, 2001).

1.1.4.2 Solid surface conditions: Some types of pipe materials are especially prone to biofilm development. The materials may include the pipes themselves, valves, joints, fittings or joint-packing materials. Pipe material may be more influential than the level of organic matter in the drinking water distribution (Volk *et al.*, 1999). Some materials provide the microbes a protective niche where growth can occur, while some provide nutrients to support microbial growth. The bacterial levels on disinfected iron pipes generally exceed those on disinfected PVC pipes (Norton *et al.*, 2000). Biofilms also develop more rapidly on iron pipes (Haas *et al.*, 1983).

Biofilm problems tend to increase when systems have iron pipes that are predominantly 50 years old or more (Geldreich, 1996). Elastomeric materials supported more abundant biofilms and higher levels of *L. pneumophilia* than did glass or steel (Rogers *et al.,* 1994). Materials that support microbial growth include rubber, silicon, PVC, Polyethylene and bituminous coatings (Schoenen *et al.,* 1988; Frensch *et al.,* 1987). Colonisation appears to increase as the surface roughness increases (Characklis *et al.,* 1990). This is because shear forces are diminished and surface area is higher on rougher surfaces. Most investigators have found that micro-organisms attach more rapidly to hydrophilic materials such as glass or metals (Fletcher *et al.,* 1979; Pringle *et al*., 1983).

1.1.4.3 Availability of nutrient: The effect of nutrient concentration in the bulk water on the development of biofilms have been the subject of a number of studies example (Bott *et al.,* 1982, 1983) in which it has been shown that nutrient enrichment of the bulk water passing over a biofilm increases both the thickness and the cellular density of the biofilm. Increases in the C: N ratio of the bulk water also led to thicker film. The C: N ratio of the water is of importance since nitrogen is essential for the production of proteins and biomass. A high ratio tends to lead to the production of large quantities of extracellular polysaccharides which may have several effects. It may enhance the adhesion of the biofilm to its substratum. It may also increase the efficiency of scavenging, both by increasing the adhesion of impacting particles and by adsorption of dissolved organic compounds which in turn increase the nutrient loading of the biofilm upon which the subsequent growth depends. Even in nutrient depleted waters the polymer matrix once developed, can scavenge nutrients, and the nutritional status of the biofilm may be far higher than that of the surrounding waters. Hence, although water chemistry has a major influence in the early stages, the fully developed biofilm is substantially buffered against short term variation (Bott *et al.,* 1983). The presence or absence of specific nutrients can select for microbial populations present in drinking water. However, some pathogens such as the opportunistic pathogen *P. aeruginosa* are especially versatile in the type of organic nutrients they can use.

1.1.4.4 Changes in temperature: Drinking water temperature affects the microbial growth rate, disinfection efficiency, pipe corrosion rates and other phenomena associated with biofilm development (LeChevallier, 1989). Where

nutrients are adequate, microbes generally grow more rapidly at warmer temperature than at colder temperature (Donlan *et al.,* 1988; LeChevallier *et al.,* 1996).Thus warmer temperatures likely facilitate the growth of opportunistic pathogens in the biofilm. Predictions are somewhat complicated by the fact that disinfectants are less efficient at lower temperature in inactivating microbes. Generally, faecal pathogens can survive longer in very warm waters with a high organic load. In general, the rate of biofilm growth increases with increased temperature up to a temperature of around 40 degree Celsius, above which it tails off (Donlan *et al.,* 1988; LeChevallier *et al.,* 1996).

1.1.5 Organisms in drinking water biofilms (community analysis)

Drinking water biofilms harbour diverse microorganisms including some pathogens. The organisms associated with potable water biofilms include bacteria, viruses, protozoa, fungi and algae.

Bacteria. The bacterial community in drinking water biofilms can be comprised of primary pathogens, opportunistic pathogens and non-pathogenic organisms. Primary pathogens which cause disease in healthy humans may persist within the biofilm. However, the survival time for many pathogens in biofilms is uncertain and varies depending on the organism. For some pathogens, the water distribution system is a physical, chemical and biological environment unsuitable for their growth. Some pathogens however, may accumulate in the drinking water biofilm, and the biofilm may extend their survival by protecting them from disinfectants. The primary intestinal bacterial waterborne pathogens include *Shigella, Salmonella, Yersinia enterocolitica, Campylobacter jejuni, Escherichia coli, Vibrio cholerae, Helicobacter pylori,* and *Legionella pneumophila* (LeChevallier, 1999; Costerton, 1989). The potential for them to attach to biofilms exists and limited growth in some circumstances can occur. Helicobacter pylori, was found to survive at least 192 hours on stainless steel coupons in a chemostat (Mackay *et al.,* 1998). Park *et al.,* (2001) have also noted the presence of *H. pylori* in biofilms of drinking water mains. In another study, two non-pathogenic *E. coli* strains injected into a pilot distribution system with a biofilm at 20 degree Celsius grew slightly in the biofilm before eventually dying out (Fass *et al.,* 1996). *Salmonella typhimurium* also was able to

grow for a short time at 24 degree Celsius in non-sterile tap water (Armon *et al.,* 1997). Some potable water bacteria may cause disease in humans under certain circumstances, especially in individuals with weakened immune system or other major underlying conditions that facilitate infections. These microbes are referred to as opportunistic or secondary pathogens. They include *P. aeruginosa, L. pneumophila, Mycobacterium avium* complex (MAC), *Acinetobacter calcoaceticus, Aeromonas hydrophila, Citrobacter spp, Flavobacterium spp, Enterobacter spp, Pseudomonas cepacia* and *Serratia marcescens.* Infective dose studies on healthy individuals and animals using the oral or intranasal route, demonstrate that very high doses (10^6-10^{10}) are needed for infection or disease for healthy individuals (Rusin *et al.,* 1997). These clinically listed significant strains of bacteria may cause diseases ranging from mild to severe pneumonia and septicemia (Toder, 1998; Thomas *et al.,* 1977). Since opportunistic pathogens affect sensitive individuals, such as hospitalised individuals the percentage of nosocomial infections caused by these organisms may provide some insights on the effects on sensitive individuals in general. There are several sources through which sensitive individuals can come into contact with these bacteria, with some cases being linked to drinking water (Thomas *et al.,* 1977).

Viruses. Viruses need a specific host (example humans) to proliferate; therefore, they may accumulate but not grow in the biofilm. A pilot-scale distribution system study demonstrates that more polioviruses1 were recovered from the biofilm than from the water column (Quignon *et al.,* 1997). In the presence of chlorine there were ten-fold more viruses in the biofilm than in the water flow (Quignon *et al.,* 1997). Viruses associated with waterborne disease that could potentially become entrained in the biofilm matrix include, *Hepatitis A virus, Hepatitis E virus, Poliovirus, Coxsackievirus, Echovirus, calicivirus, Astrovirus* and *Enteric adenovirus* (Benenson, 1995).

Protozoa. Several studies have examined the presence of protozoa in the distribution system or in pipe biofilms; and ciliates, amoebae, and flagellates have all been detected within biofilms in drinking water systems (Sibille *et al.,* 1998; Block *et al.,* 1993; Pedersen, 1990). Sibille *et al.,* (1998) found an average protozoal count of $1.0x10^3$cells/cm^2 in potable water biofilm. Since many protozoa feed on bacteria, it is likely that the protozoa population in the biofilm correlates with bacterial density. Some primary protozoal pathogens associated with drinking water biofilm include *Cryptosporidium parvum, Giardia lambia, Microspora, Acanthamoeba, Entoamoeba*

histolytica, Cyclospora cayatanensis, Naegleria fowleri and *Toxoplasma gondii* (Benenson, 1995). Available data suggest that these organisms may attach to and accumulate within the pipe biofilm and persist. Cryptosporidium oocyst attach rapidly to biofilms on glass tiles in a chemostat. There has been a report on the putative role of biofilms and amoeba in the proliferation, development and dissemination of potentially pathogenic *Legionella spp*. (Storey *et al*., 2004). The significance of protozoa as environmental reservoirs for *Legionella* is well described (Storey *et al.*, 2004). Although protozoan cysts/oocysts may attach to and accumulate within the pipe sediment and biofilm, these organisms do not likely proliferate in such environments. Several free-living protozoa have been implicated in waterborne diseases especially some *Acanthamoeba* species and *Naegleria fowleri.* Acanthamoeba is common in soil and water, including drinking water and home plumbing systems (Sawyer, 1989; Seal *et al.,* 1992).

Fungi. Fungi are ubiquitous in the environment. A diverse group of fungi has been found in water distribution systems (Rosenzweig *et al.,* 1986; Niemi *et al.,* 1982). Several studies report that filamentous fungi and yeast are common on water pipe surfaces even in the presence of free chlorine residuals (Nagy *et al.,* 1986; Doggett, 2000).Pathogenic fungi found in drinking water distribution systems and biofilms include, *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Cryptococcus neoformans, Candida albicans, Mucor, Petriellidium boydii, Sporothrix schenkii, Stachybotrys chartarium and Trichophyton* (Benenson, 1995). Although fungi have been found in drinking water distribution systems and biofilms, fungi have not been conclusively implicated in waterborne diseases. The pathogenic fungi that have been detected in the distribution system are opportunistic and infrequently cause illness. However, A. *flavus* and several other *Aspergillus* species detected in drinking water distribution systems produce potent toxins (mycotoxins) including aflatotoxins.

ALGAE. A few algal species, primarily cyanobacteria, or blue-green algae, produce algal blooms in fresh water, which can result in elevated toxin levels. An outbreak of waterborne gastrointestinal illness in the U.S. was associated with an algal bloom in an uncovered finished water reservoir (Lippy, 1976). The presence of algae or their toxins in the pipe biofilm has been demonstrated using scanning electron microscopy on a pipe surface. Diatom and other algal fragments or "microfossils" embedded in the biofilm were found (Nagy *et al.,* 1986 and Allen *et al.,* 1980).

1.1.6 Problems associated with biofilms in drinking water

1.1.6.1 Corrosion

Pipe corrosion may be caused by non-biological factors such as rapidly flowing water (causes erosion corrosion and impingement attack) or chemical oxidation processes (Schock, 1990). However, microbes in the biofilm can also play an important role in pipe corrosion. Ultimately, corrosion can lead to pipe leaks creating a pathway for pathogen intrusion into the drinking water. Biofilms on the inner surface of the pipe represent a complex and dynamic ecosystem that collectively influences corrosion process. The microbes most closely identified with pipe corrosion are the iron and sulphur bacteria. Iron bacteria such as *Gallionella* oxidize soluble reduced (Fe^{2+}), in the pipe and water to the insoluble oxidized form (Fe^{3+}), which precipitates (Costerton *et al.,* 1994). Microbes involved in the oxidation of iron and steel surfaces can deposit oxides of iron and manganese in raised hard outgrowths from the pipe known as tubercles (Costerton 1994). Sulphur-oxidizing microbes, such as *Thiobacillus spp*. generate sulphate and hydrogen ions, which lowers the pH, often resulting in a highly acidic environment that can pit and gouge metal. More importantly, sulphur-reducing microbes can generate hydrogen sulphide gas, which has a rotten odour, and which can accelerate corrosion (Costerton, 1987). Nitrifiers may decrease the pH by oxidising ammonium to nitrate and other nitrogen compounds, and thus corrode copper and other pipe materials (Schock ,1990; Keevil, 2004). Other bacteria produce polymers that may complex with pipe material or change the redox potential of the pipe surface, accelerating corrosion (Schock, 1990). Microbially-induced corrosion may penetrate 5/8 inch steel within six months (Costerton 1994).

1.1.6.2 Effect on coliform enumeration

The number of enteric coliform bacteria within drinking water is commonly used as an indicator of water quality. An extensive, well-developed pipe biofilm may compromise the effectiveness of total coliform bacteria as an indicator of drinking water quality in two major ways.

Firstly, a high level of heterotrophic bacteria in the pipe biofilm and sediments may interfere with the analysis of total coliform. This may occur when high numbers of heterotrophic bacteria detach from the biofilm and enter the water flow. As a result,

water samples collected for the analysis of total coliform may contain a large number of heterotrophic bacteria that, by competitive inhibition for nutrients and production of various toxins, may prevent the growth, and thereby detection of coliform using standard analytical media (Geldreich 1978; Seidler *et al.*, 1981).

Secondly, the conditions that facilitate microbial growth on pipes can result in the growth of coliforms including *E. coli* as part of the biofilm (Geldreich 1978; LeChevallier *et al.*, 1991). Biofilm coliform may detach into the flowing water and result in coliform positive samples. For some systems this phenomenon reduces the usefulness of the coliform test for detecting problems in water treatment or distribution system integrity.

However, a coliform positive test resulting from coliform growth in the biofilm may represent a distribution system deficiency because the conditions that permit its proliferation in the biofilm may also permit the growth of many other microbes as well, including opportunistic pathogens. In addition, an extensive coliform biofilm could reflect a high degree of pipe corrosion and deterioration, as well as water system operational systems (Geldreich 1978; Schock ,1990).

1.1.6.3 Effect on the Physico-chemical Properties of Drinking water

Aesthetic concerns such as discoloration of the water, taste and odour problems may result from a number of processes, some of which are microbially-mediated. Microbes most often linked to aesthetic problems in drinking water are the actinomycetes, iron and sulphur bacteria, and algae, especially the blue-green algae (Burlingame and Anselme 1995; Cohn *et al.*, 1999). Many algal species and some actinomycetes produce geosmin and 2-methylisoborneol, both of which produce earthy-musty odours. Some pseudomonads can also produce foul-smelling sulphur compounds. The bacteria in the genus *Hyphomicrobium*, when sloughed off from a biofilm, can cause episodes of black water (van der Wende and Characklis 1990).The percentage of complaints to water suppliers associated with aesthetic concerns is often high. According to a survey, 60% of responding utilities reported taste and odour to be their most common water quality problems, (van der Wende and Characklis 1990). Bacteria can be the starting point of a trophic food web leading to the proliferation of undesirable higher organisms.

1.2 Microbial interactions in biofilms

In most natural and industrial environments, biofilms are complex communities. The diversity in microbial communities leads to a variety of complex relationships involving inter-species and intra-species interactions. Interactions among bacterial species may have a profound influence on the initial stages of biofilm formation and development (Vieira *et al.,* 2007). The conventional analysis of drinking water micro-organisms such as plate counting, microbial biomass determination, and crude microbial metabolic measurement, unfortunately, give limited information on the complex microbial ecology of drinking water distribution systems (Keinanen *et al.,* 2004) and are unlikely to provide further evidence that can contribute to the development of effective biofilm control strategies.

Hence, the use of confocal lasers scanning microscope and other modern techniques. The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic forces, presence of microbial metabolites and molecules (cell-to-cell signalling communications) excreted by the micro-organisms and dominant microbial inhabitants in the biofilm (Keinanen *et al.,* 2004). Once cells are firmly attached to surfaces in the drinking water pipes, the activity of the community is dependent on the metabolism and growth of each member species under local surface conditions, such metabolic activities include: substrate consumption, cellular growth and replication, and synthesis of extracelluller polymeric substances (Characklis and Marshall 1990). The metabolic activities of those micro-organisms that become associated with a surface cause interfacial chemical gradients to evolve over time and space, creating conditions not normally encountered in the bulk water phase (Geesey *et al.,* 1994; Geesey 2001).

Several bacterial pathogens have been shown to associate with, and in some cases actually grow in biofilms. These include *Legionella pneumophila* (Murga *et al.,* 2001), *S. aureus* (Raad and Sabbagh 1992), *Listeria monocytogenes* (Wirtanen *et al.,* 1996), *Campylobacter jejuni* (Buswell *et al.,* 1998), *E. coli* 0157:H7 (Camper 1998), *Salmonella typhimurium* (Hood and Zottola 1997), *Vibrio cholerae* (Watnick and Kolter 1999), and *Helicobacter pylori* (Stark *et al.,* 1999). Although, all these organisms have the ability to attach to surfaces and existing biofilms, most appear incapable of extensive growth in the biofilm. This may be because of their inability to compete with indigenous organisms.

The mechanism of interaction and growth apparently varies with the pathogen. Survival and growth of pathogenic organisms within biofilms might be enhanced by the association and metabolic interactions with indigenous organisms, Camper (1998) showed that *Salmonella typhimurium* persisted in a model water distribution system containing undefined heterotrophic bacteria from an unfiltered reverse osmosis water system for >50 days, which suggest that the normal biofilm flora of this water system, provided niche conditions capable of supporting the growth of this organism.

1.2.1 Co-aggregation in biofilms

Co-aggregation is the specific recognition and adherence of genetically distinct bacteria to one another and occurs in a variety of ecosystems (Kolenbrander, 2000; Rickard *et al.,* 2003a). These specific surface interactions between different bacterial species are thought to play a major role in controlling succession during the development of a microbial community (Rickard *et al*., 2003b\; Simoes *et al*., 2007). The structure of human dental plaque is based in part on the mutual surface attraction of different bacterial species and specific molecular interactions play an integral part in the microbial organisation within some biofilms (Kolenbrander, 1988; Buswell *et al.,* 1997). Apart from the dental plaque, co-aggregation has been reported to occur in other biofilm communities such as fresh water biofilm bacteria (Buswell *et al*., 1997; Rickard *et al.,* 2004). Co-aggregation may mediate the sequential integration of species of bacteria in fresh water biofilms (Rickard *et al.,* 1999). It is highly specific in its mechanism and is thought to play a role in the development of multi-species biofilms in different environments (Kolenbrander and London 1993; Kolenbrander *et al*., 1999). Some advantages are conveyed to microorganisms through co-aggregation such as exchange of genetic information, metabolic cooperation between different species, transfer of chemical signals and protection from adverse environmental conditions (Simoes *et al*., 2007). There has been a report that in fresh water bacteria, co-aggregation between pairs of bacteria is typically mediated by a protein adhesion on one cell type and complimentary saccharide receptor on the other (Simoes *et al.,* 2008). Co-aggregation could be inter, intra, or multispecies interactions or a combination of which contributes to the overall structure and diversity of the bacterial community in fresh water biofilms (Buswell *et al.,* 1997; Rickard *et al.,* 2002; Simoes *et al*., 2008). Bacterial surface properties along with interspecies relationships are

believed to play a determinant role in the formation of multi-species biofilms in drinking water (Rickard *et al.,* 2003b; Simoes *et al*., 2008).

This project therefore aims to study how *P. aeruginosa* integrates and interacts within multi-species drinking water biofilm. This will provide new insights into mechanisms by which drinking water biofilms may harbour important pathogenic bacteria.

1.3. Microbial community analysis of drinking water biofilms using molecular and genomic approaches.

Traditional microbiological methods for testing water for the presence of pathogens rely on growth in culture media followed by isolation, and biochemical and serological identification. Majority of cells in the drinking water are not culturable even though they may be viable (Colwell and Grimes, 2000). Some cells which are otherwise culturable example (*Escherichia coli*) under certain conditions may enter a state of unculturability and these are called viable but not culturable (VBNC) (Oliver, 2005; Vieira *et al.,* 2004). Bacteria in the drinking water environment are exposed to different stresses through which they may become unculturable on the common media for these bacteria. Such bacteria may have acquired stress resistance by active mechanism, which in turn are genetically programmed but have developed recalcitrance to culture (Colwell and Grimes, 2000). The formation of VBNC cells has been proposed as a survival strategy in response to mild environmental stress such as nutrient deprivation or starvation (Ganesan *et al.,* 2007; Yamamoto *et al.,* 1996), temperature (Weichart *et al*., 1997), osmotic shock (Xu *et al.,* 1982; Asakura *et al*., 2005), sunlight radiation (Besnard *et al.,* 2002; Pommepuy *et al*., 1996), low pH (Chaveerach *et al.,* 2003) and presence of certain metal (Grey and Steck 2001).VBNC bacteria can become culturable again upon resuscitation under favourable conditions. Oliver (2010) provided a list of pathogen known to enter the VBNC state, in which all relevant water-associated bacterial pathogens are included example *Pseudomonas aeruginosa, Legionella pneumophila*, *Salmonella typhi*, or *Vibrio vulnificus*. Since culture methods will most likely not detect all active bacteria, alternative are now being sought such as DNA hybridization, polymerase chain reaction (PCR), and other proteomic and genomic methods. The advantages of such alternative methods are not only detection of non-culturable organisms but also their rapidity. Rapid detection of

pathogens in water is essential for ensuring the safety of water for consumers. Advances in biotechnology have resulted in the development of these methods. Two major categories of rapid detection methods include immunologic-based assays and genomic/molecular approaches.

1.3.1 DNA hybridization

DNA hybridization or gene probe assays consist of detection of DNA or RNA targets using complementary labelled nucleic acid probes (Afonina *et al.*, 2002; Allen *et al.*, 2001). Fluorescence in situ hybridization (FISH) has increasingly been developed for the most important species of bacteria. FISH is based on the specific binding of nucleic acid probes to specific regions on rRNA (Amman *et al*., 1997; Azevedo *et al.*, 2003). FISH methods are based on the use of conventional DNA oligonucleotide probes containing around twenty bases. Through nucleic acid hybridization, the degree of sequence identity can be determined and specific sequence can be detected and located on a given chromosome. This method can also be used for the detection of VBNC cells (Wilks and Keevil, 2006). The method comprises of three basic steps: fixation of a specimen on a microscope slide, hybridization of labelled probe to homologues fragments of genomic DNA, and enzymatic detection of the tagged target hybrids. Non-isotopic hybridization has become increasingly popular with fluorescent hybridization more than isotopic reagents for the detection of probe sequence. Protocols involving non-isotopic probes are considerably faster with greater signal resolution and provide options to visualise different targets simultaneously by combining various detection methods (Spector *et al*., 1998; Nistico *et al.*, 2009). It is possible to combine the FISH method with some viability or activity assays such as CTC (5-cyano-2, 3-ditolyltetrazolium chloride) and or DVC (direct viable count). PNA (peptide nucleic acid) probes which do not encounter electrostatic repulsion due to non-charged backbone which allows them to hybridize rapidly and tightly to nucleic acid targets are preferred to DNA probe which has to overcome a destabilizing electrostatic repulsion between the negatively charged backbones, resulting in slower and weak binding while hybridizing to complementary nucleic acid sequence. PNA probes are also preferred since these have been found useful when investigating drinking water biofilms due to their ability to penetrate even the thick biofilm layers (Wilks and Keevil, 2006).

1.3.2 Polymerase chain reaction (PCR).

The PCR is a powerful technique that has revolutionized molecular biology research and has application in diagnosis of microbial infections and genetic diseases as well as in detection of pathogens in water, food and environmental samples. The PCR is an in vitro method that employs a DNA polymerase enzyme and oligonucleotide primers to amplify a specific region of DNA. The choice of the DNA region to be amplified determines the specificity of detection (Cheng *et al.,* 2004).

Real time PCR: Conventional PCR generally involves four steps which include, nucleic acid extraction, DNA amplification, product detection and sizing by agarose gel electrophoresis and amplicon confirmation. A disadvantage of traditional PCR is that post-PCR sample handling may result in carry over contamination of the amplicon to future PCR-assay potentially causing false positive results.

Real times multiplex PCR:

In multiplex PCR assays, two or more primer pairs are used in a single reaction enabling simultaneous amplification of multi-target, resulting in saving of time, labour and cost. However, compared to PCR assays in which one sequence is amplified, multiplex assays can be tedious and time consuming to establish since more extensive optimization of reaction parameters is required to avoid the formation spurious amplification products and uneven amplification of target sequences (De vos *et al*., 1997; Morin *et al*., 2004).

1.3.3 Genomic and proteomic analysis of biofilm formation

Genomic and proteomic-based techniques such as DNA microarrays or 2-dimensional gel-electrophoresis allow analysis of the metabolic pathways that contribute to growth and survival of pathogens in water, foods, food processing environments, and in humans. These techniques also enhance the understanding of biofilm formation at the molecular level but have not revealed a common gene and /or protein expression pattern for biofilm formation in microorganisms (Ghigo, 2003; Sauer, 2003). Sauer (2003) stated that biofilm cells differ from planktonic cells in their patterns of gene expression and probably protein expression. Proteomic analysis of biofilm forming *Campylobacter jejuni* indicated that proteins involved in motility were up regulated in comparison with planktonic cells (Kalmokoff *et al.,* 2006).

DNA microarrays:

DNA microarrays are the latest in pathogen detection technology and represent a potential solution to the challenge of multiplexed pathogen detection. DNA arrays employed for pathogen detection consist of glass slide or nylon membranes onto which PCR products of target-specific sequences or oligonucleotides are bound. DNA samples analysis are chemically labelled with fluorescent dyes or are labelled during PCR amplification and are hybridized with their complementary sequences on the chip. Following hybridization and washing steps, the arrays are examined using a high-resolution scanner (Sergeev *et al.,* 2004).

1.4.1 The organism Pseudomonas aeruginosa

P. aeruginosa is the principal model organism used for biofilm research world-wide. It is a Gram-negative aerobic rod belonging to the bacterial family *Pseudomonadaceae.* It measures 0.5 to 0.8 µm by 1.5 to 3.0 µm (Rahme *et al.,* 1997). Almost all strains are motile by means of a single polar flagellum. The typical pseudomonas bacterium in nature might be found in a biofilm, attached to some surface or substrate, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in hay infusions and pond water samples (Miyata *et al*., 2003). *P. aeruginosa* isolates may produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. Clinical samples, in general yield one or another of two smooth colony types. One type has a fried-egg appearance which is large, smooth, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a mucoid appearance, which is attributed to the production of alginate slime (Miyata *et al.,* 2003; Webb *et al.,* 2004). *P. aeruginosa* secretes a variety of pigments including pyocyanin, fluorescein (yellow-green and fluorescent, known as pyoverdin) and pyorubin (King *et al.,* 1954). *P. aeruginosa* is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded opportunistic pathogen (Hachem *et al.,* 2007). The bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its outer membrane LPS. Also, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics (Donlan and Costerton 2002).

Some recent studies have shown that phenotypic resistance associated to biofilm formation or to the emergence of small-colony variants may be important in the

response of *P. aeruginosa* populations to antibiotic treatment (Oliver *et al.*, 2004). *P. aeruginosa* is ubiquitous in soil and water and on surfaces in contact with soil or water. It has a very simple nutritional requirement. It is often observed growing in distilled water (Favero *et al.*, 1971) which is evidence of its minimal nutritional needs. In the laboratory, the simplest media for growth of *P. aeruginosa* consist of acetate for carbon and ammonium sulphate for nitrogen and sulphur (LeChevallier *et al.*, 1991). Its optimum temperature for growth is 37ºC, and it is able to grow at temperatures as high as 42ºC. It is tolerant to a wide variety of physical conditions, including temperature, it is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly use antibiotics. According to Vanhaecke, pseudomonas cells adhere to stainless steel, even to electropolished surfaces within 30 sec. of exposure (Vanhaecke *et al.*, 1990). These natural properties of the bacterium undoubtedly contribute to its ecological success, and also help to explain the ubiquitous nature of the organism and its prominence as a nosocomial pathogen.

1.4.1.1 Contraction of *P. aeruginosa*

Each day we contact large numbers of *P. aeruginosa* through ingestion of food, from implements used to bathe us, and often *P. aeruginosa* may be present in low numbers in drinking water (Nelson, 2005). It has the ability to multiply in many aqueous environments, food sources, or surfaces. *P. aeruginosa* does not attack normal tissue. In order to cause infection, tissue must be damaged before exposure to virulent clones of this species (Saiman *et al.*, 1992). Specific conditions must be met for it to establish an infection: the particular *P. aeruginosa* clone must contact the target organ in large numbers and the host surface must possess certain specific defects in its defence and immune system (Duncan and Edberg 1995). Because *P. aeruginosa* is commonly found in the environment and because it causes serious infections, it has been assumed to be a primary health threat and measures have been recommended to eliminate it from water and food (Warburton, 1993; Warburton *et al*., 1994). *P. aeruginosa* has the capacity to adapt quickly to its host and environmental conditions. Its ability to undergo phenotypic variations under environmental stress has allowed the species to persist in adverse conditions such as nutrient deprivation, pH, oxygen depletion, osmolarity, and antibiotic or antiseptic substances attempting to remove them from their niche (Warburton, 1993; Warburton *et al*., 1994).

1.4.1.2 P. aeruginosa in drinking water

Heterotrophs are broadly defined as microorganisms that require organic carbon for growth. A variety of simple culture-based tests is used to recover a wide range of microorganisms from water and is collectively referred to as heterotrophic plate count (HPC) (Nelson, 2005). HPC in drinking water often include isolates from the following genera: *Pseudomonas, Acinetobacter, Moraxella, Aeromonas* and *Xanthomonas* (Rusin *et al*., 1997). Biofilms in distribution systems may provide a favourable condition for some bacteria, such as opportunistic pathogens (*Legionella spp., P. aeruginosa*, and *Mycobacterium avium*), to colonize it and may harbour pathogens, such as *Salmonella enterica serovar Typhimurium*, which have entered the distribution system (Armon *et al.,* 1997; Berry *et al.,* 2006; Keevil, 2002; Parsek and Singh 2003). Using a risk analysis approach it was determined that the presence of an opportunistic pathogen such as *P. aeruginosa* in water provided a risk of less than 1/10,000 for a single exposure to the bacterial agent for colonisation and not infection. *P. aeruginosa* needs to be ingested at a rate of 5000 to 10,000 per ml in two litres of daily intake to have a 1:10000 chance of colonisation (not infection) (Rusin *et al.,* 1997; Nelson, 2005). The estimated oral infection dose for healthy individuals is about 10^8 to 10^9 CFU ml^{-1} (Rusin *et al*., 1997). The infectious dose for immunocompromised individuals may be lower. The US environmental protection agency website for ground water rule (www.epa.gov/safewater/standard/phs/html) listed pathogens of concern in water systems and it reported that the most common waterborne bacterial pathogens are the opportunistic pathogens because they may enter the water system or may be found in situ and the most important of these opportunistic pathogens is the pneumonia and diarrhoea-causing *P. aeruginosa* (Rusin *et al*., 1997).

P. aeruginosa can occur sporadically in public drinking water systems and household installations, where it originates from the source water or may be introduced for instance during construction work (Hambsch *et al*., 2004). Once established *P. aeruginosa* is difficult to eliminate from water systems probably due to their persistence in biofilms as a result of protection being derived from this association (Bressler *et al.,* 2009*). P. aeruginosa* has been observed to be occasionally present in drinking water biofilms (Rogers *et al.,* 1994; Kilb *et al.,* 2005). It is shown that *P. aeruginosa* is able to colonise mixed-population biofilms in a drinking water

environment and can persist for extended periods in these habitats (Bressler *et al.*, 2009). The work of Bressler *et al*., (2009) has demonstrated that the opportunistic pathogen *P. aeruginosa* is capable of colonizing established mixed-population drinking water biofilms of high cell density for at least 7 days under stagnant conditions and 5 weeks under constant flow in a culturable state, indicating successful competition of *P. aeruginosa* with authochthonous biofilm microflora (Bressler *et al.*, 2009).

Bottled water represents a specific growth situation for opportunistic microbial flora. Bottled water contains no long-lasting disinfectant residual and the finished product is often stored in elevated room temperatures over a period of weeks before consumption. These conditions would allow certain microbes to grow to a level that is significantly higher than normally found in municipal water supplies. The organisms most widely isolated from mineral water are *Pseudomonas, Acinetobacter* and *Alcaligenes* (Warburton, 1993). In making a decision to include a limit of *P. aeruginosa* in bottled water, Health Canada (Warburton, 1993) found that the presence of *P. aeruginosa* can indicate serious contamination by pollution since it is associated with surface run-off, human faecal matter, domestic and agricultural effluent. *P. aeruginosa* is an important indicator of water quality since it has been cited as a major pathogen in waterborne and food borne disease by several researchers. The presence of *P. aeruginosa* may interfere with standard coliform enumeration procedures. *P. aeruginosa* contamination can degrade water colour, turbidity and taste and finally *P. aeruginos*a has been classified as "multiple antibiotic resistant" bacteria that can carry transmissible R-plasmids, and thus a major concern when found in "raw" ingested foods such as bottled water.

The WHO did not think *P. aeruginosa* can be used as an indicator of faecal contamination as it may also multiply in the aquatic environment. Its presence may be one of the factors taken into account in assessing the general cleanliness of water distribution systems and the quality of bottled water (Nelson, 2005).

1.5 Biofilm formation by Pseudomonas aeruginosa

Recently, many studies have suggested that biofilm formation in *P. aeruginosa* proceeds as a regulated developmental sequence, and five stages have been proposed (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Stages one and two are generally, identified by a loose or transient association with the surface, followed by robust

adhesion. Stages three and four involve the aggregation of cells into microcolonies and subsequent growth and maturation (figure 1.3). The biofilm structures can be flat or mushroom-shaped (figure 1.4) depending on the nutrient source which seems to influence the interactions between localised clonal growth and the subsequent rearrangement of cells through type IV pilus-mediated gliding motility in response to the nutritional cues (Klausen *et al.,* 2003). Stage five is characterised by a return to transient motility where biofilms cells are sloughed or shed. Detachment has been best described in non-mucoid *P. aeruginosa* biofilms. After initial biofilm growth, the micro colonies differentiate to form an outer wall of stationary bacteria (of biofilm phenotype), while the inner region of the micro colony liquefies, which allows motile cells to swim out of the micro colonies leaving a hollow mound (Sauer *et al.,* 2002). Liquefaction has been attributed to lysis of a subpopulation due to prophage-mediated cell death (Webb *et al.,* 2003). Significant variation has also been observed among *P. aeruginosa* colonies obtained from laboratory biofilms, as well as from persistent clinical infections caused by *P. aeruginosa*. The variants include mucoid (Deretic *et al.,* 1994; Martin *et al.,* 1995), dwarf (Martin *et al.,* 1995; Deziel *et al.,* 2001; HauBler, 2008), lipopolysaccharide-deficient (Dasgupta *et al.,* 1994), rough (Martin *et al.,* 1995), hyper-phyliated (Deziel *et al.,* 2001; HauBler, 2008) and antibiotic- resistant (Drenkard and Ausubel, 2002) colonies. There have been many studies on single species biofilm formation by clinical and laboratory strains of *P. aeruginosa*, but research on the formation and development of micro colony-based biofilms, by *P. aeruginosa* in the drinking water system is limited. Also not much is known about its biofilm formation and interactions within multi-species natural biofilms from drinking water systems. The research presented in this thesis investigates the ability of *P. aeruginosa* to form both mono-species and mixed-species biofilm in drinking water.

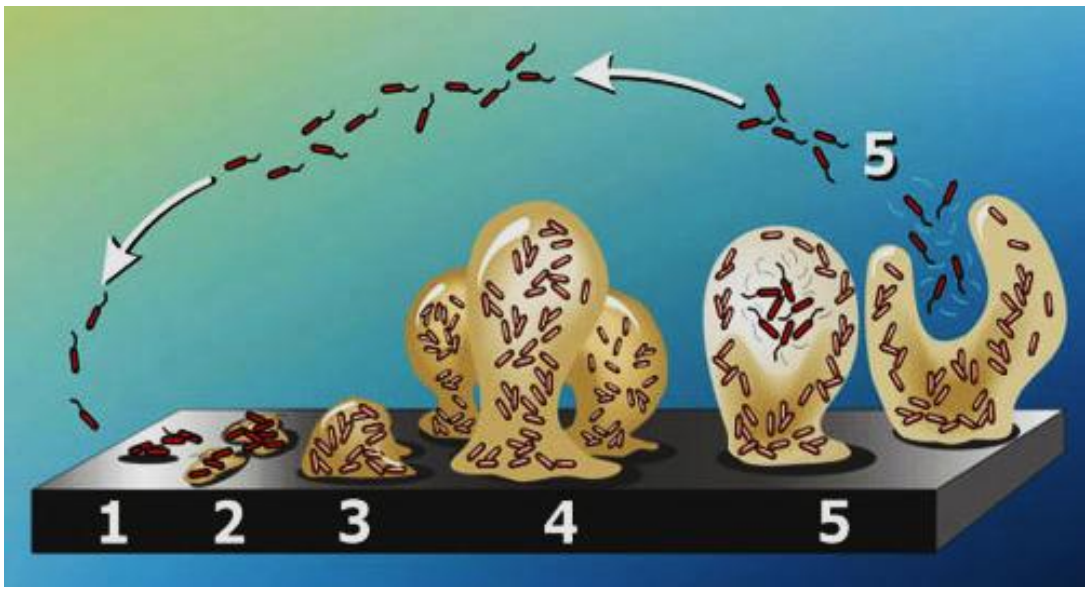


Figure 1.3 Different stages of biofilm formation in *Pseudomonas aeruginosa* 1. reversible attachment 2. irreversible attachment 3. microcolony formation 4. biofilm maturation 5. detachment (Centre for biofilm engineering at MSU-Bozeman, 2003).

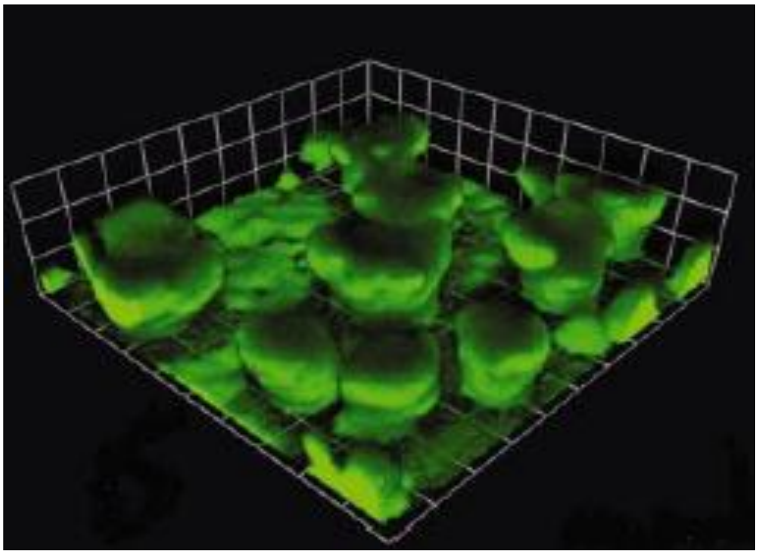


Figure 1.4 Microcolonies in a *P. aeruginosa* biofilm. (Greenberg EP, 2003).

1.6 The use of the green fluorescent protein (GFP) in the study of bacterial interactions.

Fluorescent probes have allowed investigators to study bacterial interactions by epifluorescence microscopy, laser scanning confocal microscopy and flow cytometry. Traditionally, bacterial pathogens have been detected indirectly by using fluorescent antibodies. Investors have directly coated microorganism with fluorochromes such as 3, 6-bis [dimethyl amino] acridinium chloride (acridine orange) (Kogure *et al.*, 1979; McFeters *et al.*, 1991), 4, 6-diamidino-2-phenylindole (DAPI) (Kepner and Pratt, 1994; Yu and McFeters *et al.*, 1994), SYTOX green, propidium iodide and many others to study real time interactions with live host cells (Falk *et al*., 1994). Whilst DAPI stains both living and dead cells, the discrimination between live (active) and dead cells can partly be done by the direct viable count (DVC) (Kogure *et al.*, 1997) and live/dead light assay (Boulos *et al.*, 1999). However, these approaches can be of limited value for following bacterial pathogens within live host cells or any other system; moreover, bacterial division dilutes the coated fluorescence signal during long-term infections. The GFP from *Aequorea Victoria* (Prasher *et al.*, 1992) can be employed to overcome these experimental shortcomings. Figure 1.4 shows bacteria cells expressing GFP protein. The GFP protein has several unique features that will prove advantageous to the study of bacterial interactions: (i) GFP is a cytoplasmic protein without toxicity (Chalfie *et al.*, 1994); therefore, the presence of GFP should have minimal effects on the bacterial cell-surface dynamics; (ii) GFP can be continuously synthesised, which minimizes the effect of fluorescence-signal dilution during bacterial replication; and (iii) GFP is easily imaged and quantitated (Chalfie *et al.*, 1994; Wang and Hazelrigg, 1994). GFP is now used extensively for labelling and tracking cells in biofilm experiments and for studies of gene expression where the GFP is fused to the protein/gene of interest example in the work of Conibear *et al* (2009) where the GFP reversion method was used to study mutation in *Pseudomonas aeruginosa* biofilm development.

For work in this thesis, all the *P. aeruginosa* strains were tagged with the GFP to differentiate them with the drinking water multi-species consortia.

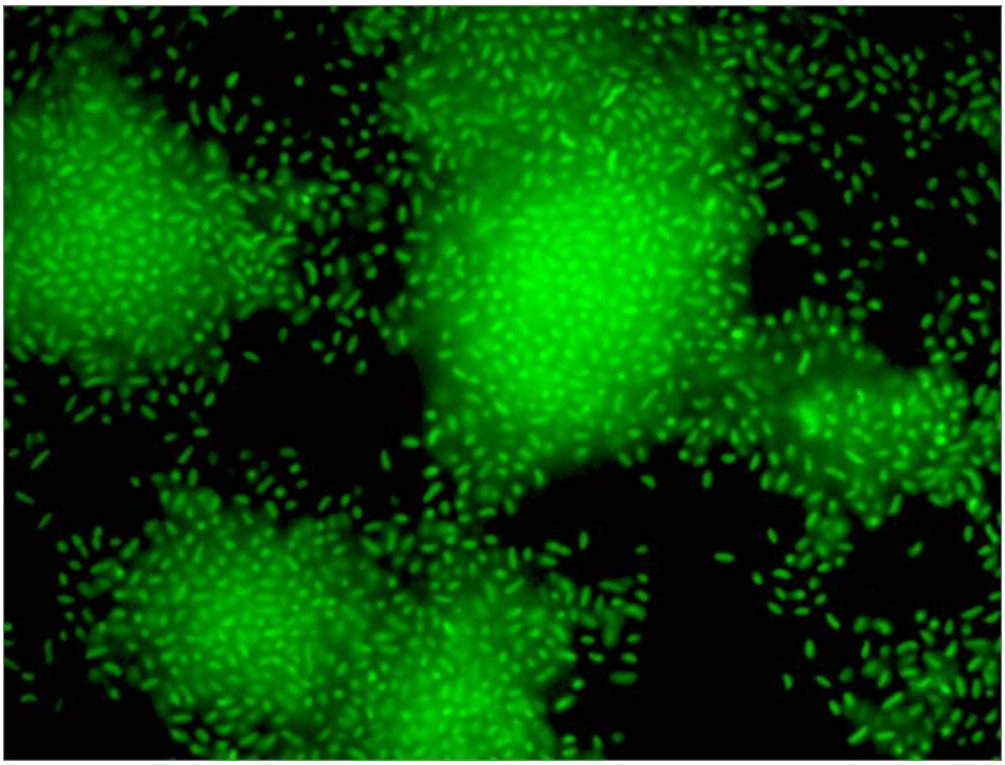


Figure 1.5 *Pseudomonas aeruginosa* biofilm expressing green fluorescent protein (GFP). X400 Conibear *et al.*, 2009

1.7 Role of mutation and genetic diversification in biofilms

Biofilm growth increases the ability of cells to persist in adverse conditions by inducing bacterial phenotype that is not apparently found in planktonically grown cells. Bacterial populations with a high level of genetic variability have a higher probability of survival in constantly changing environments (Taddei *et al.,* 1997a). Since genetic variability is generated mostly by mutagenesis, bacterial strains with high mutation rates are expected to have higher capacities for adaptation. Such strains are favoured by selection when the advantage of beneficial mutations is greater than the cost of being a mutator due to the overproduction of lethal and deleterious mutations (Mao *et al.,* 1997; Taddei *et al.,* 1997b; Tenaillon *et al.,* 1999) meaning that the acquisition of a stable mutator phenotype may confer a selective advantage for bacteria particularly in stressful and /or fluctuating environments because both high mutation rates and increased promiscuous recombination allow faster adaptation.

One mechanism that leads to defects in mutator cells is the methyl-directed mismatch repair system. Cells lacking one component of the methyl-directed mismatch repair system, *mutS, mutL, mutH, or uvrD,* are unable to repair DNA mismatches that occur as a result of replication errors, recombination or chemical damage (Chopra *et al.,* 2003). Mutator strains, having a defective mismatch repair system (MMR), have been observed in natural populations of *E. coli, S. enterica, N. meningitidis* and *P. aeruginosa* (LeClerc *et al.,* 1996; Matic *et al.,* 1997; Richardson and Stojiljkovic 2001; Richardson *et al.,* 2002). Because most of these isolates are pathogens, it has been hypothesized that mutators and hyper recombination phenotypes may accelerate the evolution of pathogenic strains by example increasing the variation of surface antigens, as well as by facilitating the acquisition of pathogenic determinants and antibiotic resistance.

Oxidative stress has also been linked to genetic variation in biofilms. Increasing the sensitivity of bacteria to oxidants increases both breaks of double stranded DNA and diversity and reducing oxidant stress has the opposite effect (Singh *et al*., 2008).

It has been observed that the levels of resistance to antibiotics were significantly

higher in mutator than in non-mutator pathogenic *P. aeruginosa* isolates (Oliver *et al.,* 2000). *P. aeruginosa* strains obtained from cystic fibrosis patients as well as in other natural bacterial populations such as drinking water are often hypermutable (LeClerc *et al.,* 1996). The most frequently involved system is the mismatch repair (MMR) system and *mutS* is the most frequently affected gene (Oliver *et al.,* 2000).
Recent *in vitro* work has shown that *mutS* deficiency in *P. aeruginosa* determines the immediate development of resistance to every single anti-pseudomonal agent due to the ascent to dominance of resistant mutants in a few hours during biocide exposure (Oliver *et al.,* 2004). The work of Conibear *et al.,* (2009) has also shown that mutator phenotypes can enhance micro-colony-based growth of *P. aeruginosa* cells. The group also reported how microcolony initiation and growth was enhanced with increased mutation frequency of *P. aeruginosa* within biofilms. The lifestyle of *P. aeruginosa* is such that the organism has to cope with a high number of stressful environments even though its genome seems to encode a vast adaptive capacity (Stover et *al*., 2000). Micro-colony-based growth can involve mutation and subsequent selection of mutants better adapted to grow on surfaces within crowded-cell environments (Conibear *et al.,* 2009). Increased mutation and homologous recombination rates may allow for the rapid adaptation to some unpredicted and /or fluctuating stressful environments. The lungs of cystic fibrosis patients and drinking water distribution systems are some of these highly stressful and fluctuating environments due to the challenges imposed by host defences and low nutrient and turbulence respectively.
This project aims to study the effect of mutation on the survival of *P. aeruginosa* within drinking water biofilms and how this affects its interactions within drinking water multi-species biofilms.

1.7.1 Mismatch repair protein (MutS)

The three-dimensional structure of MutS has been resolved for *E. coli* and *Thermus aquaticus* proteins (Bjornson *et al.,* 2003; Obmolova *et al.,* 2000). MutS is a 95 kDa protein that functions as a dimer in vivo.
Prokaryotic mismatch repair (MutS) proteins are encoded by a single gene and form homodimers (Allen *et al.,* 1997; Biswas *et al.,* 1999). MutS proteins belong to the lineage that participates in methyl-directed mismatch repair (MMR), prevents

homologous DNA recombination between heterologous sequences and mediates cell death induced by DNA damaging agents (Matic *et al.,* 1996).

All members of the MutS family possess a conserved ATPase activity (Modrich *et al.,* 1996) and a highly conserved phex-Glu motif responsible for binding DNA (Acharya *et al*., 2003). Both mismatch recognition and the ATPase activities of MutS are essential for MMR (Gradia *et al*., 1997). The MutS protein binds specifically to heteroduplexes DNA containing base mismatches (Su and Modrich, 1986), and deletion-insertions of up to four nucleotides (Parker and Marinus, 1992). Spontaneous mutation rates in bacteria are enhanced by mutation in at least 25 separate genetic loci (Miller, 1998; Horst *et al.,* 1999). The majority of mutators found so far among natural strains are due to defects in *mutS, mutH, mutL* or *uvrD* gene (LeClerc *et al.,* 1996; Matic *et al.,* 1997). These genes define the methyl-directed mismatch repair pathway, a DNA repair system that not only corrects base mismatches in newly replicate DNA but is also the main barrier for recombination of mismatched heteroduplexes in DNA (homologous recombination) (Modrich and Lahue, 1996). Thus, MMR defects relax the recombination barrier, allowing DNA exchange between species that normally do not mate. This promiscuous property unique to MMR mutants may explain the emergence and persistent of MMR mutants in natural populations.

Hypermutable strains display higher mutation frequencies than their wild type counterparts (Miller, 1996). They have been shown to play an important role in the adaptation of bacterial populations in changing and stressful environments (Taddei *et al.,* 1997a). Adaptation abilities are particularly relevant in the case of pathogenic bacteria like *Staphylococcus aureus* and *P. aeruginosa* for which conditions inside the host can be extreme and subjected to rapid changes.

The high incidence of MMR-defective mutators has been reported among *E.coli* and Salmonella pathogens (LeClerc *et al.,* 1996), and accounts of mutator subpopulations among pathogenic isolates of *E. coli* (Matic *et al.,* 1997; Denamur *et al*., 2002), *P. aeruginosa* (Oliver *et al*., 2002), *Neisseria meningitides* (Richardson *et al.,* 2001) and *Streptococcus pneumonia* (Negri *et al*., 2002) bolster the view that these mutators are positively selected in nature most likely because of the beneficial mutations they carry. Mutation rates vary widely among species (Drake, 1999) in relation to a set of population parameters such as stress. Mutation rates tend to increase with population size and frequency of sexual recombination and they tend to decline with increase in

the information content of the genome. Thus species with high mutation rates generally have small genomes, large populations, and high rates of sexual recombination (Lawrence and Roth, 1999). Organisms with high mutation rates adapt genetically to rapidly changing conditions and minimize the cost of deleterious mutations by changing very little genetic information and by having huge populations that include many individuals with impaired fitness along with some that remain largely free of deleterious mutations. Such species may be able to replicate faster by abandoning proofreading and repairs (Roth *et al.*, 2003).

Species with low mutation rates have lifestyles that depend on maintaining a considerable body of genetic information that is placed at risk by mutation. They are likely to adapt physiologically rather than genetically to stresses by regulating expression of their information. Consistent with these ideas, Drake (1999) provided evidence that for DNA-based microbes there is an inverse correlation between genome size and mutation rate that is; organisms with a large genome have lower mutation rates per base pair.

The research presented in this thesis investigates the effect of the defect in the DNA mismatch repair system in the formation of both mono-species and mixed-species biofilm by *P. aeruginosa* within a drinking water model system.

1.8 Control of biofilms in drinking water

Biofilm control is one of the important objectives for ensuring that water delivered to the consumers is of high quality. Many different methods have been used to control biofilms .The methods include nutrient control, control of contamination from materials and equipment, control and mitigation of system hydraulic problems, cross-connection control and backflow prevention, disinfectant residuals, corrosion control, infrastructure replacement and repair, and storage vessel management and alteration. Biofilm control requires the use of a variety of tools, rather than a single tool, and the relative effectiveness of a control practice may be site-specific.

1.8.1 Nutrient control

Nutrient control is recognised as one of the most effective methods for controlling microbial growth and biofilm formation. This can be accomplished by controlling the source of carbon or other nutrients (phosphorus, nitrogen), depending on the growth-rate limiting nutrients for the specific system. Control of nutrients for the subsequent control of growth in the distribution system is one of the main reasons

that systems apply biological treatment (Norton *et al.*, 2000). Some methods for nutrient control include biological treatment, coagulation, membrane filtration, and granular activated carbon (GAC). Control of nutrient levels is the most direct method of controlling biofilm growth, it is also the most difficult (USEPA, 1992a). Biofilm control through nutrient reduction may not be immediate. Several months are required for biological filtration to have an observable impact on bacterial water quality (Volk *et al.*, 1999). Careful disinfectant selection is also necessary. Oxidation with chlorine or ozone may increase the amount of biodegradable organic matter in the finished water. (Joret *et al.*, 1992 and LeChevallier *et al.*, 1992).

Since organic carbon is the primary carbon and energy source for much of the distribution system microbial activity, the rate and extent of biofilm formation can be minimised by control of the organic carbon concentration (Characklis, 1988). To control the influent organic carbon, treatment for control of assimilable organic carbon (AOC) is applied at the treatment plant. Control of the AOC concentration has proven so effective at controlling bacterial survival and growth that some systems have discontinued applying secondary disinfection. (Schellart, 1986; Van der Kooij, 1982). Biological treatment is one technology used to control AOC. Biological treatment uses microbial activity at the point of treatment to reduce the AOC concentration in the water entering the distribution system, thereby reducing the rate and extent of microbial growth and biofilm formation. Preozonation is commonly used, followed by biological filtration with GAC (Morin *et al.*, 1996). Preozonation oxidises organic matter to a more readily degradable form prior to biological treatment. Activated carbon, as both GAC and the powdered form (PAC), can effectively remove AOC from drinking water prior to distribution. GAC and PAC filter out the AOC by sorption. However, research suggests that coliforms and opportunistic pathogens can be associated with GAC particles released from GAC filters (Camper *et al.*, 1986 ; Stewart *et al.*, 1990). The GAC and PAC processes require careful control as well as careful monitoring of breakthrough of GAC particles containing organisms. The controls may include preozonation (Morin *et al.*, 1996). AOC is mainly low molecular weight non-humic substances that are difficult to remove by coagulation (Volk *et al.*, 1999). However, coagulation has been effective at removing dissolved organic carbon (DOC) and biodegradable dissolved organic carbon (BDOC) (Volk *et al.*, 1999). Both nanofilteration (NF) and reverse osmosis

(RO) have been suggested for the removal of nutrients (Crozes *et al.*, 2000). In addition to decreasing the concentration of organic matter from drinking water, NF and RO are also effective at removing microbes (Sibille,1998). The control of finished water phosphorus concentration may also help limit microbial survival, growth and biofilm formation, were phosphorus is the limiting nutrient. Systems with high organic carbon levels often have phosphorus as the growth-limiting nutrient (Mettinen *et al.,* 1997).

1.8.2 Control of contamination from materials and equipment

Control of contamination from materials and equipment can reduce the subsequent contamination of the distribution system. When microbial contamination is present on the materials or equipment used in distribution system maintenance, the microbes can become a part of the microbial community of the distribution system (provides a biological seed to the distribution system). By reducing the biological seed entering the distribution system, biofilm problems can be reduced (Trussell, 1999).

1.8.3 Poor operations and maintenance procedure

Poor operations and maintenance procedures facilitate pathogen entry into the distribution system (Berger *et al.,* 1993; Nygard *et al.,* 2007). Therefore, good distribution system maintenance techniques are viable alternatives for biofilm control (Camper, 1996). Disinfection of equipment and a high pressure flush of various tools with tap water could reduce the pathogen seed. To reduce biofilm sloughing, the most effective type of flushing is unidirectional flushing (LeChevallier, 1999a).

1.8.4 Control and mitigation of system hydraulic problems

System hydraulic control is important in controlling microbial contamination in the distribution system. Among the several measures, systems that can be used to control the hydraulic condition of the pipelines and biofilm is to flush and/ or pig (use of a water-propelled device) the pipeline at regular intervals. Flushing and pigging can remove the biofilm, sediments (Crozes *et al.*, 2000), and tuberculation, improving the system hydraulics. Where tuberculation is severe, flushing may not suffice. Pigging or the use of cable-drawn devices may be necessary (AWWA, 1987). Distribution system cleaning practices such as flushing and pigging will not prevent recolonization (Walker *et a*l., 1997). Flushing and pigging therefore are measures that many systems

should routinely conduct. The elimination of low-flow areas and dead ends can improve system hydraulics (Camper, 1996), thereby reducing microbe survival and biofilm formation. Backflow prevention devices are an important barrier against entry of contaminated water (LeChevallier, 1999b). Installation of backflow prevention assemblies or devices accompanied by the regular inspection of the assemblies and devices, as well as regular testing of the assemblies (Kirmeyer *et al.,* 2001), could prevent system contamination and biofilm formation.

1.8.5 Disinfection residuals

System provides disinfectant residuals throughout the distribution system (secondary disinfection) for protection of the finished water from microbial contamination in the distribution system. Among the reasons for secondary disinfection are the inactivation of coliforms and pathogens entering through cross-connections and line breaks, and the suppression of bacterial growth and biofilm in static water areas (Geldreich, 1996 ; Trussell, 1999). Secondary disinfection also protects against reinoculation of the flowing water by microbes trapped in the biofilm (Haas, 1999), which can occur through sloughing or erosion of the biofilm. Although contamination from cross-connections and backflow may be controlled by a disinfectant residual (Snead *et al.,* 1980), some water supply professionals believe a disinfectant residual is not effective when cross-connections result in massive contamination (LeChevallier,1999b and Snead *et al.,* 1980).The concentration of the residual disinfectant is important in determining biofilm bacterial density and composition (Norton *et al.,* 2000). Bacterial growth can be controlled with adequate disinfectant residuals (Morin *et al.,* 1996), and bacterial density will remain low (Berger *et al.,* 1993). However, many factors influence the concentration of the disinfectant residual in the distribution system, and therefore the ability of the residual to control microbial growth and biofilm formation. These factors include the AOC level, the type and concentration of disinfectant, water temperature, pipe material, and system hydraulics. These factors make the prevention of pathogens survival and growth to require strict attention to the residual disinfectant throughout the system (Trussell, 1999). Disinfectant residual penetration into biofilms can also be inhibited by corrosion products due to the reactions between the residuals and the corrosion products (LeChevallier *et al.,* 1993; LeChevallier *et al*., 1996). Various studies have obtained different results with respect to the ability of chlorine to control biofilms, depending on the chlorine level used and other factors. At higher

levels (1mg/L), free chlorine or chloramine was effective in disinfecting biofilms on galvanised iron, copper or PVC pipes (LeChevallier *et al.*, 1990b). However, some investigations have found that significant biofilms can develop in the presence of low levels of residual free chlorine, and even at high levels , biofilms are not eliminated (Characklis,1988). According to Nagy (1986), bacterial densities in biofilms were unaffected by the presence of free chlorine residuals. It was also noted that free chlorine residuals were ineffective in controlling coliform occurrences when the AOC concentrations are greater than 100 micron gram per litre (Camper, 1996). Characklis (1988) noted that biofilm sloughing has not been documented in the presence chlorine. Some research has shown that most of the free chlorine is depleted before penetrating the biofilm (Haas *et al.,* 1999). In contrast to biofilms, chlorine controls the level of heterotrophic bacteria and viruses in water (Characklis, 1988 and Quignon *et al.,* 1997). Chloramines can also be used for controlling microbial growth and biofilm formation in the distribution system. Chloramines may be preferred over free chlorine when the disinfecting objective is biofilm control (Trussell, 1999), as chloramines may be more effective than free residual chlorine at controlling biofilm formation. In support of this position, LeChevallier (1999a) found that in filtered systems, coliforms were present in 0.97 per cent of systems using free chlorine, but only 0.51 per cent of systems using chloramines. While chloramines can be used to retard biofilm formation, no disinfectant completely eliminates biofilms. One study found that neither chlorine residuals nor chloramines residuals alone were able to control biofilm development but when used in combination, biofilm were controlled (Momba *et al.*,2002).

1.8.6 Corrosion control

The ability of corrosion control to inhibit biofilm formation is widespread (Smith *et* al., 1990; LeChevallier *et al.,* 1990a). Corrosion control may include the inhibition of biofilm formation or the prevention of biofilm sloughing by coating the biofilm (Berger *et al*., 1993). Corrosion is among the fundamental factors leading to the release of biofilms into the water (LeChevallier *et al*., 1998). Several factors influence the effectiveness of corrosion inhibitors for controlling microbes and biofilm formation. One of the factors includes high concentrations of organic materials (Volk *et al*., 2000). Corrosion control can also impact disinfection effectiveness on biofilm, with an increase in free chlorine disinfection effectiveness were observed when using

corrosion control on iron pipes (LeChevallier *et al*., 1990b, Lowther *et al.*, 1984; Martin *et al*., 1982). However, the use of corrosion inhibitors can be detrimental at excessive concentrations. A legionellosis outbreak in Lanzarote Canary Island in 1993 may have been amplified due to excessive polyphosphate concentrations (Crespi *et al*., 1997).

1.8.7 Infrastructure replacement and repair

In some cases where growth is severe, replacement of pipe sections may be necessary (USEPA, 1992b). When systems have frequent contamination problems resulting from deficiencies in or due to the nature of the infrastructure, the system may opt to initiate a repair or replacement program. In general, pipe replacement should target sections of the system experiencing the greatest number of leaks (Kirmeyer *et al.*, 2001). Mains are not the only infrastructure whose failure can lead to contamination. Failure of other appurtenances, such as isolation valves, air valves and surge control devices may lead to contamination (Kirmeyer *et al*., 2001). Some materials used in drinking water distribution systems may provide limiting nutrients to biofilm organisms and enhance microbial growth. In some instances, replacement of these materials may be a viable solution. These materials may include the pipes, valves, joints, fittings, or joint-packing materials. Bacteria can colonize piping, pipe joints, valves, elbows, tees, and other fittings due to the changing water movement and stagnant areas of the distribution system (Berger *et al*., 1993).

1.8.8 Proper storage vessel management and alteration

Proper storage vessel management and alteration, when necessary, can prevent contamination of the distribution system. To reduce pathogen presence and biofilm development, systems should have a scheduled program to rehabilitate all water storage facilities (USEPA, 1997). Storage tanks and stand pipes should be pressure flushed or steam cleaned, then disinfected before returned to service (USEPA, 1992b), preferably with a disinfectant solution. This may not only remove microbial contamination from the vessel's inner surface, but also nutrients that may be present. Properly designed inlets and outlets, and the overall system design can improve problems caused by dead ends (Trussell, 1999). Pathogen contamination due to air introduction can be reduced by installing air filters to guard against pollution

entering covered water reservoirs (USEPA,1992b). Covering finished water reservoirs can protect against contamination from airborne sources, surface runoff, accidental spills and animals, such as insects and birds (USEPA,1992b). Proper turnover of the water in finished water storage facilities eliminates what amounts to dead ends and can reduce the extent to which biofilms develop, minimize nutrient availability and prevent the accumulation of sediments. To accomplish this, systems can exercise valves to reduce stagnation, and eliminate excess storage (Crozes *et al*., 2000).

1.9 Disinfection by ultra violet irradiation (UV)

Ultra violet rays are energy-rich electromagnetic rays that are found in the natural spectrum of the sunlight. They are in the range of the invisible short wave light having a wavelength ranging from 100-400nm. UV disinfection systems are an effective and economical means of water purification and water disinfection, by eliminating harmful bacteria and other microbes from drinking water supplies. Micro-organisms must be exposed to the UV light for a certain amount of time. This is called the dose or more correctly fluency, measured in joules/m^2: Ws/m^2. The amount of UV light required is dependent on the type of organism. The pathogen must be exposed for a period of time by the correct amount of light at the right wavelength of 254nm. The relationship between right light volume and time is critical for effective reduction. *E. coli* requires approximately 6mWs/cm2 to achieve 90% reduction (Chang *et al.,* 1985). When UV energy is absorbed by the reproductive mechanisms of bacteria and viruses, the genetic materials (DNA/RNA) is rearranged and they can no longer reproduce. The mutation caused is brought about by two chemical changes. The first one is the formation of pyrimidine dimers which distort the shape of the DNA molecule and thereby interferes with the normal base pairing. A second change is a hydration of pyrimidine residues. The bacterial response to DNA damage caused by UV light, ionising radiation involves the repair of DNA by several processes. Two products of UV damage, pyrimidine cyclobutane dimers and pyrimidine 6’ to 4’ photo products, are known to be involved in cellular lethality and both are handled by the nucleotide excision mode of repair in most organisms (figures 1.6 and 1.7).

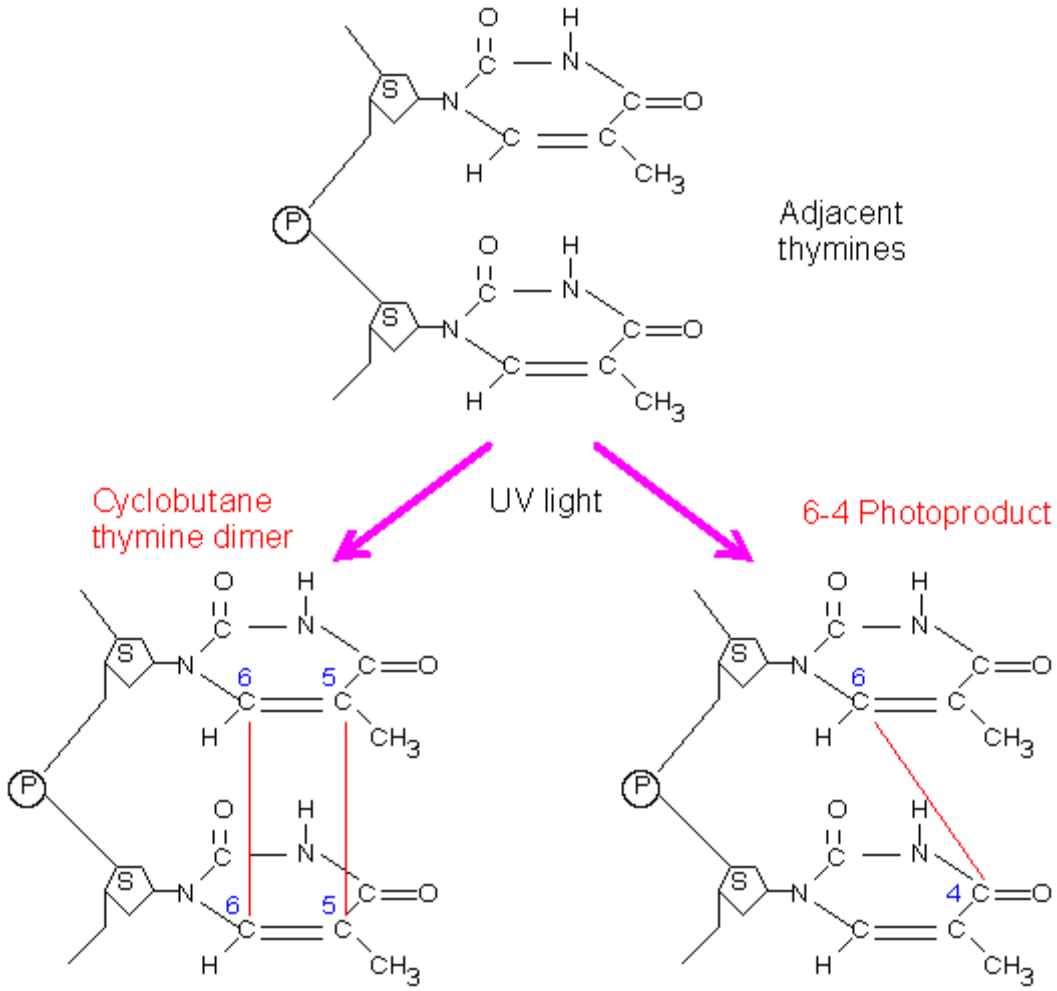


Figure 1.6 bacterial responses to UV damage by the formation of pyrimidine cyclobutane thymine dimer in bacterial DNA. This figure uses thymine as an example. Cytosine may form a similar dimer.
(http://www.webbooks.com/MoBio/Free/Ch7F5.htm).

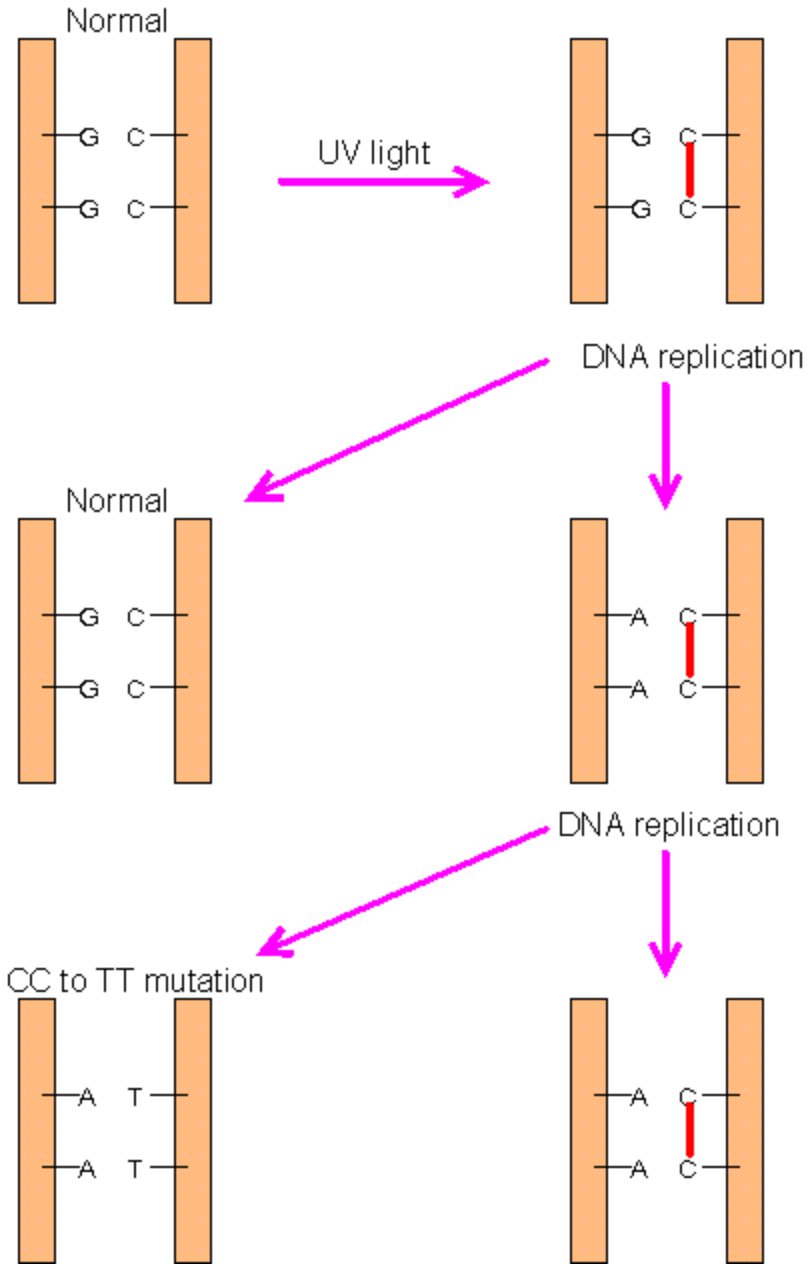


Figure 1.7 A possible mechanism for the mutation induced by UV light. (http://www.web-books.com/MoBio/Free/Ch7F5.htm).

In the above figure, the UV light causes two adjacent cytosine residues to form a dimer. During DNA replication, both strands are used as templates to synthesize new strands. The cytosine dimer could cause adenine instead of normal guanine to incorporate into the new strand. Subsequent DNA replication will produce CC to TT mutation. Although the cytosine dimer may eventually be corrected, the mutation cannot be detected by the DNA repair system.

1.9.1 Susceptibility of Micro-organisms

UV irradiation destroys the genetic structure of microorganisms and inhibits its ability to reproduce and ultimately causing its death. The germicidal wavelength of UV ranges from 200nm to 285nm. Low doses of radiation may not produce any adverse effects on cells. However, if the exposure time or the intensity is of the UV light is increased an increase in the number of unpaired dimers and mutation probably occur. The selection of UV wavelength is very important for efficient disinfection process. The optimum microbial killing efficiency ranges from 254-260nm wavelength (Johnson *et al*., 2010). Generally, the UV dose required to inactivate viruses and moulds is much higher than for bacteria (Chang *et al.*, 1985; Morgan, 1989). However, resistance of bacteria differs between the species and also depends on age of the organisms, presence of spores and size of the bacteria with the smallest bacteria being most resistant. The reason may be due to the amount of UV light absorption per cell. In general, Gram-positive bacteria tend to be more resistant to UV radiation than Gram-negative organisms and spore formers are more resistant than non-spore formers. Vegetative bacteria tend to be most resistant to UV radiation just prior to active cell division, during the lag phase. It has also been demonstrated that a given dose will become less effective with a higher number of cells and the absence of oxygen increases microbial resistance to UV irradiation (Jay, 2000). The UV dose required to destroy *P. aeruginosa* is 5.5 mWs/cm^2 for 90% killing (1 log reduction) and 10.5 mWs/cm^2 for 99% killing (2 log reduction) (Johnson *et al*., 2010). However, in water treatment the UV intensity, water quality, contacts time, flow rate, surface area of the disinfection chamber etc. are very important to provide the adequate UV dosage for microbial disinfection. If these are not carefully controlled and validated then, bacteria may escape unharmed or become partially irradiated and may latter undergo DNA repair within the drinking water biofilms which could give rise to mutator strains that possess enhanced adaption and survival within the drinking water biofilms thereby making them difficult to eliminate. The research presented in this thesis aims to evaluate the susceptibility and mutability of UV irradiated *P. aeruginosa* in mixed-species drinking water biofilms.

1.9.2 Aims and objectives

Pseudomonas aeruginosa is an important pathogen of humans having the ability to form biofilms in drinking water. They are often hypermutable when obtained from natural biofilms due to defective DNA error repair systems (Oliver *et al*., 2000). However, the role of mutation frequency in determining their survival and fitness within environmental multi-species natural biofilms such as drinking water distribution pipelines and the effect of some micro-environmental conditions as well as some drinking water disinfection practices on their mutation frequency, survival and persistence has not been explored.

This research therefore aims to:
1. Examine the role of mutator phenotypes in *P. aeruginosa* survival within multi-species drinking water biofilms.
2. Study the effect of bacterial interactions on the mutation frequency of *P. aeruginosa* within polymicrobial biofilms derived from drinking water and
3. To evaluate the influence of ultra violet (UV) treatment on *Pseudomonas aeruginosa* mutability and survival within mixed-species drinking water biofilms. `

Chapter Two

Role of mutator phenotypes in *Pseudomonas aeruginosa* survival in drinking water biofilms

Abstract

The survival of bacteria in nature is greatly enhanced by their ability to grow within surface-associated communities called biofilms. Biofilms provide a protective stronghold for harmful pathogenic bacteria within the environment, for example biofilms in drinking water distribution pipelines can harbour bacteria such as the opportunistic pathogen *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Much research has been carried out on *P. aeruginosa* single species biofilm formation and its role in human infections, but little is known about the interactions that can occur within complex and mixed-species bacterial communities within the environment. A recent finding is that strains of *P. aeruginosa* obtained from natural biofilms are often hypermutable due to defective DNA error repair systems. However, the role of mutation in determining survival and fitness of *P. aeruginosa* within the environment has not been explored. Analysis of mixed species biofilms showed that the average biomass generated by the mutator strain was 32-fold higher than that of the environmental isolate and 230-fold more than the wild type strain. In terms of average microcolony development there was 6-fold increase in the number of microcolonies developed by *mutS* strain more than the environmental isolate and 20-fold increase more than the wild type strain, while the average biofilm thickness of the *mutS* strain was 25-fold higher than the average thickness of the environmental isolate and 242-fold increase more than the wild type. Our results therefore showed that *Pseudomonas aeruginosa* mutator strains are better able to integrate into drinking water biofilms compared to non-mutator strains and also can persist longer within these multi-species biofilms. Thus the *mutS* mutator phenotype can enhance the integration, survival and persistence of *P. aeruginosa* in laboratory grown, drinking water multi-species biofilms.

2.1 Introduction

Biofilms serve as a reservoir of bacteria that can affect animal and human health. Any microorganism (including some pathogens) present in drinking water may attach, or become enmeshed in the biofilm and primary pathogens, which cause disease in healthy humans, may survive for a time in the biofilm (Camper *et al.,* 1986; LeChevallier *et al.,* 2003). A wide range of primary and opportunistic pathogens have demonstrated the ability to survive, if not grow, in drinking water biofilms. These pathogens are of both faecal and non-faecal origin, and have a multitude of pathways through which they can enter the water distribution system. Some of the pathogens identified as growing or potentially surviving in biofilms include *Legionella spp., Mycobacterium avium complex, P. aeruginosa, Poliovirus 1, Coxsackie virusB* and several species of fungi (Hall-Stoodley *et al.,* 2004). Although, all these organisms have the ability to attach to surfaces and existing biofilms, most appear incapable of extensive growth in the biofilm. This may be because of their inability to compete with indigenous organisms. In the aquatic environment, the survival of pathogenic micro-organisms such as *Salmonella spp.* and *P. aeruginosa* may represent a risk to public health. *P. aeruginosa* are ubiquitous bacteria. They are constantly shed into the water distribution pipelines through the environment as a result of pipe leakages and water treatment lapses. The ability of *P. aeruginosa* to be transmitted by this route depends on its resistance to environmental factors that control survival.

Under natural conditions, true mono-species biofilms are rare and in most natural and industrial environments, biofilms are complex communities. Diversity in microbial communities leads to a variety of complex relationships involving interspecies and intraspecies interactions (Vieira *et al.* 2007). Interactions among bacterial species may have a profound influence on the initial stages of biofilm formation and development. The conventional analysis of drinking water microorganisms such as plate counting, microbial biomass determination, and crude microbial metabolic measurement, unfortunately give limited information on the complex microbial ecology of drinking water distribution systems (Keinanen *et al.,* 2004) and are unlikely to provide further evidence that can contribute to the development of effective biofilm control strategies.

Survival and growth of pathogenic organisms within biofilms may be enhanced by the association and metabolic interactions with indigenous organisms. Camper *et al* showed that *Salmonella typhimurium* persisted in a model water distribution system containing undefined heterotrophic bacteria from an unfiltered reverse osmosis water system for >50 days, which suggests that the normal biofilm flora of this water system, provided niche conditions capable of supporting the growth of this organism (Camper, 1998).

P. aeruginosa is an important opportunistic pathogen of humans and also has the ability to form biofilms in drinking water (LeChevallier *et al.,* 1987). Much research has been carried out on *P. aeruginosa* single species biofilm formation and its role in human infections, but not much is known about its biofilm formation and interactions from an ecological perspective. *P. aeruginosa* obtained from natural biofilms are often hypermutable due to defective DNA error repair systems (Oliver *et al.*, 2000) Recent in vitro work has shown that *mutS* deficiency in *P. aeruginosa* determines the immediate development of resistance to every single anti- pseudomonal agent due to emergence of resistant mutants in a few hours during biocide exposure (Oliver *et al.*, 2004). The effects of mutation rates on the fitness of bacterial cells have been studied in *E. coli* and *Salmonella typhimurium* serotype under various growth and starvation conditions (Hughes and Anderson 1997;Taddei *et al*., 1995). Competition experiments between mutator and wild type bacteria sharing the same environment have demonstrated an adaptive advantage for the mutator population. This advantage arises from an increased probability of acquiring adaptive mutations, compared to that of the wild type population (Giraud *et al*., 2001; Tenaillon *et al*., 2001). Cells under conditions of cellular crowding within biofilms are constrained by intensely competitive and nutrient limited growth conditions. In these circumstances micro evolutionary processes such as mutation selection are often important for growth. *P. aeruginosa* in the drinking water distribution pipelines also have the ability to form biofilms with other drinking water bacteria (van der Kooij *et al*., 1982), but its mutability and fitness under these conditions have not been explored. To obtain further insight into the role of mutator phenotypes in bacterial fitness, we hypothesized that hypermutators have a fitness advantage over the wild type.

This project therefore investigated the effect of mutation on the survival of *P. aeruginosa* within a multi-species drinking water biofilm model system.

2.1.1 Materials and Methods

2.1.2 Preparation and storage of a multi-species drinking water standard bacterial inoculum

The inoculum for this study was prepared from tap water by filtering tap water (approximately 1000 L) from the Southampton municipal water supply using a filter unit containing an Ultipor nylon membrane of 0.2 µm pore size of 142 mm in diameter, at a flow rate of 3 L min^{-1}. The filter membrane was suspended in a 500 ml sterile bottle containing 50 ml of milliQ water and some glass beads. This was vortexed for 30 min to shake off the bacteria attached to the filter membrane. The volume of water was filtered using several fresh sterile filters to avoid clogging, until enough bacterial suspension was generated from the tap water. This was pooled together to form a bacterial suspension stock. The number of bacterial cells in the pooled suspension was enumerated by carrying out serial dilution and plating 100 µl of each dilution onto R2A (Oxoid United Kingdom) and Nutrient agar (Oxoid United Kingdom) (Kalmbach *et al.,* 1997) by using the spreading method. This was incubated for 24 or 48 h at 22ºC. Sterile 20% (w/v) glycerol was added to the pooled bacteria stock and mixed thoroughly by shaking, which was then aliquot into tubes (5 ml per tube) and stored at -80ºC for subsequent use throughout the experiments described here.

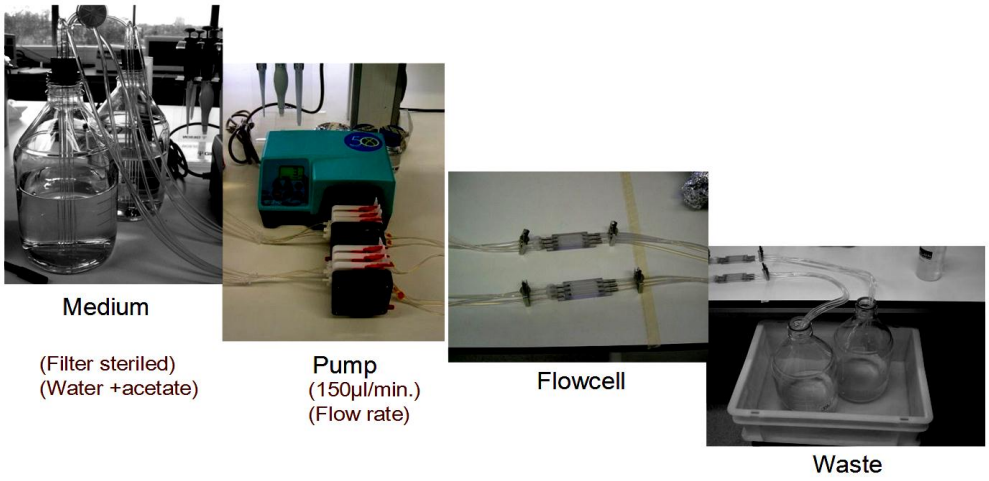
2.1.3 Biofilm media preparation

Tap water was stored in a reservoir (20 L Nalgene aspirator) and stored for 24 h in order to allow for chemical breakdown of chlorine. This tap water was then sterilized by passing the water (2 x 2 L aliquots) through a sterile filter unit containing 0.2 µm pore sized polycarbonate membrane disk of 47 mm in diameter (Whatman) with the aid of a vacuum pump. Sodium acetate was added to sterile tap water to serve as carbon source. Sodium acetate was used because of its importance in water treatment plants as a carbon source and in the de-nitrification of waste water.

2.1.4 Biofilm experimental setup and the formation of drinking water biofilms using drinking water filtrates as inoculum.

Drinking water biofilms were grown under continuous flow culture conditions at sodium acetate concentrations of 30 mg L^{-1} and 200 mg L^{-1} to evaluate the extent of biofilm growth using different carbon concentrations. These concentrations yielded 8.8 mg L^{-1} and 58.7 mg L^{-1} of carbon respectively. A flow cell channel dimension of 1 x 4 x 40 mm was used and incubated at room temperature of approximately 22 ^{o}C. Using a flow cell apparatus and set up (figure 2.1) (Moller *et al.*, 1998), the channels were inoculated with 0.5 ml of thawed stock culture of drinking water filtrate containing approximately 2.0 x 10^4 cells ml^{-1} and incubated without flow for 1 h, then allowed to flow at a rate of 150 µl min^{-1} (Laminar flow) (Stoodley *et al.*, 2002). Biofilms were visualised on glass surface of flow cells after 24 h of growth using both epifluorescence and confocal scanning microscopy.

A



B

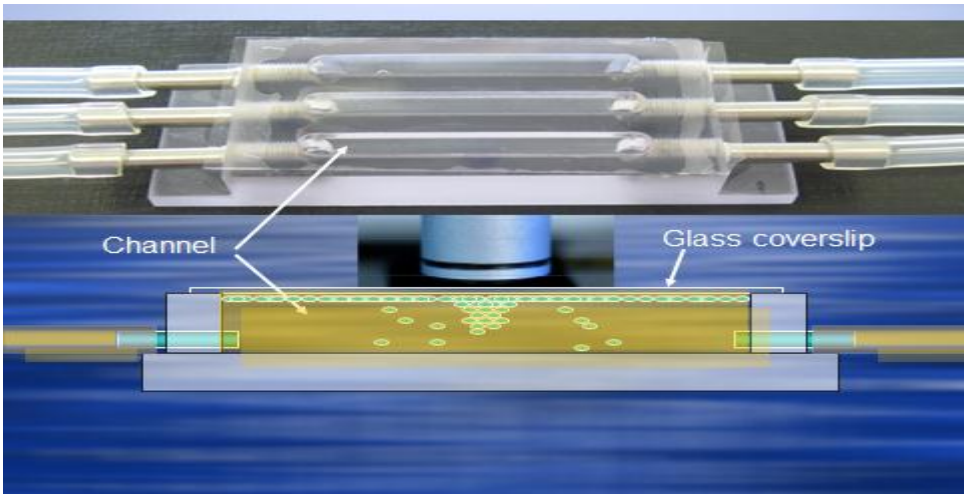


Figure 2.1 A. Biofilm experimental setup showing two flow cells connected to the peristaltic pump and effluent bottles using silastic rubber tubing. **B.** Enlarged image of the flow cell showing the attachment of cells onto its glass surfaces.

2.1.4.1 Fluorescent labelling of Pseudomonas aeruginosa PAO1 WT and ΔmutS strains

Escherichia coli and *P. aeruginosa* were maintained on LB agar (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of Bacto Agar per litre). A 1:1 mixture of pseudomonas isolation agar (Difco) and LB agar was used to select *for P. aeruginosa* following mating with *E. coli*. Antibiotics were used at the following concentrations (per mL): gentamicin at 100 µg and carbenicillin at 300 µg.

DNA manipulation. General DNA manipulations were performed by Nivens *et al* (2001) using the method of Maniatis (Maniatis *et al*., 1982). Restriction endonucleases were purchased from Boehringer Mannheim. Triparental matings was used to mobilize plasmids from *E. coli* and *P. aeruginosa* using the conjugative helper plasmid pRK2013 (Figurski and Helinski 1979). Oligonucleotide primers were synthesized on an applied Biosystems 380B DNA synthesizer. To visualise bacteria with SCLM, a plasmid that constitutively expressed the green fluorescence protein GFP was introduced into each of the *P. aeruginosa* strains. The gene for the GFP containing the *mut2* mutation (Cormack *et al*., 1996) was amplified by PCR from plasmid pBCgfp (Stratton. 1998). The PCR primer used in the amplification is as follows: GFP Sal3, 5'-GCGCGTCGACAGGAGAAGAAAAAATGAGTAAAGGAGAAGA-3'; and GFP Hind4, 5'-GTACCTGGAATTCTACGAAGCTTATTTGTATAGTTCATCC-3'. The primer was designed to introduce a *P. aeruginosa* ribosomal binding site upstream of *gfp*. The PCR product was digested with Sall and HindIII and ligated into pUC19. The Xbal and fragment from pUC19, containing the *GFP mut2* mutation was ligated into vector pMF36 (Franklin and Ohman 1993) behind the trc promoter forming plasmid pMF230. pMF230 contained an *oriT* site and the stable application fragment and therefore could be mobilized into *P. aeruginosa* by triperental mating where it was maintained. Since pMF230 does not contain the *lacI* repressor, *gfp* was expressed.

2.1.5 Isolation of environmental strains of Pseudomonas aeruginosa

Water samples used for the isolation of *P. aeruginosa* were obtained aseptically from a nearby stream on Southampton Common using sterile 50 ml centrifuge bottles

wrapped with aluminium foil. Plates of cetrimide agar selective for *P. aeruginosa* were prepared and inoculated with 100 µl of the samples and plates were incubated at 37^{o}C for 48 h. *P. aeruginosa* PAO1 and *E. coli* JM109 were also inoculated on cetrimide agar which served as both positive and negative controls, respectively. Cetrimide agar was used because of its inhibitory action on other bacteria other than *P. aeruginosa* and its use in the production of fluorescein and pyocyanin.

2.1.6 Direct detection and Identification of the environmental strains of Pseudomonas aeruginosa by multiplex PCR

The *P. aeruginosa* colonies on the cetrimide agar plates were further screened by multiplex PCR using the method of (De Vos *et al.*, 1997) based on the identification of two outer membrane genes *oprL* (504bp) which is *P. aeruginosa* specific, and *oprI* (249bp) which is specific to fluorescent *Pseudomonads*. Two colonies of each strain were suspended in 100 µl sterile water. PCR microcentrifuge tubes were filled with 1 µl of the above colony suspension and 19 µl of sterile water. The thermo cycler was used for the lysis of cells using the following cycling profile: 65^{o} C for 15 sec, 96^{o} C for 2 min, 65^{o} C for 4min, 96^{o} C for 1 min, 65^{o} C for I min, 96^{o} C for 30 sec and 20^{o} C hold. Total 20 µl PCR mix was prepared in PCR tubes containing 10 µl Gotaq Master mix (Promega), 6.6 µl sterile water, 1 µl of template DNA (from above lysis of cells), 0.6 µl 10 µM PSI primer, 0.6 µl 10 µM PS2 primer, 0.6 µl 10 µM PAL1 primer, 0.6 µl 10 µM PAL2 primer. The mixture was run on the thermo cycler using the following cycling profile: 94^{o} C for 40 sec, 30 cycles of : 94^{o} C for 40 sec, 57^{o} C for 50 sec, 72^{o} C for 1 min, 72^{o} C for 2 min and 4^{o} C ∞. Agarose gel 1.5% (w/v) was prepared and allowed to cool before 1 µl concentration of ethidium bromide was added and was allowed to set in a tray. Each sample of 5µl in volume including both the positive and the negative controls which were PAO1 DNA and a well without DNA, respectively, were loaded into separate wells with 5 µl easy ladder in the first well. The electrophoresis was run for 1 h 30 min, at 90 V and 300 amps and DNA bands were viewed using a UV Trans illuminator.

2.1.7 Minimum inhibitory concentration (MIC) of carbenicillin for environmental strains of Pseudomonas aeruginosa

A stock solution (5 mL) of carbenicillin of 100 g L^{-1} was prepared by dissolving 0.5 g of the antibiotic in 5 mL of sterile distilled water, which was sterilised with a filter membrane of 0.2 µ m pore size by using a 5 mL syringe. LB agar (500ml) was prepared and distributed in 25 mL aliquots into 9 cm Petri dishes containing different volumes of the antibiotic stock solution ranging from 1 µg mL^{-1} to 7 µg mL^{-1}. These were mixed thoroughly by gentle shaking and allowed to set at room temperature. Plates were dried so that no drop of moisture remained on the surface of the agar. An antibiotic-free LB agar plate was also prepared as control. Plates were inoculated by spreading with 100 µL of an overnight culture of bacteria grown in LB broth and adjusted to optical density of 0.1 at a wavelength of 660nm and then incubated at 37^{o} C for 24 h. MIC was defined as the lowest concentration of drug that inhibited visible growth after 24 h of incubation at 37 ^{o} C.

2.2. Tagging of the environmental strains of Pseudomonas aeruginosa

Genetic tagging of *P. aeruginosa* carried out using *E. coli* strain DH5X containing the plasmid pMF 230 which incorporates the gfp gene pMF 230 was kindly provided by Michael Franklin, Centre for Biofilm Engineering Montana State University. *E. coli* was inoculated onto LB agar, containing ampicillin at a concentration of 100 mg ml^{-1} for plasmid maintenance and incubated overnight at 37^{o} C. A bright fluorescing colony of the *E. coli* was then inoculated into LB broth and incubated overnight at 37 ^{o} C by shaking. Plasmid DNA was then extracted from the cell suspension using the Quiagen plasmid miniprep kit according to the manufacturer's instructions.

2.2.1 Preparation of competent cells

The environmental strains of *P. aeruginosa* were inoculated into 10 ml of LB broth and incubated overnight at 37° C by shaking. The overnight culture of *P. aeruginosa* in LB broth was distributed into 4 microcentrifuge tubes and the cells were harvested by centrifugation at room temperature for 1-2 min at 16,000 x g. The cell pellet in each tube was washed twice with 1 ml of 300 mM sucrose and re-suspended in a combined total of 100 µl of 300 mM sucrose, containing an average of 10^9-10^{10} viable bacteria.

2.2.2 Electroporation

Electro competent cells (100 µl) were mixed with 11 ng of plasmid DNA purified via the QIA amp kit. The mixture was transferred to a 2 mm gap width electroporation cuvette and pulse was applied (setting: 25 µF; 200 Ω; 2.5 kV on a Bio. Rad gene pulser). One millilitre of room temperature LB medium was added and cells were immediately transferred to a 2.0 ml tube and shake for 2 h. Cells were harvested in a micro centrifuge and 900 µl of the supernatant was discarded. The cell pellet was re-suspended in the residual medium and the entire mixture was plated on an LB agar containing carbenicillin at a concentration of 100 mg ml^{-1} and incubated at 37° C for 24 h. Cells pulsed without added DNA served as the control. Plates were observed under the microscope using the x4 objective for bright fluorescing colonies which were then sub cultured until pure fluorescing colonies were obtained. A subsequent PCR check (see section 2.6.3) was carried out on the environmental strains tagged with *gfp* to confirm that they are *P. aeruginosa* and the overnight cultures of the GFP tagged environmental strains of *P. aeruginosa* were stored in 60% glycerol at -80° C for subsequent use.

2.3.1 Bacterial strains, plasmid and culture condition

P. aeruginosa strain PAO1-GFP, one PAO1 mutant strain, Δ*mutS-GFP* (Oliver *et al.*, 2004; Stover *et al.,* 2000; Lewenza *et al.,* 2005) and environmental isolate (Envpa-GFP) were used in this work.

All the strains of *P. aeruginosa* used in this work carry the *gfp* plasmid pmf230 (Nivens *et al*., 2001). However, the environmental isolates had their gfp proteins tagged in our laboratory using pmf230 plasmid.

PAO1 wild type, Δ*mutS* and environmental strains of *P. aeruginosa* cultures were prepared by removing the vials containing the strains from the -80 ºC freezer and streaking onto LB agar plates containing the appropriate antibiotic (100 mg ml^{-1} gentamycin for Δ*mutS* and 100 mg ml^{-1} carbenicillin for pMF 230). Plates were viewed under the microscope to check for maintenance of the GFP plasmid pMF 230. Starter cultures (10 ml) were then prepared using the most brightly fluorescent colonies in LB broth and incubated overnight at 37ºC. The flow cell channels were then inoculated in triplicates with 0.5 ml of the overnight cultures after the adjustment of the cultures to optical density of 0.1 at a wavelength of 660 nm. The biofilm medium was made up of filter sterilised tap water containing 30 mg L^{-1} sodium acetate as carbon source. Δ*mutS* strains were kindly provided by Antonio Oliver, Hospital Son Durcia, and Spain.

2.3.1.1 Comparison of monospecies biofilm formation by three environmental isolates (Envpa1-GFP, Envpa2-GFP and Envpa3-GFP) of Pseudomonas aeruginosa

The method used in the formation of monospecies biofilms by the three environmental isolates of *P. aeruginosa* is the same as previously described (2.3.1)

2.3.2 Enumeration of Pseudomonas aeruginosa attachment in glass flow cell

P. aeruginosa PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains were cultured in shake flasks overnight at 37ºC in LB broth (180 rpm), and then diluted to an optical density of 0.1 at 660 nm in the same medium. The diluted culture (100 µl) was then inoculated into the flow cell and incubated at room temperature without flow for 1 hour. The unattached cells where flushed out by switching on the peristaltic pump for 30 min at a flow rate of 150 µl min^{-1}. The number of GFP cells attached to the flow cell was observed and enumerated using the epifluorescence microscope at different fields of view with the aid of the x100 objective.

2.4. Formation of mono-species biofilms by Pseudomonas aeruginosa PAO1-GFP wild type ΔmutS-GFP, and Envpa1-GFP strains in drinking water

Flow cells were inoculated in triplicate with 0.5 ml of the overnight broth cultures of PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains of *Pseudomonas aeruginosa* (OD 660nm 0.1) prepared as previously described (2.3.1) (the reason for selecting Envpa1-GFP out of the three environmental isolates for this experiment was because of its ability to produce more biomass and its biofilm forming tendency (figure 2.3b) tested by using same method of pure culture biofilm formation at sodium acetate concentration of 30 mg L^{-1}). The flow cell was incubated for 1 h in an inverted position without flow to allow the attachment of the cells onto the glass surface. The peristaltic pump was then switched on at a flow rate of 150 µl min^{-1} equivalents to 3 rpm and the biofilm formation continuously monitored.

Biofilms were observed *in situ* by transmitted bright field and green fluorescence microscopy using an Olympus BH2 microscope. A COHU 4612-5000 charge-coupled-device camera was used to capture images. Image capture, processing and analysis were done using NIH-image J. Distance and area measurements were calibrated using a 1 mm graticule with 10 µm divisions (C5990; Graticule Ltd, Tonbridge, Kent, United Kingdom).

2.5 Mixed-species biofilm formation by PAO1-GFP wild type, ΔmutS-GFP and Envpa1-GFP strains of Pseudomonas aeruginosa and drinking water bacteria

PAO1-GFP wild type, Δ*mutS-GFP* and environmental strains inoculum of *P. aeruginosa* prepared as previously described (2.3.1) were inoculated into the flow cells containing 10-day old drinking water biofilm (2.1.4) using filter sterilised biofilm medium containing 30mg L^{-1} acetate concentration. Mixed species biofilm formed by the PAO1 Δ*mutS-GFP* strain of *P. aeruginosa* after integration within established drinking water biofilms was compared with that of the PAO1-GFP wild type and the environmental strains (Envpa1-GFP) under the same conditions.

2.5.1 GFP plasmid stability test

This test was carried out in order to estimate the percentage of *gfp* plasmid loss in *P. aeruginosa* (PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP) within the biofilm since the biofilm medium contained no antibiotics to maintain the *gfp* plasmids in *P. aeruginosa* due to its antimicrobial effect on other drinking water bacteria. The essence of this experiment was to ensure that the disappearance of *P. aeruginosa* cells from the drinking water biofilm and any difference in their biofilm characteristics (thickness, biomass and number of microcolonies) is not attributed to *gfp* fluorescence loss.

Serial dilution of the biofilm effluents of PAO1-GFP WT, Δ*mutS-GFP* and Envpa1-GFP collected on days 1, 5 and 26 were made to 10^{-7} dilution and plates of cetrimide agar containing the appropriate antibiotics (100 mg mL^{-1} gentamycin for Δ*mutS* and 100 mg mL^{-1} carbenicillin for pMF230) were inoculated with 100 µl of the effluents using the spread plate method and incubated overnight at 37 ^{o}C. This was repeated in triplicates. Total numbers of *gfp* fluorescing (FC) and non-fluorescing (NFC) *P. aeruginosa* colonies were counted with the aid of epifluorescence microscope. Percentage *gfp* loss was calculated as the total number of non-fluorescing cells as a fraction of total number of *P. aeruginosa* cells (FC + NFC) expressed as a percentage.

2.5.2 Confocal laser imaging of the mixed-species biofilms and COMSTAT analysis

From each flow cell channel, an average of 5 stacks was acquired at different time points. After inoculation images were acquired from random positions in the upper part of the flow channel, at a distance of 5-10 mm from the inlet. Images were acquired at 1.0 to 2.0 µm intervals down through the biofilm and therefore the number of images in each stack varied according to the thickness of the biofilm. Confocal laser microscopic observations and image acquisition were carried out using a Leica TCS 4D microscope. Images were obtained with an x40 oil immersion objective. The biofilm images acquired were analysed using the COM STAT program which was written as a script in MATLAB 5.1 (Math Works), equipped with the image processing toolbox. The following parameters were measured, biomass, thickness, surface to volume ratio and number of microcolonies formed.

Statistical analysis: The mean and standard deviation within samples were calculated for all cases and statistical analysis was performed using the one way analysis of variance (ANOVA) based on a confidence level equal or higher than 95% ($P<0.05$) as statistically significant and the Tukey's test to identify which means are different from which.

2.6 Results

2.6.1 Development of drinking water biofilm

Drinking water bacteria were tested for their ability to form microcolony-based biofilms in a flow cell model of a drinking water system. Figure 2.2b shows the biofilm formed by the drinking water bacteria at different concentrations of sodium acetate (30 mg L^{-1} and 200 mg L^{-1}). There was a development of microcolonies within 24 hours of inoculation. The initial interaction with the surface was reversible, since some cells were microscopically observed to detach during the developmental stage or were observed to attach only briefly (data not shown). The second stage of development (irreversible attachment) was observed to occur when cell clusters commenced their development, as visualised multiple cells in contact with one another and the substratum. The result of biofilm based microcolony image analysis, using image J software revealed an increase in growth of microcolonies expressed as mean percentage area coverage from day 1 to day 10 in two different flow cells containing different concentrations of sodium acetate (30 mg L^{-1} and 200 mg L^{-1}). In the first flow cell (200 mg L^{-1} acetate), the mean percentage area coverage per field of view was 20.9% ±1.0 (n=10) on day 1. This increased to 24.1% ±1.0 (n=7) on day 10 when the biofilm morphology became stabilized. In the second flow cell (30 mg L^{-1} acetate) the majority of the biofilm biomass was established after 24 h, after which time relatively small increases in biomass were observed from 11.7% ±1.0 (n=6) on day 1 to 11.9% ±1.0 (n=10) on day 10. However, biofilm and microcolony growth was more pronounced in the first flow cell (200 mg L^{-1} acetate) when compared with the second flow cell (30 mg L^{-1} acetate). The overall result showed a significant increase ($P<0.05$) in the percentage area coverage of biofilm between the higher acetate concentration at different all intervals (figure 2.2a).

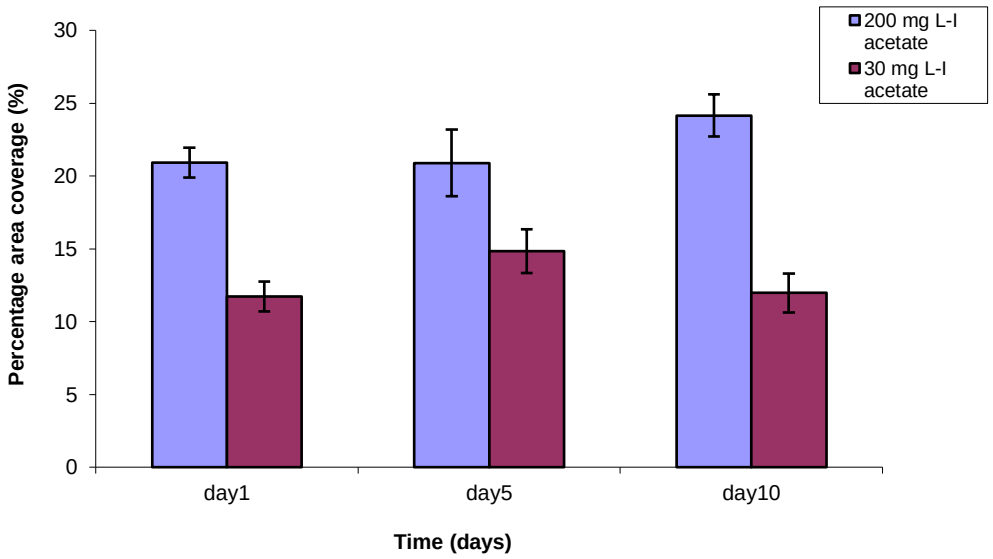


Figure 2.2a Comparison of mean percentage surface coverage of mixed species drinking water biofilms grown in drinking water supplemented with different concentrations of sodium acetate (30 mg L^{-1} and 200mg L^{-1}). Result shows a significant increase ($P<0.05$) in the percentage area coverage of biofilm between the higher acetate concentration at different all intervals.

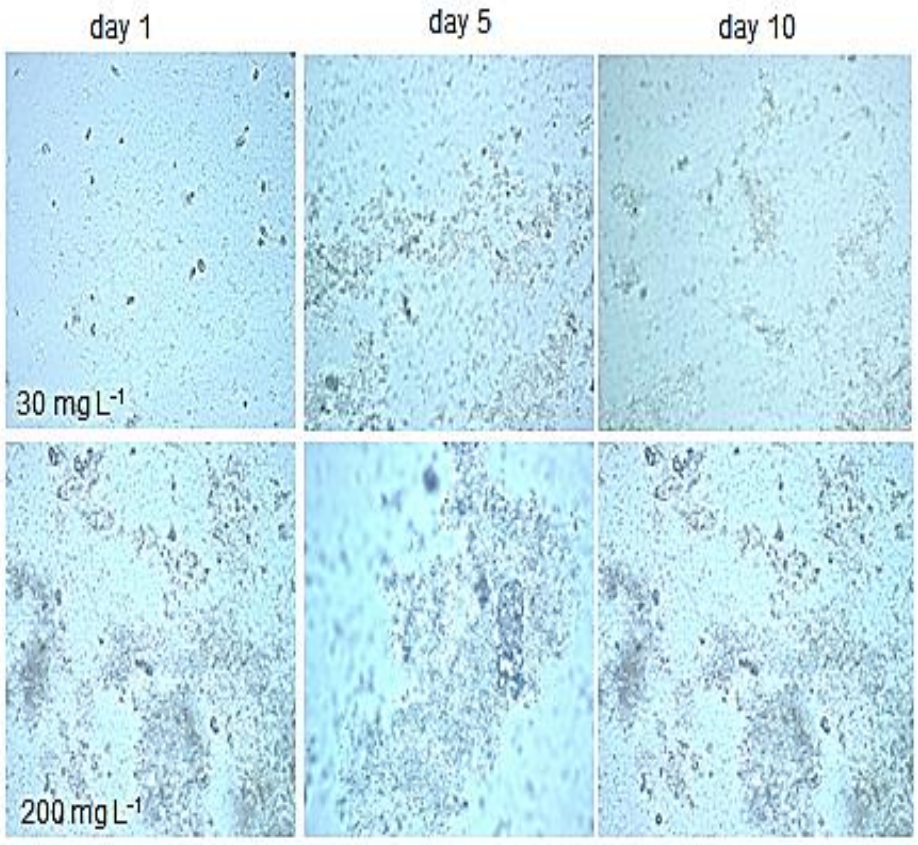


Figure 2.2b Bright field images of mixed species biofilms formed by drinking water bacteria grown in continuous flow cell culture at different acetate concentrations and examined using Epifluorescence microscopy. Scale bar represents 10 µm

2.6.2 Isolation of environmental strains of Pseudomonas aeruginosa

Environmental water samples screened for the presence of *P. aeruginosa*, showed colony growth of *P. aeruginosa* selective cetrimide agar with a characteristic *P. aeruginosa* smell and the presence of blue green coloration suspected to be pyocyanin pigment associated with *P. aeruginosa.* There was growth on the positive control plate of cetrimide agar inoculated with the laboratory *P. aeruginosa* PAO1 strain while there was no growth on the negative control plate of cetrimide agar inoculated with *E. coli* JM 109.

2.6.3 Identification of the Pseudomonas aeruginosa isolates.

A PCR check based on identification of the outer membrane protein genes oprI and oprL was carried out on four isolates suspected to be *P. aeruginosa*; figure 2.3 shows that the multiplex PCR (M-PCR), as visualised after agarose gel electrophoresis, is positive for 3 out of the four isolates (lanes 1, 2, and 4). Two amplification bands of 249 bp (*oprI*) and 504 bp (*oprL*) are observed when *P. aeruginosa* colony lysate is used as the template for the PCR.

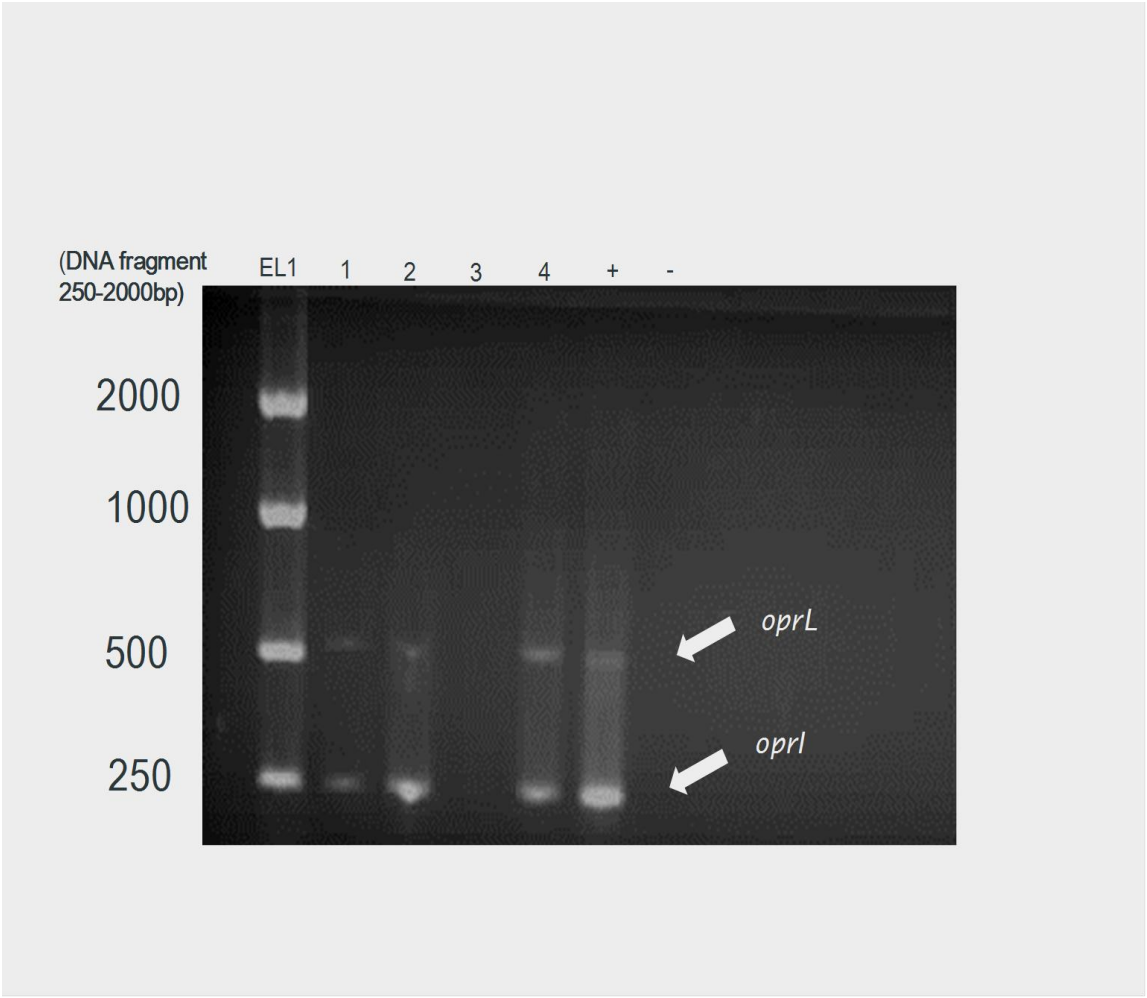


Figure 2.3a M-PCR for the identification of *P. aeruginosa* based on the amplification of two outer membrane lipoprotein genes oprI and oprL after agarose gel electrophoresis. Lane1 (Easyladder1) showing DNA fragment of 200-2000 bp, Lane 1,2 ,3, and 4 show *P. aeruginosa* M-PCR of the environmental isolates while the last two lanes show the positive and negative controls respectively.

2.6.4 Minimum inhibitory concentration of carbenicillin on environmental strains of Pseudomonas aeruginosa

In order to assist with molecular experiments involving antibiotic marker selection, the minimum inhibitory concentration of carbenicillin for strains was determined (table 2.2). Based on these data, 6μg mL^{-1} of the antibiotic was used as the acceptable concentration for molecular experiments with the environmental strains of *P. aeruginosa.*

2.6.5 Green fluorescent protein (GFP) tagging of the environmental strains of Pseudomonas aeruginosa

Microscopic examination of the electroporated mixture on LB agar after incubation showed many fluorescing colonies that had successfully been transformed using the pMF 230 plasmid. Colonies were sub cultured several times until pure fluorescing colonies were obtained that showed no segregated non-fluorescent cells (figure 2.4).

2.6.6 Comparison of monospecies biofilm formation by environmental isolates (Envpa1-GFP, Envpa2-GFP and Envpa3-GFP) of Pseudomonas aeruginosa

The results obtained from the comparison tested on single species biofilm forming ability of the three environmental isolates showed that the Envpa1-GFP isolate had the ability to form biofilms and produce biomass more than Envpa2-GFP and Envpa3-GFP isolates ($P<0.05$)(figure 2.3b). Based on this result, the Envpa1-GFP was chosen as our environmental isolate for subsequent experiments.

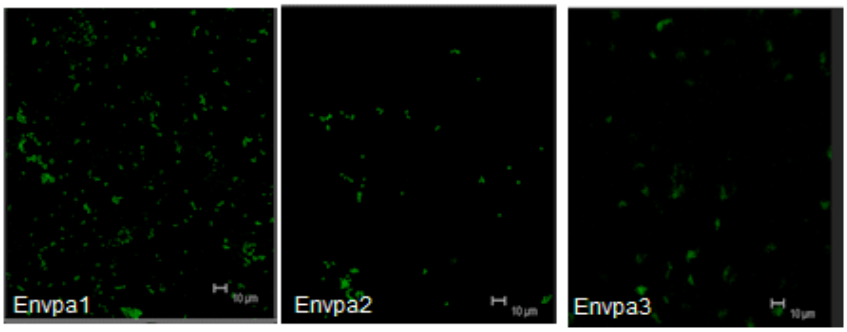


Figure 2.3b (i). Epifluorescence images of five day old biofilms of *P. aeruginosa* environmental isolates grown in continuous flow cell culture of filter sterilised tap water containing 30 mg L^{-1} acetate. Scale bar represents 10µm.

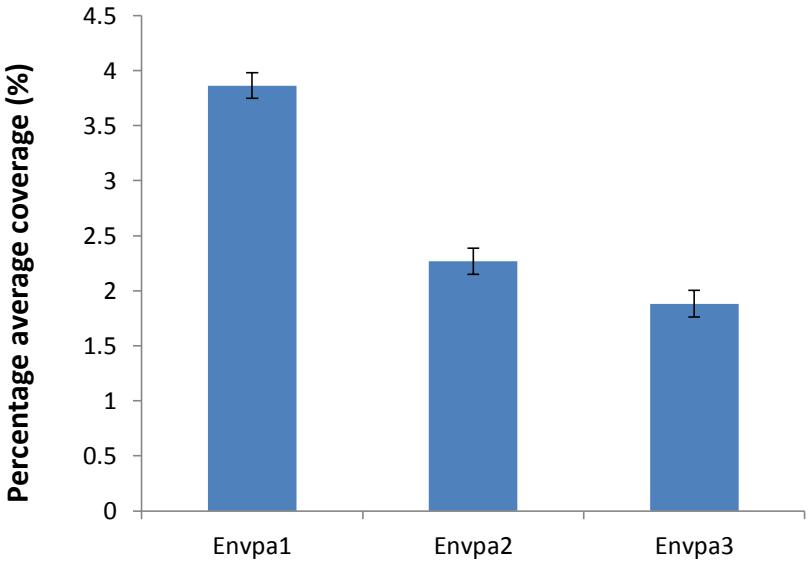


Figure 2.3b (ii). Comparison of the percentage average coverage of five day old biofilms of *P. aeruginosa* environmental isolates grown in continuous flow cell culture of filter sterilised tap water containing 30 mg L^{-1} acetate using image J

2.7 Pseudomonas aeruginosa attachment in glass flow cells

The rationale for the enumeration of *P. aeruginosa* attachment was to ensure that an approximately equal number of cells (wild type PAO1-GFP, Envpa1-GFP and Δ*mutS-GFP*) strains attached to the glass surface prior to their biofilm formation so that any differences in biofilm formation will not be attributed to difference in cell attachment. The average number of *mutS* cells attached to the surface per mm^2 field of view was 94.9 ± 8.6, 94.1± 18.2 for the wild type and 94.7± 8.8 for the Envpa. There was no significant difference in attachment between *P. aeruginosa* WT, Envpa and *mutS* strain in the glass flow cell ($P>0.05$) (figure 2.5).

	Antibiotic (carbenicillin) concentration (µg mL^{-1})/CFU mL^{-1}						
Environmental strains	1	2	3	4	5	6	7
Envpa1	Tntc	Tntc	Tntc	51	NG	NG	NG
Envpa2	Tntc	Tntc	Tntc	42	NG	NG	NG
Envpa3	Tntc	Tntc	Tntc	11	NG	NG	NG

TNTC- Too numerous to count **NG-** No growth

Table 2.1 The minimum inhibitory concentrations of carbenicillin effective against environmental strains of *P. aeruginosa.*

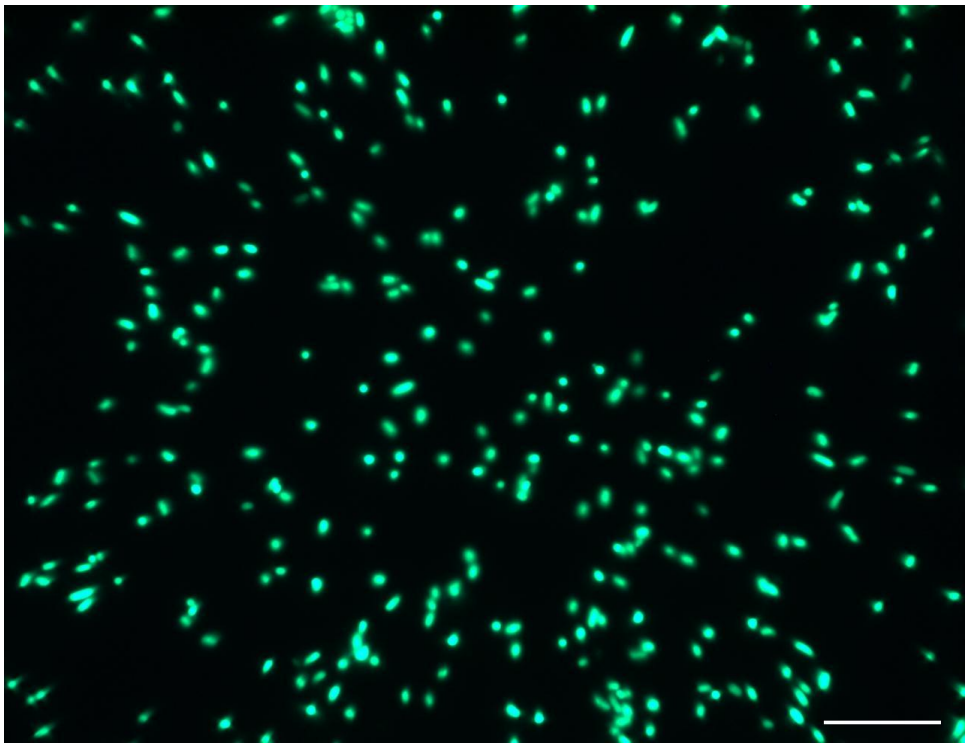


Figure 2.4 Microscopic image of environmental *P. aeruginosa* cells tagged with *gfp*. Scale bar represents 10 µm.

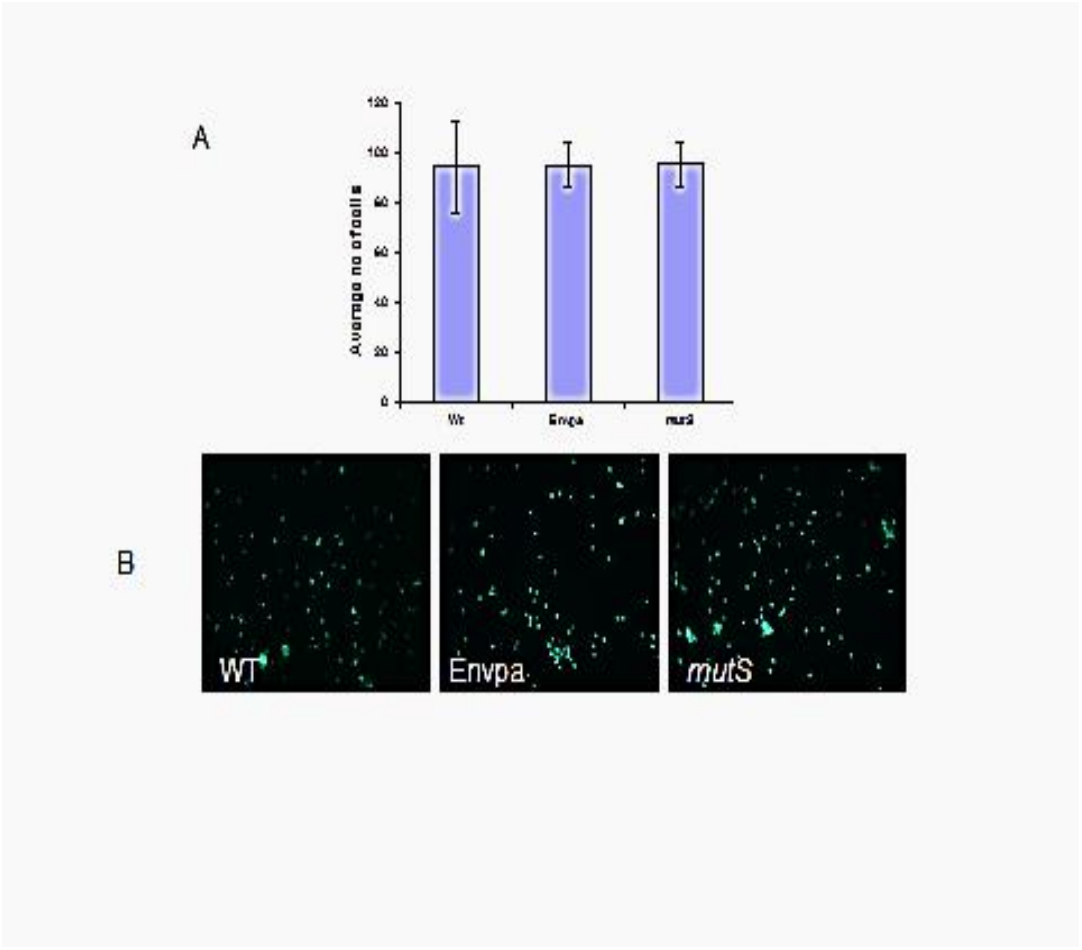


Figure 2.5 Attachment of PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains in glass flow cells 1 h after inoculation (A), the result shows no significant difference in attachment between the three strains (P>0.05), and (B), representative images of *P. aeruginosa* cells attached to the glass surfaces of the flow cells.

2.8 Computer-based image analysis of mono-species biofilm data

P. aeruginosa was tested for its ability to form microcolony-based mono-species biofilms in the drinking water system using the flow cell. Figure 2.6a shows the biofilms formed by *P. aeruginosa* wild type, environmental and *mutS* in a sodium acetate concentration of 30 mg L^{-1} at different time intervals. The assay revealed that *P. aeruginosa* formed microcolony-based biofilms immediately from day one of inoculation into the drinking water flow cell system. A number of large microcolony structures were observed in all the biofilms especially the environmental and the *mutS* strains. The *mutS* strain produced thicker and more complex architectural biofilm structures than the environmental and wild type strains. In all cases however, the biofilms grew at high rate and it was possible to identify distinct microcolony structures. The mean percentage area covered by the micro-colonies formed by the *mutS* strains were 30.80% ±3 (n=8), on day 1. This increased to 34.34% ±2 (n=8) on day 5 and decreased to 29.78% ±5 (n=8) on day 12. The maximum growth was observed on day 5 which gave mean percentage area coverage of 34.34%. This strain persisted in the system for over 31 days (data not shown). The environmental strain on the other hand showed a percentage coverage of 15.33 % ±3 (n=8) on day one, 10.21% ±3 (n=8) on day 5 and 18.5% ±1.5 (n=8) on day 12. Growth persisted within the flow cell for less time than the *mutS* strain, lasting for approximately 45 days in the system. The wild type also showed a growth from day 1 with a mean percentage area coverage of 5.51% ±2 (n=8) on day 1and a sharp fall of 0.96% ±0.4 (n=8) on day 5 which later increased to 1.57% ±0.5 (n=8) on day 12. Its maximum growth occurred on day1 (figures 2.6b). The wild type also persisted in the system for quite some time but for a short period of time than the environmental and *mutS* strains at approximately 28-35 days. In summary, these data show a significant difference in the percentage coverage between the wild type, environmental and the *mutS* strains ($P<0.05$), with the mutS strain exhibiting the greatest extent of biofilm formation in drinking water.

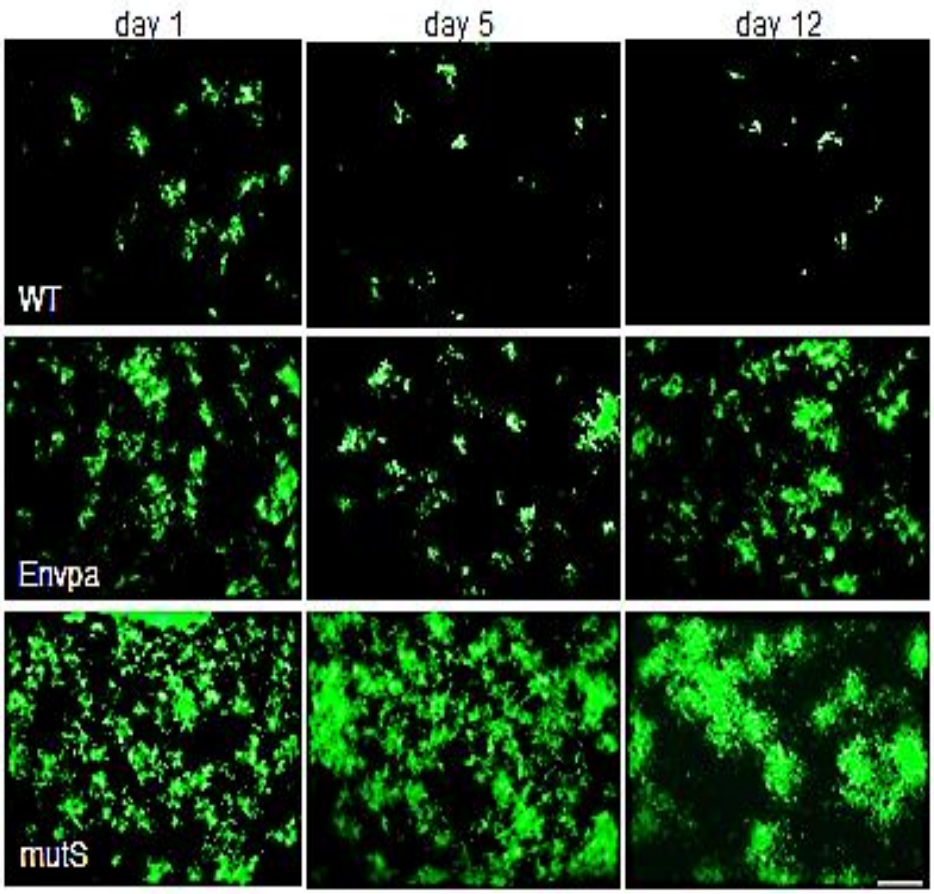


Figure 2.6a. Epifluorescence images of mono-species *P. aeruginosa* PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains grown in continuous culture flow cell at acetate concentration of 30 mg L^{-1}. Scale bar represents 10µm

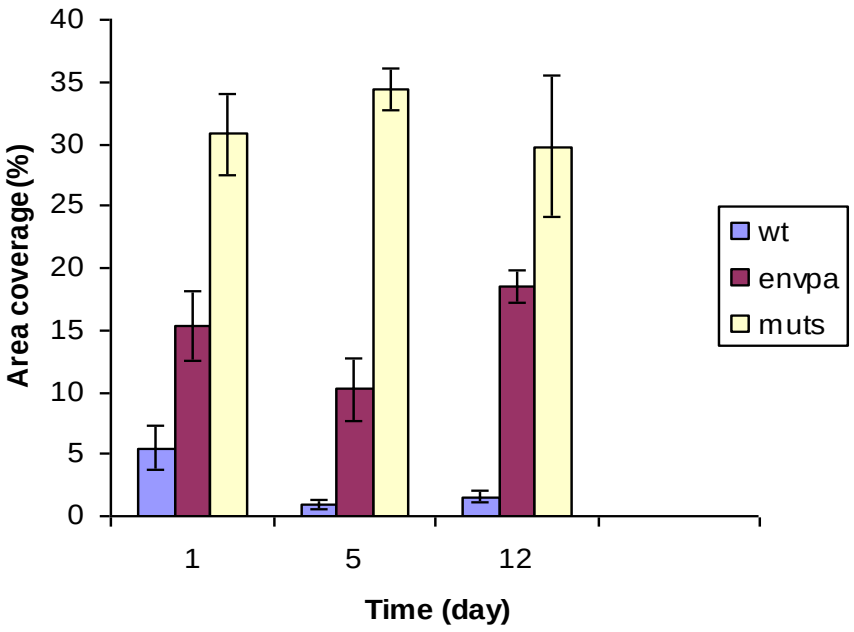


Figure 2.6b Comparison of mean percentage surface coverage *P. aeruginosa* PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains grown in continuous culture flow cell within mono-species drinking water biofilms at acetate concentration of 30 mg L^{-1} using image J.

2.8.1 Mixed species biofilm formation by Pseudomonas aeruginosa strains within drinking water biofilms

The *mutS*, environmental and wild type strains have the ability to form mixed-species biofilms when inoculated with drinking water bacteria inoculum within the drinking water system. All biofilms formed had similar 3-D architecture, observed microscopically. Figure 2.8- show spatial biofilm distribution and 3-D structures of the three strains of *P. aeruginosa* within the drinking water mixed-species biofilm for days 1, 5, and 26. The pattern of biofilm formation for the wild type as observed in the flow cell was quite distinct. Initially, the wild type cells attached individually with the formation of less number of microcolonies until day 5 when microcolonies at the substratum began to diminish. On the other hand, the environmental and *mutS* strains showed more tendencies to form more integrated mixed-species biofilms with distinct microcolonies within the flow cell more than the wild type. The biofilm association of *mutS* strain and environmental isolate with drinking water bacteria also lasted longer within the system more than the wild type before they began to diminish. However, the *mutS* strain exhibited more tendencies to integrate and produce more microcolonies more than the wild type and environmental strains and also lasted longer within the system for approximately 45-55 days (data not shown).

2.8.2 GFP plasmid stability test

Result of *gfp* fluorescence loss between *P. aeruginosa* PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains (figure 2.7) carried out on days 1, 5 and 26 using their biofilm effluents showed that there was no *gfp* fluorescence loss on day 1 for all the strains. However, there was *gfp* fluorescence loss over time with an average *gfp* plasmid loss of 6.2% ± 0.3 (n=3) by the PAO1-GFP wild type, 9.3% ± 0.4 (n=3) by Δ*mutS-GFP* and 7.0% ± 0.5 (n=3) by the Envpa1-GFP isolate on day 5. On day 26 however, the PAO1-GFP wild type exhibited a *gfp* fluorescence loss of 16.6% ± 1.0 (n=3) while the Δ*mutS-GFP* and Envpa1-GFP strains lost their *gfp* fluorescence by 17.4% ± 0.3 (n=3) and 16.7% ± 0.6 (n=3) respectively. This result has shown no significant difference ($P>0.05$) in the average *gfp* loss between these three strains on the three occasions the experiment was carried out.

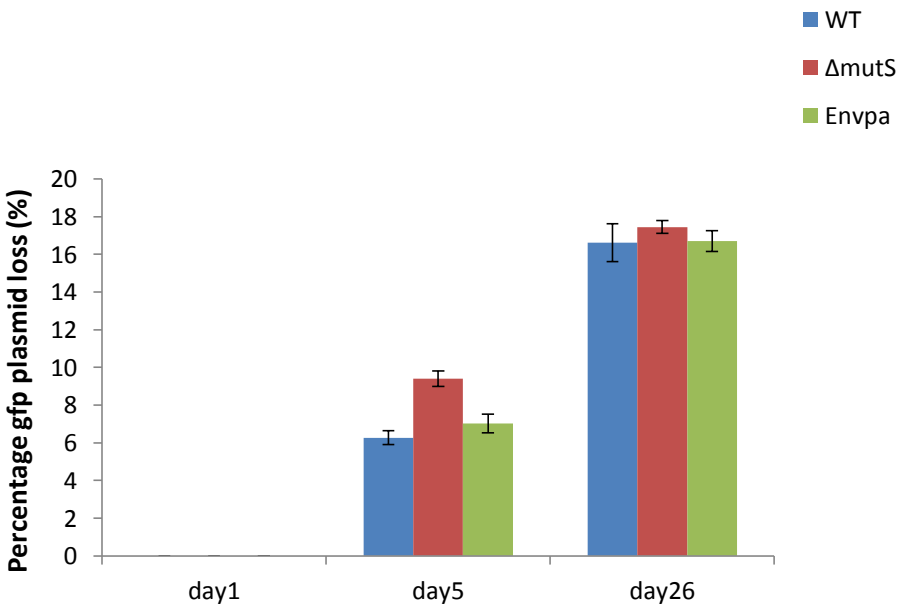


Figure 2.7 Comparison of *gfp* fluorescence loss of *P. aeruginosa* PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains over time within drinking water biofilms using their biofilm effluents (n=3) (P>0.05).

2.8.3 Confocal laser imaging and COMSTAT analysis of the mixed-species biofilm.

The computer program (COMSTAT) was used in the analysis of three-dimensional biofilm image data, acquired using the CSLM. In this study the mean biofilm thickness, mean biomass, average surface to volume ratio and average number of micro-colonies were chosen to characterize the biofilm structures developed by *P. aeruginosa* wild type, environmental and *mutS* strains. Mean thickness indicates the spatial dimensions of the biofilms; the average biomass relates to the total number of cells within the biofilm while the surface to volume ratio tells how large a portion of the biofilm is exposed to the nutrient flow. These four variables were calculated by COMSTAT for biofilms developed by the three strains of *P. aeruginosa* after acquiring images as described in the methods. The listed values were determined as means of 24 images stacks (six image stacks from two channels in two independent experiment rounds). From the results it is clear that the biofilm structures of the *mutS* , environmental and wild type strains were different in all the four parameters tested, with the *mutS* strain having a stronger tendency to form biomass more than the environmental isolate and the wild type strain (day 20). The mean thickness of the biofilm formed by the *mutS* strain was approximately nine times higher than the wild type and the environmental isolate on day 5, while the surface to volume ratio was averagely higher in the wild type than the *mutS* and the environmental isolate. The number of micro-colonies formed by the *mutS* strain was higher than the wild type and the environmental strains in overall. On the average, the biomass developed by the *mutS* strain from day 1-26 was 230-fold more than the average biomass of the wild type and 32-fold more than the environmental isolate however; the environmental strain had a 7-fold increase in biomass more than the wild type. In terms of the number of micro colony development, the *mutS* strain had an average of 20-fold increase in number of micro colonies more than their wild type counterparts and 6-fold increase more than the environmental isolate while the environmental isolate gave an average of 3-fold increase in the number of micro colonies more than the wild type. The thickness of the biofilm developed by the *mutS* was 242-fold more than that of the wild type and 25-fold more than the environmental isolate whereas the

environmental isolate gave a 10-fold increase in biofilm thickness more than the wild type (figures 2.8 and 2.9).

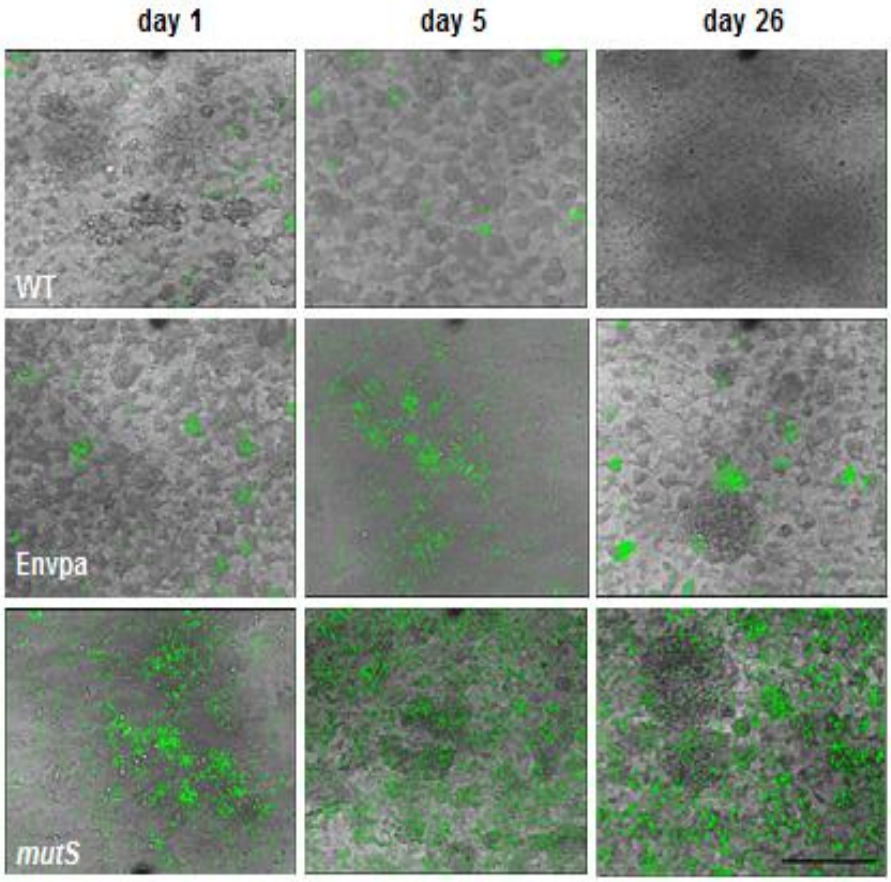


Figure 2.8a Confocal images of biofilms formed by PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP strains of *P. aeruginosa* grown in continuous flow cell culture within drinking water biofilms at acetate concentration of 30 mg L^{-1}. The green colouration represents *P. aeruginosa* biofilms while the bright field represents the drinking water biofilms Scale bar represents 10 µm

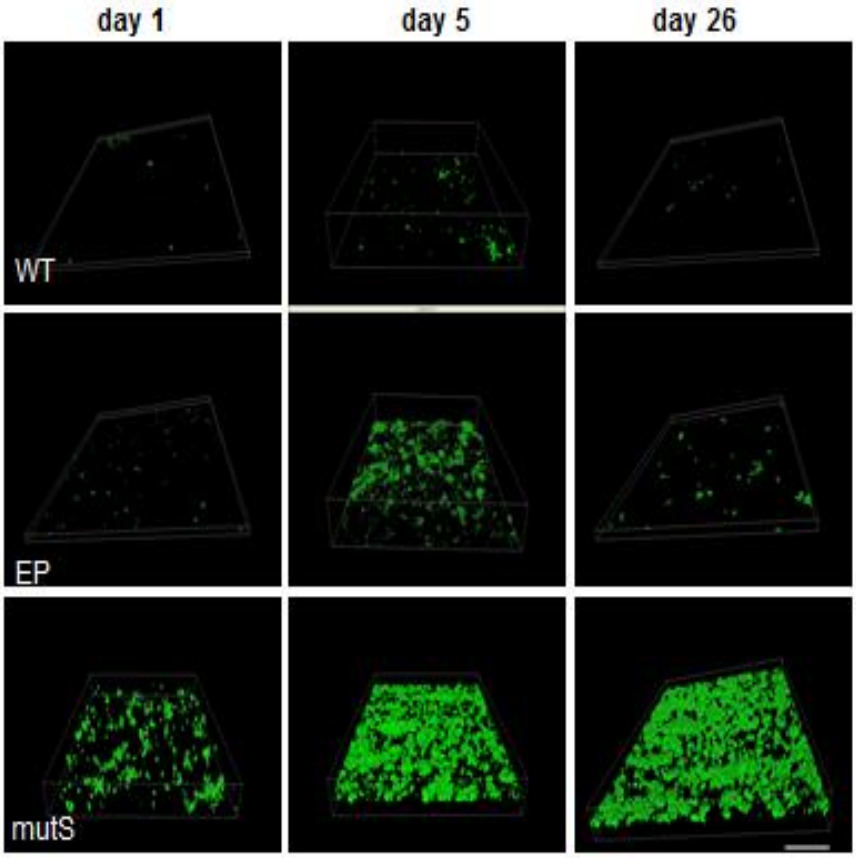


Figure 2.8b 3-dimensional rendering of confocal microscopic image stacks of biofilms formed by PAO1-GFP wild-type, Δ*mutS-GFP* and Envpa1-GFP strains of *P. aeruginosa* grown in continuous flow culture within drinking water biofilms at acetate concentration of 30 mg L^{-1}. Scale bar represents 10 mμ

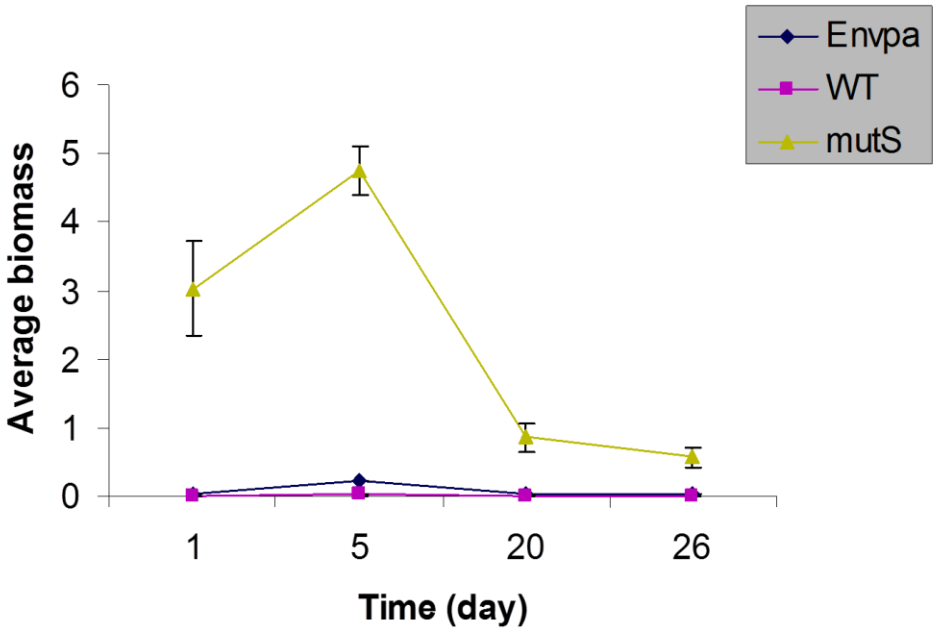


Figure 2.9a Average biomass of *P. aeruginosa* (PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP) biofilms grown in continuous culture flow cell within mixed-species drinking water biofilms at acetate concentration of 30 mg L^{-1} and examined using confocal scanning laser microscopy.

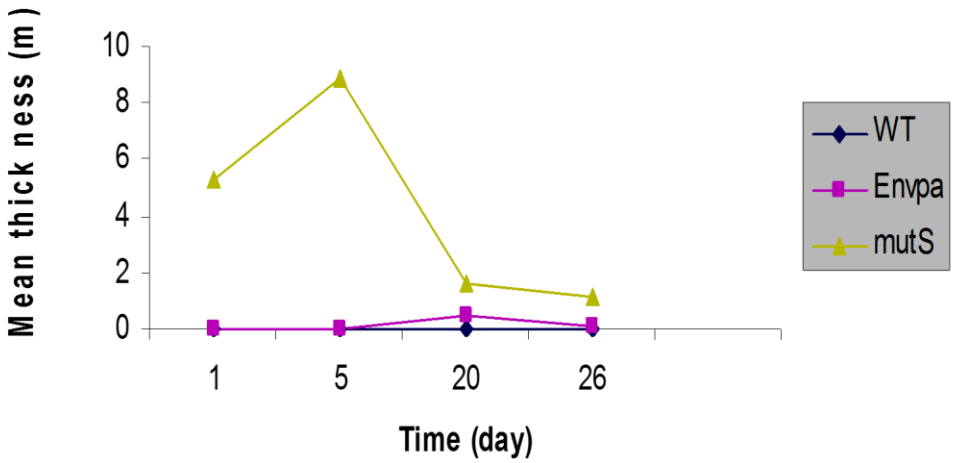


Figure2.9b. Average thickness of *P. aeruginosa* (PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP) microcolonies grown in continuous culture flow cell within mixed-species drinking water biofilms at acetate concentration of 30 mg L^{-1} and examined using confocal scanning laser microscopy.

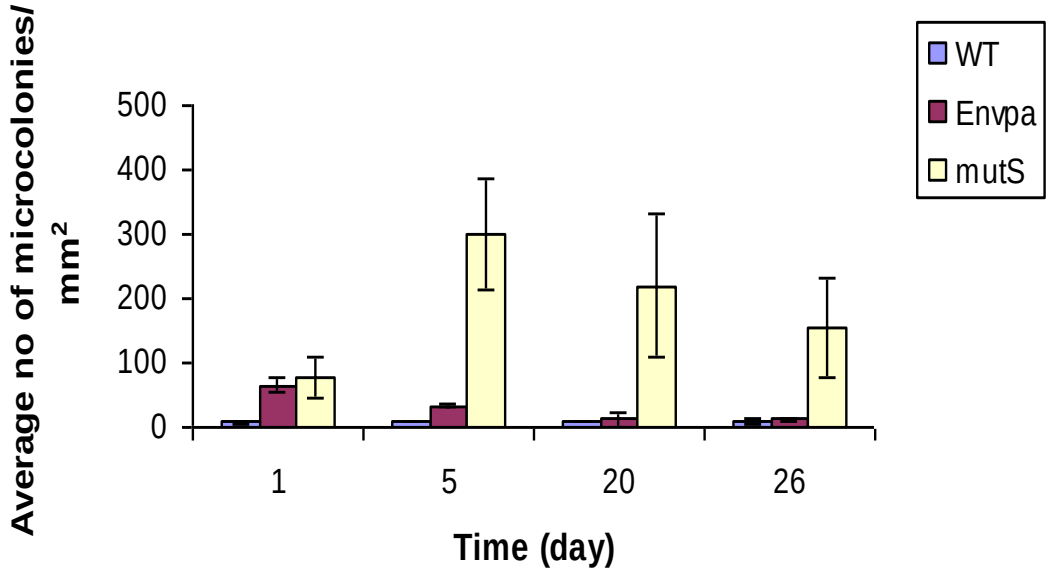


Figure 2.9c Average number of microcolonies of *P. aeruginosa* (PAO1-GFP wild type, Δ*mutS-GFP* and Envpa1-GFP) grown in continuous culture flow cell within mixed-species drinking water biofilms at acetate concentration of 30 mg L^{-1} and examined using confocal scanning laser microscopy.

2.9 Discussion

In this study we isolated bacteria from the Southampton municipal drinking water supply and showed that this multi-species bacterial community inoculum was able to form microcolony-based biofilm in an artificial drinking water flow cell system. We further showed that laboratory and environmental isolates of *P. aeruginosa* were able to colonise and integrate within multi-species drinking water biofilms, and that *mutS* strain of *P. aeruginosa* deficient in DNA mismatch repair and exhibiting a mutator phenotype exhibit an enhanced ability to establish and survive within drinking water biofilms.

The extent of biofilm formation by the drinking water bacterial inoculum was compared using two different concentrations of supplemented sodium acetate; 30 mg L^{-1} and 200 mg L^{-1} of these concentrations, the optimal for biofilm growth was found to be 200 mg L^{-1}. In addition to enhancing growth and biomass production the carbon source present within biofilm media can influence biofilm development in many ways. For example quorum sensing signals, which regulates motility and other biofilm-relevant factors in certain species influenced by the carbon sources available and can have a strong effect on biofilm formation (Shrout *et al.,* 2006). Biosynthesis of other signal substances, cell-surface appendages and EPS might also be affected by the presence or absence of certain carbon sources. In the present work the biofilm reached a steady state without further increases in biomass after 2 weeks. By comparison, the work of Lehtola *et al* found that at room temperature in the laboratory, poly-microbial biofilms may reach steady state within 3 weeks in ozonised drinking water (Rogers *et al.,* 1994; Lehtola *et al.,* 2001). The ability of *P. aeruginosa* to form biofilm, interact, and persist within the drinking water mixed-species biofilm in this study has further supported the previous findings that several bacteria pathogens have been shown to associate with, and in some cases actually grow in biofilms in a variety of systems (Keevil, 2001; Raad and Sabbagh 1992; Wirtanen 1996; Buswell 1998; Camper 1998; Hood and Zottola 1997; Watnick and Kolter 1999 and Stark *et al.*, 1999). Recently, Banning *et al* (2003) reported the incorporation and persistence of *P. aeruginosa* in groundwater-derived biofilms on glass surfaces in flow-through

reactors that were perfused with high nutrient treated wastewater effluent or low–nutrient groundwater. Bourion and Cerf (1996) observed rapid biofilm formation of *P. aeruginosa* in pure and mixed culture on previously un-colonised rubber material, and also on Teflon and steel surfaces under flow conditions in an artificial nutrient medium. These observations suggest that *P. aeruginosa* can colonise both clean surfaces and pre-established mixed-population biofilms under flow conditions independent of the available nutrient and the type of substratum. The role of *P. aeruginosa* as an opportunistic pathogen for which the source of contamination comes mainly from the environment, resulted in a rapidly growing interest in its biofilm formation in a range of settings. We isolated *P. aeruginosa* from the environment in order to investigate its interaction and biofilm formation in drinking water biofilms and to evaluate whether environmental strains exhibit enhanced fitness and survival within polymicrobial biofilms compared to laboratory strains. We also investigated biofilm formation by a mutator strain of *P. aeruginosa* deficient in mismatch repair because mutator strains have recently been shown to exhibit enhanced biofilm formation compared to wild-type strains (Conibear *et al.,* 2009) and are commonly isolated from medical and environmental settings (Ishii *et al* 1989; Oliver *et al*., 2000). Although both the wild type, environmental and *mutS* strains were capable of extensive growth in the biofilm, *P. aeruginosa* Δ*mutS* formed more micro-colonies, biomass, thickness and surface to volume ratio more than the wild type and the environmental strains within the drinking water mixed-species biofilm community and also persisted longer in the drinking water biofilm for longer than the wild type and environmental strains. This result is supported by our plasmid stability test carried out on the three strains of *P. aeruginosa* used in this study which showed no significant difference ($P > 0.05$) in their *gfp* fluorescence loss. This therefore, means that the fitness and persistence of Δ*mutS* more than the PA01 wild type and Envpa1 strains is not attributed to difference in *gfp* fluorescent loss within the drinking water biofilms. This result has also shown that the rate of *gfp* loss over time is not as high as to attribute the disappearance of *P. aeruginosa* cells from our drinking water biofilm system to *gfp* loss but probably out competition by drinking water bacteria. Mutator phenotype in biofilm can promote microcolony initiation. Experiment involving *P. aeruginosa* Δ*mutS* strain show that enhanced mutation frequency in *P. aeruginosa* can promote microcolony growth within biofilms (Conibear *et al.,* 2009). Mutator strains are those with an increased mutation rate compared with those commonly found in a

particular species. These strains usually have alterations in genes participating in the DNA error avoidance systems (Miller 1996). The acquisition of a stable mutator phenotype may confer a selective advantage for bacteria particularly in stressful and or fluctuating environments because high mutation rates allow faster adaptation (Leigh 1970; Ishii *et al.,* 1989; LeClerc *et al.,* 1996; Sniegowski *et al.,* 1997; Taddei *et al.,* 1997). The lung of the cystic fibrosis patients is one of the highly stressful and fluctuating environments in addition to the challenge imposed by host defences or medical intervention; *P. aeruginosa* has to cope with very high doses of different antibiotics administered for prolonged periods of time. This may explain why a high proportion of *P. aeruginosa* isolates (20%) from the lungs of cystic fibrosis patients have been reported to be mutator strains (Oliver *et al.,* 2000). The present work has shown that in the stressful environment of the drinking water system which is characterised by low nutrient concentration, and competition for nutrient by multiple species drinking water bacteria, the mutator strains of *P. aeruginosa* exhibit enhanced integration and have a fitness and survival advantage over the course of these biofilm experiments. In addition, the environmental strain formed more microcolonies, biomass, thickness and surface to volume ratio more than the wild type within the drinking water mixed species biofilm community. Perhaps reflecting an adaptation to survival in complex polymicrobial communities that has been lost in domesticated wild-type strains. The acquisition of new genetic traits may cause a pathogen to become more virulent or more resistant to antimicrobials and other environmental stresses and hence longer persistence within the biofilms (Atlas 2002). It has been reported that environmental microorganisms like *P. aeruginosa, Legionella* or *Mycobacteria* adapted to oligotrophic aquatic conditions can persist over long time periods in biofilms and possibly even multiply in these environments (Wingender *et al.,* 2011). Also, although not tested in this study, the isolated environmental strain could develop a mutator phenotype trait within the biofilm which could have contributed to its ability to form more biofilm than the wild type. The occurrence of mutator strains in natural populations has mostly been studied in *E. coli, Salmonella* and *Neisseria meningitidis* (LeClerc *et al.,* 1996; Matic *et al.,* 1997; Richardson and Stojiljkovic 2001) and has been estimated to be around 1% in both pathogenic and commensal strains (LeClerc *et al.,* 1996; Matic *et al.,* 1997). In addition to the role of mutability in *P. aeruginosa* biofilm interactions, many other factors may affect biofilm formation and persistent of *P. aeruginosa* within the drinking water mixed-

species biofilm community. These may include the metabolism and growth of each member species, their substrate consumption, cellular growth and replication, and synthesis of extra cellular polymeric substances (Bryer and Ratner 2004; Vieira *et al* 2007).

Competition for substrate is considered to be one of the major evolutionary driving forces in microbial communities, and experimental data obtained under laboratory conditions has previously shown how different micro-organisms may effectively out compete others because of better utilization of a given energy source (Christensen *et al.,* 1982; Miller 1998 Vieira *et al.,* 2007). There is also evidence that biofilm community diversity can affect disinfectant efficacy and pathogen survival within biofilms (Vieira *et al.*, 1993; Keevil, 2002 and Burmolle *et al.,* 2006; Wingender *et al.,* 2011). Most research into interspecies interactions within biofilms has focused on the beneficial, aspects of these relationships to bacteria. However, antagonistic interactions may also play an important role in the development and formation of biofilm. The production of antimicrobial compounds including toxins, bacteriolytic enzymes, bacteriophages, antibiotics and bacteriocins could determine the fate of both the environmental and wild type strains of *P. aeruginosa* within the drinking water system. According to (Riley 1998), the production of substances antagonistic to the growth of other bacteria within a mixed species community seems to be a generic phenomenon for most bacteria.

Another mechanism of interaction between bacteria in biofilms is co-aggregation which is the specific recognition and adherence of genetically distinct bacteria to one another via adhesion/receptor interactions. Co aggregation is a widespread phenomenon which has been observed amongst bacteria from other biofilm communities in several diverse habitats. More recently, a few reports on the co-aggregation abilities of fresh water biofilm bacteria have also been published (Keevil, 2004; Rickard *et al.,* 2004; Vieira *et al.,* 2007).

In the present work all strains of *P. aeruginosa* were eventually out competed within the drinking water biofilm and were either lost completely (laboratory PAO1 wild-type strain) or were declining significantly (Envpa1 and Δ*mutS*) after 26 days of biofilm culture. This may be attributed to their inability to compete for so long with the indigenous bacteria. Studies on the integration of other pathogens within biofilms may indicate that *P. aeruginosa* competes comparatively poorly in multispecies biofilm communities for example, the work of Camper (1998) showed that

Salmonella typhimurium persisted in a model water distribution system containing undefined heterotrophic bacteria from an unfiltered reverse osmosis water system for >50 days, which suggest that the normal biofilm flora of this water system, provided niche conditions capable of supporting the growth of this organism. Further studies would be required to determine why *P. aeruginosa* does not persist for longer in our system. In summary, biofilm formation and integration with multi-species drinking water biofilms was greatly enhanced in a *P. aeruginosa* Δ*mutS* strain compared to environmental and laboratory strains. This suggests an important role for mutability in the persistence and survival of pathogens in drinking water that will be investigated in further detail within the subsequent chapters of this thesis.

Chapter three

Influence of bacterial interactions and oxidation on the mutation frequency of *Pseudomonas aeruginosa* within polymicrobial biofilms derived from drinking water

ABSTRACT

Little is known about the role of environmental and biological factors influencing the frequency of mutation in complex polymicrobial biofilms. This section of work therefore aimed to investigate whether the mutability of the wild type strain of *P. aeruginosa* may be influenced by interactions with other microorganisms or by chlorine treatment within our model drinking water biofilm system. We also examined the effect of oxidative stress and chlorination on the mutation frequencies and persistence of *P. aeruginosa* strains within drinking water biofilms. Our results showed that co-culture of *P. aeruginosa* strains within mixed-species biofilms led to a higher frequency mutation to rifampicin resistance than in mono species biofilms. We also found that the mutation frequency exhibited by the wild type in the presence of antioxidants (L-proline and N-acetyl-cysteine) added to the biofilm was reduced in all cases ($P<0.05$), suggesting that this interaction may in part be due to an increase in oxidative stress or DNA damaging processes in the biofilms. Addition of chlorine (1ppm) had no significant effect on the mutation frequencies of *P. aeruginosa* within mixed species drinking water biofilms.

Our data suggest that interactions within complex multispecies biofilms can lead to elevated mutation frequencies in mixed species biofilms and may therefore contribute to enhanced pathogen survival and genetic adaptation of microbial pathogens.

3.1 Introduction

Recent findings show that biofilm growth can rapidly lead to extensive genetic diversity in bacterial populations. Diverse genetic variants are generated by biofilms of *P. aeruginosa, Pseudomonas fluorescence, Vibrio cholera*, and *Staphylococcus aureus* (Conibear *et al.*, 2009; Boles *et al.*, 2004; Kirisits *et al.*, 2005; Deziel *et al.*, 2001). Biofilm growth increases the ability of cells to persist in adverse conditions by inducing bacterial phenotypes that are not apparent in planktonically grown cells (Stoodley *et al.*, 2002). Research with *P. aeruginosa* biofilm has shown that the diversity produced occurs within many bacterial traits including colony morphology, pigment formation; exopolysaccharide production; nutritional requirements, antibiotic resistance and other phenotypes (Boles *et al.*, 2004). Population diversity can reduce the impact of environmental upsets because the presence of diverse subpopulations extends the range of conditions in which the community will thrive (McCann, 2000). Diversity is a consequence of mutation and the amplifying effects of selection. Measuring the mutation frequency of a pathogen such as *P. aeruginosa* grown within a biofilm in a stressed environment such as the drinking water system could help in the evaluation of the rate of diversification in such bacteria and within such environment.

In chapter 2, we studied how mutation/mutators can increase fitness of *P. aeruginosa* within drinking water multispecies biofilms. This chapter aimed to study the factors that influence mutation frequency in the biofilms, principally the interactions between *P. aeruginosa* and of the members of the microbial community, as well as role of Oxidative stresses and chlorine treatment.

A recent study has shown that oxidative stress can contribute to genetic variation in biofilms. Increased sensitivity of bacteria in biofilms to oxidants increases both DNA breaks and diversity and reducing the oxidant stress has an opposite effect (Singh, *et al*., 2008).

Driffield *et al.*, (2008) has also shown that the down-regulation of antioxidant enzymes in *P. aeruginosa* biofilms may enhance the rate of mutagenic events due to accumulation of DNA damage.

According to conventional knowledge, bacteria in biofilms predominantly in stationary phase should be in a state of transient genetic instability in relation to the RpoS-dependent stress response thus increasing mutation frequencies of bacteria in

biofilms (Lambardo *et al*., 2004). The question that needs to be answered therefore is whether mutation frequency which could be a measure of the rate of bacterial diversification in biofilms could be affected by oxidative stress in both the wild type and mutator strains of *P. aeruginosa*. We hypothesized therefore that oxidative stress increases the mutation rate of bacteria within biofilms. This work investigated the effect of antioxidant compounds on the mutation frequencies of *P. aeruginosa* wild type and *mutS* strains grown within mixed species drinking water biofilms.

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Cells under conditions of cellular crowding are constrained by intensely competitive and nutrient limited growth conditions. In these circumstances micro evolutionary processes such as mutation selection are often important for growth. We hypothesized therefore that species competition increases the mutation rate of bacteria in biofilms. In addition to antagonism where each population pursues individual fitness at the cost of others, various forms of synergism between bacteria can increase the fitness of all partners in biofilms. An interesting way for bacteria to do this is through cooperation which typically leads to an organisation wherein all species are spatially located close together. Such cooperation can evolve if the interest of two or more parties is directly aligned (Buckling and Brockhurst 2008). In cooperative interactions, all species profit in some way from the presence of others leading to an enhanced overall fitness of the biofilm consortium. This can be through the formation of substances that can serve as nutrients for the co-inhabiting species or by removing metabolites that would otherwise slow down growth or by any combination of the two. Kives *et al., (*2005) reported the co-cultivation *of Lactococcus lactis ssp. Cremoris* and *Pseudomonas fluorescens* in milk. Compared to each monospecies biofilm, the mixed species biofilms showed a more developed structure in which both species were maintained. Hansen *et al* (2007) has shown that in a system of two or more interdependent species, mutation led to adaptation towards enhanced cooperation.

Chlorination is the most widely employed method of disinfection for community water distribution systems and reservoirs. In aqueous environments, uncombined chlorine in the form of unionized hypochlorous acid (HOCl) is an extremely potent bactericidal and virucidal agent, even at a concentration of less than 0.1 mg L^{-1} (0.1 ppm). The most sensitive bacteria are readily killed by chlorine concentration of 1.0 mg L^{-1} (1ppm) (Hu *et al*., 2005; Ridgway *et al.,* 1982). Chlorine is known to exert

disruptive effect on a variety of subcellular components and metabolic processes (Haas *et al.,* 1980) including in vitro formation of chlorinated derivatives of purine and pyrimidine nucleotide bases (Dennis *et al.,* 1979), oxidative decarboxylation of amino acids (Pereira *et al*., 1973) and other naturally occurring carboxylic acids (Pereira *et al*., 1973), inhibition of enzymes involved in intermediary metabolism (Camper *et al.,* 1979), inhibition of protein biosynthesis (Shih *et al*., 1976b), introduction of single-and double stranded lesions into the bacterial chromosomes (Shih *et al*., 1976b), production of bacterial mutations (Shih *et al*., 1976a), inhibition of membrane mediated active transport processes and respiratory activity (Camper *et al.,* 1979), and uncoupling of oxidative phosphorylation accompanied by leakage of macromolecules from the cell (Wenkobachar *et al.,* 1975, 1977). Some halogenated aldehydes and ketones are potent inducers of mutation in bacteria (Ridgway *et al.,* 1982). Some proposed mechanisms by which bacteria may develop resistance to chlorine include, modification of cell surface structures which may lead to increased aggregation or clumping of cells in situ (Dennis *et al*., 1979), microbial adhesion to pipe surfaces or to suspended particulate matter such as detritus or clay particles (biofilms) (LeChevallier *et al.,* 1980), extrusion of protective extracellular capsular or slime layers (Seyfried *et al.,* 1980), and formation of resistant spores (Haas *et al.,* 1979; Bissonnette *et al*., 1975).While the chlorine disinfectant residue in the drinking water distribution system may not kill the bacteria in biofilms (LeChevallier *et al*., 1988; Stewart *et al.,* 1994), it can stress them chemically. An additional source of stress for such bacteria is limited nutrient available in treated drinking water. These factors together can enhance the induction of heat-shock and SOS responses and in turn induce the expression of the error-prone polymerase, causing an increased rate of bacterial mutation (Foster, 2005). While these mutations are random, one possible mutant phenotype that could arise is increased antibiotic and disinfectant resistance. *P. aeruginosa* often survives and grows within the biofilms in the water distribution system (Allen *et al.,* 2004), they are resistant to chlorination and this is a major concern when found in drinking water (Wilkinson *et al.,* 1998).

We therefore investigated the effect of chlorine on the mutation frequencies of *P. aeruginosa* wild type and mutator phenotypes grown within mixed species drinking water biofilms which provides new insights into mechanism of pathogen resistance to disinfection in drinking water distribution systems.

In summary, this chapter investigated the impact of multispecies interactions, environmental factors such as oxidation stress and water disinfection practice (chlorination) on the mutation frequency of *P. aeruginosa* within drinking water biofilms.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

GFP tagged PAO1-GFP wild type, Δ*mutS-GFP* and Envpa-GFP isolates 1,2 and 3 strains of *P. aeruginosa* maintained in vials frozen at -80 ºC were streaked on LB agar plates (25% tryptone, 12.5% yeast extract, 25% NaCl and 37.5% agar powder) using a sterile wire loop and incubated overnight at a temperature of 37ºC. Colonies were inoculated into LB broths and incubated overnight at 37ºC on rotary shaker operating at 100 rpm.

3.2.2 Minimum inhibitory concentration (MIC) of rifampicin to *Pseudomonas aeruginosa*

The minimum inhibitory concentrations of rifampicin were determined for *P. aeruginosa* laboratory strains (PAO1-GFP wild type and Δ*mutS-GFP*) and three environmental isolates (Envpa1-GFP, Envpa2-GFP and Envpa3-GFP) by using the agar dilution method.

A 5 ml stock solution of rifampicin having a concentration of 100 mg L^{-1} was prepared by dissolving 0.5 g of the antibiotic in 5 ml of dimethyl-sulfoxide (DMSO). Cetrimide agar (Enzymatic digest of gelatin 20 g L^{-1} , MgCl 1.4 g L^{-1} , KCl 10 g L^{-1} , Cetrimide 0.3 g L^{-1} final pH: 7.2 ± 0.2 at 25 ºC) containing rifampicin concentrations in the range 50-300 µg mL^{-1} by adding the antibiotic to molten agar at a temperature of 60ºC. These were thoroughly mixed and allowed to set at room temperature. Plates were air dried without lids under sterile conditions to eliminate any condensation of water droplets on the surface of the agar. A rifampicin free cetrimide agar plate was also prepared as control. The experiment was carried out in triplicates and the average counts were taken.

MIC was defined as the lowest concentration of drug that inhibited visible growth after 24 h of incubation at 37 ^{o} C.

3.2.3 Measurement of resistance to rifampicin mutation frequency

The rifampicin resistance method (Oliver *et al.*, 2000) was used to estimate the mutation frequency of all strains (PAO1-GFP WT, Δ*mutS-GFP*, Envpa1-GFP, Envpa2-GFP and Envpa3-GFP). Cells were grown overnight in LB broth containing tryptone 1.0 g L^{-1}, yeast extract 0.5 g $L^{-1,}$ NaCl 10.0 g L^{-1} (Luria broth Fore medium, UK) at 37ºC on rotary shaker operating at 100 rpm. A tenfold serial dilution of the overnight cultures containing approximately 10^9 CFU ml^{-1} of the bacteria were carried out in sterile sodium chloride solution (0.9%) and 100 µL of appropriate dilutions were spread on either cetrimide agar or cetrimide agar with rifampicin (final conc.300 µg ml^{-1}) to select rifampicin resistant cells. Plates were incubated for 24-36 h at 37ºC. The spontaneous mutation frequencies were calculated as the density of colony-forming unit (CFU) on cetrimide agar with rifampicin divided by the density of CFU on cetrimide agar. The mutation frequency of a strain was taken as the median value from three replicates.

3.2.4 Single and mixed-species biofilm formation by the Envpa1-GFP strains of Pseudomonas aeruginosa

Flow cell channels were inoculated in triplicates with 1) 0.5 ml of the overnight culture of Envpa1-GFP containing approximately 10^9 CFU ml^{-1} mixed with 0.5 ml of the drinking water inoculum containing $2x10^4$ CFU ml^{-1} drinking water bacteria. We have previously established (Chapter 2) that this ratio of inoculae allowed both *P. aeruginosa* and the multispecies drinking water community to establish in co-culture within flow cell biofilms. 2) A second flow cell was inoculated with 0.5 ml of the overnight culture containing approximately 10^9 CFU ml^{-1} this time without drinking water filtrate. Both treatments were carried out in triplicates flow cell channels in each of three replicate experiments. Flow cells were incubated at room temperature for 1 h with inversion of the flow cell and without flow to allow for bacterial attachment to the glass substratum. In order to ensure that an equal density of attached *P. aeruginosa* cells were used to initiate biofilm formation in both flow cells, the number

of *P. aeruginosa* strains attached to both flow cells were enumerated after 1 h adhesion period. The culture medium (filter sterile tap water) containing 30 mg L^{-1} of sodium acetate (carbon source) was allowed to flow at the flow rate of 150 µl min^{-1}. (3rpm). The biofilm growth and development was monitored daily under both bright field and epifluorescence microscopic examination of GFP fluorescence using Olympus BX 61 microscope equipped with a GFP filter set (BP460-480 nm/BA 495-540 nm, Olympus) and captured using a digital camera (infinity 2-2C, Lumenera Ltd).

3.2.5 Harvesting of biofilm biomass for determination of mutation frequency analysis

Biofilm was harvested from each channel by squeezing the flow cell tubing into a 25 ml centrifuge tube. To ensure that the biofilm attached to the glass surface of the flow cells were also harvested, the in and out flow tubing of the flow cells were clamped and treated with 70% ethanol and then cut off with a sterile razor blade. The biofilm medium (500 µL) was injected into the flow channels and sucked up again using a syringe. The rinsing was repeated three times to ensure that the majority of biomass attached to the glass was sheared from the surface and washed out of the flow cell. This was followed by aseptically scrapped off the entire glass slide covering the flow cell into the centrifuge tube. Cells were concentrated by centrifugation at room temperature for 1-2 min. at 16,000 x g and were suspended in 1 ml of sterile physiological saline. Aliquots (100µl) of appropriate dilutions were spread on *P.aeruginosa* selective cetrimide agar (Oxoid, United Kingdom) with or without 300 µg/mL rifampicin for *P. aeruginosa* cell count, and on R_2A agar (Oxoid, United Kingdom) to determine the heterotrophic plate count (HPC) in the case of the mixed-species biofilm. The cetrimide agar inoculated plates were incubated at 37ºC for 24-36 h while the R_2A agar plates were incubated at room temperature (21ºC). All experiments were repeated in triplicates and the mutation frequency means were calculated.

3.2.6 The effect of chlorine on the mutation frequency of PAO1-GFP wild type and ∆mutS-GFP strains of Pseudomonas aeruginosa multispecies biofilms.

Aliquots (0.5 mL) from overnight cultures were mixed with 0.5 mL of drinking water bacteria inoculum containing approximately $2x10^4$ CFU ml^{-1} cells inoculated into the flow cells and incubated at room temperature without flow for 1 h to allow the attachment of bacteria cells onto the surface of the glass. Three peristaltic pump was switched on to supply the biofilm medium which was made up of filter sterilized tap water plus 30 mg L^{-1} of sodium acetate at a flow rate of 150 µl min^{-1}. The mixed species biofilms of the wild type and the *mutS* strains were allowed to reach a stable state after 5 days of growth before the biofilm growth medium was made to 1 ppm of chlorine prepared by dissolving 1.8 mg of Haz-tab which gives 1 mg of available chlorine (according to the manufacturer's directive 1 tablet of 1.8 g of Haz-tab gives 1 g available chlorine and 1000 ppm of chlorine L^{-1} which equals to 1000 mg L^{-1}) in 1 L of filter sterilized tap water. The biofilm was maintained for another 5 days using the chlorine containing growth medium. Biofilm was harvested as described in section 3.2.5 and the mutation frequencies of the wild type and *mutS* strains within the biofilm were then analysed based on their resistance to 300µg ml^{-1} of rifampicin in cetrimide agar. The mutation frequencies of the wild type and the *mutS* mixed-species biofilms grown without chlorine residue were also analysed as control.

3.2.7 The effects of antioxidants on the mutation frequencies of PAO1-GFP wild type and ∆mutS-GFP strains of Pseudomonas aeruginosa multispecies drinking water biofilms.

Aliquots (0.5 mL) from overnight cultures were mixed with 0.5 ml of drinking water bacteria inoculum containing approximately $2x10^4$ CFU ml^{-1} cells inoculated into the flow cells and incubated at room temperature without flow for 1 h before the biofilm medium containing filter sterilized tap water + 30 mg L^{-1} acetate + filter sterilized 0.55 mM of N-acetyl–cysteine or 2 mM of L-proline was allowed to the flow by switching on the peristaltic pump at a flow rate of 150µl min^{-1}. The biofilm was harvested after 12 days as described in section 3.2.5 and the mutation frequencies of *P.*

aeruginosa wild type and *mutS s*trains within the drinking water mixed species biofilms were analysed based on their resistance to 300 µg ml^{-1} of rifampicin in cetrimide agar. The mutation frequencies of *P. aeruginosa* wild type and *mutS* within the mixed species drinking water biofilms grown without antioxidant were also analysed as control experiment.

Statistical analysis:

The mean and standard deviation within samples were calculated for all cases and statistical analysis was performed using the one way analysis of variance (ANOVA) and student's t-test based on a confidence level equal or higher than 95% ($P<0.05$) as statistically significant.

3.3 Results

3.3.1 Minimum inhibitory concentration of rifampicin effective against P. aeruginosa

Based on the below results, 300 µg ml^{-1} of the antibiotic was used as the MIC value for rifampicin because this was capable of inhibiting all strains of *Pseudomonas aeruginosa* used in our experiment.

P. aeruginosa strains	Rifampicin concentration (µg ml^{-1}) and CFU^{-1} $100µl^{-1}$			
	50	100	200	300
WT	6	3	0	0
ΔmutS	Tntc	305	1	0
Envpa1	9	2	2	0
Envpa2	15	6	0	0
Envpa3	183	11	4	0

TNTC- Too numerous to count

Table 3.1 Inhibitory concentrations of rifampicin for the different strains of *P. aeruginosa* determined by colony forming unit (CFU) counts.

3.3.2 Measurement of rifampicin resistance (RifR) mutation frequency in planktonic culture

The results of the mutation frequencies of the planktonic cultures of the different strains of *P. aeruginosa* were first determined in order to make comparison with mutation frequencies of *P. aeruginosa* in both the mono and mixed-species biofilms. The result of the tests (figure 3.1) shows that the *mutS* exhibited approximately 12 fold higher mutation frequency than the wt by showing a mutation frequency of $6.49x10^{-6} \pm 3.9x10^{-6}$ which is due to its hyper mutable nature (defect in the DNA repair mechanism). The wild type strain showed a mutation frequency of $5.5x10^{-7} \pm 4.2x10^{-7}$. The environmental strain Envpa1 showed a mutation frequency of $4.55x10^{-7} \pm 3.5x10^{-7}$, Envpa2 showed a mutation frequency of $5.7x10^{-5} \pm 3.0x10^{-7}$ while the mutation frequency of Envpa3 was $5.9x10^{-7} \pm 4.6x10^{-7}$. This means that *mutS* strain exhibited approximately 10 fold higher mutation frequency than the average mutation frequencies of the environmental isolates. Statistically, there was a significant difference between the mutation frequency exhibited by the *mutS* strains, the wild type and the environmental isolates ($P<0.05$) whereas there was no significant difference between the mutation frequency exhibited by the wild type and the environmental isolates($P>0.05$).

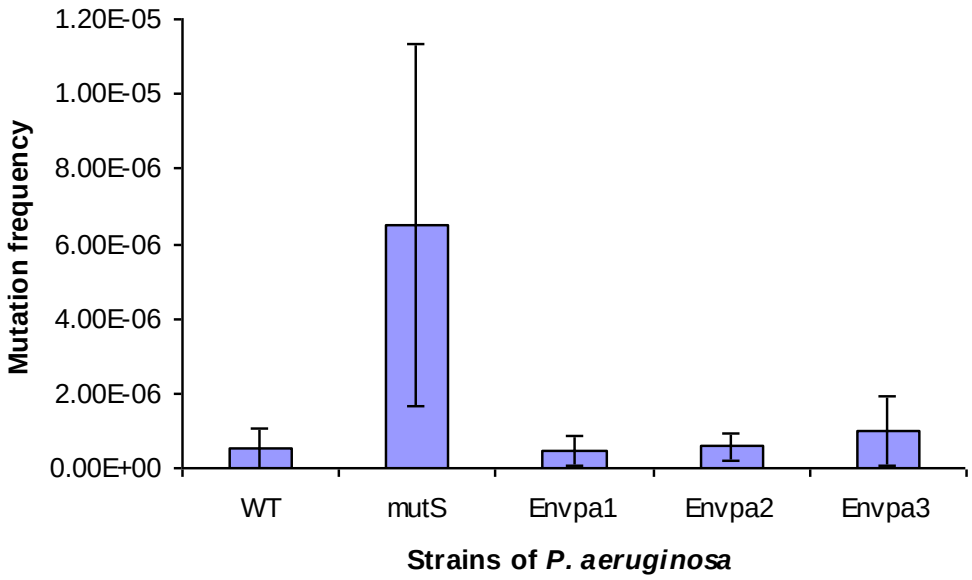

Figure 3.1 Mutation frequencies for the selection of rifampicin resistant mutants of *P. aeruginosa* strains grown in their planktonic cultures in Luria Bertani broth at 37°C (n=5).

3.3.3 Mono and mixed-species biofilm formation by the envpa1-GFP strains of P. aeruginosa

3.3.4 Attachment of the cells onto the glass surfaces of the flow cells

The rational for the attachment experiment was to ensure that an approximate number of cells attached to the glass surface prior to both mono and mixed species biofilm formation in both flow cells so that any difference in mutation frequency was not attributed to difference in the number of cell attachment. The result shows that the average number of *P. aeruginosa* attached to the glass surface per mm^2 field of view in the mixed-species was 99.1 and 90.6 in the mono-species. The statistical analysis of the result shows no significant difference in attachment ($P>0.05$).

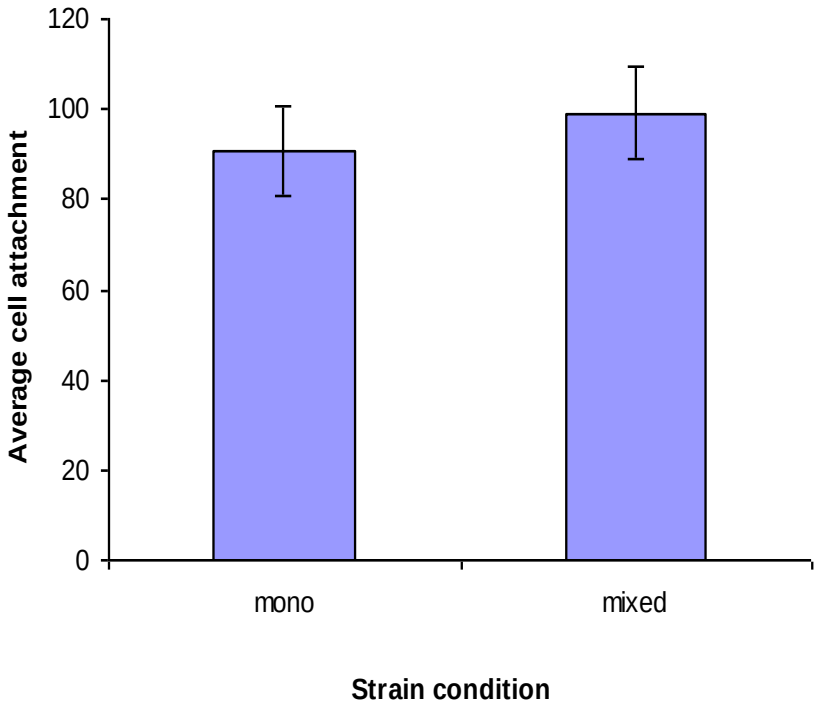

Figure 3.2 Analysis of initial cell attachment per mm^2 field of view of the Envpa1-GFP strains of *P. aeruginosa* (carrying the pmf230 gfp gene) onto glass surfaces of flow cells in both pure and mixed-species cultures. The result shows no significant difference in attachment in both the mono and the mixed-species cultures ($P>0.05$).

3.3.4 Mutation frequency analysis of Pseudomonas aeruginosa (Envpa1-GFP strains) in its mono and mixed-species biofilms

Having read some reports on the enhancement of the mutation frequency of the mono-species clinical biofilm cultures of *P. aeruginosa* (Oliver *et al.,* 2000), this work examined the mutation frequency of *P. aeruginosa* Envpa1 strain within a mixed-species biofilm in the drinking water system compared with its mono-species biofilm under the same conditions. Within the biomass extruded from the flow cell tubing, *P. aeruginosa* generated average cell densities of $6.9x10^{9}\pm 1.4x\ 10^{8}$ CFU ml^{-1} within the mixed species biofilms and average heterotrophic bacterial count of $1.2x10^{11}\pm 1.8x\ 10^{10}$ CFU ml^{-1} while the single species biofilm generated an average cell density of $9.1x10^{9} \pm 4.2x\ 10^{8}$ CFU ml^{-1}. The result obtained shows that *P. aeruginosa* within the mixed-species biofilm exhibited a mutation frequency of $4.2x10^{-7} \pm 3.4x10^{-7}$ on day 8 and a mutation frequency of $2.7x10^{-6} \pm 1.1x\ 10^{-6}$ on day 12. Conversely, a mutation frequency of $5.3x10^{-8}\pm 4.2x10^{-8}$ was exhibited within its mono-species biofilm on day 8 and $1.0x10^{-7} \pm 6.4x10^{-8}$ on 12 day. From this result it has shown that *P. aeruginosa* within the mixed-species biofilm had 8-fold increase in mutation frequency compared with its mono-species biofilm on day 8 and a 26-fold increase on day 12 (figure 3.3). Comparing this result with the mutation frequency of the planktonic cultures of the wild type strains it has been shown that the mutation frequency of *P. aeruginosa* within the mixed-species biofilm was 5-fold higher than the average mutation frequency of the planktonic cultures where as it was 2-fold higher in its mono-species biofilm. Thus co-culture of *P. aeruginosa* with the drinking water microbial communities can lead to an increase in mutation frequency to a level that is greater than that of both *P. aeruginosa* planktonic and continuous biofilm cultures.

Table 3.3 shows total *P. aeruginosa* cell count in both mono and mixed-species biofilms on cetrimide agar and table 3.4 shows the total bacterial count on R_2A agar.

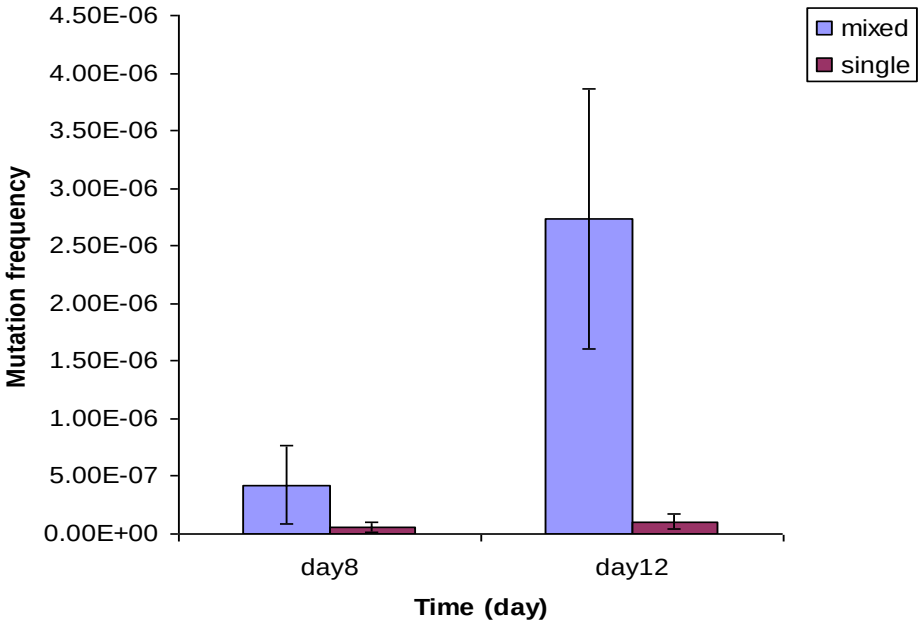

Figure 3.3 Mutation frequencies for rifampicin resistance of planktonic cells of *P. aeruginosa* (Envpa1-GFP) grown in continuous culture flow cell within mono and mixed-species drinking water biofilms at acetate concentration of 30 mg L^{-1}.

Dilution factor	Mixed-species (CFU ml^{-1})	Mixed-species (CFU ml^{-1})	Mono-species (CFU ml^{-1})	Mono-species (CFU ml^{-1})
		Replicate		Replicate
1.0E-06	6.65E+09	7.71E+09	8.74E+09	8.55E+09
1.00E-07	7.10E+09	6.30E+09	9.00E+09	8.60E+09
1.00E-08	6.00E+09	8.00E+09	1.10E+10	9.00E+09
1.00E-09	0.00E+00	0.00E+00	0.00E+00	0.00E+00

Table 3.2 Total *P. aeruginosa* count within mono and mixed-species drinking water biofilms on cetrimide agar.

Dilution factor	Mixed-species (cfu/100µl)	CFU ml^{-1}	Mixed-species (cfu/100µl)	CFU ml^{-1}
			Replicate	
1.00E-07	1251	1.25E+11	1132	1.13E+11
1.00E-08	135	1.35E+11	120	1.20E+11
1.00E-09	15	1.50E+11	11	1.10E+11

Table 3.3 Total heterotrophic bacterial count within mixed-species drinking water biofilms on R_2A agar

3.3.5 The effect of chlorine on the mutation frequency of PAO1-GFP wild type and ∆mutS-GFP strains of Pseudomonas aeruginosa multispecies biofilms

P. aeruginosa wild type and *mutS* strains established stable mixed species biofilm in the drinking water system using the flow cell. The treatment of the biofilms with 1 ppm of chlorine incorporated into the biofilm medium after 5 days of biofilm growth did not kill the biofilm cells. *P. aeruginosa* wild type mixed species biofilm treated with chlorine gave an average of 1.2-fold increase in mutation frequency compared with the non-treated by showing a mutation frequency of $1.0x10^{-6} \pm 1.6x10^{-7}$ and $8.3x10^{-7} \pm 1.6x10^{-7}$ respectively ($P>0.05$). On the other hand, the *mutS* mixed species biofilm treated with chlorine showed an average mutation frequency of $3.7x10^{-5} \pm 1.7x\ 10^{-6}$ 1.1-fold reduction compared with the non-chlorine treated which exhibited an average mutation frequency of $4.1x\ 10^{-5} \pm 1.2x10^{-6}$ ($P<0.05$). Comparing these results with the mutation frequencies *of P. aeruginosa* wild type and *mutS* planktonic cultures showed that chlorine has no major effect on the mutation frequencies of *P. aeruginosa* within mixed species drinking water biofilms (figure 3.4).

3.3.6 The effects of antioxidants on the mutation frequencies of PAO1-GFP wild type and ∆mutS-GFP strains of P. aeruginosa mixed-species drinking water biofilms.

In the presence of the antioxidants L-proline and N-acetyl-cysteine, *P. aeruginosa* PA01 wild and *mutS* strains were able to form well established mixed species biofilms. The mutation frequency analysis of *P. aeruginosa* wild type biofilm cells after 12 days of biofilm growth showed a mutation frequency of $3.3x10^{-7} \pm 6.1x10^{-8}$ when grown with L-proline and a mutation frequency of $1.3x10^{-6} \pm 3.7x10^{-7}$ without L-proline showing that wild type mixed-species biofilm growth without proline exhibited approximately 4-fold increase in mutation frequency more than when grown in the presence of proline. The *mutS* strain gave an average mutation frequency of $1.1x10^{-5} \pm 6.9x10^{-7}$ with L-proline and an average mutation frequency of $1.2x10^{-5} \pm 9.9x10^{-6}$ without proline showing an average of 1.1-fold increase in mutation frequency when grown without proline more than when grown with proline. On the

other hand, the wild type exhibited 4.6-fold reduction in average mutation frequency when grown with the addition of N-acetyl-cysteine in the biofilm by showing a mutation frequency of $2.5x10^{-7} \pm 7.9x10^{-8}$ and an average mutation frequency of $1.1x10^{-6} \pm 6.9x10^{-8}$ without N-acetyl-cysteine, while the *mutS* strain gave an average mutation frequency of $1.3x10^{-5} \pm 1.7x10^{-7}$ with N- acetyl-cysteine and $1.5x10^{-5} \pm 1.2x10^{-6}$ without N-acetyl-cysteine showing just approximately 1.2-fold reduction in mutation frequency. These data show a significant difference ($P<0.05$) between the average mutation frequencies of the wild type grown with and without antioxidants, and between the average mutation frequencies of the *mutS* strain grown with and without antioxidants (figure 3.5).

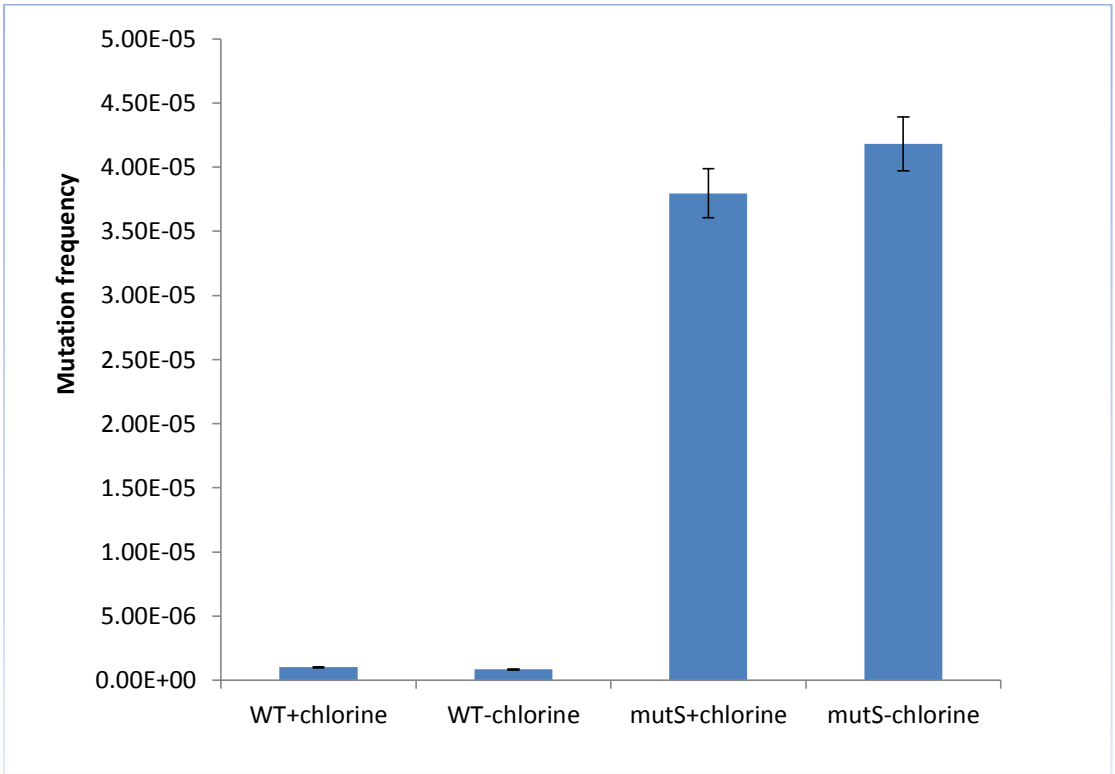

Figure 3.4 Effect of chlorine on the mutation frequency of *P. aeruginosa* PAO1-GFP wild type and Δ*mutS-GFP* grown in continuous culture flow cell within mixed-species drinking water biofilms at acetate concentration of 30 mg L^{-1}.

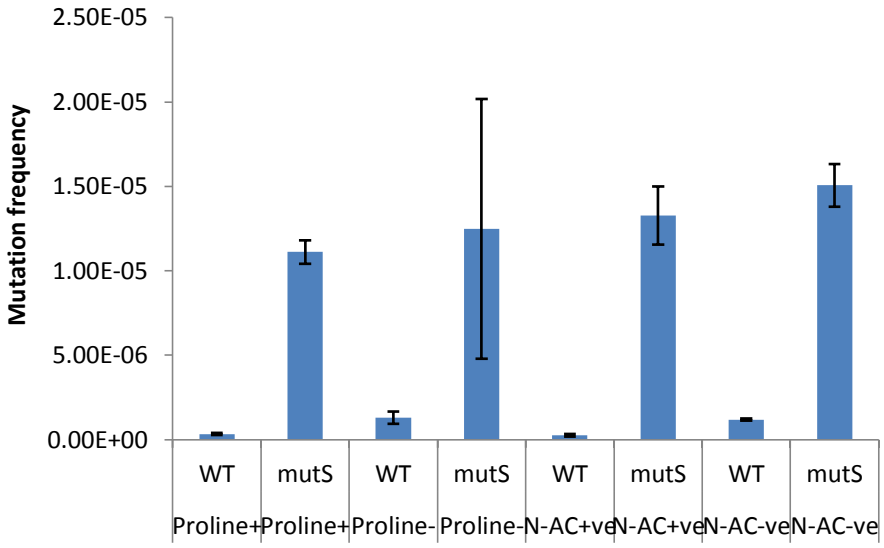

Figure 3.5 Effect of antioxidants (proline and N-acetyl-cysteine (N-AC) on the mutation frequency of 12 day old PAO1-GFP wild type and Δ*mutS-GFP strains of P aeruginosa* grown in continuous culture flow cell within mixed-species drinking water biofilms at acetate concentration of 30 mg L^{-1}

3.4 Discussion

In the drinking water distribution environment, the fitness and persistence of pathogenic microorganisms such as *P. aeruginosa* may pose a risk to public health. This study has shown that *P. aeruginosa* present within mixed-species drinking water biofilm are capable of generating a 5-fold increase in mutation frequency as a result of interactions with the microbial community within complex biofilms that was not observed in single species biofilm under the same conditions. Hansen *et al*., (2007b) have shown that in a mixed biofilm containing two species, mutants can be selected that respond specifically to the presence of the other organism. Therefore factors that relate to competition or other interactions with other drinking water bacteria within the system are capable of influencing the mutation frequency of this pathogen. Understanding the role of inter-bacterial interactions in the fitness of *P. aeruginosa* within this system should provide new insight into the pathogenicity of the organism and may have important implications for its control. For example the higher mutation frequency exhibited by *P. aeruginosa* within the drinking water mixed-species biofilms may lead to altered antibiotic resistance or virulence traits, or may promote the persistence and long-term survival of the pathogen in drinking water. Our result also showed that the mutation frequency of *P. aeruginosa* within mixed species drinking water biofilms increases with the age of the biofilm. This has supported the work of Alison *et al* (2009) which showed that the relative fitness of cells obtained from aged biofilms heritably increases over time, indicating that adaptive evolution occurs.

In the last two decades, studies of bacterial responses to environmental stresses have become a major theme not only in bacterial physiology but also in microbial ecology to provide better understanding of the behaviour of specific microorganisms (for example allochtonous pathogens) in natural environments (Munro *et al.,* 1995; Barcina *et al.,* 1997; Manuel and Roberto 1998). Giao *et al., (*2008) has demonstrated the generation of different cells of *Helicobacter pylori* within drinking water biofilms as a result of environmental stress (temperature). Selection pressure might dominate in selecting for strains with high mutation rates and could observe a correlation to

resistance. For example, in the drinking water environment the exposure of bacteria to a continuous changing environment could conceivably cause enrichment for mutation. Our result has shown that, *P. aeruginosa* integrated within other drinking water bacteria exhibited enhanced mutation frequencies compared with its single species biofilms which suggest that *P. aeruginosa* in mixed-species biofilm may play an important role as a foci for genetic adaption and evolution. It has been suggested that interaction between species facilitated by environmental structure generates biodiversity. These interactions can span from antagonistic (possibly due to inhibitory or antimicrobial activity of other organisms within the biofilm) to mutualistic (for example metabolic cooperation) (Kuramitsu *et al*., 2007; Poltak *et al*., 2011). It has also been suggested that strains of bacteria with high mutation frequencies can acquire resistance to disinfectants more rapidly than the wild-type (BlÃ¡zquez 2003; Macia *et al.,* 2005; Wingender *et al.,* 2011) and can also exhibit enhanced horizontal acquisition of exogenous DNA (Townsend *et al.,* 2003). This further suggest that *P. aeruginosa* in mixed-species biofilm can acquire higher resistance to disinfectant more rapidly than the single species biofilms. The high mutation frequency experienced in the mixed-species biofilm of *P. aeruginosa* could also be a consequence of locally induced DNA-damaging stresses within the micro-colonies (Conibear *et al.,* 2009). There are many sources of endogenous DNA damage that can cause mutation within cells, including toxic oxidative products of normal metabolism (Burcham 1999; Marnett and Plastaras 2001; Conibear *et al.,* 2009). Mixed-species biofilms generate steep gradient stresses caused by the accumulation of metabolites.

Oxidative stress has previously been linked to the occurrence of hyper mutable *P. aeruginosa* strains in cystic fibrosis infections (Ciofu *et al.,* 2005; Boles *et al*., 2004). Although we do not understand the mechanism by which the drinking water bacteria can induce increased mutation frequency in our experiment. There is evidence that the source of environmental stress in this instance is related to oxidative stress because when antioxidants was included in the biofilm medium a lower mutation frequency was obtained relating to the work of Boles *et al.,* 2004 which reported diversity of *P. aeruginosa* in biofilms as a result of oxidative stress. We can hypothesise that perhaps other members of the microbial community may produce inhibitory compounds that produce oxidative damage against *P. aeruginosa*. This is similar to the production of the Alp protein by marine bacterium *Pseudoalteromonas tunicata* (Mai-Prochnow *et*

al., 2004), which catalyses the production of hydrogen peroxide in biofilms as an inhibitory compound.

Given that oxidative stress within biofilms can increase diversity in *P. aeruginosa* (Frinkel *et al*., 1999; Bjedov *et al.,* 2003). We considered the possibility that antioxidant compounds could affect the mutation frequency of *P. aeruginosa* within mixed species drinking water biofilms. Our results showed a significant reduction in the mutation frequency of the wild type when grown in a biofilm medium incorporated with antioxidant compounds than when grown without antioxidants ($P<0.05$) .The antioxidants could have increased the carbon and energy sources within the biofilms thereby reducing starvation stress within the microenvironment (Frinkel *et al*., 1999; Bjedov *et al.,* 2003). The utilization of proline by *P. aeruginosa* in tap water has been reported by Van der Kooij *et al.,* (1982) .Secondly, since antioxidant neutralises oxidative stress within the biofilms the reduction in the mutation frequency of the wild type grown with antioxidant medium could have resulted due to a reduction in oxidative stress within the biofilm. The *P. aeruginosa mutS* strain grown with the antioxidant also showed a reduction in its mutation frequencies but less reduction compared with the wild type strains. This could be a result of the inactivation of the *mutS* genes by the antioxidant compounds thereby overcoming the mutation and diversity-inhibiting effects of the antioxidants thus increasing the mutation frequencies. A similar work carried out by Singh *et al*., (2008) has reported the production of extensive diversity in *mutS* strain of *P. aeruginosa* biofilm grown in antioxidant medium. Secondly, the less effect of the antioxidants on the mutation frequencies observed in the *mutS* could also be attributed to the development of antioxidant defences such as superoxide dismutase by this strain. This is a class of enzyme that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide in cells. A report has described the enhancement of antioxidant metabolism and their transcribing genes within biofilms (Resch *et al.,* 2005).

With this result it has shown that selective pressure responsible for genetic variation in biofilms is independent of the oxidant- mediated mechanism generating mutation and variation within biofilms because according to Singh *et al.,* (2008) diversity is generated even with the blockage of oxidative stress in biofilms.

Bacteria growing in biofilms can remain in drinking water distribution system because they are resistant to the level of disinfectants residuals present in the water

(Lechevallier *et al*., 1988; Stewart *et al*., 1994; Giao *et al.,* 2010). In this study, we used the flow cell to study the effect of chlorine on the mutation frequency of resident cells of *P. aeruginosa* within drinking water mixed species biofilms, but not to study biofilm specific resistance to chlorine.

According to World Health Organisation (WHO) guidelines for water quality (2004), there should be a residual concentration of free chlorine in the range of 0.2 to 0.5 mg L^{-1} for effective disinfection. Ridgway and Olson (1982) have reported that the most sensitive bacteria including *P. aeruginosa* are readily killed by chlorine concentration of $\leq$ 1.0mg L^{-1}. Laboratory experiments using different strains of *P. aeruginosa* in distilled water showed that only the resistant strain survived chlorine treated at a dose of 500 µg L^{-1} (Shrivastava *et al*., 2003). Our result has shown that *P. aeruginosa* can maintain its mixed species biofilms in the presence of 1ppm chlorine. Some investigations have found that significant biofilms can develop in the presence of low levels of residual free chlorine, and even at high levels, biofilms are not eliminated (Characklis, 1988; Giao *et al.,* 2010). According to Nagy (1986), bacterial densities in biofilms were unaffected by the presence of free chlorine residuals. Stewart *et al.,* (2001) have reported that bacteria in the biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* are highly resistant to killing by both alkaline hypochlorite and chlorosulfamates. Some research has shown that most of the free chlorine is depleted before penetrating the biofilm (Haas *et al*., 1999). LeChevallier demonstrated that biofilm associated bacteria may be 150-3000 times more resistant to free chlorine (LeChevallier, 1988). He also reported that a germicide flushed through the water distribution systems kill free-floating microbes, but it cannot touch bacteria embedded in the slimy biofilm according to him, when bacteria are in a film, they are very resistant to biocides and they often produce more exopolymers after biocide treatment. Our data agree with these observations as we found that 1ppm chlorine has no significant effect on the viable counts of *P. aeruginosa* harvested from the biofilm tubing.

The subsequent analysis of *P. aeruginosa* cells recovered from the biofilm showed that these cells have undergone mutation that allowed for an increase in their resistant to antibiotics as planktonic cells compared with the results of the non- chlorine treated experiments. The mutation frequency exhibited by the chlorine treated mixed species biofilm of the wild type strain compared with the non-treated was not significant ($P > 0.05$) which shows a weak mutagenic response to chlorine treatment. Such barriers

could include extracellular polysaccharides polymer (EPS). However, there was a significant difference ($P<0.05$) in the mutation frequency exhibited by the *mutS* chlorine treated and non-treated mixed species biofilm but in this case, the non-chlorine treated biofilm exhibited a high mutation frequency more than the chlorine treated. This means that the chlorine treated mixed species biofilms of the *mutS* strain produced less rifampicin-resistant isolates than the non-chlorine biofilm. This outcome may due to the fact that chlorine disinfection can decrease glucose metabolism (Chen *et a*l., 1993), or decrease bacterial diversity (William *et al*., 2005). Overall, *P. aeruginosa mutS* mixed species biofilm showed approximately 40- fold increase in mutation frequency more than the wild type mixed species biofilms in all cases. This could be due to the hyper mutable nature of the *mutS* strain having a defect in its DNA mismatch repair systems since stress and starvation within the biofilm can activate error-prone polymerase (Foster, 2005) more in the *mutS* than in the wild type. On the other hand, the mutation frequencies exhibited by the wild type and the *mutS* mixed species biofilms treated with or without chlorine supersede the mutation frequencies of their overnight planktonic cultures.

3.4.1 Conclusions

The higher mutation frequencies observed in *P. aeruginosa* mixed-species biofilm compared with mono-species biofilms has revealed the role of inter-bacterial communication in fitness and diversification of *P. aeruginosa* within mixed species biofilms. This observation may suggest that *P. aeruginosa* mixed-species biofilm may play an important role as foci for genetic adaptation and evolution which could lead to the development of alternative treatment and control strategies of this pathogen. We also found that chlorine has no major effect on the mutation frequency of *P. aeruginosa* within mixed-species drinking water biofilms and the mutation frequency exhibited by the mutS mutator strains of *P. aeruginosa* within mixed-species drinking water biofilm is independent of oxidative stress within the biofilms. Our data suggest that studies to understand the survival of *P. aeruginosa* present in mixed species drinking water biofilms in relation to its enhanced mutation frequency need to be explored in order to better understand the degree of health risk these bacteria pose within the drinking water distribution system in particular and the environment in general.

Chapter four

Influence of ultra violet (UV) treatment on *P. aeruginosa* mutability and survival within mixed-species drinking water biofilms.

Abstract

Ultra-violet (UV) light treatment can be an effective method for improving the microbiological quality of drinking water. However, its effectiveness becomes questionable if the water contains suspended solids, turbidity, or soluble organic matter such that the penetration of the water by the UV-radiation is limited and therefore the UV lethal dose is compromised. Because we have shown that increased mutability can enhance survival of *Pseudomonas aeruginosa* in drinking water, we hypothesise that mutagenesis caused by sublethal exposure of this organism to UV light may similarly influence its survival within biofilms. The work in this chapter therefore uses *P. aeruginosa* as a model organism to investigate the effect of sublethal doses of UV irradiation on mutation and survival in a drinking water biofilm system. Our results show that UV treatment can lead to an increase in the mutation frequency of *P. aeruginosa* compared with non-irradiated cells, and that UV-treated cells can exhibit enhanced colonization and survival within the mixed species biofilms. Our data therefore suggest that partial sublethal UV irradiation may enhance persistence and survival of this pathogen within the drinking water system.

4.1 Introduction

Ultra violet (UV) radiation has been used effectively for sterilising air (Hart, 1960; Riley *et al.,* 1962; Rubbo *et al.,* 1965; Wedum *et al.,* 1961), vaccines (Collier *et al.,* 1955; Hable *et al.,* 1947), and water (Ricks *et al*., 1955; Zelle *et al*., 1955; Rubbo *et al*., 1965; Straford, 1963). The UV unit for water treatment consist of a specialised low pressure mercury vapour lamp that produces UV radiation at 254nm. The optimal wavelength for disinfection is close to 260nm (USEPA, 2011). The UV lamp never contacts the water, it is either housed in a quartz glass sleeve inside the water chamber or mounted external to the water which flows through the transparent UV tube. It is mounted so that water can pass through a flow chamber, and UV rays are admitted and absorbed into the stream (Wolfe, 1990).

UV radiation at 254nm can be absorbed by most purine and pyrimidine bases and as such can cause direct damage to DNA. When this happens the microbial cells are rapidly killed and there is a high rate of mutation among the surviving cells if the damage is not corrected. The genetic information may be permanently mutated. The mutation caused by UV lights is a CC to TT mutation, caused when a CC dimer is mispaired with two adenine bases during replication (Friedberg, *et al.,* 1995; David, 2001; Rajesh, *et al.,* 2010). *P. aeruginosa*, which is an important micro-organism from both environmental and sanitary points of view, possesses mechanisms of error prone DNA repair (e.g. DinB polymerase) that are very similar to SOS- response system in *Escherichia coli* (Miller and Kokjohn, 1990). Such error prone polymerases are considered to be the principal source of UV induced mutation. Mutations occur when error prone DNA polymerase reaches a dimer where incorrect bases are inserted. The nucleotide excision repair UvrB gene (which up-regulate SOS response and error prone polymerase) also plays a role in UV-induced mutation repair in *P. aeruginosa*. The UvrB protein must be functional in *P. aeruginosa* cells, because a UvrB defective mutant is extremely sensitive to UV radiation (Walker, 1984; Rivera *et al.,* 1996; Rudd, 2000).

The lethality and the mutagenicity of the UV depend upon the types of the organisms, wavelength of the UV light, time of exposure etc. Disinfecting drinking water with UV makes good sense. It is economically safe because no known toxic or significant non-toxic by product is introduced and many pathogenic micro-organisms are killed or rendered inactive. However, UV radiation is not suitable for water with high levels of suspended solids, turbidity, colour or soluble organic matter. These materials can react with UV irradiation and reduce disinfection performance. Turbidity makes it difficult for radiation to penetrate water (Wolfe, 1990; Gadgila, 1997) and pathogens can be shadowed, protecting them from the UV light. In view of this, some bacteria may escape irradiation or are sub-lethally irradiated which may trigger bacterial mutation and persistence within drinking water systems in view of this we hypothesized that UV light may influence mutation frequency and therefore survival of bacteria in biofilms Secondly, with the absence of residual disinfectant in UV treated water, the water is subject to recontamination via the distribution system, prior to consumption. In addition, biofilm formation and coliform regrowth may occur.

P. aeruginosa forms biofilms with other bacteria in medical infections which tend to enhance their mutability within these biofilms and therefore poses a challenge to decontamination (Markus and Colin, 2005).

Few studies have examined the impact of UV disinfection on *P. aeruginosa* multispecies biofilm growth in water distribution systems.

The purpose of this study therefore is to evaluate the mutability and survival of UV treated planktonic cells of *P. aeruginosa* within multi-species drinking water biofilms.

4.2 Materials and methods

4.2.1 Bacterial strains and culture conditions

Wild type PAO1-GFP strains of *P. aeruginosa* stored at -80 ºC was removed from the vials and streaked onto LB agar plates containing the appropriate antibiotic (100 mg ml^{-1} carbenicillin for pMF 230). Plates were viewed under the microscope to check for maintenance of the GFP plasmid pMF 230. Starter cultures (10 ml) were then prepared using the most brightly fluorescent colonies in LB broth and incubated overnight at 37ºC. Preparation and storage of multi-species drinking water standard bacterial inoculum and biofilm media preparation are the same as previously described in chapter two (2.1.2 and 2.1.3) but sodium acetate concentration of 30 mg L^{-1} was used in this case.

4.2.2 Irradiation procedure

The UV energy source was a 30 W Westinghouse low-pressure mercury germicidal lamp (Sterilamp model 782L-30) having a wavelength of 254 nm. The lamp was placed in a cabinet (105 by 30 by 45 cm). The distance between the lower surface of the UV bulb and the Petri plate was 22 cm and a constant UV intensity of 176.5 $\mu W/cm^2$ (17.65 erg/mm^2) incident UV dose at each target spot was maintained. UV dose was defined as the measurement of the energy per unit area that falls upon a surface. It is the product of UV intensity and exposure time.

4.2.3 UV survival by planktonic cells of P. aeruginosa

Duplicate sets of 1 ml amount of the overnight culture of *P. aeruginosa* (PAO1-GFP wild type) having a cell density of approximately 10^9 CFU ml^{-1} were dispensed into Petri dishes having a depth of 1.5 cm. This was irradiated simultaneously with incremental doses of UV light in seconds (0, 30, 60, 90 and 120). A 10-fold serial dilution of the UV exposed cell suspensions were carried out and 100µl of the dilution containing 10^7 CFU ml^{-1} were plated out in duplicates on LB agar, while the un-irradiated cells of the same dilution factors were also plated as control. These were incubated at 37ºC for 24 h and survivor colonies were counted. The survival rate is

expressed as the number of UV irradiated cells forming colonies as a fraction of the colonies formed by non -irradiated cells expressed in percentage.

4.2.4 Mutation frequency of the irradiated planktonic cells

Duplicate sets of 1 ml amounts of the overnight culture of the wild type *P. aeruginosa* were dispensed into Petri dishes of the same depth as previously described. These were irradiated simultaneously for increasing UV exposure period of 0, 30, 60, 90 and 120 seconds. Tenfold serial dilutions of both UV irradiated and un irradiated cells were carried out and 100 µl of the dilution containing 10^7 cfu ml^{-1} were plated out on LB agar containing 300 µg ml^{-1} of rifampicin while the same dilution of the non-irradiated cells were also plated on LB containing 300 µg ml^{-1} of rifampicin and LB without rifampicin as control. Plates were incubated at 37 ºC for 36-48 h and the mutation frequencies were expressed as the number of rifampicin resistant colonies as a fraction of the colonies formed by LB agar without rifampicin.

4.2.5 Biofilms experiments

The survival rate of *P. aeruginosa* on exposure to UV-irradiation determined the time of exposure appropriate for this experiment. Overnight cultures of the PAO1 wild type strain were exposed to UV-irradiation of appropriate time of exposure (30 s) at 254 nm. Then 0.5 ml of the UV-irradiated culture and 0.5 ml of the UV-irradiated culture mixed with 0.5ml of the drinking water bacteria were inoculated into the flow cell and allowed to attach onto the glass surface by incubating the flow cell in an inverted position for 1 h without flow and then with flow after 1 h in order to form mixed-species biofilms. Biofilm medium was made up of filter sterilised tap water containing 30 mg L^{-1} acetate. The growth and development of the biofilms were monitored under the microscope using the green fluorescent light, since the *P. aeruginosa* carries gfp plasmid. Non-irradiated overnight cultures (0.5 ml) were inoculated either as pure cultures or mixed with drinking water bacteria to serve as control and the biofilm development was also monitored.

4.2.6 Confocal laser imaging of the biofilms and COMSTAT analysis

Image acquisition. The *P. aeruginosa* strains of the PAO1-GFP wild type irradiated and non-irradiated single and mixed species biofilms were grown in separate channels. From each channel, an average of 5 stacks was acquired after a period of five days from the date of inoculation. Images were acquired from random positions in the upper part of the flow channel, at a distance of 5-10 mm from the inlet. Images were acquired at 1.0 to 2.0 μm intervals down through the biofilm and therefore the number of images in each stack varied according to the thickness of the biofilm. All microscopic observations and image acquisition were performed by Confocal scanning laser microscope (CSLM) (TCS 4D microscope; Leica Laser technik). Images were obtained with a x40 oil immersion objective.

COMSTAT analysis of data: The biofilm images acquired were analysed using the COM STAT program which was written as a script in MATLAB 5.1 (The Math Works), equipped with the image processing toolbox. The following parameters were measured viz, biomass, thickness and number of micro-colonies formed.

4.2.7 Biofilm harvesting and mutation frequency analysis

The biofilm was harvested from each channel by squeezing the flow cell tubing into a 25 ml centrifuge tube. To ensure that the biofilm attached to the glass surface of the flow cells were also harvested, the inlet and outlet flow tubing of the flow cells were clamped and treated with 70% ethanol and then cut off with a sterile razor blade. The biofilm medium (500μl) was injected into the flow channels and sucked up again using a syringe. The rinsing was repeated three times to ensure that all cells were washed out. This was followed by aseptically scrapped off the entire glass slide covering the flow cell into the centrifuge tube. Cells were concentrated by centrifugation. The pellets were suspended in 1 ml of sterile physiological saline, and 100 μl of the dilution containing 10^7 CFU ml^{-1} of *P. aeruginosa* were spread on cetrimide agar containing a concentration of 300 μg/ml rifampicin for total mutant cell count, on cetrimide agar for total viable *P aeruginosa* count and on R2A agar for total bacterial count in the case of the mixed-species biofilm. These were incubated at 37ºC for 24-36 h. All experiments were repeated in triplicates and the relevant mutation frequency means were calculated.

Statistical analysis:

The mean and standard deviation within samples were calculated for all cases and statistical analysis was performed using the one way analysis of variance (ANOVA) based on a confidence level equal or higher than 95% ($P<0.05$) as statistically significant and the Tukey's test was used to identify which means are different from which.

4.3 Results

4.3.1 Rate of UV survival by planktonic cells of Pseudomonas aeruginosa (killing curve)

Survival of UV-irradiated planktonic cells of *P. aeruginosa* diluted to 10^7 CFU mL^{-1} and exposed to increasing UV periods in the range 0-120 s is summarised in figure 4.1. The result shows an average of 48.5% survivor at exposure time of 30 s, 19.6% at 60 s, 7.6% at 90 s and 0% at 120 s meaning that the maximum rate of inactivation 90-99% (4 log killing) occurred between 90 and 120 s.

4.3.2 Mutation frequency of the irradiated planktonic cells

Mutation frequency analysis for the selection of rifampicin resistant mutants was carried out on wild-type *P. aeruginosa* exposed to increasing UV irradiation periods of 0-90 s. These data showed a mutation frequency of $1.1x10^{-7}\pm1.1x10^{-8}$ at 0 s (control), $2.3x10^{-7}\pm1.1x10^{-8}$ at the exposure time of 30 s, $7.3x10^{-8}\pm5.6x10^{-9}$ for 60 s, and $1.1x10^{-8}\pm 2.8x10^{-9}$ for 90 s. This shows a 2-fold increase in mutation at 30 s exposure time compared with the unexposed bacterial cells. However, there was a 1.5-fold and 10-fold decrease in mutation frequency at the exposure periods of 60 and 90 s respectively compared with the mutation frequency of the non-irradiated cells (control). From this result, the irradiation period of 30 s was chosen as the ideal exposure time for subsequent experiments because it offered optimal killing of the cells coupled with the highest mutation frequency exhibited by the irradiated cells at this time of exposure.

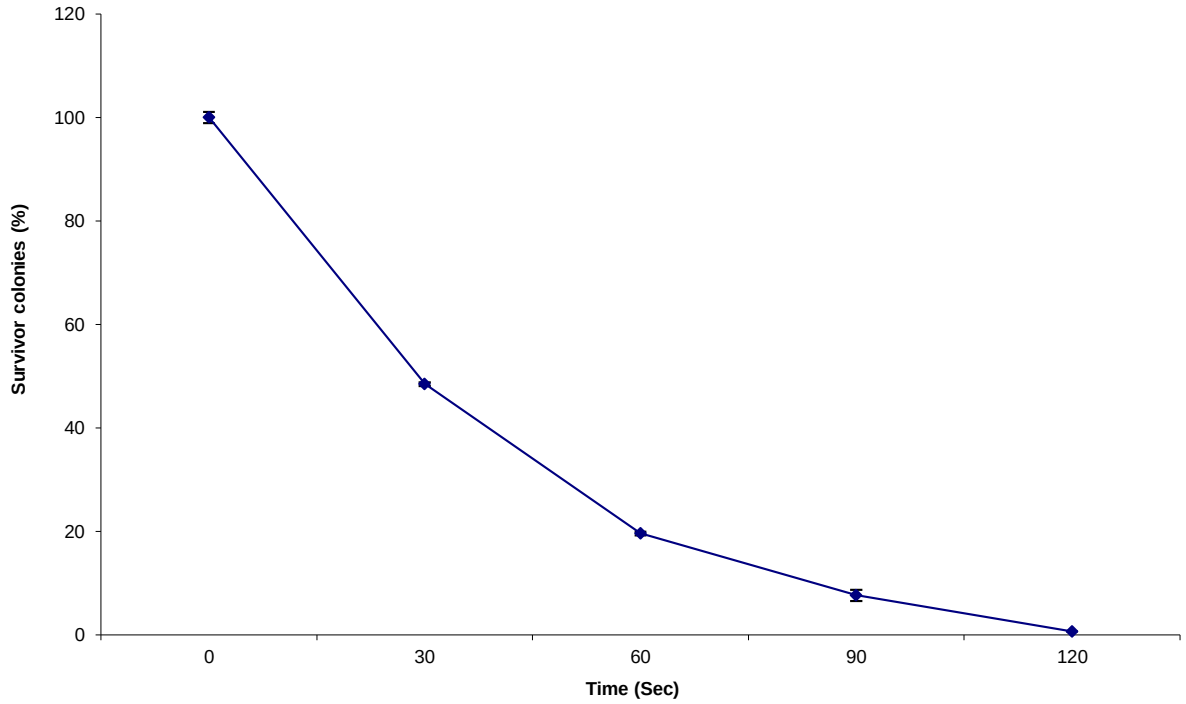

Figure 4.1 Dose-survivor curves (%) of PAO1-GFP wild type *P. aeruginosa* cells exposed to a UV intensity of 176.5µW/cm^2 at different time periods of irradiation.;

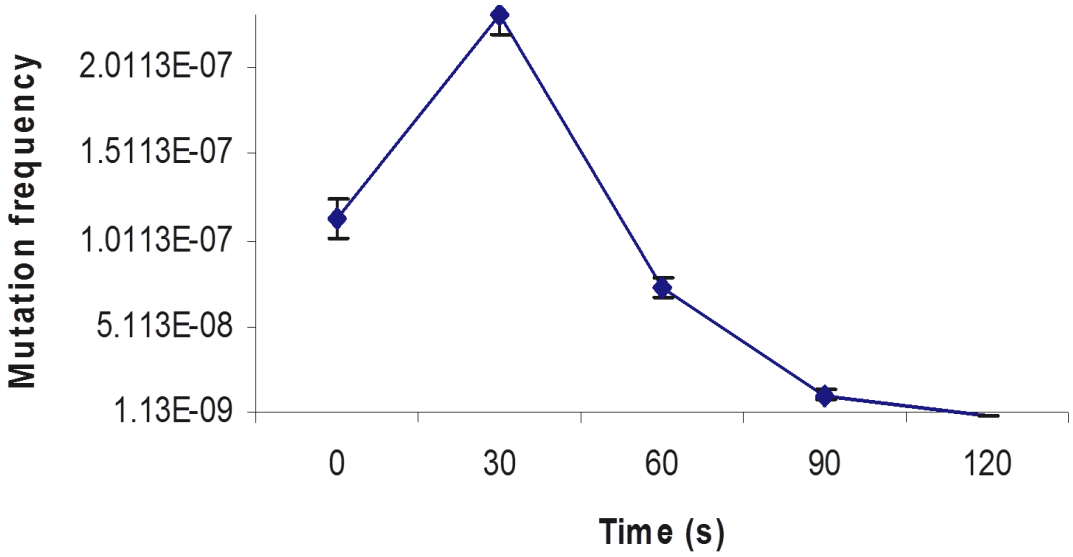

Figure 4.2 Resistance to rifampicin mutation frequencies of UV irradiated planktonic PAO1-GFP wild type strains of *P. aeruginosa* containing a cell density of 10^7 CFU mL^{-1} at different time of exposure and at a wavelength of 254nm.

4.3.3 Biofilm formation by single and mixed-species biofilms containing UV-irradiated PAO1 wild type P. aeruginosa.

In this study, the mean biofilm thickness, mean biomass and average number of micro-colonies were chosen to characterize the biofilm structures developed by the irradiated and un-irradiated PAO1-GFP wild type *P. aeruginosa* single and mixed species biofilms. Mean thickness indicates the spatial dimensions of the biofilms; the average biomass shows the total number of cells within the biofilm. These three variables were calculated by COMSTAT for biofilms developed by the single and mixed species biofilms formed by the irradiated and un-irradiated PAO1-GFP wild type strains of *P. aeruginosa* after acquiring images as described in methods. The results are summarized in figures 4.3, 4.4 and 4.5. From the results, the biofilms formed by *P. aeruginosa* UV treated biofilms (WT+DWB+UV and WT+UV) had a stronger tendency to form more biomass, increased biofilm thickness and more number of micro colonies than the UV untreated strains (WT and WT+DWB). However, there was no significant difference **(**$P>0.05$) in the mean thickness and mean biomass formed between the PAO1-GFP wild type *P. aeruginosa* mixed and pure species biofilms formed by the irradiated and non-irradiated biofilms whereas the difference in the number of micro colonies formed between the mixed and single species biofilms formed by the UV-irradiated and non-irradiated PAO1 wild type *P. aeruginosa* were statistically significant ($P<0.05$).

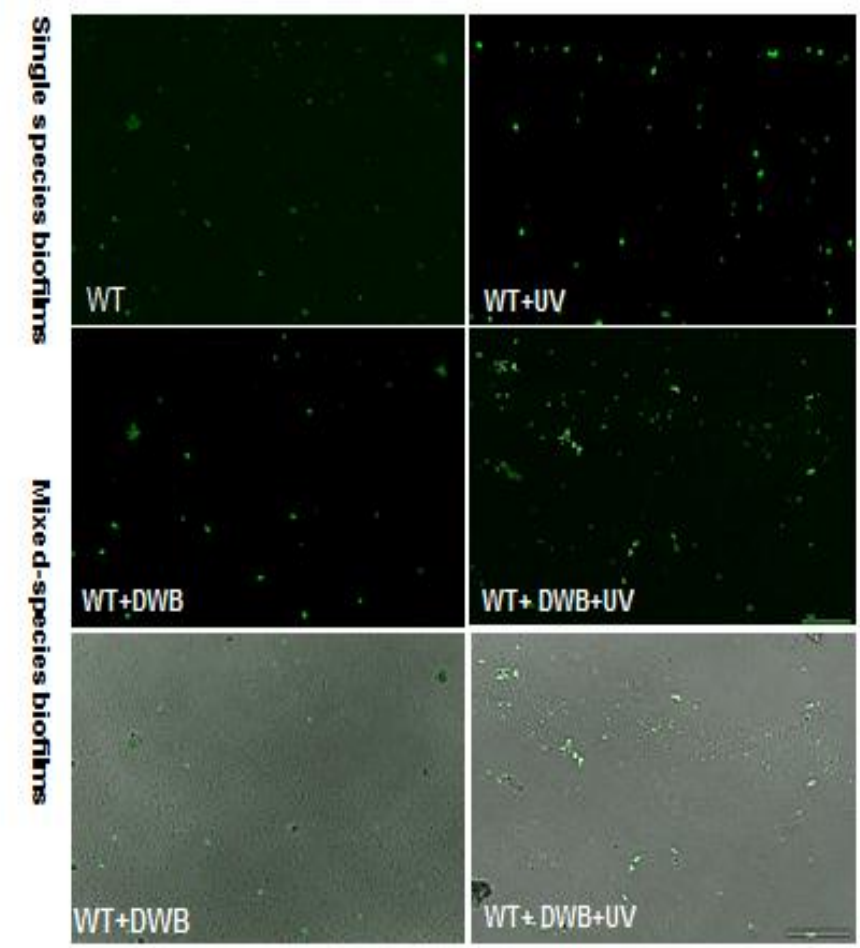

Figure 4.3 Confocal images of five day old single and mixed species biofilms formed by PAO1-GFP wild type *P. aeruginosa* strains grown in continuous flow cell culture after exposure to UV light (254nm) for 30 sec at the intensity of 176.5 µW/cm2 Confocal images are overlaid on the corresponding bright field images (DWB-drinking water bacteria). Scale bar represents 10µm.

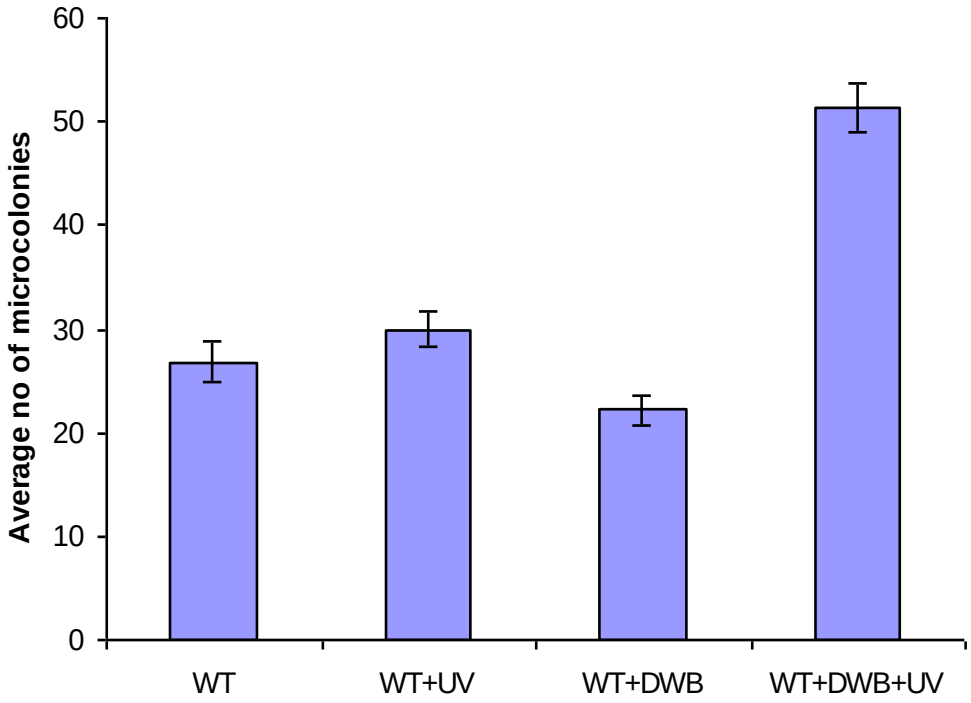

Figure 4.4 Comparison of the average number of micro-colonies formed by five day old single and mixed species biofilms of PAO1-GFP wild type *P. aeruginosa* grown in continuous flow cell culture after exposure to UV light (254nm) for 30 sec at the intensity of 176.5 µW/cm^2 (DWB-drinking water bacteria).

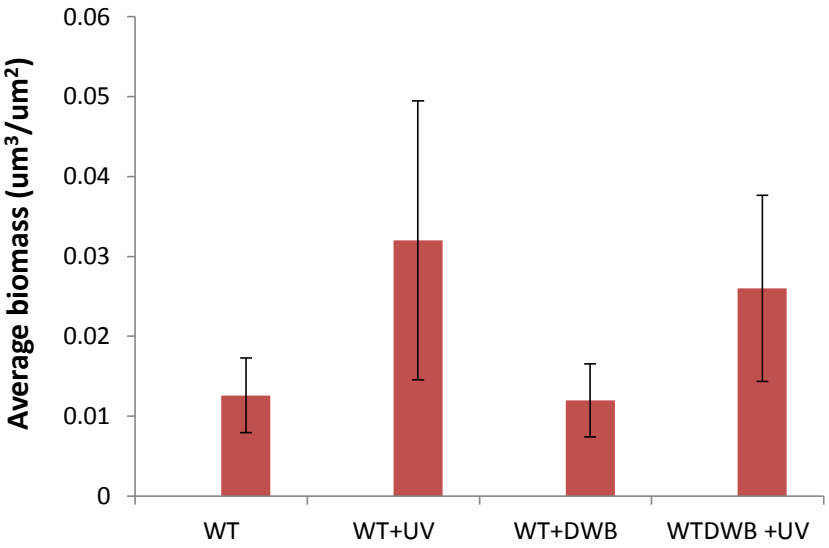

Figure 4.5a Average biomass (µm^3/µm^2) of five day old single and mixed species drinking water biofilms formed by PAO1-GFP wild type *P. aeruginosa* grown in continuous flow cell culture after exposure to UV light (254nm) for 30 sec at the intensity of 176.5 µW/cm^2 (DWB- drinking water bacteria) **(**P>0.05).

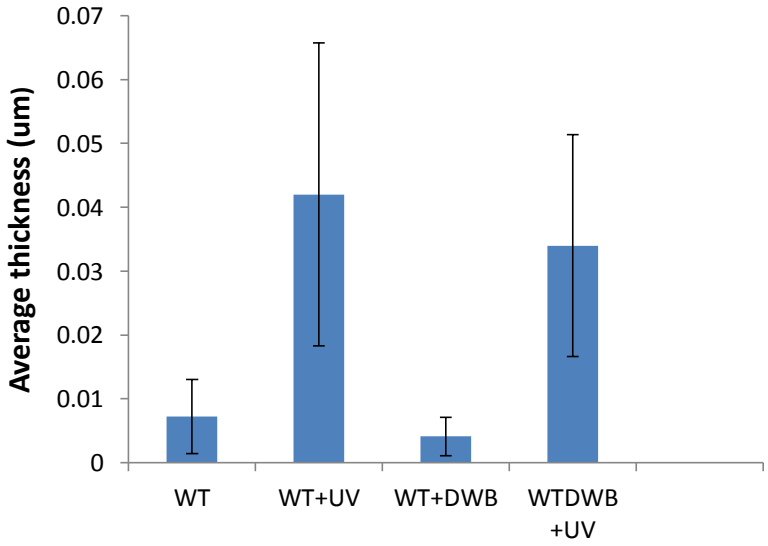

Figure 4.5b Average thickness (µm) of five day old single and mixed species drinking water biofilms formed by PAO1-GFP wild type *P. aeruginosa* grown in continuous flow cell culture after exposure to UV light (254nm) for 30 sec at the intensity of 176.5 µW/cm^2 (DWB-drinking water bacteria) **(**P>0.05).

4.3.4 Mutation frequency analysis of biofilm cells of PAO1-GFP wild type Pseudomonas aeruginosa

Mutation frequency analysis for the selection of rifampicin resistant mutants of UV-irradiated and non-irradiated *P. aeruginosa* within both single and mixed species biofilms was carried out. Our results showed that the planktonic cells of UV-irradiated *P. aeruginosa* within single species biofilms (WT+UV) exhibited a mutation frequency of $1.3x10^{-6} \pm 3.3x10^{-7}$ while the control (non-irradiated) gave an average mutation frequency of $3.3x10^{-7} \pm 8.3x10^{-8}$. On the other hand, the planktonic cells of *P. aeruginosa* non-UV treated wild type within mixed species biofilm (WT+DWB) (control), exhibited an average mutation frequency of $4.5x10^{-7} \pm 5.0x10^{-8}$ while the planktonic cells of UV-treated *P. aeruginosa* within mixed species biofilms (WT+DWB+UV) showed an average mutation frequency of $1.3x10^{-6} \pm 3.3x10^{-7}$. This result shows a 3-fold increase in the mutation frequencies of the planktonic cells of UV-irradiated *P. aeruginosa* within single species biofilms (WT+ UV) more than the non-UV irradiated (WT). An increase in mutation frequency by 1.5-fold was exhibited by the planktonic cells of *P. aeruginosa* non-UV treated mixed species biofilm (WT+DWB) more than the non-UV treated single species biofilm (WT). There was a 5-fold increase in the mutation frequency exhibited by the planktonic cells of UV-irradiated *P. aeruginosa* mixed species (WT+ DWB+UV) more than the non-UV treated single species biofilm WT. This has demonstrated that the UV irradiation enhanced the mutation frequencies of *P. aeruginosa* within both the single and the mixed species biofilms (figure 4.6).

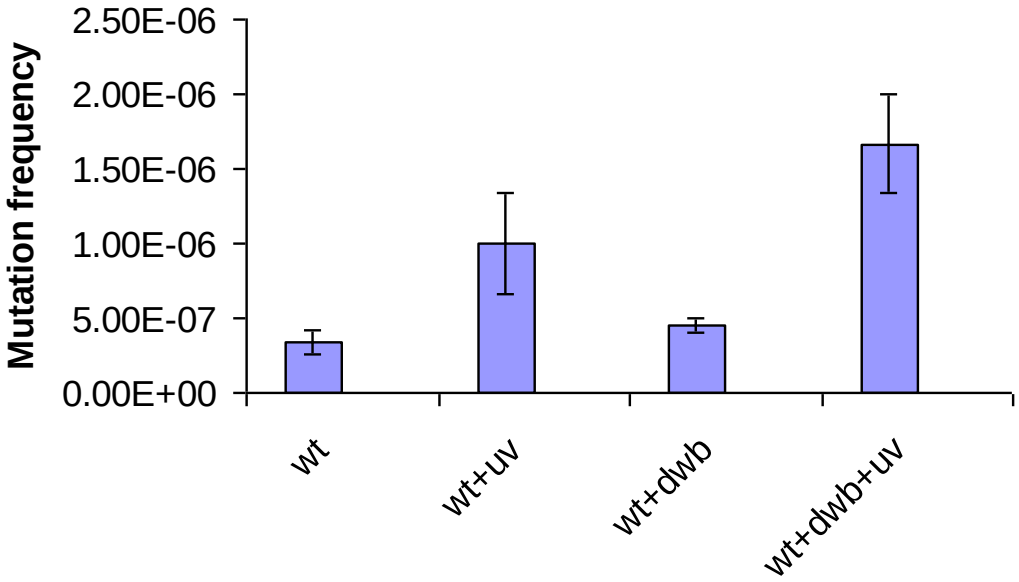

Figure 4.6 Mutation frequencies exhibited by five day old single and mixed species biofilms formed by PAO1-GFP wild type *P. aeruginosa* grown in continuous flow cell culture after exposure to UV light (254nm) for 30 sec at the intensity of 176.5 $\mu W/cm^2$. The difference in mutation frequency exhibited by UV-irradiated and non-irradiated *P. aeruginosa* biofilm is statistically significant ($P<0.05$).

4.4 Discussion

Survival studies were performed to determine how *P. aeruginosa* would respond to ultra violet (UV) irradiation which is one of the common and modern methods of drinking water treatment. We obtained a 4 log killing at the exposure time of 90s.This result is consistent with results reported by (Collins, 1971) which showed that a 4-log reduction (99.99%) of 10^9 cells of *P. aeruginosa* occurred after inoculae were exposed for 90 s (100 $\mu W/cm^2$). It has been indicated by other researchers that UV- energy inactivates bacteria exponentially and thus the typical death curve for micro-organisms treated with UV irradiation is often sigmoidal (Bachmann, 1975; Sastry *et al.,* 2000; Yousef *et al.,* 1988). The result of the inactivation of *P. aeruginosa* gave a curve typical of other micro-organisms exposed to UV irradiation. However, the tail of inactivation curves could be attributed to multiple hit phenomena which states that "the sigmoidal survival curve is accounted for on the basis of multiple UV hit on a single cell or single UV hits on multiple cells". The inconsistences in the percentage killing in relation to time of exposure could be explained by lack of homogeneity in the population of the cells (Block, 1991). This occurs when the bacteria are not uniform in structure or composition throughout the culture due to the fact that they might be composed of several different strains. These strains could all have distinctive characteristics that could make them different and which may cause one strain to be more resistant to UV energy than another (Sastry *et al.,* 2000).

During the period of UV exposure, as the UV dose increases, mutation occurs in the DNA of the *P. aeruginosa* cells which obstructs cellular replication that blocks DNA transcription and replication as a result of the formation of cyclobutane pyrimidine dimers (Sano *et al*., 1987), and therefore, compromise cellular functions which eventually leads to death (Sastry *et al.,* 2000). Some cells that experienced sublethal UV strike also suffered DNA damage (Sano *et al*., 1987), which interferes with the DNA replication. If the damage is done when the cell has just finished a reproduction cycle, its most likely to repair itself by the help of the UvrB gene (a gene which belongs to the SOS network which are transcriptionally induced by treatments (example UV light) inhibiting DNA replication, before the next splitting occurs. However, if the damage is inflicted on a cell that has just started splitting or doubling

the DNA, it will not be able to complete the cycle and will subsequently die depending on the dose of the sublethal UV exposure (Garriga *et al.,* 1992).
Our result shows that the mutation frequency of the UV treated overnight planktonic cells of *P. aeruginosa* increased with an increase in exposure time up to 30 s before a sharp decrease in mutation frequency was experienced. This increase could be as a result of multi target response at lower doses which necessitated mutation caused by the UV damage. This in other words means increase rate of mutation response begins at the dose where UV light is absorbed by the purine and pyrimidine molecules present in the DNA chain which interferes with normal base pairing of DNA either as altered bases or single strand break (Garriga *et al*., 1992). This work agrees with that of Doudney *et al.,* 1961 which reported the increase in the mutation frequency of *E. coli* WP3 strain from 0-20 s exposure time. Witkin (1961) proposed that in the lower dose range of UV exposure an increase in dose not only cause an increase in the number of pro mutants but also affect an increase in the duration of the sensitive period allowing more time for the continued synthesis of UvrB protein (SOS-response protein) to influence the establishment of a large fraction of potential mutants.
Our result has also shown that at a UV dose greater than 30 s the mutation frequency began to decrease sharply which may be that higher radiation doses have damaged the capability of *P. aeruginosa* to carry out error prone DNA repair: thus a decline in mutation frequency. Therefore, the actual mutation frequency at any given dose of UV depends on both the frequency of causing damage at the genetic region, and the probability that this genetic region will be additionally damaged by UV so that misincorporation of bases during DNA replication which can result to mutation can occur.
There was a significant difference in the mutation frequency of the UV exposed and the unexposed planktonic cells of *P. aeruginosa* within both the single and mixed-species biofilms. The UV exposed *P. aeruginosa* within both the single and mixed-species biofilms exhibited a higher mutation frequency than the unexposed under the same conditions. This could be attributed to the mutation triggered by the UV irradiation which was possibly maintained within the biofilm as a result of the selection pressure experienced within the biofilm. There could be however, more pressure within the mixed–species biofilm (WT+DWB+UV) due to mixed-species competition and the extra stress subjected to the cells by the UV light, which could explain the higher mutation frequency exhibited by *P. aeruginosa* within the mixed-

species biofilm more than the single species biofilms. This observation could be a possible explanation of what happens within the drinking water distribution pipelines when some bacteria are sub-lethally irradiated due to the presence of suspended solids and turbidity (Sastry *et al.,* 2000) which may cause UV light to be scattered and have less contact with the microbial cells that could result to genetic lesion rather than cell death. The most commonly used UV lamps for water disinfection are low pressure (wavelength 260nm). Most large municipal drinking water facilities that currently employ UV disinfection use low pressure high output (LPHO) and medium pressure (MP). Both lamps have an initial burn-in period that is required for the lamp to stabilize and render a regular germicidal output (Wolfe *et al.,* 1990). The output however, slowly diminishes over the lifetime of the lamp which is a significant limitation of this method that could result to partial disinfection of bacteria which could otherwise trigger mutation as the bacteria survive.

Another shortcoming of this disinfection method is photo-reactivation. This is a repair mechanism in damaged DNA caused by exposure to ultraviolet light which can occur when the organisms are exposed to visible light and with the help of DNA repair enzyme called photolyases. However, DNA damage repair can also occur in the dark both of these regeneration mechanisms vary from organism to organism and can easily be prevented if sufficient doses of UV are applied and exposure to direct sunlight is prevented. In the absence of these measures which depends on a variety of factors as earlier explained such as shadowing, suspended solids, cell clumping and UV absorption, microbial resistance to UV may occur, which poses a health risk to drinking water consumers.

Irradiated cells can also find a safe haven in biofilms where they could rejuvenate, strive and undergo mutation. Microbial resistance to UV irradiation matches that of chemical disinfection with double stranded DNA viruses least susceptible, followed by MS2 coli phage, bacterial spores, double stranded RNA enteric viruses and lastly vegetative bacteria (Maier *et al*., 2003).

The bacterial response of DNA damage caused by UV light involves the UVrA and the UvrB genes which belong to the SOS network comprising at least 20 different genes which are transcriptionally induced by a variety of treatments damaging the chromosome or DNA replication (Rivera *et al.,* 1996). However, in *P. aeruginosa,* the UvrB gene is not DNA damage inducible (Rivera *et al*., 1996) which could be the

reason why our result showed extreme sensitivity to UV radiation due to the inactivation of the UvrB gene in this pathogen.

The increase in the mutation frequency of the UV-irradiated *P. aeruginosa* compared with the non-irradiated cells within the biofilms could suggest that non-lethal irradiation may lead to mutation that can provide adaptive advantages to bacteria within the drinking water system. However, the role that the inability to induce UvrB gene transcription plays in producing high sensitivity to UV radiation of *P. aeruginosa* cells compared with other UV less sensitive bacteria such as *E.coli* requires further investigation.

Chapter five

General conclusions and future work

Hypermutable (mutator) bacteria characterised by increased spontaneous mutation rates due to alterations of the DNA mismatch repair (MMR) system are commonly isolated from natural populations of bacteria associated with human pathogenesis (Oliver *et al*., 2002; 2004). The importance and role of the mutator phenotypes in disease is well understood: for example *mutS* deficiency in *P. aeruginosa* can determines the immediate development of resistance to every single anti-pseudomonal agent due to the emergence of resistant mutants within a few hours following drug exposure (Oliver *et al*., 2004). However, the role of mutator phenotypes for bacterial survival and fitness of this organism in environmental and industrial settings is less well researched.

In view of the public health risk imposed by *P. aeruginosa* in drinking water systems we investigated the extent of biofilm formation by *P. aeruginosa* and the impact of mutator phenotypes of this organism on fitness within a multispecies drinking water biofilms model compared with their wild type strains under the same environmental conditions.

Our results revealed that *P. aeruginosa* mutator phenotype (*mutS*) is capable of enhanced integration and fitness into the drinking water biofilms more than the wild type. Several reasons may explain why the enhanced establishment of the *mutS* strain within this drinking water system. Enhanced competitive biofilm formation in MutS strains may be mediated by a complex adaptive process whereby new mutations lead to physiological changes produced by the activation of specific regulatory pathways, including the induction of the biofilm mode of growth more than the wild type (Ventre *et al.*, 2006). There may also be selection for an important number of adaptive mutations required for long term persistence (Smith *et al.*, 2006). Laboratory and theoretical approaches have shown that under particular circumstances such as exposure to new environments or stress conditions including our drinking water system, mutator cells are selected in bacterial populations by hitchhiking with the adaptive mutations that they produce more frequently than the regular cells, therefore playing a role in evolution (Mao *et al.*, 1997; Sniegowski *et al*., 1997; Taddei *et al.*, 1997b; Karine *et al.*, 2002). Various in vivo models have also shown that

hypermutation may favour the adaptation and persistence of bacterial pathogens. Giraud *et al* (2001) using a murine model of *E. coli* intestinal colonisation, found that hypermutation was initially beneficial because it allowed a faster adaptation to the mouse gut environment. It has also been shown that the inactivation of the MMR system in *E. coli* favours the persistence of urinary tract infections in a mouse (Labat *et al*., 2005).

The result of this work has also shown that PAO1 wild-type *P. aeruginosa* within multispecies drinking water biofilms exhibited higher mutation frequencies than occurred within pure culture biofilm. It has been proposed that genetic change and diversification of bacteria within a biofilm community provide an insurance policy against future antimicrobial challenges or environmental changes, with the diversified population predicted to be more robust than a single parent strain (Drenkard *et al*., 2002; Boles *et al*., 2004). Possibly, the environmental conditions present within multispecies biofilms are such that create additional stresses and pressures on *P. aeruginosa* that may further enhance diversification. Other investigators have suggested that phenotypic variation generates cells that are more adept at surface colonization (Yildiz *et al.,* 1999; Deziel *et al.,* 2001). It has also been reported that in mixed species biofilms, genetic changes during biofilm development can lead to the evolution of new species interactions (Hansen et al., 2007a). When populations of single cells are subjected to certain forms of strong selection pressure such as in our case the drinking water multispecies biofilm interactions, variants can emerge bearing changes in DNA sequence that bring about an appropriate change in phenotype (Cairns *et al.,* 1988). Thus within complex multispecies biofilms, selective pressure produce a range of mutations that enhance survival in the presence of complex interacting organisms which may lead to many new bacterial strains with mutations that confer the correct phenotype that will alleviate this pressure. Many recent studies have demonstrated that during the growth of bacteria in biofilms, variant subpopulations often emerge (Allegrucci *et al.,* 2006; Haussler, 2004; Webb *et al.,* 2004; Conibear *et al*., 2009). Following the growth of the parent strain in biofilms proportion of isolated strains exhibit colony morphologies that are distinct from the parent strain, for example the small rough or winky variants of *P. aeruginosa* (Kirisits *et al*., 2005). The small non-mucoid variants of *Streptococcus pneumoniae* (Allegrucci *et al.,* 2006) and the rugose colony type of *Vibrio cholerae* (Yildiz *et al.,* 1999). Our results suggest that mutation and diversification are further enhanced in

multi-species drinking water biofilms than in single species pure culture drinking water biofilms. When considering the mechanistic processes that may drive enhanced mutation within multi-species biofilms, competition among microorganisms for space and nutrients is a powerful selective force, and which ultimately has led to the evolution of a variety of effective strategies for colonizing and growing on surfaces (Burgess *et al*., 1999; Szewzyk *et al*., 2000). The ecology of a biofilm is a complex equation of physicochemical and biological parameters. As with an all levels of evolution, a complex web of interactions is central to the structure, composition and function of these communities (Hansen *et al*., 2007b; Vieira *et al.,* 2007).

Mature biofilms contain concentration gradients of metabolic substrates and products. Oxygen is the best studied and most familiar example (Debeer *et al.,* 1994; Zhang *et al*., 1995; Xu *et al.,* 1998). Oxidative stress has been demonstrated to affect diversification within biofilm (Singh *et al*., 2008). In this study, the reduction of oxidative stress within the multi-species drinking water biofilm by the incorporation of antioxidants into the biofilm medium reduces the mutation frequency of *P. aeruginosa* within the biofilms suggesting that oxidative damage to DNA may indeed play a role in generating mutations in our multispecies biofilms as described in the study. However, the nature and extent of the mutations arising across and within the genome of *P. aeruginosa* within biofilms remains to be characterised fully and will be an interesting topic for further studies.

Although several methods eliminate disease causing microorganisms in water, chlorination and UV irradiation are the most commonly used methods. Chlorination is effective against many planktonic pathogenic bacteria, but when pathogens grow in multi-species biofilms such as the drinking water mixed species biofilms, chlorination becomes partially effective or completely ineffective (Lechevallier *et al*., 1988).

P. aeruginosa that grow within biofilms are said to be Hypermutable and more resistant to antibiotics (Oliver *et al.,* 2004) and other microbial disinfectants (Lechevallier *et al.,* 1988).

In light of our data that links enhanced mutation frequencies and improved survival of *P. aeruginosa* in biofilms it was deemed important to investigate the control of *P. aeruginosa* pathogen in the drinking water system by disinfection (chlorination and UV irradiation) and the implication of such treatments for the mutability of *P. aeruginosa*

Our investigation into the effect of free chlorine on the mutation frequency of the wild type and mutator phenotype (*mutS*) strain of *P. aeruginosa* within drinking water biofilms has shown no significant difference in the mutation frequency of the wild type compared to the non-chlorine (control) and between the mutation frequency of the *mutS* treated compared with the non-chlorine treated (control). This shows that free chlorine has little effect on *P. aeruginosa* mutability when growing within drinking water multi-species biofilms, irrespective of the genetic state (wild type or mutator phenotype) of the pathogen.

Ultraviolet (UV) radiation has been used effectively for sterilizing water (Ricks *et al.*, 1955; Stratford, 1963). However, there has been evidence that shielding by particles within drinking water systems can often result in sublethal irradiation of pathogens and consequently the survival of UV susceptible bacteria (Nicki *et al*., 2004). It was therefore in view of this that we investigated the mutability *of P. aeruginosa* UV irradiated and surviving cells within multi-species drinking water biofilm. A comparison made between the mutation frequency exhibited by the overnight planktonic culture of *P. aeruginosa* and that of the irradiated and un-irradiated biofilm planktonic cultures revealed that the UV irradiated planktonic survivor cells within mixed species biofilm cultures exhibited higher mutation frequency more than the un-irradiated planktonic cells within mixed species biofilm cultures and the overnight planktonic cultures. However, the un-irradiated *P. aeruginosa* biofilm planktonic culture exhibited more mutation frequency more than the overnight planktonic culture. These results signify that in addition to biofilm-mediated increases in mutation frequency of *P. aeruginosa,* UV irradiation can also contribute to further significant increase in the mutation frequency (and potentially enhanced adaptation and survival) of this pathogen within this system.

This finding therefore calls for appropriate caution and further study of microbial adaptive responses to UV light in water treatment in particular because the response of bacterial pathogens to sublethal UV doses may make them more difficult to eliminate and increase the health risk to drinking water consumers.

SUMMARY OF KEY FINDINGS

1) Mutator phenotypes of *P. aeruginosa* can enhance biofilm fitness and survival of this opportunistic pathogen in drinking water multi-species biofilm systems (Chap 2).

2) Growth within multi-species drinking water communities led to an enhanced mutation frequency of *P. aeruginosa* relative to cells within mono-species biofilms (Chap 3)

3) The causes of increased mutation frequency of *P. aeruginosa* within mixed-species biofilm may include damaging oxygen species since the incorporation of antioxidant to the biofilm medium reduces the mutation frequency of *P. aeruginosa* within the biofilms (Chap 3).

4) Chlorination has no major effect on the mutation frequency of *P. aeruginosa* within drinking water multi-species biofilms however; treating *P. aeruginosa* pathogen with sublethal UV irradiation can increase its mutation frequency and fitness within drinking water multi-species biofilms.

Future work

The results in this thesis lay the groundwork for understanding *P. aeruginosa* interactions within drinking water multi-species biofilms. Following these investigations, a number of projects could be taken up involving biofilm growth of *P. aeruginosa* within drinking water multi-species biofilms such as;

1. Do mutator strains (e.g. *mutS*) occur naturally in environmental and industrial *P. aeruginosa* populations, as they do in clinical settings? Identification of factors within drinking water biofilms that may lead to the evolution of high mutation rates in *P. aeruginosa* populations within multi-species biofilms;
2. Competition studies between co-evolving mutants and clonal lineages: - identification of *P. aeruginosa* genetic factors that determine survival in multispecies biofilms;
3. Studies of other possible causes of mutation in *P. aeruginosa* including other genetic systems involved in DNA metabolism and repair;
4. Next generation sequencing studies of the nature and extent of mutations occurring throughout the entire *P. aeruginosa* genome as a result of genetic adaptation to the biofilm lifestyle;
5. The effect of the physicochemical and hydrodynamic properties of drinking water systems on the development and integration of *P. aeruginosa* within multi-species drinking water biofilms and
6. Identification and analysis of the autochthonous drinking water microbial community involved in the formation of mixed-species biofilms with *P. aeruginosa* and studies of the dynamic interactions within this community.

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