GROWTH OF *MYCOBACTERIUM AVIUM* IN DUAL SPECIES BIOFILMS WITH *PSEUDOMONAS AERUGINOSA*

by

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ABSTRACT

Interest in the growth of *M. avium* in biofilms has increased in the last few years. Research has shown that *M. avium* cells in biofilms are more resistant to disinfectants than their planktonic counterparts. Although *M. avium* has been detected in biofilms in *in situ* and laboratory models, information available on *M. avium* is limited compared to biofilm model species such as *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus* and *Streptococcus*.

The main objective of the present research was to study the growth of *M. avium* in biofilms in the presence of *P. aeruginosa*. Biofilms were grown in sterile tap water on stainless steel coupons in batch mode. Two kinds of reactors were used; mason jars and a recirculation system. Each experiment lasted from 27 to 35 days depending upon the nature of the experiment.

The two strains were inoculated in isolation (monospecies) and also in combination (dual species). When inoculated simultaneously, in jar reactor experiments, *M. avium* density was found lower in dual species than in monospecies biofilms and the difference was statistically significant. However the growth of *P. aeruginosa* in monospecies did not differ significantly from the dual species biofilms. *P. aeruginosa* reduced the growth of *M. avium*.

In sequential inoculation experiments an established biofilm of *P. aeruginosa* did not prevent biofilm formation by *M. avium*. The growth of *M. avium* and *P. aeruginosa* was similar whether they were inoculated as base or invading species. The density of *P. aeruginosa* remained higher than the density of *M. avium* in the dual species biofilm, likely due to the higher growth rate of *P. aeruginosa* compared to that of *M. avi*um.

It is important to understand their growth in mixed species biofilms, in order to begin to develop effective methods to both monitor and eventually control this opportunistic pathogen

INTRODUCTION

Background Introduction

The majority of studies on drinking water microbiology have focused on pathogens of fecal origin (Steinhart et al., 2002), for example, *Escherichia coli, Salmonella, Shigella, Vibrio cholerae*, helminthes, protozoans and enteric viruses. These remain the primary health concern in parts of the world where hygiene and sanitation is substandard. However, there are other opportunistic pathogens like *Legionella*, mycobacteria and *Pseudomonas aeruginosa* which are also common and may thrive in natural and man made water systems (Norton et al., 2004). The research described here examined the growth of the bacterium *Mycobacterium avium*, belonging to the group *Mycobacterium avium* complex (MAC), in biofilms.

The MAC includes *M. intracellulare, M. avium* sub sp. *avium* (*M. avium*) and *M. avium* sub sp. *paratuberculosis* (*M. paratuberculosis*), the etiologic agent of Johne's disease of cattle, which has also been implicated in Crohn's disease in humans. Genome analysis reveals that the two subspecies *avium* and *paratuberculosis* have 98% overall sequence homology. Approximately 385 genes of *M. avium* sub sp *avium* have no counterpart in *M. tuberculosis* (Cangelosi et al. 2004). Serotyping of MAC is based on the presence of serovar specific glycopeptidolipid (ssGPL). ssGPL consists of core nonspecific GPL common to many environmental mycobacteria modified by the addition of serovar-specific oligosaccharide side chains (Laurent, 2003; Saad et al., 1997). The synthesis of serotype specific GPL distinguishes MAC from *M. tuberculosis* (Cangelosi et al., 2004).

M. avium as the Etiologic Agent

Mycobacterium avium is an opportunistic pathogen, mainly infecting immunocompromised people such as AIDS patients. In non-AIDS patients *M. avium* causes lymphadenitis in children and pulmonary infection in people with preexisting pulmonary diseases (Inderlied et al. 1993). Mycobacterial lymphadenitis (swelling of lymph nodes) occurs mainly in cervical and facial regions. In a study of 105 patients over 32 years, it was found that before 1978 the etiologic agent of lymphadenitis was mostly *M. scrofulaceum.* However, after 1978, MAC strains replaced *M. scrofulaceum* (Wolinsky 1995). *Mycobacterium avium* can infect individuals via gastrointestinal or pulmonary tracts (Inderlied et al 1993). Because mycobacteria colonize water surfaces and are readily aerosolized, pulmonary infection is probably caused by the mycobacteria released from water into the air (Wendt et al., 1980). For example, employees working in a hot water therapy pool suffered from respiratory problems and at least two of the patients were diagnosed with pulmonary infection by *M. avium*. Abundance of mycobacteria was found to be higher in the air than in the pool (Angenent et al., 2005). Pulmonary diseases caused by MAC usually occur in patients with chronic lung diseases like chronic obstructive pulmonary disease, tuberculosis, bronchiectasis and pneumoconiosis. However, pulmonary diseases caused by MAC in patients without predisposing conditions have been increasing (Prince et al., 1989). As much as 50% of the general population could be exposed to MAC in some areas of the US. However, the incidence of clinical disease is remarkably low, less than 10 per 100,000 people (Inderlied et al. 1993).

Before the AIDS epidemic in the early 1980s, disseminated infection with *M. avium* was extremely rare. Since then it has increased among AIDS patients and has caused

extensive mortality (Horsburgh 1991). In the US, prior to the availability of more potent antiretroviral medications, more than 30% of AIDS patients developed disseminated MAC infections (more than 95% are caused by *M. avium*). However, after the introduction of the antiretroviral therapy, infections were reduced to 2% among those individuals with access to the therapy (www.emedicine.com/med/topic1532).

Much of the morbidity and mortality in cystic fibrosis patients is caused by chronic pulmonary infections with *P. aeruginosa* (Ohman and Chakrabarty 1981). Nontuberculous mycobacteria (NTM) are also potential respiratory pathogens in cystic fibrosis patients. Approximately 13% of sputum samples from CF patients have been shown to be NTM positive. *M. avium* complex has been detected in 72-75% of NTM positive sputum samples (Olivier et al., 2003a,b).

M. avium in the Environment

M. avium is an acid-fast, rod shaped, aerobic and non motile bacterium. It is characterized by its slow growth, thick hydrophobic cell wall and antimicrobial resistance. Because of its hydrophobicity, it tends to become concentrated at air-water interfaces (Wendt et al., 1980). Its slow growth is attributed to the presence of a single rRNA gene, high energy consumption for long chain fatty acid synthesis, and impermeability of the lipid rich cell wall (Primm et al., 2004; Cangelosi et al.,2004). It has also been found to grow at high temperatures (above 40^{o}C), low oxygen tension and low pH environments (Schulze-Robbecke and Bochholtz 1992; Norton et al., 2004; Bodmer et al., 2000; George and Falkinham 1986). Growth at high temperature and resistance to disinfectants may select for *M. avium* growth in drinking water and in hot water supplies (LeChevallier, 2004).

Mycobacteria inhabit a wide variety of environments like water, soil, aerosols, protozoans, birds, animals and humans (Primm et al., 2004) but the specific reservoir for human infection has not been conclusive (Reed et al., 2006). Members of MAC have been isolated from natural and treated waters (Goslee and Wolinsky 1976). Falkinham et al., (2001) observed mycobacterial growth in drinking water distribution systems. During monitoring of 42 drinking water supply systems from 21 states in the US, 35 % of the samples showed the presence of non-tuberculous mycobacteria. The greatest occurrence of the mycobacteria was observed in samples from large buildings and hospitals (Covert et al., 1999). Recirculating hot water systems found in many hospitals and schools appear to be good habitats for *M. avium* (du Moulin et al., 1988; von Reyn 1994). Identical strains of mycobacteria were isolated from the infected patients and the drinking water they consumed (Du Moulin and Stottmeier 1978; Mansfield and Lackner 1997).

Morphotypes

It appears that the *M. avium* complex can exist in different morphotypes. They segregate into smooth-transparent and smooth-opaque and rough colony types (Cangelosi et al., 2001). Transparent variants are more virulent and more drug resistant than their opaque counterparts. Rough mutants lack glycopeptidolipids in their walls (Cangelosi et al., 2004). Transition of smooth transparent to smooth opaque has been shown to occur at a frequency of 10^{-4} to 10^{-5} while smooth opaque to smooth transparent occurs at a frequency of 10^{-4} to 10^{-6} (Rastogi et al., 1981).

Cell Walls of Mycobacteria and Antimicrobial Resistance

The mycobacterial cell wall is rich in lipid content which forms a protective barrier against antimicrobials. The outermost layer consists of glycopeptidolipids (GPLs) and other kinds of lipids. GPL is linked to long-chain mycolic acids. Mycolic acids are long chain length branched fatty acids typically containing 70-90 carbon atoms (Lambert, 2002). The mycolic acids are covalently linked to an arabinogalactan layer which is linked to peptidoglycan. The mycolic acids layer is interrupted by porins as in gram negative outer membranes providing access for hydrophilic nutrients.

Lipoarabinomannan (LAM), a glycolipid, protrudes from the cytoplasmic membrane, spanning the cell wall and extending to the cell surface. LAM has been implicated in virulence and pathogenesis of mycobacteria (Chan et al. 1991). Lipoarabinomannans of *Mycobacterium* species in their various forms have been implicated in immune response such as suppression of T-cell proliferation and induction of IL-12 expression (Kaur et al., 2002).

The high lipid content of mycobacterial cell walls is assumed to act as a major barrier to the penetration of antimicrobial agents. Lipophilic agents should pass through the lipid rich membrane. However, low fluidity and unusual thickness of the cell wall may slow down such a process (Jarlier and Nikaido, 1994). Hydrophilic agents can migrate through the cell wall through porins, but the porins are few in number and also are less efficient in transporting antimicrobial agents into the cell due to their structure (Englehardt, 2002). However, the cell wall barrier alone does not account for the antimicrobial resistance of mycobacteria (Li et al., 2004). It is likely that other factors like inactivating enzymes, target site alteration and drug efflux pumps also contribute to resistance (Jarier and Nikaido 1994; Lambert, 2002). Several

classes of drug efflux pumps have been identified in the genome sequences of mycobacteria (Li et al., 2004).

Mycobacteria in Biofilms

Occurrence of bacteria in sessile forms in biofilms has long been recognized (O,Toole et al., 2000) and is considered as a survival strategy of the microbes (Costerton et al., 2003). A biofilm is a biologically active matrix of cells and extracellular products attached to a solid surface. Formation of biofilms has been recorded in environmental habitats and in human organs and tissues. Microbes can form biofilms both on abiotic surfaces as in water pipes, dental water unit lines, implants, medical devices, and in pathological environments as in cystic fibrosis, otitis media, endocardtitis and dental plaque (Costerton et. al., 1999).

Biofilm formation involves microbial attachment to the surface, movement of the cells along the surface to form a monolayer, microcolony formation and exopolysaccharide (EPS) production leading to three dimensional mature biofilms. A mature biofilm is a heterogeneous environment with gradients of oxygen and nutrients (Lazazzera, 2005).

Mycobacterium avium has been detected in drinking water systems in biofilms. Studies have shown that mycobacteria are significant inhabitants of biofilms in natural and clinical environments (Covert et al. 1999; Angenent et al. 2005; Schulze-Robbecke and Fischeder 1989). Schulze-Robbecke and Fischeder (1989) found mycobacteria in biofilms in water distribution systems and hypothesized that biofilms are an important habitat and site for proliferation of aquatic mycobacteria. Biofilms in drinking water can be a significant source of mycobacteria (Falkinham et al., 2001). Interestingly, in a laboratory study, *M. avium* formed more biofilm in water than in M7H9 broth medium (Carter et al., 2003).

Norton et al., (2004) studied *M. avium* biofilms in a model drinking water distribution system. There was a significant effect of pipe surface, nutrient level and disinfectants on the survival and growth of *M. avium* in biofilms.

Mycobacteria were observed in greater numbers in biofilms than in the planktonic phase of the hot water of a therapy pool in a hospital (Angenent et al., 2005). A central venous catheter- related sepsis was found to be related to MAC biofilms (Schelonka, 1994). Biofilms have also been linked to the middle ear infection, chronic otitis media (Costerton et al., 1999). *M. chelonae* and MAC have been found to be the causal agent of many cases of otitis media and the bacteria were resistant to antibiotics (Lowry et al., 1988). Because *Mycobacterium avium* can bind to pulmonary epithelia, can cause chronic infection, has only partial response to drugs, and can be antibiotic resistant, *M. avium* has been suspected of forming biofilms in epithelial layers of human airways (Carter et al., 2004; Carter et al., 2003). As mentioned earlier, *M. avium* complex species have also been isolated from cystic fibrosis patients (Inderlied et al., 1993). *M. avium* grown under conditions of high osmolarities and low oxygen tension have been found to be more virulent than *M. avium* controls. These environmental conditions somewhat mimic the environment in the intestine (Bermudez et al., 1997). It is possible that they face a similar environment in drinking water biofilms, where oxygen tension may be low, particularly in deeper parts of biofilms (Ford 1999) and osmolarity may be relatively high due to absorbed salts and nutrients.

In general, bacteria growing in biofilms have been found to be more resistant to antimicrobial agents (Mah and O'Toole 2001). This is believed to be due to lower penetration of the agents and slower growth of the bacteria. A biofilm is also a heterogeneous environment. Certain environment in biofilm may select for antimicrobial resistance. Even in

its planktonic form, mycobacteria are resistant to many antimicrobial agents because of low cell wall permeability (Engelhardt et al., 2002) and slow growth. Moreover, their antimicrobial resistance has been further compounded by sparse distribution of porins (50 times fewer than in the Gram negative bacteria) in the cell wall (Engelhardt et al., 2002). Therefore, growth within biofilms should further enhance their intrinsic antimicrobial resistance. In addition to these factors, mycobacteria also possess several types of drug efflux pumps (Li et al., 2004) and it is possible that the efflux pumps are also involved in drug resistance. *M. avium* has been found to be 50 to 500 times more resistant to various disinfectants than *E. coli* (Taylor et al., 2000). In addition, planktonic cells of *M. avium* have been found to be two times more susceptible to chlorine than biofilm detached cells and four times more susceptible than those in biofilms (Steed and Falkinham, 2006). Clarithromycin has been used to treat *M. avium* infection in humans and in an in vitro study it has been found to inhibit initial stages of biofilm formation but has no effect against established biofilms (Carter et al., 2004).

A "biofilm phenotype" has been found to be associated with an increase in virulence of several species, including *Staphylococcus* (Cucarella, 2001), *S. epidermidis* (Li et al. 2005), *P. aeruginosa* (Yadav et al., 2004, Prithiviraj et al., 2005), *Enterococcus fecalis* (Seno et al., 2005) and *Streptococcus pyogenes* (Cho and Caparson 2005). In an interesting study, Yamazaki et al. (2006) observed that *M. avium* cells that are unable to form biofilms have a significantly lower ability to enter into bronchial cells than the wild type.

Genetics of Biofilm Formation

Since a sessile life style would be different from planktonic growth in various aspects, gene expression is also expected to be different. Biofilm formation is believed to be

a genetically programmed process (Lazazzera, 2005). Genetic analyses carried out so far have shown diversity of genetic factors involved in biofilm formation (Beloin and Ghigo, 2005). These studies have revealed the role of surface structures like pili and flagella (Pratt and Kolter 1998; O'Toole and Kolter 1998), adhesins like fimbriae and antigen 43 (Schemberi et al., 2003), cell to cell signaling (Davies et al., 1998) and exopolysaccharide. The biofilm matrix is made up of mainly polysaccharide which provides three dimensional structure to the biofilm. Different kinds of polysaccharides are produced by different species. For example, *P. aeruginosa* produces alginate (Donlan and Costerton, 2002), *E. coli* produces colanic acid (Danese et al. 2000), and *Staphylococcus epidermidis* and *S. aureus* produce polysaccharide intercellular adhesin/poly-*N*-acetylglucosamine polysaccharide (PIA/PNAG) (Cucarella et al., 2001). There is also evidence that bacteria develop stress responses in biofilms. *S. aureus* expresses acid neutralizing pathways (Beenken et al., 2004); *E. coli* expresses *soxS* and *RecA* (Beloin et al., 2004; Ren et al., 2004) and *P. aeruginosa* expresses *sodB* and *Pf1* (Beloin and Ghigo, 2005).

Martinez et al. (1999) demonstrated that *M. avium* could show surface motility on the growth medium. They move by sliding on the surface. Glycopeptidolipid presence on the outermost layer of the cell wall of mycobacteria was found to correlate with the ability to translocate over the surface. In *M. smegmatis,* a strain that was unable to form glycopeptidolipid was also defective in surface motility and biofilm formation on polyvinyl chloride. In *M. smegmatis* the *mps* gene was responsible for GPL synthesis and an *mps* mutant had no GPL and could not form biofilms (Recht et al., 2000). Biofilm formation of *M. avium* has also been linked to several genes (Yamazaki et al., 2006). Transposon mutagenesis showed four genes which, when interrupted, impaired the ability to form biofilms. It was also

concluded that the GPL biosynthesis was the most important pathway involved in the production of *M. avium* biofilm.

GroEL1 and GroEL2 are the two forms of the HSP 60 chaperone in *M. smegmatis.* They are the homologs of GroEL of *E. coli*. GroEL2 does the regular chaperone function and GroEL1 modulates synthesis of mycolic acid specifically during biofilm maturation. Unlike other bacteria mycobacteria have fatty acid synthase (FAS) I and II systems. FAS I makes short chain mycolic acids and FASII adds to the product of FAS I and makes it longer. GroEL1 is physically associated with KasA which is a part of fatty acid synthase II enzymes. KasA is involved in assembly of FAS II. A GroEL1 mutation was found to allow normal planktonic growth but prevented formation of mature biofilms (Ojha et al., 2005). *M. tuberculosis* also encodes a similar GroEL1 protein to that in *M. smegmatis* and the *M. tuberculosis* mutants defective in mycolic acid synthesis have attenuated phenotypes. Ojha et al., (2005) expressed the possibility that the GroEL1 of *M. tuberculosis* in biofilm related pathogenesis in humans is an area for exploration. Zambarno and Kolter (2005) suggested that biofilm formation by mycobacteria does not follow the typical mode shown for other bacteria. In mycobacteria no surface proteinaceous extensions have been identified in initial surface attachment and no exopolysaccharide components of their extracellular matrices are known. Mycobacterial genome analyses suggest that they do not possess the capability for exopolysaccharide production (Zambarno and Kolter 2005) . One possibility is that the mycolic acids, formation of which is mediated by GroEL1, may be released to form a hydrophobic extracellular matrix in mature biofilms (Zambarno and Kolter 2005).

Fibronectin is an extracellular matrix of glycoprotein produced by human and animal tissues. *M. avium* binds to fibronectin by fibronectin-attachment protein. It is believed

that binding to fibronectin enhances initial colonization of the mucosal surface (Schorey et al., 1996). The Mammalian Cell Entry (*mce*) gene is considered to be a virulence factor because it enhances ability to survive in macrophages. Several mycobacteria including *M. avium* possess this gene (Li et al., 2005). Sec A plays a major role in protein translocation. Limia et al. (2001) found a direct correlation between the formation of biofilm on tissue culture plates and expression of *secA* in *M. avium*. It was hypothesized that *Sec A* possibly secretes protein or peptides associated with biofilm formation.

Mixed Species Biofilms

The predominant mode of microbial life in nature is connected with surface bound communities called biofilms (Molin et al., 1999; Costerton et al., 1995). The majority of the bacteria in freshwater are found growing as biofilms on the surface of submerged substrata or sediments. When substrata become coated with chemical constituents, a conditioning layer is formed and the adhesion of bacteria from the water column begins (Costerton et al., 1987). As the communities develop, more species may be added and the biofilm will ultimately reach a steady state where further formation is counterbalanced by processes reducing or removing biofilm (Banks and Bryers, 1991).

Biofilms in nature are rarely found in monospecies. Biofilms in industrial and natural environments are usually a consortium of complex communities of microorganisms (Tait and Sutherland, 2000; Skillman, 1997; Christensen et al., 2002, Cowan et al., 2000). A variety of interactions can occur among different populations and the interactions can be competitive, commensal, mutualistic or amensal. An established biofilm may be beneficial, harmful or neutral to the growth and maintenance of an invading species and vice versa (Kolmos et al., 2005). Ciardi et al. (1987) found that bacteria attached to a substratum help attachment of a

second species which otherwise cannot attach by itself. Synergistic interspecies interactions may allow a poor colonizer to become established (Cowan et al., 1991). Increased biofilm formation was observed in binary species compared to monospecies biofilms (Filoche 2004; Jones and Bradshaw, 1997; Skillman et al., 1998). The sequence of attachment can also determine the nature of dual or multispecies biofilm formation (Bibel et al., 1983). *P. aeruginosa,* a base species, greatly reduced the adherence of *S. aureus* which was inoculated subsequently. When the sequence of inoculation was reversed *S. aureus* dominated. The nature of biofilm formation by *Acinetobacter, Staphylococcus* sp. and *S. aureus* depended upon the sequence and simultaneous inoculation of the three species (McEldowney and Fletcher 1987). Also, the nature of interaction among the bacterial species can be different between the planktonic phase and biofilm phase (McEldowney and Fletcher 1987, Tait and Sutherland 2002; Kolmos et al., 2005; Buhler et al. 1998). Bacterial attachment to a surface may be different in pure culture and mixed culture. In cystic fibrosis patients, *Burkholderia cepacia* usually colonizes already formed *P. aeruginosa* biofilm (Bakri et al. 2004). In some cases synergistic communities have been found to be more tolerant to antimicrobials than the bacteria in isolation (Tait and Sutherland, 2000; Whitely et al., 2001).

In mixed species biofilms of *Klebsiella pneumoniae* and *P. aeruginosa*, although *K. pneumoniae* had five times higher growth rate than *P. aeruginosa*, K. *pneumoniae* did not dominate the microbial population (Siebel and Characklis, 1991; Stewart et al., 1997). However, Banks and Bryers (1991) reported that the species with higher growth rate dominated the biofilm, but the slow growing organism also remained established. When *E. coli* and *K. pneumoniae* were used to form dual species biofilm, the species were competitive and one species dominated the biofilms (Skillman et al., 1998). It was also observed that *E.*

coli and *Serratia marcescenes* stably coexisted in biofilms and did not influence the growth of each other. Camper et al. (1996) reported that inoculum growth rate had a dramatic effect on the ability of coliforms to remain on surfaces. Interestingly, in another study, *Acinetobacter* and *P. putida* showed competitive interactions because they used the same carbon source and they were also commensal because *P. putida* utilized benzoate excreted by *Acinetobacter* (Christensen et al., 2002). In some cases the interaction could be antagonistic. When *B. cepacia* biofilms were challenged with *P. aeruginosa* PAO1, the immigrant population rapidly displaced the established biofilms because PAO1 produces substances that inhibit *B. cepacia* (Bakri et al., 2004).

Establishment of mixed species biofilms depends upon surface composition and the growth environment besides the species combination (Brading et al., 1997; Moller et al., 1997). Environmental factors like nutrient source and dissolved oxygen can significantly influence biofilm formation (Mc Eldowney and Fletcher 1987; Jang et al. 2002; Camper et al., 1996). The relative abundance of each organism in a dual species biofilm has been found to depend upon the high and low substrate concentrations in the growth medium (Komlos et al., 2005). Nutrients and oxygen would be present in higher concentrations in the periphery of a microcolony than in the interior. However in the interior of the microcolony, higher concentrations of metabolites and anaerobic respiration or fermentation would be encountered (McLean et al., 1999). At different concentrations of oxygen in bulk water, distribution of different functional groups of bacteria may be different. Heterotrophs dominated at 2 mg DO/L whereas nitrifiers increased significantly at 10 mg DO/L (Jang et al., 2002). Biofilm thickness reached a steady state sooner in the higher DO concentrations in the bulk water (Jang et al., 2002).

Rationale, Hypothesis Statement and Objective

M. avium and *P. aeruginosa* have been found in similar environments. *P. aeruginosa* causes morbidity and mortality among cystic fibrosis patients. *M. avium* has also been detected in a significant number of cystic fibrosis patients. In the environment, both *M. avium* and *P. aeruginosa* have been found in natural water, soil, tap water and hot water systems. *P. aeruginosa* is well known for its ability to form biofilms and it has been shown that, in cystic fibrosis patients, *P. aeruginosa* forms biofilms in the lungs. It would be a matter of interest and benefit to know whether *M. avium* and *P. aeruginosa* occupy similar niches and whether they interact when grown together. The two species have very different growth rates. *M. avium* has a generation time of above 20 hours whereas that of *P. aeruginosa* is 1 hr in laboratory media (Mashburn et al. 2005; Falkinham et al. 2004).

In natural biofilms, microorganisms are found in communities of multiple species and a variety of interactions can occur among the different populations. Mixed species biofilm formation can vary depending on the species composition and sequence of inoculation and environmental factors. In some cases the base biofilm has also been found to be beneficial to the invading species, whereas it has been detrimental in other cases and yet in others no effect has been observed.

This study is designed to investigate *M. avium* in dual species biofilms with tap water as the primary growth medium. The present investigation aims to determine whether the growth of *M. avium* could be influenced by the presence of the second bacterial strain, *P. aeruginosa,* in biofilms. The hypothesis is that *P. aeruginosa* will dominate the biofilms because of its much higher growth rate. Since mycobacteria have been found to survive in laboratory grade water for a considerable period of time and grow in very low organic carbon

content, *M. avium* will also exist in biofilms although at lower densities than that of *P. aeruginosa*.

The specific objectives are:

- To characterize the growth of *M. avium* in monospecies and dual species biofilms with *P. aeruginosa*.
- To study the growth of *M. avium* when coinoculated with *P. aeruginosa*.
- To study the growth of *M. avium* when *P. aeruginosa* is inoculated after formation of an *M. avium* biofilm.
- To study the colonization of *M. avium* on established biofilms of *P. aeruginosa.*

Approach

Three different experiments were carried out in batch mode with tap water as the growth medium.

Experiment I

Reactors were inoculated simultaneously with *M. avium* and *P. aeruginosa.* Bacterial growth was monitored weekly for four weeks and the growth of *M. avium* in monospecies and dual species was compared. The objective was to see whether the presence of *P. aeruginosa* in the dual species biofilm influenced the growth rate of *M. avium* when the two bacteria were inoculated simultaneously.

Experiment II

The reactors were first inoculated with *P. aeruginosa* (base species) and a biofilm was allowed to form for one week. *M .avium* (invading species) was then inoculated and growth of *M. avium* was monitored every 5 days for the next 20 days. The objective was to see if *M. avium* can colonize and grow on a previously established *P. aeruginosa* biofilm.

Experiment III

The reactors were first inoculated with *M. avium* (base species) and a biofilm was allowed to form for 2 weeks. *P. aeruginosa* (invading species) was then inoculated and growth of *M. avium* subsequently monitored over the next 20 days at 5 day intervals. The objective was to see if *M. avium* growth changes after the inoculation of *P. aeruginosa*.

Recirculation System

The growth of *M. avium* and *P. aeruginosa* was also monitored under nutrient rich conditions and at a higher temperature. A stainless steel recirculation system was used to grow the biofilms. The growth of both the species was monitored in order to see if the growth patterns change in a different environment over a period of several weeks.

METHODS

The dual species biofilm growth of *M. avium* and *P. aeruginosa* was studied in batch mode in mason jar reactors and a recirculation reactor.

Mason Jar Reactors

These reactors were used to study the dual species biofilms in tap water under low nutrient conditions. The reactor consists of a 450 ml capacity wide mouth glass jar with a stainless steel lid (Cargill et al., 1992). Stainless steel coupons of size 7.0cm x 1.4cm, supported by silicon stoppers, were suspended into the jar through holes made in the lid of the jar (Fig.1). Before placing the coupons into the reactors they were washed in RBS35 (PIERCE Inc.), abraded with sand paper (grit size 600), rinsed in hexane and submerged in 95% ethanol for 30 min. They were then dried in a laminar flow hood. The coupons were oriented parallel to the wall of the reactor to give less turbulent flow during incubation on an orbital shaker. Only about two thirds of the length of the coupon was submerged in the growth medium to provide enough head space for aeration. The reactor was filled with 350 ml of tap water as the growth medium (low nutrient condition). In order to ensure the homogeneity of water added to the reactor, the laboratory tap was run for 2 min before the water was collected in a plastic bucket and poured into the jar. The lid was covered with aluminum foil before it was autoclaved at 121^{o}C for 30 min. The autoclaved reactors were cooled to room temperature, the foil cover removed and the reactor inoculated with the respective strains of bacteria inside the laminar flow hood, the foil cover replaced and the reactors incubated on an orbital shaker at 125 RPM and 25^{o}C.

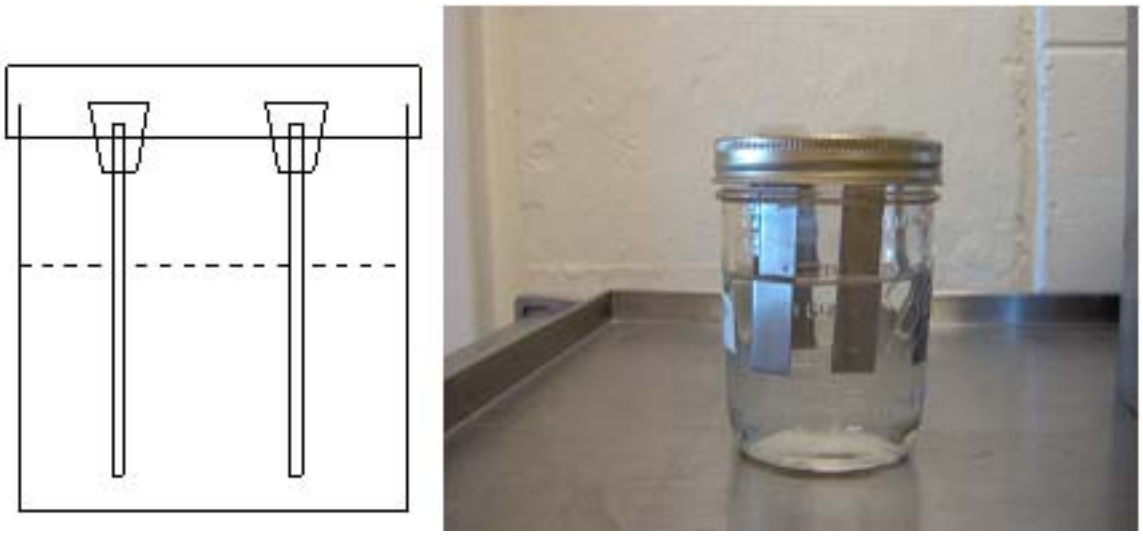

Figure 1. Jar reactor, the coupons were suspended through the holes on the stainless steel lids supported by the silicon stoppers.

Strains of Microorganisms

P. aeruginosa and *M. avium* were used to study the mycobacterial growth in dual species biofilms. An environmental isolate of *P. aeruginosa* was obtained from the Pyle Lab, Montana State University, Bozeman, Montana. *M. avium* W2001, also an environmental isolate, was obtained from T. Ford, (Montana State University, Bozeman, MT). Stocks of both the strains were prepared in 2% peptone glycerol broth and stored frozen at -78^{o}C.

Frequency and Duration

Experiments lasted for time periods from 27 days to 35 days. The experiment that involved simultaneous inoculation of the species into the reactor was repeated three times and the reactors were set up in duplicate. Coupons were removed for cell counting each week for a 5 week period.

For experiments with sequential inoculation, the reactors were set up in duplicate but the experiments were carried out only once. One coupon was harvested after 24 hr of

inoculation of the base species and 24 hr prior to the inoculation of the invading species. One coupon was harvested after every 5 days for 20 days after the invading species was inoculated.

Water Quality

After autoclaving, water quality of the growth medium was tested for pH (Beckman SS-3) and dissolved organic carbon (DOC) was measured in mg/L (Dohrman Carbon Analyzer).

Inoculum Preparation

Fresh cultures of *P. aeruginosa* and *M. avium* were grown by streaking the frozen culture onto R2A and Middlebrook 7H10 agar plates respectively. R2A plates were incubated at 30^{o}C for 24 hours and M7H10 plates were incubated at 37^{o}C for 21 days. Middlebrook agar was enriched with OADC (10% v/v, oleic acid, albumin, dextrose and catalase) and glycerol (0.5% v/v), and cycloheximide (0.0005% w/v) was used to inhibit the growth of fungi. After incubation, colonies were harvested into sterilized deionized water. The suspensions were vortexed for 1 min and allowed to settle to break up bacterial clumps for 15 min. The supernatant from the culture suspensions was sonicated (Sonogen, Model D-50, Branson Instruments Inc.) for 90 seconds (30 sec sonication/30 sec rest, 3 cycles). The sonicated samples were again vortexed and diluted to 25 Klett Units (KU) before they were inoculated into the reactors.

Inoculation of Reactors

For simultaneous inoculations (experiment I), 6 reactors were set up at a time, with duplicates for each of; 1) *M. avium* monospecies; 2) *P. aeruginosa* monospecies; and 3) *M.*

avium and *P. aeruginosa* (dual species). For sequential inoculation (experiments II and III) six reactors were set up. One reactor was inoculated with *M. avium* only and another with P. *aeruginosa*. In two of the reactors *M. avium* was inoculated first to form a base biofilm and after 14 days *P. aeruginosa* was inoculated as an invading species. Similarly, in the last two reactors *P. aeruginosa* was inoculated first for base biofilm formation and after 7 days *M. avium* was inoculated as an invading species. The autoclaved and cooled reactors were inoculated with the appropriate 25 KU cell suspensions in a laminar flow hood to give approximately 10^5 CFU/ml at time zero in the bulk water.

Sample Collection and Processing

The reactors were placed on a shaker after introduction of the culture suspension for 15 minutes to allow for cell distribution in the bulk water. A sample of bulk liquid was then taken for time zero plating. For sequential inoculations, 24-hour samples of both fluid and coupons were collected to determine bulk water counts and degree of biofilm formation.

The reactors were transferred to the laminar flow hood just before sample collection, and transferred back to the shaker immediately afterwards. Coupons were removed aseptically by using sterile technique under the laminar flow hood and transferred to vials with sterile water. All bulk water samples were also collected aseptically in sterile vials. The coupons were rinsed by dipping them in the vials to remove loosely attached cells. They were then scraped with a Teflon policeman for 8 to 10 minutes each to remove the attached cells, and the scrapings collected in 3 ml of sterile water in a beaker. The scraped coupons were then rinsed on both sides with 6 ml water and the resulting 9 ml volume was poured into a sterile vial. The beaker was rinsed with an additional 2 ml water; thus the total volume of diluent was 11 ml. Both scraped biofilm samples and bulk water samples were sonicated in

ice cold water in a bath sonicator for 90 sec (3 cycles: 30 sec sonication followed by 30 sec rest). The dual species biofilm and bulk samples were divided into two portions and diluted in 10-fold series. For *M .avium* cultures one portion of the sample was treated with an antimicrobial, cetyl pyridinium chloride (CPC) to 0.005% (Schulze-Robbecke et al., 1991) for 15 min in order to inhibit growth of *P. aeruginosa*. This ensured that the growth of *M. avium* would not be masked by faster growing *P. aeruginosa*. The decontaminated samples were filtered through a 0.45 μm Millipore filter which was rinsed with 300 ml of deionized sterile water in order to wash away residual CPC. The filters were placed on M7H10 agar plates and *M. avium* colonies were counted after 21 days of incubation at 37^{o}C. The samples were plated in duplicate. A second portion of the sample was used to culture *P. aeruginosa.* The samples were then plated on R2A agar (without adding any antimicrobial agents because *M. avium* is considerably slower-growing than *P. aeruginosa* and also does not grow well on R2A agar) by a drop plate method described below and the plates were incubated at 30^{o}C for 20-24h before counting the colonies.

All results were tabulated on Excel spreadsheets and the density of the biofilm cells was normalized to CFU per cm^2, and bulk water concentration was expressed in CFU/ml.

Drop Plate Method Protocol

This method was used as an alternative to spread plating (Herigstad et al. 2001; Yu et al. 1993). For each sample, three dilutions and two Petri dishes (8.5cm diameter) were used. The outside of the base of each Petri dish was marked into three equal sectors with the corresponding dilution labeled in each sector. A volume of 200 μl of a dilution was pipetted and 10 drops of 10μl were dispensed on the first plate and the next 10 drops were dispensed

on the second plate. This was repeated for the remaining two dilutions. The plates were then dried in the laminar flow hood until the drops were absorbed and the plates then incubated.

Recirculation System

The growth of *M. avium* in dual species biofilms was also studied in a recirculating reactor with a different set of growth conditions. The recirculation reactor consists of a 10L stainless steel tank (reservoir) with a lid (Fig. 2) connected via a peristaltic pump to a stainless steel circulation system. There were two openings on the sides of the tank which were connected to the two ends of the piping system. The piping system and the tank were connected to each other by a piece of silicon tubing fitted with a quick-disconnect. The piping system had pipe sections of varying lengths (5.5", 7.5", 11" and 18") and a diameter of 0.39". Pipe sections were connected with Swagelok^{R} (Swagelok Company) connectors. There were 8 Swagelok^{R} valves in the circulation system designed to facilitate the removal of coupons. Stainless steel coupons (3/4" X 3/8") are attached in series on a length of stainless steel wire and inserted into the pipes. There is a port on top of the reservoir for taking bulk water samples and an opening fitted with a 0.2 μm filter for aeration. The growth medium in the system is circulated by a peristaltic pump and the flow rate maintained at 38 ml/min.

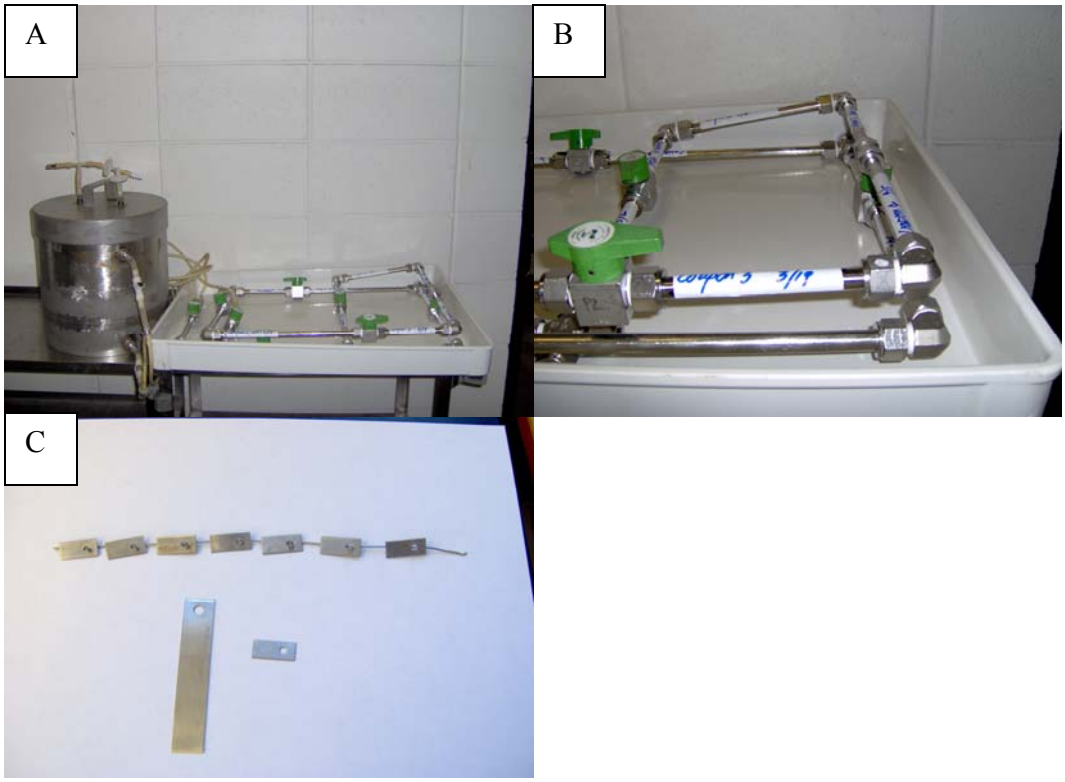

Figure 2. Recirculation reactor. (A) the reservoir on the top left and distribution pipe on the top right. (B); close view of distribution pipe and (C) coupons attached to a string which is placed inside the pipe section (bigger coupons were used in jar reactors).

Growth Conditions

Nine liters of autoclaved tap water supplemented with 100 ml of full strength TSB, i.e. 0.03% TSB, were used as the growth medium. In order to provide a continuous flow of oxygen, the medium was aerated by a fish tank aerator. The recirculation system was incubated at 38^{o}C.

Bacterial Strains and Inoculum

M. avium WOpBEN (a GFP strain) was obtained from the Seattle Biomedical Research Institute, Seattle, WA. pBEN is the plasmid that codes for GFP in *M. avium*. For the last several years the *gfp* gene encoding green fluorescent protein (GFP) has been used as

reporter gene. Wild type *gfp* is obtained from jelly fish *Aequorea victoria*. Plasmid pMV261 contains the mycobacterial origin of replication and hsp60 heat shock protein promoter. pFPV2 was created by inserting *gfp* into pMV261 (Valdivia et al., 1996). pBEN is a plasmid made by inserting a mutant of gfp (*gfpmut*3) into pFPV2 (Zafer et al., 2001). pBEN incorporated a kanamycin resistant gene as the selective marker.

The *DsRed* gene includes a constitutively active *E. coli* promoter and a transcriptional terminator collectively called pLOW2 which was excised from pTTN54 and ligated into pBluescript SK(+) to give pCO32. The pLOW2 fragment was excised from pCO32 and ligated into pBBR1MCS-3. This vector was designated pHKT3. From pHKT3 the pLOW2 fragment was cloned into pBR1Tp and the new vector is called pHKT4 (Tomlin et al., 2004). The vector pHKT4 which encodes RFP was transformed from *E. coli* to *P. aeruginosa* by triparental mating. pHKT4 incorporated a trimethoprim resistant gene as a selective marker.

A strain of *E. coli* DH5a (pHKT4) containing a plasmid coding for red fluorescent protein was graciously donated by the Biofilm Research Group, Ceri Lab, University of Calgary. *P. aeruginosa* (the same strain that was used for jar reactor experiments) was transformed with the RFP from *E. coli* by triparental mating using *E. coli* pRK2013 as the helper strain (graciously donated by the Franklin Lab, Montana State University).

Transformation of RFP into *P. aeruginosa* by Triparental Mating
(Adapted from the protocol provided by the Franklin Lab, Montana State University)

- *P. aeruginosa* was grown in Luria broth (LB) overnight at 37^{o}C
- Two ml of the culture was transferred to 25 ml LB in a flat bottom flask; the flask was incubated at 125 rev/min for 12 hrs at 42^{o}C

- 100 µl of thawed frozen stock of pRK2013 (helper strain in *E.coli*) was inoculated into 5 ml LB
- 100 µl of thawed frozen stock of pHKT4 (donor strain in *E.coli* DH5a) was inoculated into 5 ml LB
- Both the cultures were incubated in a roller at 37^{o}C for 6 hrs
- 200 µl of each culture was transferred to a test tube containing 2.5 ml LB and mixed
- The resulting suspension was added to a sterile syringe and was filtered through a 0.22 µm polycarbonate filter (type GTTP, Millipore Inc.)
- The filter was transferred to a Luria agar plate
- The plate was incubated at 42^{o}C for 6 to 10 hours
- The filter was placed in a test tube with 5 ml saline and vortexed to suspend the cells
- An appropriate volume of the cell suspension was plated on *Pseudomonas* isolation agar with the appropriate antibiotic (Trimethoprim, 1.5mg/ml final concentration in media (stock: 100mg/ml in N,N-dimethyl acetamide), dilutions used were 50 µl, 100 µl, 200 µl and 500 µl
- The plates were incubated at 37^{o}C for 18 to 24 hrs
- Pure cultures of RFP *P. aeruginosa* were isolated by repeated streaking of colonies on TSA plates. Pure culture of *P. aeruginosa* was confirmed by the API test (Biomurieux, MO, USA).

  Both the GFP *M. avium* and RFP *P. aeruginosa* were stored as frozen stock (2% peptone glycerol) at -80^{o}C until use. The excitation and emission values of RFP are 543 and 585 nm (Tomlin et al., 2004) and those for GFP are 490/10 nm and 520/10 nm respectively (Zafer, et al., 2001).

In order to prepare the inoculum, the frozen GFP *M. avium* was streaked on M7H10 agar plates supplemented with OADC and glycerol (with kanamycin as a selective antibiotic) and incubated at 37^{o}C for 3 weeks. The colonies were transferred to deionized sterile water and washed three times (centrifugation for 10 min at 10,000 RPM at 4^{o}C) and resuspended in sterile deionized water for an approximate bacterial concentration of 10^8 CFU/ml. The washed suspension was sonicated on ice (30sec/3 cycles) and inoculated into the reactor to the desired concentration of approximately 10^6 CFU/ml. Similarly, frozen RFP *P. aeruginosa* culture was streaked on a TSA plate with trimethoprim as the selective antibiotic and was incubated at 30^{o}C for 24 hours. The colonies from the plate were suspended in sterile deionized water. After washing three times they were resuspended and inoculated into the reactor to the desired concentration of approximately 10^6 CFU/ml.

Sample Collection and Processing

The whole recirculation system was placed in the laminar flow hood for sample collection. Bulk samples were taken from the port at the top of the reservoir using a syringe. To remove the coupons, the Swagelok^{R} connectors that connect the pipe sections were disconnected and coupons removed with the help of sterile forceps The coupons were then dip rinsed in sterile water and transferred into a vial with 3 ml sterile water. The cells were removed from the coupons by sonication for three one-minute intervals with 30 seconds rest periods in an ice bath in 3 ml water. Diluent from two of the coupons were pooled to make one sample. The sample was then processed and plated as described previously for the mason jar reactors.

RFP Expression by *P. aeruginosa*

Although RFP was quite well expressed by *P. aeruginosa* on TSA agar plates it was not well expressed in biofilms and bulk water when grown in tap water. An experiment was conducted to see if RFP expression could be increased by altering the growth environment, namely nutrient and temperature. A total of 6 jar reactors were set up, each containing 350 ml of growth medium. Two of them had tap water, two had tap water with 3ml full strength TSB and two with 30ml TSB as growth medium. The autoclaved growth medium was inoculated with RFP *P. aeruginosa* suspension to give an approximate concentration of 10^5 CFU/ml. One reactor each of tap water only, with 3ml TSB added, and 30 ml TSB added was incubated in a shaker operated at 25^{o}C and at 125 RPM. The remaining three reactors were incubated in a shaker operated at 37^{o}C at 125 RPM. The cell density of RFP *P. aeruginosa* was estimated by direct count on coupons before and after staining with SYBR Green.

Method Optimization

In order to see the difference between viable count and direct count, cells were enumerated by plate count (see sample collection and processing above) and counted after SYBR Green staining. Also cells on the inner side and the outer side of the coupons were directly counted after SYBR Green staining in order to see if the cell densities on the two sides of the coupons were different. Effects of treatment of CPC and sonication were estimated by doing viable counts before and after the treatment of the cells.

Staining and Imaging

Different fluorescent DNA stains including Live-Dead/BacLight (Molecular Probe), SYBR Green (Invitrogen), Propidium Iodide (Molecular Probe) and a fluorescent acid-fast stain, auramine/rhodamine, were used for visualization and imaging of biofilms. A Zeiss epifluorescence microscope (Axioskop) with a digital color camera (Axiocam) and associated software were used to take the biofilm images. The three dimensional images of biofilms were taken by Benjamin Klayman, a confocal microscopy expert using a confocal microscope (Leica, TCS/SP2/AOBS) in the Center for Biofilm Engineering, Montana State University. Attempts to take images of cryosections prepared with a cryostat were not successful. Had this been successful, sectioned images could show relative positions of *M. avium* and *P. aeruginosa* in biofilms from which we might be able to generate additional hypotheses about their interactions.

SYBR Green

SYBR Green stains nucleic acids and gives a bright green color to the cells. Stock SYBR Green (10,000x) was diluted to 1000X in DMSO and filter-sterilized. The working SYBR Green was made by dilution of the 1000X solution in filtered and autoclaved deionized water (FAMQ) to 100X and 300 μl of the working dye was applied to the coupons. Before applying the stain the coupons were fixed on a glass slide by using a piece of a double sided tape. The coupon was left in the dark at room temperature (25^{o}C) for 15 minutes before observation under the epifluorescence microscope.

LIVE/DEAD Staining

LIVE/DEAD BacLight, Kit L7012 (Molecular Probes), was used for the visual observation of cell viability, however, it was not used to quantify the cells. BacLight has SYTO 9 (3.34 mM) which stains DNA green and propidium iodide (20 mM) which stains DNA red. SYTO 9 stains all bacterial cells in a population whether the membrane is intact or damaged whereas propidium iodide penetrates only those bacteria with damaged membranes; the red counterstain overpowers the green SYTO 9 fluorescence. Thus, when SYTO 9 and propidium iodide are used together, live bacteria appear green and dead bacteria red when viewed with a broadband filter. A working solution of the BacLight stain was prepared by mixing 3 µl each of SYTO 9 and propidium iodide in 1ml of FAMQ. 300 µl of the working dye solution was applied to the surface of a coupon which was then left in the dark for 15 minutes before viewing under the epifluorescence microscope.

Fluorescent Acid-fast

Fluorescent acid-fast staining (Strahl et. el., 2001) was used to stain *M. avium* in biofilms. Coupons were flooded with auramine/rhodamine stain [Auramine 10.5g (ICN Biomedicals, Ohio), Rhodamine B 5.25g (Alfa Aesar Inc.), glycerol 525 ml, phenol 70 ml, deionized water 350 ml] for 20 min. Excess stain was washed away with deionized water and treated with acid alcohol (0.5% HCl) for 5 minutes to decolorize. Acid alcohol was washed away with water and the coupon was flooded with potassium permanganate (0.5% in water) for 1 minute. The coupon was air dried in the laminar flow hood and then viewed under the epifluorescence microscope (100X objective).

Propidium Iodide

Propidium iodide is a DNA binding stain which can only penetrate cells with a damaged membrane. Therefore cells stained with propidium iodide are stained red. RFP *P. aeruginosa* did not express the fluorescent protein well. Therefore, in order to distinguish between the red fluorescent RFP *P. aeruginosa* and green fluorescent GFP *M. avium* cells on a dual species coupon, the coupon was first exposed to 0.01% cetyl pyridinium chloride (CPC) for 30 min. Then the coupon was rinsed with sterile water and treated with propidium iodide (25 μg/ml, Molecular Probes). Probably pretreatment with CPC damaged the cell membrane (because CPC acts on the cell membrane of bacterial cells) of *P. aeruginosa* and allowed the passage of propidium iodide into the cells but the *M. avium* remained unaffected (*M. avium* cell density was not affected by treatment with CPC, Results, Table 5). Confocal images were taken to obtain the three dimensional image of the biofilm with red and green cells.

PCR Restriction Enzyme Pattern Analysis (PRA)

PRA is a method which involves PCR amplification of a gene and subsequent fragmentation of the amplification products by restriction enzyme digestion. PRA methods have been applied for rapid detection of MAC species. PRA of the heat shock protein gene called *Hsp*65 (also called *gro*EL2) of MAC shows finger prints that can distinguish among the strains of MAC (Telenti et al., 1993). In order to confirm that the isolate obtained for this study was indeed *M. avium*, PRA of the *hsp*65 gene was carried out.

A pure culture from the frozen stock of *M. avium* W2001 was streaked on a Middlebrook 7H10 agar plate and incubated at 37^{o}C for 21 days. A few colonies were taken to make a suspension in sterile water. Genomic DNA was extracted from the suspension by

using the Fast DNA^{R} Spin Kit (Bio 101) and associated protocol. In brief, 500 µl of the suspension was placed in a bead tube and 978 µl of sodium phosphate buffer and 122 µl of MT buffer were added. The bead tube was processed in a bead beater for 45 sec at 6.5 speed to lyse the cells and the lysis product was centrifuged at 10,000 RPM for 1 min. Supernatant from the bead tube was transferred to a centrifuge tube and 240 µl of PPS solution was added. The mixture was mixed by hand for 2 min before it was centrifuged at 10,000 RPM for 10 min. The supernatant was then transferred into a 15 ml fisher tube and 1 ml diatomaceous earth binding matrix added. The contents of the tube was mixed by inverting for 2 min. 600 µl of the mixture was transferred to a spin filter and was centrifuged at 6,000 RPM for 1 min and the catch tube was emptied. 500 µl of SEWSM solution was then added to the spin filter and centrifuged at 10 000 RPM for one minute. The catch tube was emptied and the spin filter was centrifuged at 6,000 RPM for 5 min. The spin filter was then put in a fresh catch tube and air dried for 10 min under the laminar flow hood. 150 µl of DES solution was added to the spin filter and the matrix on the filter membrane was stirred gently to resuspend the silica for efficient elution of the DNA. Great care was taken not to damage the filter. The filter was then centrifuged at 6,000 RPM for 3 min. The solution in the catch tube contained the extracted DNA of *M. avium*. The presence of DNA in the extracted solution was confirmed by agarose gel electrophoresis.

Polymerase Chain Reaction (PCR)

A DNA sample of 5 µl was added to the PCR mixture (final volume 50 µl) that contained 50 mM KCl, 10mM Tris HCl, 1.5 mM MgCl2, 10% glycerol, 200 µM dNTP, 0.5 µM of each primer and 1.25 U Taq Polymerase (Fisher Bioreagents FB600010). The reactants were subjected to 45 cycles of amplification (denaturation at 94^{o}C for 1min;

annealing at 60°C for 1 min; elongation at 72°C for 1min). This was followed by an extension of 10 min at 72°C (Telenti et al., 1993). The primers used for the amplification of *hsp*65 were Tb11 (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT). The presence of amplified product was confirmed by agarose gel electrophoresis.

Restriction Fragment Length Polymorphism (RFLP)

Restriction enzymes *Bst*EII and *Hae*III (Fisher Scientific, USA) were used to digest the amplification products. 20 µl of the amplified product was added to a mixture containing 2 µl of *Bst*EII or *Hae*III, 5 µl of restriction buffer and 25 µl of sterile water. The mixtures were incubated for 90 min at 60°C for *Bst*EII and at 37°C for *Hae*III digestion. A mixture of 4 µl of loading dye and 14 µl of the restriction digestion product was loaded on to a 3% agarose gel and restriction fragments were separated by electrophoresis in 1x Tris-borate-EDTA buffer. The restriction fragment sizes were compared with a 50bp internal size marker.

Statistical Analysis

Statistical analyses were performed using SAS. The Wilcoxon Rank Sum test was used to compare means between experiments. A p-value of <0.05 was considered statistically significant. In the graphs, error bars represent the standard error. Error bars were plotted by using Excel software. For each dataset, e.g. Fig. 8 below, all 15 results for *M. avium* in monospecies biofilms were pooled and compared with the results for *M. avium* in dual species biofilms. The same approach was taken to statistical analysis of results for Figs. 9, 11, and 13.

RESULTS

In this chapter the results have been presented in two parts. In the first part the images of biofilms and the results of validation of the different techniques used in this research have been described. Since enumeration by plating *M. avium* takes at least 3 weeks of incubation, direct counting of stained or fluorescent cells on coupons was initially undertaken (Figs. 3-6) . Although image quality on initial cultures was excellent, fluorescence techniques were not reproducible over the time course of each experiment and the plating method was adopted resulting in long analyses times. In addition, verification tests were carried out for identifying the effects of antimicrobial treatment and sonication on the bacterial cells. In the second part of the results, data on simultaneous inoculation, sequential inoculation and the recirculation system have been described.

The following are some biofilm images that were obtained after staining by using epifluorescence and confocal microscopy. The monospecies and dual species images are all from the jar reactor coupons. Different DNA staining fluorescent dyes like SYBR Green, BacLight and propidium idodide were used to stain the cells in biofilms. BacLight stains dead cells red and live cells green. However, it was not used to quantify the viable cell counts. Fluorescent acid-fast stains auramine and rhodamine were used for *M. avium* only.

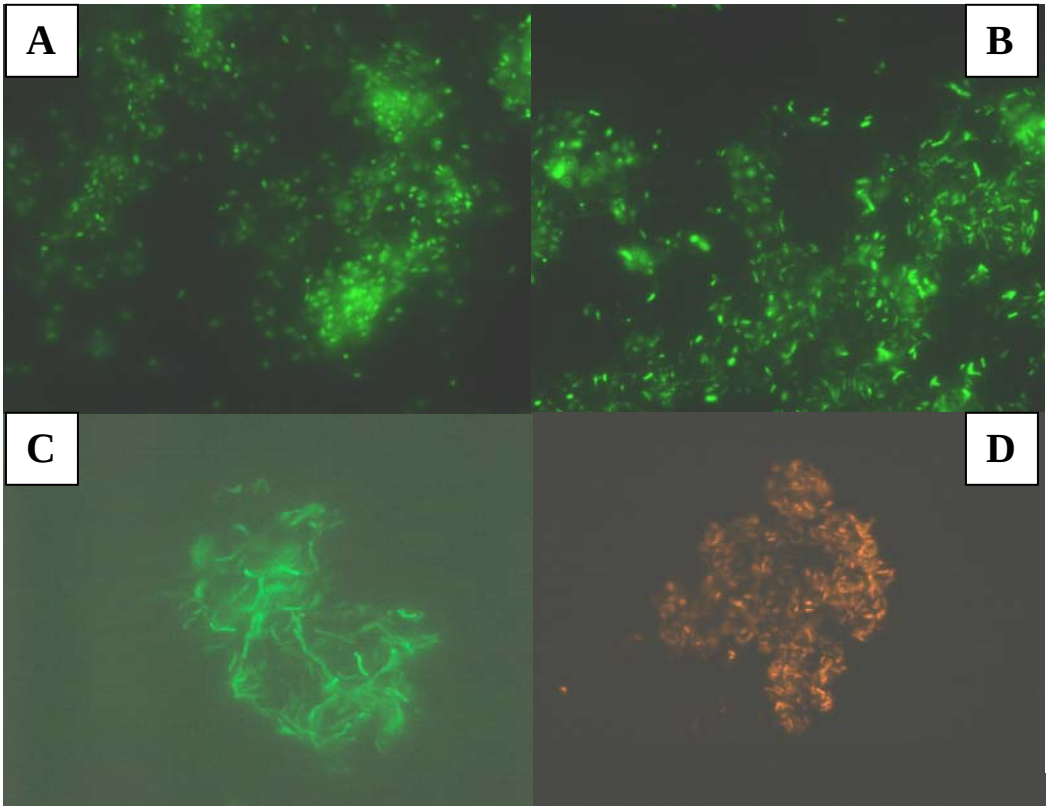

Figure 3. *P. aeruginosa* and *M. avium* in biofilms in jar reactors. (A) SYBR Green stained *P. aeruginosa* cells in biofilms near the air-water interface (one week old biofilms). (B) SYBR Green stained cells of *M. avium* W2001 in 17 day old biofilms. (C) Pseudofilaments of GFP *M. avium* WOpBEN in 13 day old biofilms. (D) Fluorescent acid-fast (auramine/rhodamine) stained cells of *M. avium* in 21 day old biofilms.

SYBR Green staining (Figs. 3A and 3B) shows that the cells of both *P. aeruginosa* and *M. avium* near the air-water interface were concentrated mainly in clusters or microcolonies, with bare areas of coupon surface in much of the space between clusters. These areas of coupon near the air-water interface were the most heavily colonized (Tables 8 and 9 below). GFP *M. avium* appeared to form chains or pseudofilaments (Fig. 3C). Fluorescent acid-fast staining (Fig. 3D) also revealed clearly-defined clusters of *M. avium*.

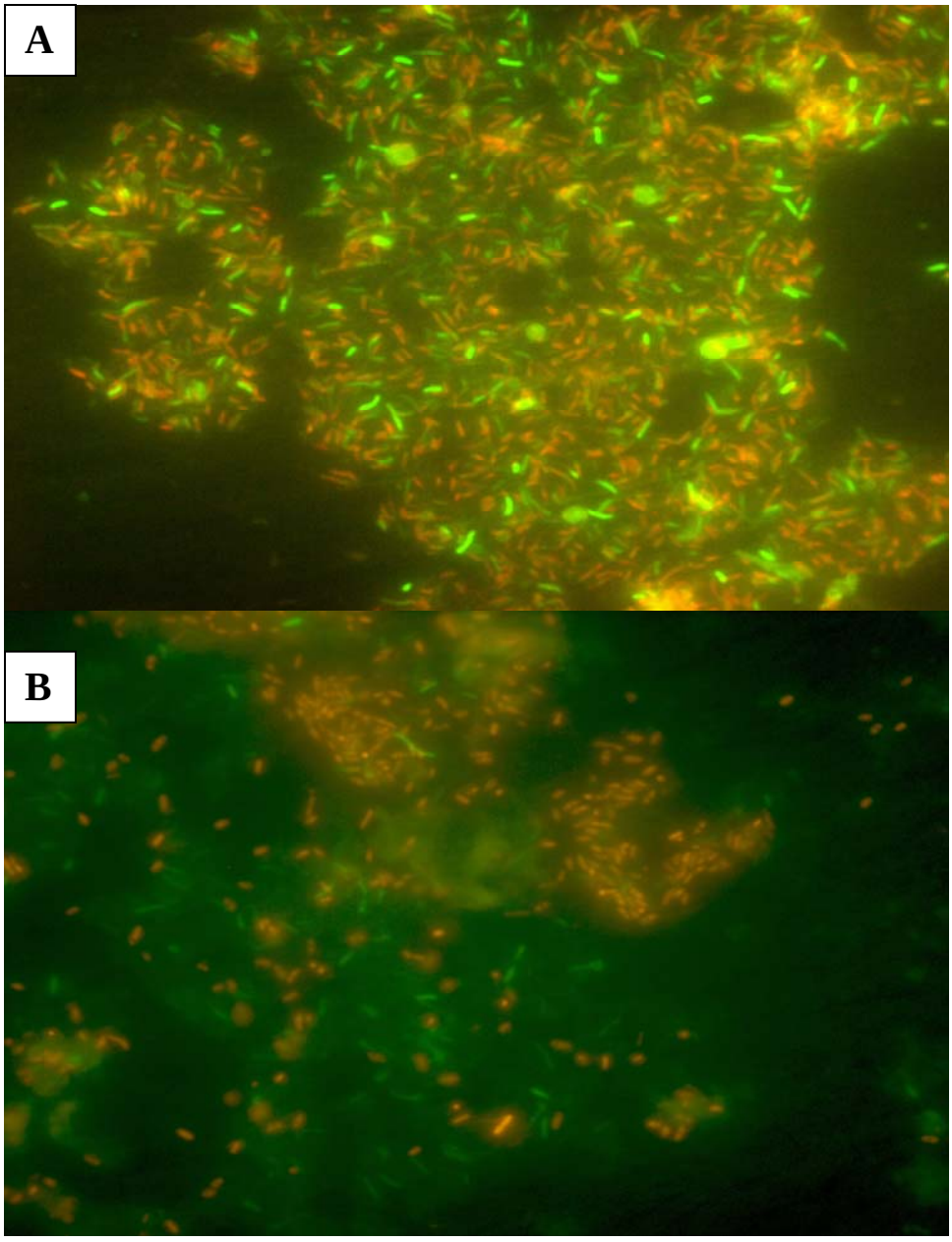

Fig 4. (A) LIVE/DEAD BacLight stained *M. avium* cells in jar reactor in seven days old biofilms. Green cells are live and red cells are dead. (B) GFP *M. avium* (green) and RFP *P. aeruginosa* cells (red cells) in 34 days old biofilm .

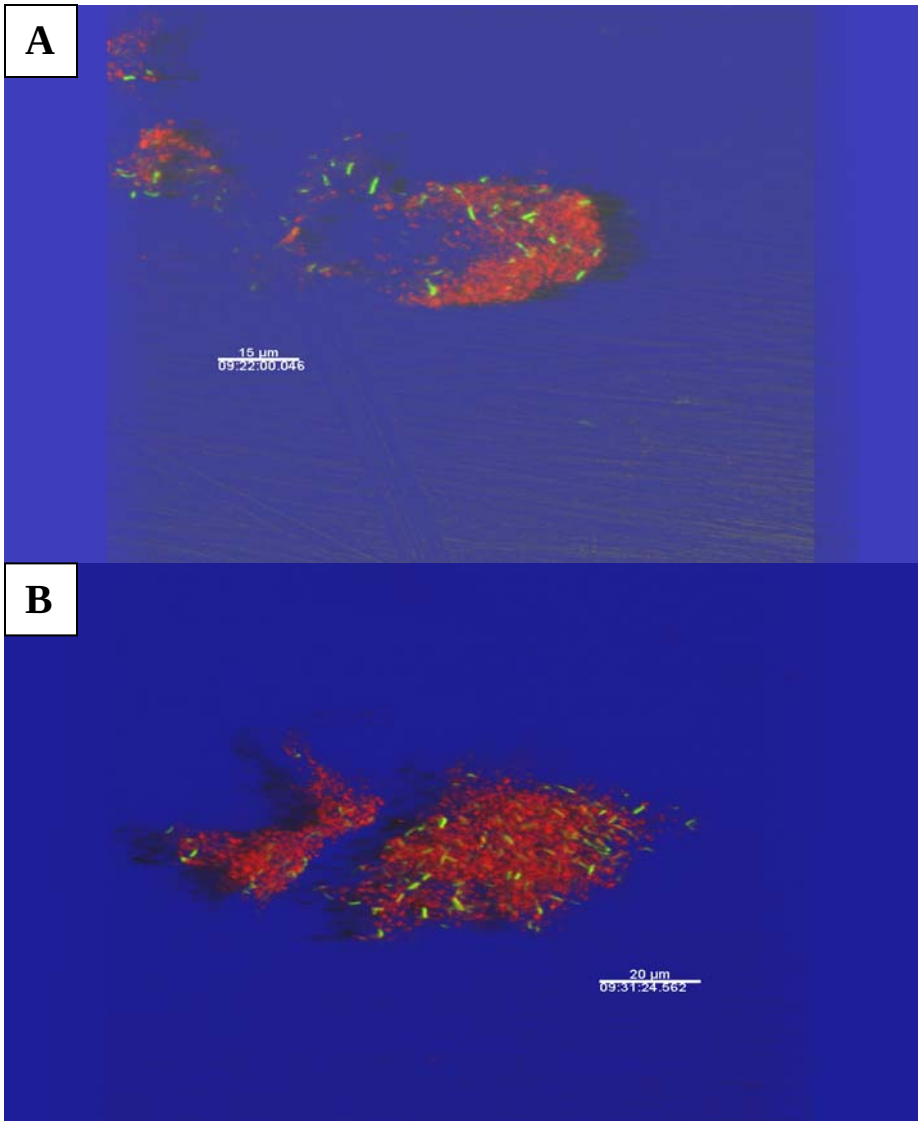

Fig 5. (A) & (B) Confocal images of propidium iodide stained biofilm of RFP *P. aeruginosa* (red cells) and GFP *M. avium* (green cells) in 20 day old jar reactor biofilms.

GFP *M. avium* cells were distributed over and through the biofilm. Some of the propidium iodide red cells may be dead or membrane damaged *M. avium* because the coupons were treated with CPC to kill *P. aeruginosa*. The depth of the biofilm was between 10 and 20 µm, a moderately thick biofilm. The two images shown above were taken from the two different areas of the same coupon.

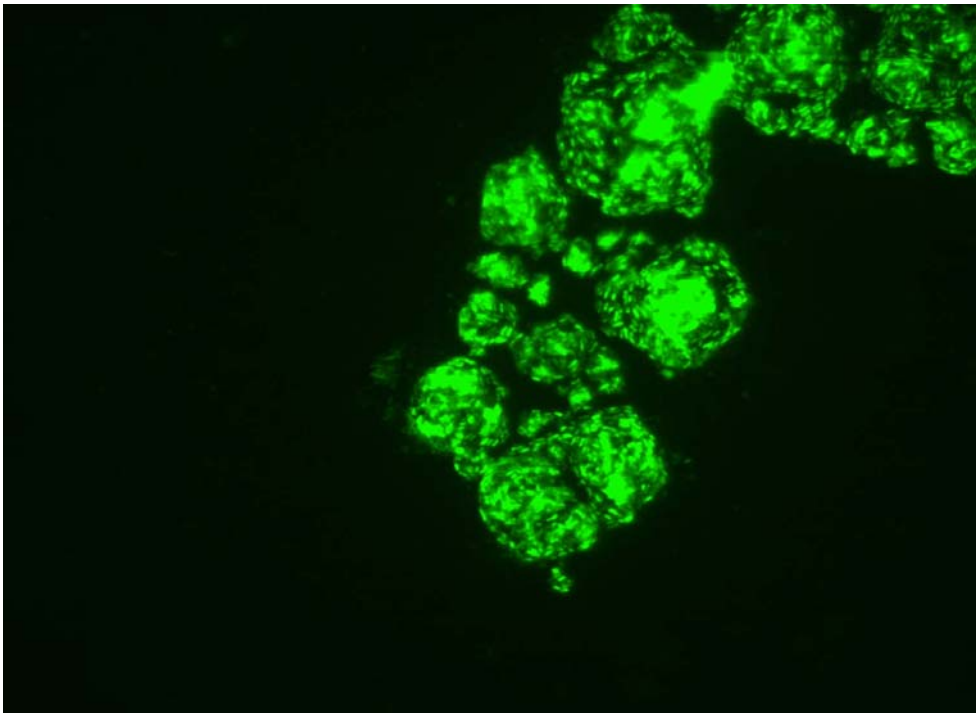

Figure 6. SYBR Green stained cell clusters (likely microcolonies) of *M. avium* W2001 in 17 day old jar reactor biofilms.

PCR-Restriction Enzyme Pattern Analysis (PRA)

An *M. avium* isolate was obtained from the Ford lab. The isolate was verified with the acid-fast stain (Fig. 3C) and PRA. Restriction digestion patterns of *hsp*65 are shown in Fig. 7. *Bst*EII divides *hsp*65 into approximately 240 and 210 bp fragments and *Hae*III divided it into 130 and 105 bp fragments. Band patterns were interpreted by comparison with published tables and patterns available at the following PRASITE internet database (http://www.hospvd.ch:8005).

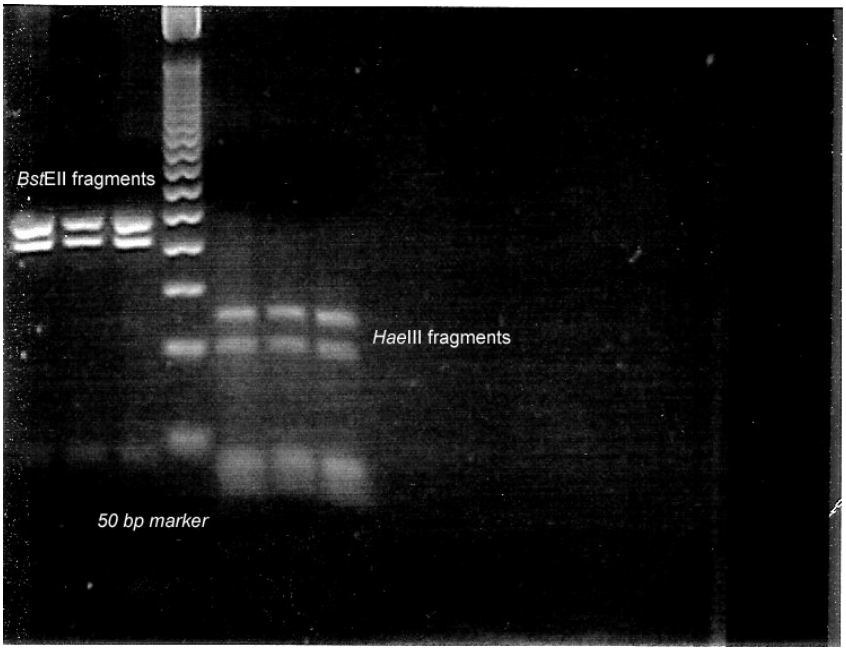

Fig 7. Restriction digestion patterns of *hsp*65 of *M. avium*. *Bst*EII fragments are in the first three columns from left, fourth column is 50bp marker and the three columns on the right side of the marker are *Hae*III fragments.

Validation of Techniques

Below are the test results of validation of some of the techniques used to quantify the cells in biofilm and bulk water. Staining of the coupons showed that the cells concentrated in the area around the air-water interface.

Direct Count on Coupons Versus Plate Count of *P. aeruginosa*

Coupons were taken from the monospecies *P. aeruginosa* reactor for comparison of cell density between direct count on coupons (from here onwards called direct count) and plate counts. For direct counts, SYBR Green (Fig. 3A) was used to stain the cells and 30 microscopic fields were counted on each coupon. For plate counts, a biofilm sample from the second coupon was processed and plated. Altogether 10 pairs of coupons were analyzed.

The results show that the direct count gave, on average, over one log higher cell density than the plate count. The direct count showed 5.4 X 10^5 cells/cm^2 and plate counting gave 5.2 X 10^4 CFU/cm^2, a 10 fold difference that was statistically significant (Table 1) (p = 0.0001).

Table 1: Direct count after SYBR Green staining and plate count of *P. aeruginosa* in biofilms.

Sample no.	Direct count cells/cm^2	Plate count CFU/cm^2	Percent Cultured
1	7.40E+05	1.20E+04	1.62
2	5.90E+05	6.60E+04	11.19
3	6.90E+05	5.70E+04	8.26
4	3.80E+05	8.10E+04	21.32
5	5.20E+05	7.40E+04	14.23
6	2.70E+05	1.40E+04	5.19
7	3.70E+05	5.00E+04	13.51
8	2.50E+05	9.90E+03	3.96
9	1.00E+06	8.50E+04	8.50
10	6.10E+05	6.90E+04	11.31
Average	5.42E+05	5.18E+04	9.56
Log	5.73	4.71	

Direct Count of GFP *M. avium* Versus Plate Count

Several pairs of coupons were harvested from GFP *M. avium* monospecies reactors (Fig. 3B). One coupon from a pair was used for direct count of GFP cells after staining with SYBR Green and the second coupon was used for plating. On average, direct count gave 1.0 X 10^4 log cells/cm^2 and plate counts gave 3.6 X 10^2 CFU/cm^2 (Table 2). The direct counts were on average 28 fold higher than plate counts which was statistically significant at (p=0.0005).

Table 2: Comparison of direct count and plate count of GFP *M. avium*

Sample no.	Direct count cells/cm^2	Plate count CFU/cm^2	Percent Cultured
1	2.70E+03	5.60E+02	20.74
2	1.00E+04	3.90E+02	3.90
3	5.30E+03	3.10E+02	5.85
4	1.00E+03	2.40E+02	24.00
5	2.60E+03	5.60E+02	21.54
6	1.50E+04	1.70E+02	1.13
7	3.40E+04	2.90E+02	0.85
Average	1.01E+04	3.60E+02	11.14
Log	4.00	2.56	

Comparison of Cells on Inner and Outer Surfaces of the Coupons

Coupons from monospecies reactors of *M. avium* and *P. aeruginosa* and also the dual species reactors were harvested and used to compare the cell density on two sides of a coupon. The side facing the wall of the jar is called the outside surface and that facing the center of the jar is called the inside surface. A pair of coupons were taken at each sampling time and one coupon was used for direct counts on the outside surface and the second for direct counts on the inside surface. Altogether 9 pairs of coupons were harvested, SYBR Green treated and the cells were counted using the epifluorescence microscope. On average, inner surfaces gave approximately 4.6 X 10^5 cells/cm^2 and outer surfaces 3.6 X 10^5 cells/cm^2 (Table 3). The difference was not statistically significant ($p = 0.15$)

Table 3: Cell densities on inner and outer surfaces of coupons after SYBR Green staining

Sample no.	Inner surface cells/cm^2	Outer surface Cells/cm^2	Ratio Outer/Inner
1	4.10E+05	4.20E+04	0.10
2	8.40E+04	5.20E+04	0.62
3	5.00E+05	3.70E+05	0.74
4	2.60E+05	2.40E+05	0.92
5	7.40E+05	2.70E+05	0.36
6	5.90E+05	3.70E+05	0.63
7	6.90E+05	2.50E+05	0.36
8	3.80E+05	1.00E+06	2.63
9	5.20E+05	6.10E+05	1.17
Average	4.64E+05	3.56E+05	0.84
Log	5.67	5.55	

Direct Count Before and After SYBR Green Staining

Coupons from a dual species reactor with GFP *M. avium* and RFP *P. aeruginosa* were harvested for direct counting. Cells were counted both before and after staining with SYBR Green. Three pairs of coupons were taken and for each pair, one was counted before SYBR Green staining and the other counted after SYBR Green staining. Counts before staining gave 1.0 X 10^3 cells/cm^2 on average whereas counts after staining gave 2.0 X 10^4 cells/cm^2 (Table 4). Therefore comparatively, enumeration by SYBR Green staining, gave 1.3 logs higher density than the estimation from naturally fluorescing cells.

Table 4: Direct count of fluorescent cells of *M. avium* and *P. aeruginosa* before and after staining with SYBR Green

Sample no.	SYBR Green Stained cells/cm^2	Unstained cells/cm^2	Ratio Unstained/stained
1	4.70E+04	2.63E+03	0.06
2	6.00E+03	3.70E+02	0.06
3	8.00E+03	1.60E+02	0.02
Average	2.03E+04	1.05E+03	0.05
Log	4.31	3.02	

Effect of CPC on *M. avium* Cell Viability

Cetyl pyridinium chloride (CPC) was used to kill the *P. aeruginosa* cells in dual species samples in order to avoid overgrowth of cells on the plates. CPC is an antiseptic used for general mouth and throat infections. CPC kills the bacteria by attacking the cell membrane. A concentration of 0.005 % (w/v) killed 100% *P. aeruginosa* cells over a 2 min contact time. Two biofilm samples of *M. avium* were plated before and after CPC treatment. (Table 5). CPC had little effect (up to ca. 20% decrease in viability) on *M. avium*.

Table 5: Effect of CPC on *M. avium* cell viability

	Before CPC CFU/cm^2	After CPC CFU/cm^2	Ratio After/Before
Biofilm	2.20E+02	1.70E+02	0.77
	6.00E+02	5.80E+02	0.97
Average	4.10E+02	3.75E+02	0.87
Log	2.61	2.57	

Effect of Sonication

Sonication reduced *M. avium* concentrations by 0.1 log, but did not affect *P. aeruginosa* density (Table 6). This test was conducted only on bulkwater samples.

Table 6: Effect of sonication on cell viability

	Non-sonicated CFU/ml	Sonicated CFU/ml
M. avium	6.00E+04	5.20E+04
Log	4.8	4.7
P. aeruginosa	4.60E+08	4.50E+08
	4.30E+05	4.00E+05
	1.90E+05	1.30E+05
Average	1.54E+08	1.50E+08
Log	8.2	8.2

Expression of RFP by *P. aeruginosa*

RFP *P. aeruginosa* did not express well in biofilms grown in tap water. The objective of this experiment was to see if RFP *P. aeruginosa* expresses its fluorescence differently at higher nutrient concentrations and higher temperature. Six reactors were set up with different growth conditions detailed in Chapter II. One pair of coupons was harvested from each reactor after 10, 15 and 35 days. One of the pair was used to count fluorescent cells before staining, and the second was used to count the cells after staining with SYBR Green. Less than between 1 and 6 percent of the unstained cells expressed RFP (Table 7).

Table 7: RFP expression of *P. aeruginosa*. Only a small fraction of the cells expressed RFP as shown in the last column

	Temp	Unstained CFU/cm2	Stained cells/cm2	% RFP Expression
Tap water	25C	<4.1E+01	3.6E+04	
	25C	<4.1E+01	1.3E+03	
	25C	8.2E+01	8.1E+03	1.01
	37C	1.0E+03	1.3E+05	0.77
	37C	2.3E+03	4.3E+05	0.53
	37C	1.8E+02	1.1E+04	1.64
3% TSB	25C	3.3E+02	6.0E+05	0.06
	25C	2.3E+03	2.5E+05	0.92
	25C	8.2E+01	2.8E+04	0.29
	37C	<4.1E+01	1.7E+05	
	37C	2.7E+03	1.1E+05	2.45
	37C	6.5E+02	1.1E+04	5.91
10% TSB	25C	2.3E+03	3.2E+05	0.72
	25C	1.7E+03	5.2E+05	0.33
	25C	1.6E+02	3.2E+04	0.005
	37C	<4.1E+01	1.2E+06	
	37C	3.0E+03	4.1E+05	0.73

Spatial Distribution of Cells on Coupons

In the jar reactors, most of the cells of *M. avium* and *P. aeruginosa* were concentrated at the air water interface. Other parts of the coupons showed very little colonization. However in the recirculation system, the coupons were colonized throughout the coupon surface. The tables below shows the distribution of cells in the coupons of jar and recirculation reactors. Thirty fields were counted on the jar reactor coupons and 21 fields were counted on recirculation reactor coupons.

Table 8. Direct counts on recirculation coupons of dual species (*M. avium* and *P. aeruginosa*) biofilms after SYBR Green staining

	View Fields						
	1	2	3	4	5	6	7
Cell counts	27	28	31	19	39	7	42
	36	25	22	13	13	13	7
	1	1	7	1	1	12	23
Total	64	54	60	33	53	32	72

Table 9. Direct count of SYBR Green stained GFP *M. avium* cells in monospecies biofilms in jar reactor

	View Fields									
	1	2	3	4	5	6	7	8	9	10
Cell counts	0	0	14	0	0	0	0	0	0	0
	0	0	5	20	0	0	0	0	0	0
	0	39	23	0	0	0	0	0	0	0
Total	0	39	42	20	0	0	0	0	0	0

Results of Biofilm Reactor Experiments

Simultaneous Inoculation of Mason Jar Reactors

In simultaneous inoculation experiments, only tap water was used as the growth medium. *M. avium* and *P. aeruginosa* were inoculated together and results of the experiments are shown in Figs. 8, 11 and 13. Samples were taken weekly after the inoculation of approximately 10^5 CFU/ml *M. avium* and approximately 10^5 CFU/ml *P. aeruginosa* into jar reactors both separately and together. Fig. 8 shows that monospecies *M. avium* density was 7.1×10^3 CFU/cm^2 in biofilms at the end of the first week after inoculation. Density reached a maximum of 3.3×10^4 CFU/cm^2 at weeks 3 and decreased slightly to about 2.0×10^4 in weeks 4 and 5. The density of *M. avium* in the dual species biofilms was 6.0×10^3 CFU/cm^2

after the first week and increased to 1.1 X 10^4 CFU/cm^2 in week 3. The figure also shows that the density of *M. avium* was higher in monospecies than in dual species biofilms. The difference between monospecies and dual species density was statistically significant (p=0.006). A monospecies biofilm of *M. avium* is shown in Fig. 3D. potential microcolonies of *M. avium* in biofilm are shown in Fig. 6. The data shown in the figures 8 and 9 are an average of 3 independent experiments and the error bars represent the standard errors.

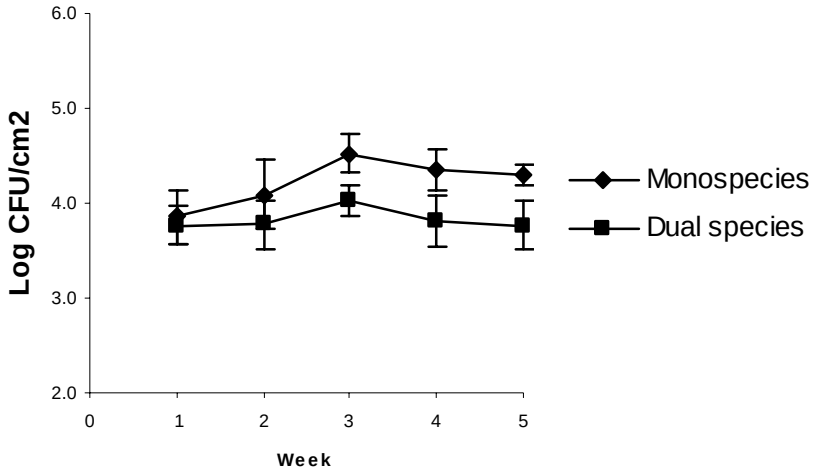

Figure 8. Density of *M. avium* in monospecies and dual species biofilms

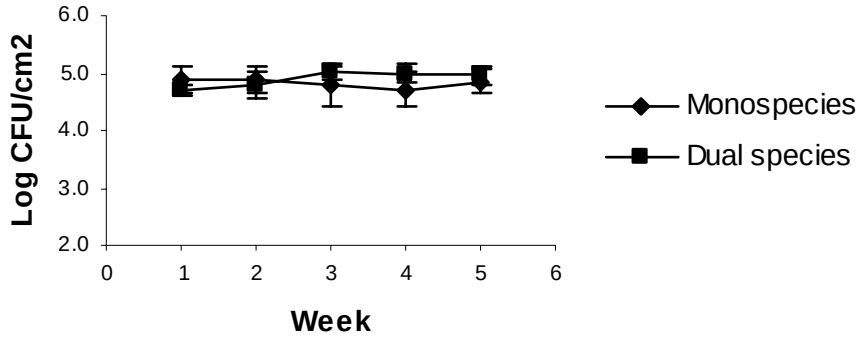

Figure 9. Density of *P. aeruginosa* in monospecies and dual species biofilms

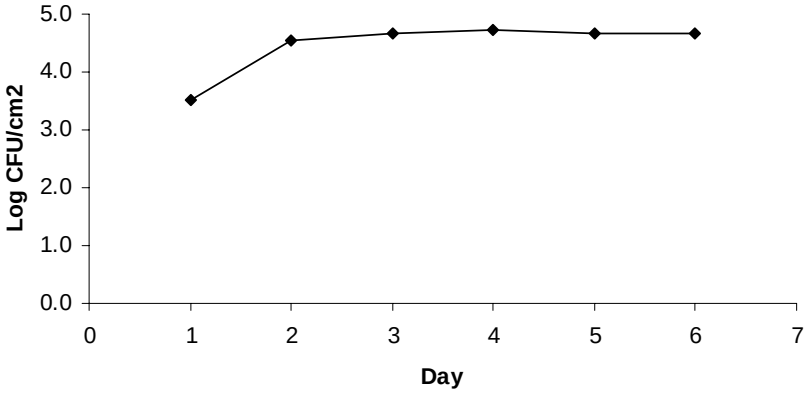

Fig 10. Density of *P. aeruginosa* in monospecies biofilm.

P. aeruginosa density in both monospecies and dual species biofilms did not change much throughout either trial (p=0.52) (Fig. 9). The density remained close to 1.0 X 10^5 CFU/cm^2 in both monospecies and dual species biofilms. When daily growth of *P. aeruginosa* was monitored the results showed that the density increased from 5.4 X 10^3 CFU/cm^2 at 24 h to 1.8 X 10^5 CFU/cm^2within 48 h, after which the density remained more or less stable (Fig. 10). *P. aeruginosa* density was about 1 log higher than that of *M. avium* in dual species biofilms although they were inoculated at similar cell densities (Fig. 11). The

difference was statistically significant (p=<0.001). *P. aeruginosa* monospecies biofilms and dual species biofilm with *M. avium* are shown in Fig. 4A and Fig. 5A respectively.

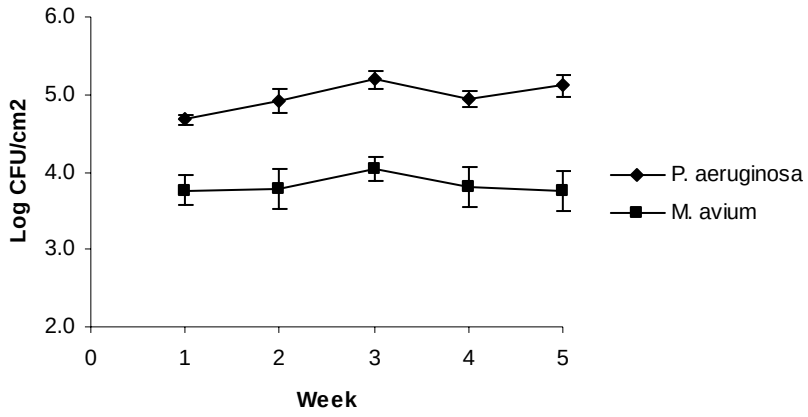

Figure 11. Densities of *M. avium* and *P. aeruginosa* in the dual species biofilms

In an additional experiment (Fig 12), *P. aeruginosa* was inoculated to a time zero density of 1.0 X 10^3 CFU/ml and *M. avium* was inoculated to 6.2 X 10^3 CFU/ml and the growth on the coupons was monitored after every 24 hours for 6 days. *M. avium* density remained more than 3.5 X 10^2 CFU/cm^2 after 24 hours whereas *P. aeruginosa* density was about 3.3 X 10^3 log CFU/cm^2 after 24 hr and stabilized just above 3.0 X 10^4 CFU/cm^2 from 48 hours onwards, which was nearly two logs higher than that of *M. avium.*

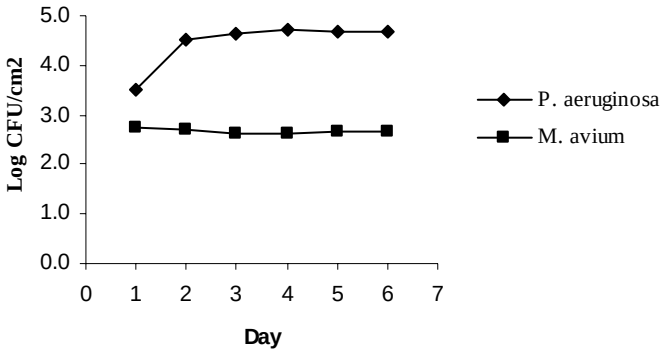

Figure 12. Densities of *P. aeruginosa* and *M. avium* in dual species biofilm

When the concentration of *P. aeruginosa* was compared with the concentration of *M. avium* in dual species bulk water, the concentration of *P. aeruginosa* was about 3 logs higher than that of *M. avium* (Fig 13). The difference was statistically significant (p=<0.001).

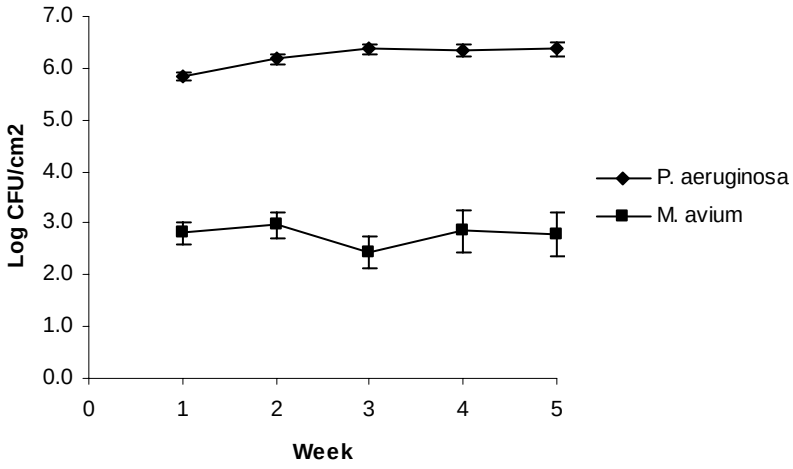

Figure 13. Densities of *M. avium* and *P. aeruginosa* in bulk water

Sequential Inoculation

For sequential inoculation, first one species (the base species) was allowed to form biofilms and then the second species (the invading species) was inoculated. Monospecies density was also monitored along with the sequential density . The growth medium had a DOC level of 3.7 mg/L. Figs. 14 to 18 show the results of simultaneous experiments.

In monospecies biofilms of *M. avium*, the density at 24 h was about 1.5 X 10^3 CFU/cm^2 which increased to 2.8 X 10^3 CFU/cm^2 at the end of 5 days (Fig.14). For subsequent time points, it remained fairly stable around 1.0 X 10^4 CFU/cm^2 .

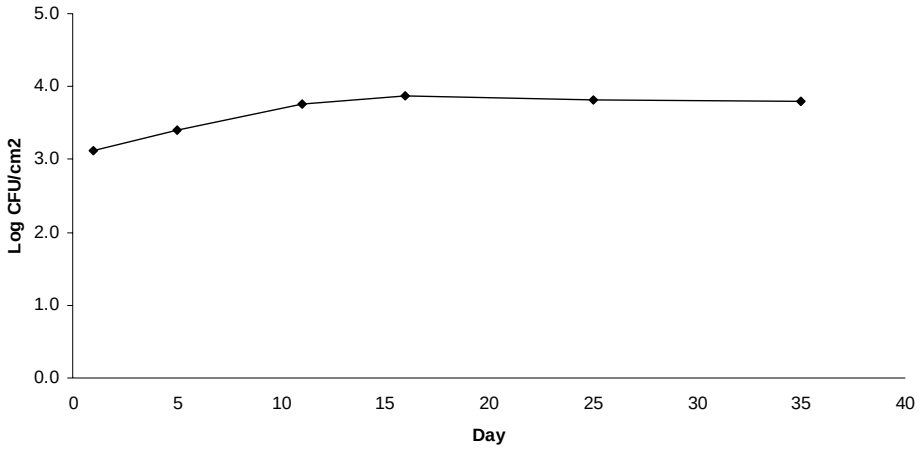

Figure 14. Density of *M. avium* in monospecies biofilms

M. avium as Base Species. In one set of experiments, *M. avium* was first allowed to grow biofilms and then *P. aeruginosa* was inoculated. The base biofilm of *M. avium* had a density of 1.8 X 10^3 CFU/cm^2 after 24h and remained close to 1.0 X 10^4 CFU/cm^2 until the end of the experiment at day 35 (Fig. 15). The result was close to that observed in monospecies growth. *P. aeruginosa* was inoculated on day 15 and samples were taken 4 times, every 5 days for 20 days. *P. aeruginosa* density was 8.3 X 10^4 CFU/cm^2 after 5 and

10 days and was about 3.5 X 10^5 CFU/cm^2 after 15 and 20 days of inoculation. Even though the time points in which the samples were taken in monospecies and base species biofilms of *M. avium* were somewhat different, their trend of growth remained fairly close over a period of 35 days. The density of *P. aeruginosa* was about 1 log higher than that of *M. avium*. An established biofilm of *M. avium* did not prevent the growth of *P. aeruginosa* in the biofilm.

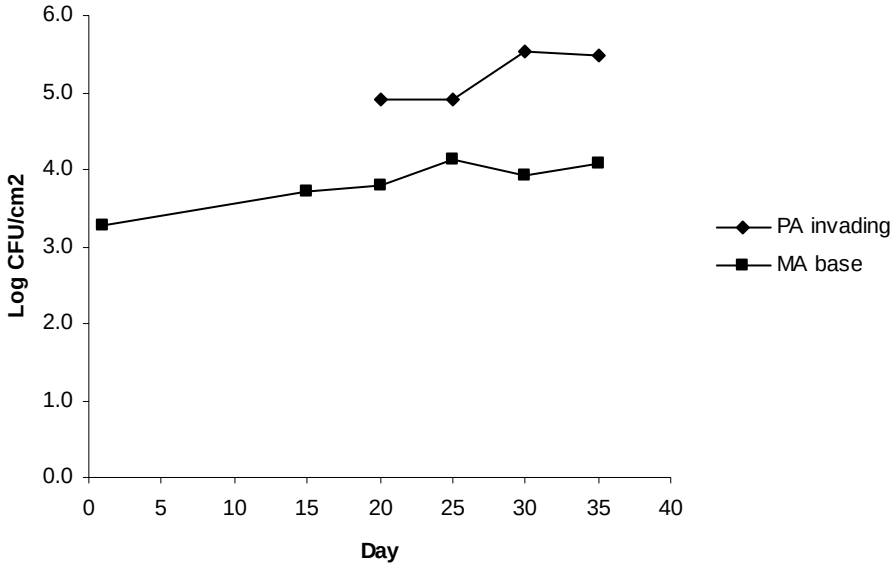

Figure 15. Density of *M. avium* as base and *P. aeruginosa* as invading species in sequential experiment. The arrow indicates the point of inoculation of *P. aeruginosa*.

In a monospecies, *P. aeruginosa* density was 5.7 X 10^3 CFU/cm^2 after 24h of incubation (Fig. 16). Growth stabilized by day 16 with a density of about 3.5 X 10^5 CFU/cm^2.

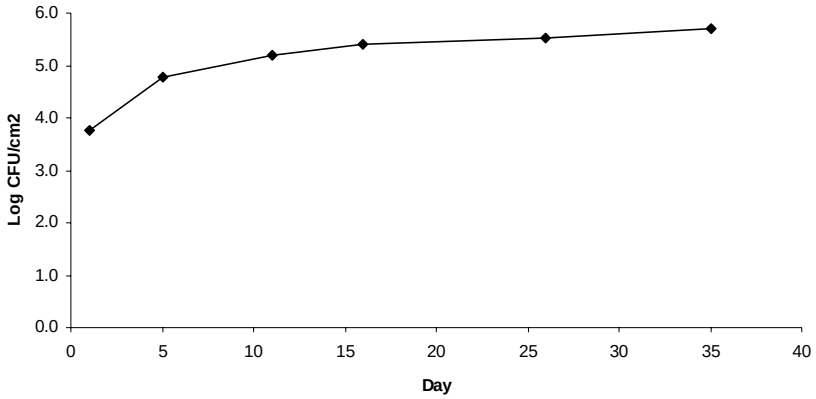

Figure 16. Density of *P. aeruginosa* in monospecies biofilm

M. avium as Invading Species. When *P. aeruginosa* was grown as the base species, its density was 6.5 X 10^3 CFU/cm^2 after 24h and then stabilized to around 1.0 X 10^5 CFU/cm^2 from day 7 (Fig 17). *M. avium* (invading species) was inoculated on day 7 and enumerations were conducted after every 5 days. *M. avium* density remained stable at about 1.0 X 10^4 CFU/cm^2. The base species and invading species density of *M. avium* remained close to 1.0 X 10^4 CFU/cm^2 (Fig. 18). For *P. aeruginosa*, also the base and invading species density was close to about 1.0 X 10^5 CFU/cm^2. An established biofilm of *P. aeruginosa* did not prevent the growth of *M. avium* in the biofilm. There was only a minimal difference regardless of whether the strains were grown as base species or invading species (Fig. 18).

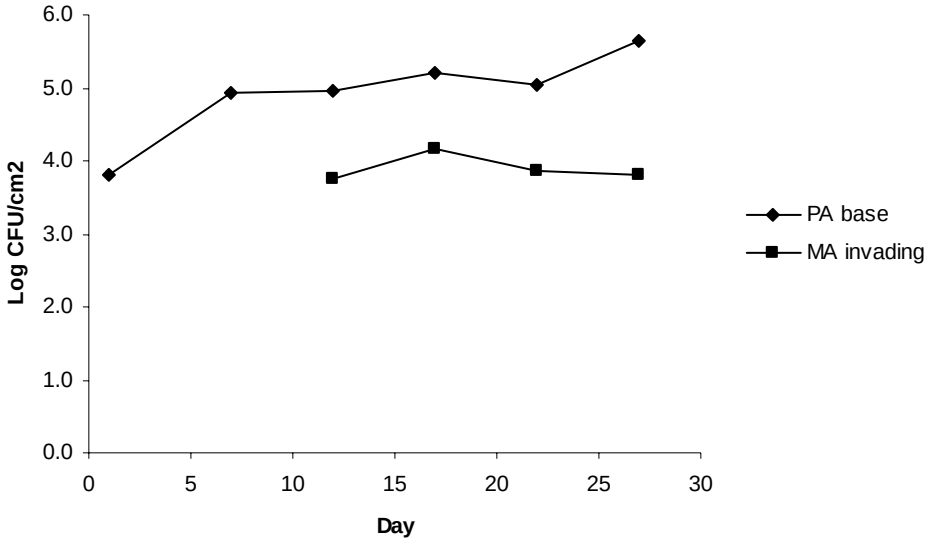

Figure 17. Growth of *P. aeruginosa* as base and *M. avium* as invading species in sequential experiment. The arrow indicates the point of inoculation of *M. avium*.

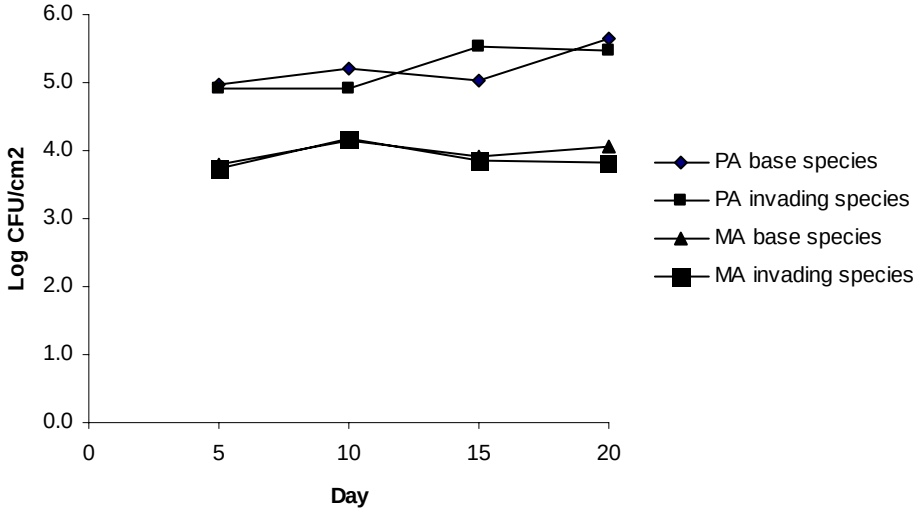

Figure 18. The density of *M. avium* (MA) *P. aeruginosa* (PA) as base and invading species in dual species biofilms

Recirculation System. Biofilm formation by *M. avium* and *P. aeruginosa* was also observed for an extended period in high nutrient (DOC 180 mg/L) and high temperature (38^{o}C) conditions in a stainless steel recirculation system. *P. aeruginosa* was inoculated as the base species. After 24 hours, the density of *P. aeruginosa* was 5.7 X 10^5 CFU/cm^2 (Fig. 19). The density gradually decreased and stabilized after 32 days to about 1.0 X 10^5 CFU/cm^2. *M. avium* was inoculated after 30 days from the day of inoculation of *P. aeruginosa*. The density of *M. avium* remained stable at close to 1.0 X 10^4 CFU/cm^2 24 days after inoculation. *P. aeruginosa* density did not change after the introduction of *M. avium*. The density was close to that observed in jar reactor experiments of about 1X10^5 CFU/cm^2.

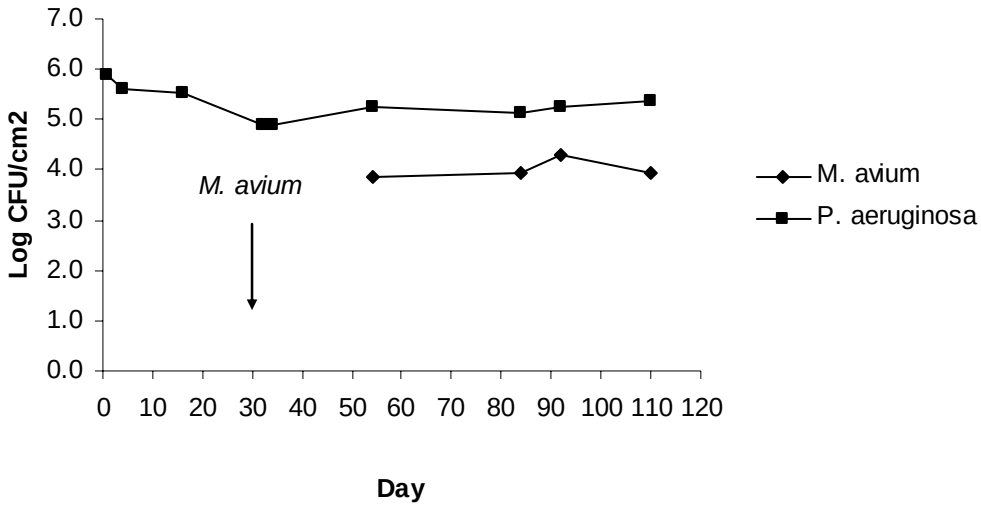

Figure 19. Density of *P. aeruginosa* as base species and *M. avium* as invading species in biofilms in a recirculation system.

DISCUSSION

Because of several steps involved in sample processing, plate counts of *M. avium* biofilm may give an underestimation of the actual numbers of cells present. It also takes three weeks of incubation before *M. avium* can be counted on M7H10 agar plates. Therefore, direct counting of cells on coupons was initially undertaken to estimate the cell density. If this study had involved only monoculture, it would have been possible to do a direct count on coupons by using an efficient DNA stain such as SYBR Green. However, for dual species biofilms the two strains have to be distinguished. AntiLAM (anti-Lipoarabinomannan) antibodies were obtained from Colorado State University, but the attempt to bind antibodies to cell surface LAM of *M. avium* was not successful. Therefore, a GFP strain of *M. avium* was obtained from Seattle Biomedical Research Institute and *P. aeruginosa* was transformed with RFP which was obtained from the University of Calgary. Unfortunately, neither of the strains expressed the fluorescent proteins consistently. Fig 4B demonstrated the fluorescing cells of both the species in biofilms in jar reactors. The fluorescent proteins expressed relatively more in the jar reactors than in the recirculation system experiment. Use of antibiotics in the growth medium can increase the fluorescent activity. However, in order to use the antibiotic in the growth medium, the selective marker would need to be identical for both *P. aeruginosa* and *M. avium*. Presence of the selective antibiotic in the growth medium can decrease loss of the plasmid.

When RFP *P. aeruginosa* was grown in biofilms in tap water, it did not fluoresce well in either biofilms or bulk water. In one experiment fewer than 1-6% of RFP cells fluoresced (Table 11). Exposure of RFP *P. aeruginosa* to higher nutrient concentration and temperature did not increase the fluorescence. Since a selective antibiotic was not used in the

growth medium (the two strains had different selective antibiotics), the bacteria could have lost the plasmids. However, Tomlin et al. (2004) reported up to 80% of plasmid (pHKT4) retention after 3 days in biofilms in the absence of a selective antibiotic. The lack of fluorescence could also be due to other confounding factors.

Various techniques used in this research were validated. Direct counts (cells/cm^2) of *P. aeruginosa* on coupons after SYBR Green staining gave one log more than the plate count (CFU/cm^2). Only about 10% of the total number of cells on the coupon were cultured at any given time. Yu et al. (1993) also observed at least one log higher cell density by direct count than by plate count of biofilm cells. The comparison of cell density between outer and inner sides of the coupon in the jar reactors showed no statistical difference. Sonication has been used as a standard practice to disaggregate cells from biofilm. The 3 cycles of 30 sec used in this study disaggregrated the cells and did not appear to reduce viability. Decontamination of a mixed species sample by using CPC (0.005%, 15 min contact time) reduced viability of *M. avium* up to only about 20%, while *P. aeruginosa* was 100% killed within 2 min of contact time. Thus, CPC had a relatively small effect on *M. avium*; almost complete inactivation of *P. aeruginosa* facilitated plating and growth of *M. avium* on Middlebrook 7H10 agar without overgrowth by *P. aeruginosa.*

In the environment, microorganisms live in communities with interspecific and intraspecific interactions, in addition to interactions with abiotic factors. *P. aeruginosa* and *M. avium* both are found in natural environments and have also been recovered from drinking water systems (Covert et al. 1999; Angenent et al. 2005; Schulze-Robbecke and Fischeder 1989). *P. aeruginosa* and *M. avium* may interact with each other if they grow together. These two species have different growth rates, *P. aeruginosa* having a significantly higher growth

rate (Mashburn et al. 2005; Falkinham et al. 2004). *P. aeruginosa* has been shown to affect the growth and activities of other bacteria (McKenney et al., 1995, Bakri et al., 2004).

Species composition and growth rate have been found to affect biofilm formation by bacteria (McLean et al., 1999, Palmer et al. 2001, Sturman et al., 1994 and Bremer et al., 2001). In the present study, the hypothesis that *P. aeruginosa* will dominate *M. avium* in biofilms is based on the fact that it is well known to form biofilms (Davies et al., 1998; Bibel et al., 1983; Siebel and Characklis, 1991; Stewart et al., 1997) and it is significantly faster-growing. *M. avium* will also grow in biofilms, as has been demonstrated previously and in the present study. Mycobacteria have been found to grow under low nutrient conditions (Norton et al., 2004; George et al., 1980; Carson et al., 1978), and its cell wall is hydrophobic which may promote attachment to substrata (Falkinham et al., 2004). However, it is a slow growing organism so it might not be expected to compete as well in mixed species biofilms with the faster growing *P. aeruginosa*. This appeared the case in this study.

This study has shown that *M. avium* and *P. aeruginosa* can form biofilms in drinking water. Further, imaging of biofilms has shown that they form microcolonies (Fig. 6). An inoculation of about 1.0 X 10^5 CFU/ml in bulk water of *M. avium* alone gave a stable biofilm density of around 1.0 X 10^4 CFU/cm^2. In dual species, the density of *M. avium* was lower than the density in monospecies and the difference was statistically significant. For *P. aeruginosa*, the monospecies and dual species densities were similar at around 1.0 X 10^5 CFU/cm^2. The average dissolved organic carbon (DOC) was 1.3 mg/L in the tap water used in these simultaneous inoculation experiments. Norton et al., (2004) reported similar densities when DOC in the growth medium was 0.42 mg/L in a laboratory model of a drinking water distribution system. However, addition of humics up to 10 mg/L did not increase the density

significantly. In another *in situ* study of eight drinking water systems, a density of only 0.3 CFU/cm^2 of *M. avium* was found (Falkinham et al., 2001). The same research found that an increase in mycobacterial numbers correlated with dissolved organic carbon. After 4 weeks of incubation of tap water with 10% M7H9 broth, a density of $2.9x10^4$ CFU/cm^2 of *M. avium* was found (Steed and Falkinham, 2006). A biofilm density of $2.3x10^3$ to $4.6x10^5$ CFU/cm^2 of nontuberculous mycobacteria was found in a drinking water distribution network by September (2002). The density of *M. avium* observed in drinking water biofilms in the present study in a laboratory model is more or less comparable to that found in previous studies. Mycobacteria have been found to grow in extremely low nutrient concentrations. For example, *M. chelonae*, a fast growing nontuberculous mycobacteria, multiplied in commercial distilled water at a level of 10^4 to 10^5 CFU/ml and the population declined only slightly over a period of 1 year (Carson et al., 1978).

In a study involving *Acinetobacer calcoaceticus, Staphylococcus* sp. and *S. aureus* the attachment of one species was either decreased, increased or not affected by sequential or simultaneous inoculation of the second species (McEldowney and Fletcher, 1987). The attachment depended on the species composition and the nature of the substratum. The present study found a significant difference in mycobacterial densities in monospecies and dual species biofilms after simultaneous inoculation, suggesting that *P. aeruginosa* growth has a negative influence on mycobacterial growth. *P. aeruginosa* density was approximately one log higher than *M. avium* density in biofilms when the bulk liquid was inoculated with about the same log concentrations. The density of *P. aeruginosa* did not change in monospecies and dual species biofilms and therefore *M. avium* does not appear to have an influence on *P. aeruginosa* growth. Banks and Bryers (1991) reported that the species with

higher growth rate, *P. putida*, dominated *Hyphomicrobium* sp. in biofilms. McLean et. al., (1999) also found that species composition and growth rate affected the ability of aquifer bacteria to form biofilms. According to Stewart et. al. (1997), after simultaneous inoculation of the two strains, because of its rapidly colonizing ability, *P. aeruginosa* formed biofilms even in the presence of *K. pneumoniae* which had a higher growth rate. And, in a different study, species interaction was not observed in a dual species biofilm of *P. aeruginosa* and *K. pneumoniae* and the organism with higher growth rate did not dominate the biofilms (Siebel and Characklis, 1991). Kolmos et al. (2005) reported that in the planktonic phase the organism with higher growth rate (*Klebsiella oxytoca*) dominated but in biofilms at low nutrient concentration the proportion of the slow growing organism (*Burkholderia cepacia*) was higher.

Interspecies interactions can be mutualistic, competitive, neutral, commensal, amensal or antagonistic. Simultaneous inoculation experiments showed that the presence of *P. aeruginosa* reduced density of *M. avium* in biofilms. This suggests that their interaction is competitive. Also as mentioned earlier in dual species biofilms, *P. aeruginosa* density was found to be about one log higher than the *M. avium* density. However the presence of *M. avium* did not affect the density of *P. aeruginosa*. However the competition does not exclude the growth of *M. avium*. Steed and Falkinham (2006) also reported that presence of water's normal flora reduced the density of *M. avium* in biofilms grown on glass beads in laboratory growth medium (M7H9 broth).

In laboratory growth medium, *M. avium* has a doubling time of 20 hrs and *P. aeruginosa* doubles in 1 hr. The higher density of *P. aeruginosa* in biofilms is likely due to its higher growth rate, although the effect of other biotic and environmental factors cannot be

ruled out. In addition, the substrate and nutrient concentrations in the present study were far lower than those in laboratory growth media. For jar reactor experiments tap water was used, and for the recirculation system an effective dilution of 0.03% (1:3333) TSB culture medium was present. For this reason, it is possible that the growth rates of the two organisms in these experiments may be more similar to each other which may also affect biofilm formation.

Growth of *M. avium* and *P. aeruginosa* in biofilms could also be influenced by several other factors. *P. aeruginosa* can produce antimicrobial agents that can inhibit the growth of other species (Bakri et al., 2004). Whether *P. aeruginosa* produced any antimicrobial agent and whether it has any effect on *M. avium* growth in this study is not known. *P. aeruginosa* produces autoinducer I, the quorum sensing molecule, and communicates within its population and with other populations (McKenney, 1995; Riedel et al., 2001). Preliminary results of a recently conducted study suggests that autoinducer II increased biofilm formation by M. avium (Geier et al. 2006). Sometimes the interspecies interaction is also influenced by the metabolites the species produce (Nielson et. al., 2000; Christensen et al., 2002).

In sequential inoculation, *M. avium* and *P. aeruginosa* were tested both as base and invading species. When *M. avium* was grown as the base species, the subsequent presence of *P. aeruginosa*, unlike in the simultaneous inoculation experiments, did not affect the growth pattern of *M. avium* significantly. Similarly, when *P. aeruginosa* was grown as the base species and *M. avium* was subsequently inoculated as the invading species, the density of *P. aeruginosa* did not change. The density of *P. aeruginosa* was about one log higher than that of *M. avium*. Therefore, based on the one time study there was virtually no difference in growth patterns in dual species biofilms regardless of which species was inoculated as base

or invading species. Sturman et al., (1994) observed the influence of growth rate in sequential inoculation. *P. aeruginosa* populations dropped by 1 log after *Klebsiella pneumoniae* was inoculated as an invading species, but invading *P. aeruginosa* did not change *K. pneumoniae* populations.

In the recirculation system, the stable density of *P. aeruginosa* was about 1.0 X 10^5 CFU/cm^2 and the density of *M. avium* was 1.0 X 10^4 CFU/cm^2. The density found in the recirculation system is comparable with the results of the jar reactor experiments. Direct observation on the coupons from the recirculation system showed that cells were not concentrated in any particular area of the coupons, in contrast to the finding in the jar reactor experiments that cells concentrated near the air water interface. The coupons were completely submerged in the recirculation system.

In water filled environments, colonization of the interface between liquid and gas phases can be selectively advantageous (Rainey and Travisano, 1998). Overproduction of cellulose increased the ability of *P. fluorescens* to colonize the air water interface resulting in formation of a thicker biofilm (Spiers et al., 2003). In the present study, in batch reactor experiments, most cells of both species accumulated in the area near the air-water interface. This could be due to more oxygen availability for metabolic activity towards the surface of the growth medium. However Wendt et al., (1980) suggested that the concentration of *M. avium* at the air water interface is because of the hydrophobicity of the cell surface. It is also possible that cells could have coaggregated due to some other biological or physico-chemical factors. It is expected that oxygen tension throughout the water column would be similar because of oxygen diffusion. If oxygen was the only limiting factor contributing to high concentrations of microbes at the air-water interface, then supply of additional oxygen could

increase cell densities over the entire coupon surface. Availability of oxygen has been found to increase the activity of *P. aeruginosa* in biofilms (Xu et al., 1998 and Werner et al., 2004). Since both the species were concentrated within a limited area near the liquid/air interface it would be logical to argue that there could be competition for oxygen, nutrients and space.

CONCLUSIONS AND FUTURE DIRECTIONS

In the experimental environments tested, *M. avium* can form biofilms in isolation or in coculture with *P. aeruginosa*. The attachment and growth of *P. aeruginosa* affected the growth of *M. avium*. However, *P. aeruginosa* growth was not affected by *M. avium*. The interaction therefore appears to be competitive. In biofilms and bulk water, *P. aeruginosa* density/concentration was higher than that of *M. avium* likely because of its higher growth rate.

Cell concentrations near the air water interface on coupons can be due to availability of oxygen, since the cells were not concentrated in specific areas of coupons collected from the recirculation system. However, the role of other physico-chemical and biological factors can not be ruled out. It can also be argued however that the role of oxygen could be minimal because of oxygen diffusion as a result of mixing on the shaker. It has been reported that hydrophobicity can also contribute to mycobacterial concentration near the air-water interface. The sequential experiments indicated that *M. avium* can colonize established biofilms of *P. aeruginosa*.

M. avium has been recovered from drinking water systems and raw source waters. This and other studies have also shown that *M. avium* can grow and survive in biofilms. Their occurrence in microcolonies indicates that biofilms may be an important site for their proliferation. MAC has been listed on the contaminant candidate list by the USEPA for possible regulation in drinking water. Occurrence of mycobacteria in drinking water may not be due to or related to fecal contamination and the bacteria are more resistant to chlorine than fecal indicator bacteria. Also, the detection and control of *M. avium* in drinking water alone is not sufficient because this may not reflect its occurrence in biofilms. Exposure to *M. avium* in

drinking water is potentially a major concern for immunocompromised individuals. Control and prevention of *M. avium* contamination in potable water therefore may require unconventional and careful management of water quality that will remove this opportunistic pathogen both from bulk water and biofilms.

Studies of mixed species interactions is important because microorganisms in nature are never found in monoculture. Growth of microorganisms in biofilms have been found to be affected by the presence of other microorganisms. How the growth of *M. avium* in biofilms is affected by the presence of other microbes present is not yet clear although it has been shown that *M. avium* can survive inside amoeba (Miltner et al. 1998). This study indicates that presence of *P. aeruginosa*, which is also an opportunistic pathogen, affects the growth of *M. avium* under the conditions in which it was grown. It is hoped that this study will provide a reference point for future investigations involving interaction of *M. avium* with other pathogens and heterotrophs in biofilms.

A wide variety of ecological and environmental factors can affect the attachment, growth and persistence of bacteria in biofilms. Only a few studies have been carried out to identify the effect of environmental factors on mycobacterial colonization and growth on surfaces. Survival and growth of *M. avium* can depend on the nature of the attachment surface, temperature of the surrounding environment, access to nutrients and the consortium of other species in the community with which it interacts. Therefore, effects of variations in temperature, pH and oxygen on the growth and distribution of *M. avium* in biofilms should be further investigated. Understanding the dynamics of growth of *M. avium* in biofilms and determining the effect of abiotic and biotic factors in growth and proliferation of *M. avium* may help in providing solutions to its control in potable water systems. Laboratory based and

in situ studies (in natural water bodies and drinking water systems) need to be carried out that take into account different abiotic and biotic factors.

Traditional interventions which mainly include disinfection, have focused on primary pathogens of fecal origin and aesthetic quality of water. In doing so, only the pathogens in bulkwater are removed. Because of slow growth rates and exopolysaccharide barriers in biofilms, a conventional disinfection dose may not eliminate microbes in biofilms. The problem is compounded by the fact that the *M. avium* cell wall is much more impermeable to antimicrobials than the cell walls of traditional indicators of fecal pollution. Application of increased doses of chlorine to provide a stronger residual in water can be harmful to human health and the water supply pipes. Therefore, an alternative disinfection strategy needs to be developed. Use of higher doses of chlorine and subsequent dechlorination that results in low levels of residual chlorine could be one approach to treat *M. avium* contaminated water. In addition, exposure of water at high temperatures could be an alternative for hot water systems, as has been proposed for *Legionella* control.

The presence of one species in a biofilm can influence the effect of disinfection on a second species. According to Skillman et al. (1997) mixed species biofilms were more resistant to disinfection than single species biofilms. It would be beneficial to carry out disinfection studies in bulk water and biofilms involving mycobacteria and other species. It could also be of interest to determine whether the removal of pathogens and heterotrophs in water could be selective for mycobacterial growth in biofilms.

Mycobacteria including *M. avium* have been detected in hot water systems of large buildings. They were also found to grow in public water distribution systems. Monitoring of drinking water for opportunistic pathogens is important in order to accurately assess human

health risk. Monitoring should include both hot and cold water in hospitals, senior centers and day care centers as these institutions are likely to have immunocompromised individuals. Also infectious dose data and risk assessment models of *M. avium* are needed to manage public health safety. The unusual and impermeable structure of its cell wall, slow growth rate and antimicrobial resistance of these opportunistic pathogens require new and innovative approaches to drinking water monitoring and treatment.

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