Adherence and Biofilm Formation of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium abscessus* in Household Plumbing

By

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Abstract

Nontuberculous mycobacteria (NTM) are ubiquitous in the environment and found in drinking water distribution systems and household plumbing. They are opportunistic pathogens of humans, causing lung disease. Their ability to adhere and form biofilm is attributed to a waxy, lipid-rich, cell envelope. This highly hydrophobic envelope also contributes to the characteristic antibiotic-, chlorine-, and disinfectant- resistance of NTM.

NTM in household plumbing reside primarily in biofilms and the ability to form biofilm has been linked to virulence. Shedding of cells from biofilm and the subsequent aerosolization of microorganisms through showerheads presents a significant public health risk, particularly to those individuals with associated risk factors.

Three species of NTM, *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium abscessus*, were examined for adherence and biofilm formation on surfaces common to household plumbing systems, including glass, copper, stainless steel, polyvinyl chloride, and galvanized steel. All experiments were conducted with sterile, Blacksburg tap water in a CDC Biofilm Reactor.

Highest adherence was observed by *M. avium* on galvanized steel surfaces, reaching 15,100 CFU/cm^2 surface at 6 hours incubation at room temperature. After 3 weeks incubation at room temperature, biofilm formation of *M. avium* was also highest on galvanized steel surfaces, reaching 14,000,000 CFU/cm^2 surface. Lowest adherence was observed by *M. abscessus* on polyvinyl chloride (PVC) surfaces, reaching 40 CFU/cm^2. Lowest biofilm formation was observed by *M. intracellulare* on glass surfaces, reaching 5,900 CFU/cm^2.

Surfaces, such as galvanized (zinc), on which high adherence and biofilm formation was observed, should be avoided in household plumbing systems of NTM patients and individuals at risk for developing NTM disease. Additionally, surfaces such as copper, harbor fewer NTM and may provide a safer alternative for household plumbing of NTM patients.

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Introduction

Nontuberculous mycobacteria. The genus *Mycobacterium* are members of the phylum *Actinobacteria*, which also include *Micrococcus*, *Nocardia, Streptomyces, Corynebacteria* and over 300 other genera (Zhi, *et al.*, 2009). The phylum contains microorganisms with high G+C content (>55 mol% in genomic DNA) (Gao & Gupta, 2012). The >150 species of *Mycobacterium* can be placed into two groups, slow-growers and fast-growers. The fast-growing species are those that form colonies within 3 days, whereas the slow growers require 7-10 days. All members of the genus *Mycobacterium* have a thick, lipid-rich (C_{60}-C_{90}) outer membrane that is a major determinant of their growth, physiology, and ecology (Brennan and Nikaido, 1995). The thick, lipid-rich outer membrane is responsible for the characteristic acid-fastness of mycobacteria. The mycobacterial genome contains only 1-2 rRNA operons, which is significantly less than other, faster growing microorganisms, such as *Escherichia coli*. This limited number of rRNA operons, coupled with the high demand for ATP required for long-chain fatty acid synthesis, contributes to the slow growth of mycobacteria.

Nontuberculous mycobacteria are non-motile bacilli. However, previous research has shown *M. smegmatis* to be capable of sliding motility, which is a flagellum-independent method of spreading across surfaces and involved in biofilm formation (Martinez, *et al.* 1999). Other research has also shown *M. marinum* to employ actin-polymerization and actin-based motility while inside macrophages (Stamm *et al.*, 2003).

The NTM cell envelope. NTM have a complex cell envelope consisting of inner and outer membranes separated by a thin peptidoglycan cell wall (Minnikin, 1982; 1991). As a result of the presence of long chain fatty acids in the outer membrane (i.e., mycolic acids), NTM cells are waxy and hydrophobic. There are approximately 250 enzymes involved in fatty acid metabolism in *M. tuberculosis* compared to only 50 in *E. coli* (Cole *et al.*, 1998; Riley and Labedan, 1996). However, some of the most effective anti-mycobacterial drugs affect biosynthesis of cell-wall components (Chatterjee, 1997).

The presence of these long-chain fatty acids (C_{60}-C_{90}) results in a low permeability, leading to low rates of transfer of hydrophilic nutrients and antibiotic, disinfectant, chlorine, and heavy metal resistance (Brennan and Nikaido, 1995; Norton *et al.,* 2004). The highly hydrophobic nature of these organisms may contribute to their ability to quickly adhere to surfaces and form biofilms in piping systems (Steed and Falkinham, 2006). The ability for early formation of biofilms in piping systems has resulted in the hypothesis that mycobacteria are biofilm "pioneers" (Steed and Falkinham, 2006).

Risk factors for NTM disease. Nontuberculous mycobacteria are opportunistic pathogens and typically cause pulmonary infection through the inhalation of aerosols or aspiration (Marras and Daley, 2002). They are also known to cause bacteremia in immunocompromised individuals, probably through gastrointestinal tissues (Chin and Hopewell, 1992). Initially, individuals infected with NTM were predominantly older males with occupational risk factors such as mining and others that involved contact with dust and particulate matter. Other risk factors

included smoking and alcoholism. NTM infection, principally *Mycobacterium avium*, rose in the 1980's, due to bacteremia in individuals with compromised immune systems, such as patients with acquired immunodeficiency syndrome (AIDS). With the advent of highly active antiretroviral therapy (HAART), and the reconstitution of the immune system, the infections declined. Now, the major NTM infections are seen in patients who are not immunocompromised and have no predisposing pulmonary condition, causing cervical lymphadenitis in children and pulmonary alveolar proteinosis (Wolinsky, 1995; Witty, *et al.*, 1994). Elderly, particularly Caucasian, women with a low body mass index (BMI) are also at high risk for developing NTM infection (Kennedy and Weber, 1994). NTM cause pulmonary infections in patients with chronic obstructive pulmonary disease, interleukin-12 receptor deficiency (IL-2-deficiency), emphysema, bronchiectasis, and cystic fibrosis (Ebihara and Sasaki, 2002; Yamazaki *et al.,* 2006). Peribronchiolar granulomas are another typical symptom of disease, which suggests that *M. avium* is capable of crossing the bronchial mucosa (Rubo *et al.,* 1998; Yamazaki *et al.*, 2006). In the United States, *M. avium* and *M. intracellulare* are among the three most common causes of NTM infection (Marras and Daley, 2002). The prevalence of *M. abscessus* infections in cystic fibrosis patients is on also on the rise (Kilby, *et al.*, 1992). A study of CF patients, by Olivier, *et al.*, in 1996 showed *M. abscessus* to be the second most common isolate after *M. avium* complex.

NTM ecology. NTM are found in soil, surface and ground waters, in drinking water systems, and household plumbing (Ichiyama, *et al*.,1988; Fry, *et al*.,1986; Falkinham, *et al.*, 1980). Previous studies showed that drinking water contains between 1 and 1000 cfu of *M. avium* per 100 mL of water and 600 CFU/ cm^2 of biofilm (Falkinham *et al.,* 2001). In a study by Briancesco, *et al*. (2010) 62% of water samples collected from hospitals and private residences in the Latium and Calabria regions of Italy were positive for NTM. In another study by Covert *et al.* (1999) NTM were isolated from 38% of drinking water systems tested in 21 states in the U.S.

NTM, such as *M. avium* and *M. intracellulare*, readily adhere to surfaces and form biofilms, and have previously been isolated from biofilms in drinking water distribution systems as well as household plumbing (Falkinham *et al.,* 2008; Falkinham, 2011). There is much interest in the role biofilms in household plumbing play in human NTM infections. A previous study by Falkinham *et al.* (2008) identified household plumbing as the source of NTM infection by matching DNA fingerprints from the patient to the patient's shower. A more recent study showed 59% of water samples collected from the homes of NTM patients to contain NTM (Falkinham, 2011). It has been suggested that release of NTM from existing biofilms may serve as a mechanism for shedding and infection of susceptible hosts (De Groote and Huitt, 2006).

NTM biofilms and virulence. The formation of biofilms plays a role in infection by many pathogenic organisms. The Centers for Disease Control (CDC)

estimates that approximately 65% of human infections are at least partially due to biofilms (Potera, 1999). Pulmonary infections due to *M. avium* are typically antibiotic-resistant and chronic, which is suggestive of biofilm presence (Yamazaki *et al.,* 2006). In a study by Torriani *et al.* (1994) of AIDS patients with disseminated *M. avium* complex (MAC) infection, or bacteremia, tissues were found to contain the highest numbers of mycobacterial cells. Evidently, mycobacterial biofilm formation and virulence are linked as a study, using both *in vitro* and *in vivo* techniques showed that biofilm-deficient mutants of *Mycobacterium* species to be incapable of invading bronchial epithelial cells (Yamazaki *et al.,* 2006).

Factors Influencing adherence and biofilm formation. Biofilms, defined by the Centers for Disease Control (CDC) as a microbial community tightly attached to a surface and enclosed in a matrix of extracellular polymers, appear to be the preferred state for many bacteria. The first step in biofilm formation is attachment. Attachment to a surface is dependent upon many factors including substratum effects, hydrodynamics, ionic conditions, and the cell surface. (Donlan, 2002). The substratum or surface has many properties that effect attachment of microorganisms. The physico-chemical properties of the substratum, such as hydrophobicity, surface free energy/charge, and surface roughness (Fang, *et al.*, 2002; Muller, *et al.*, 1992; Palmer, *et al.*, 2007).

Hydrophobicity. Many have studied the role of hydrophobicity in bacterial surface attachment. Examples of hydrophobic surfaces include PVC, Teflon, and

other plastics. Hydrophilic surfaces include glass, or metals such as stainless steel. In a study by Liu, et al, in 2004 a model for microorganisms was developed to show that high hydrophobicity of the cell favored surface adhesion. It has been demonstrated as a factor in not only bacterial adhesion, but also in the adherence of the water-borne pathogens *Cryptosporidium parvum* and *Giardia lamblia* to polymer-coated glass surfaces (Dai, *et al.*, 2004). In a previous study, *Mycobacterium avium* biofilm levels were higher on iron and galvanized pipe surfaces as opposed to copper or polyvinyl chloride (PVC) (Norton *et al.,* 2004). In another study, *Mycobacterium fortuitum* was shown to develop more biofilm on stainless steel, PVC, and polycarbonate (PC) than copper or glass surfaces (Williams, *et al.*, 2005).

Surface free energy/charge A positive correlation has been seen between surface free energy and adsorption or attachment rate in *Pseudomonas* species. The study employed copper, stainless steel 316, silicon, and glass surfaces and also found the highest surface free energy to be copper with 31.2 dynes cm^{-1} and silicon to the be the lowest with 25.1 dynes cm^{-1} (Mueller, *et al.* 1992).

Surface roughness. Surface roughness has been observed in many studies to be linked to microbial attachment. A rougher surface yields shelter from shear and turbulent flow while also providing a larger surface area for colonization. (Donlan, 2002). A study by Pedersen in 1990 found that matt steel, a rougher surface, had 1.44 times more microorganisms than the smoother, electro-polished steel. Another study by Percival *et al.*, 1998 showed higher colonization

of the rougher stainless steel grade 304 than stainless steel grade 316. Other studies have shown increased surface roughness to increase not only initial bacterial attachment but also colonization or biofilm formation (Geesey, *et al.*, 1996; Tebbs, *et al.*, 1994) A study by Arnold and Bailey (2000) showed significantly less bacterial attachment on electro-polished stainless steel than other sand-treated stainless steel surfaces, which were more rough. Another study in 2006 observed that surfaces with pits had higher bacterial adherence, but only if the pits were similar in size to the microbial cells. Pits larger than the microbial cells did not retain as many cells (Verran and Whitehead, 2006).

Hydrodynamics. Hydrodynamics also play a role in bacterial adhesion. The area immediately adjacent to the substratum, deemed the hydrodynamic boundary layer, is a zone of significantly decreased flow (Lawrence, *et al.*, 1987). The width of the zone is determined by the linear velocity (Pittner, 1988). A higher linear velocity may result in increased bacterial adhesion for numerous reasons, including a boundary layer that is thinner and more easily crossed by bacterial cells (Donlan, 2002). Higher linear velocity also increases distribution of nutrients throughout the system (Lehtola, *et al*., 2006).

Ionic conditions. The characteristics of the cell suspension, such as pH, temperature, the presence of cations, and other microorganisms, and nutrient concentrations can also affect bacterial adhesion and biofilm formation (Donlon, 2002). Another study showed *Pseudomonas fluorescens* adhesion to glass surfaces to be increased by the higher concentrations of sodium, calcium, and ferric iron (Fletcher, 1988).The presence of cations such as Ca^{2+}, Mg^{2+}, and Zn

$^{2+}$, which is attributed to water hardness, has been shown to increase *M. avium* biofilm formation, while humic acid, a common component in drinking water, has been shown to decrease biofilm formation (Carter *et al.,* 2003). A previous study by Torvinen *et al.* in 2007 showed *M. avium* biofilms to be negatively influenced by competition with other heterotrophic bacteria. This study also showed the culturability of *M. avium* to be higher at lower phosphorus concentrations. Higher phosphorus levels could increase competition between mycobacteria and heterotrophic bacteria, as phosphorus has been shown to be the limiting nutrient in growth and biofilm formation (Lehtola *et al.,* 2004). A study by Kim and Frank in 1994 examined biofilm formation of *Listeria monocytogenes* on stainless steel under a variety of nutrient conditions. Concentrations of nutrients including phosphate, amino acids, and carbohydrates were tested. The study, among other findings, observed that the carbohydrates mannose and trehalose increased biofilm formation. Many biofilm studies have been conducted using mycobacteria grown in nutrient-rich media, typically Middlebrook 7H9 broth (M7H9). However, previous studies have found biofilm formation to be higher when mycobacteria are placed in water, rather than nutrient rich media (Carter *et al.*, 2003; Limia, *et al.*, 2001).

Cell surface characteristics. A study by Bendinger *et al.* (1993) found that microorganisms with cell envelopes containing mycolic acids, such as *Mycobacterium* and *Corynebacterium* were more hydrophobic than other microorganisms lacking mycolic acids. Other characteristics such as the

presence of fimbriae (Corpe, 1980) and flagella influence cell adhesion. A study conducted using *Pseudomonas fluorescens* showed flagellated cells to adhere in higher numbers than those lacking flagella (Piette and Idziak, 1991).

Surface charge of the bacterial cell is another factor that has been implicated in cell adhesion (Kiremitci-Gumustedereliogl and Pesman, 1996; Hallab, *et al*., 1995). Surface charge varies based on a variety of factors including pH of the medium (Husmark and Ronner, 1990), age of the cell culture (Walker, *et al*., 2005), and other culture conditions. However, bacterial cells typically have an overall negative surface charge at a neutral pH (Rijnaarts, *et al.*, 1999). In contrast, a study by George, *et al*., in 1986 showed NTM, including *Mycobacterium avium* complex (MAC) to have a net positive charge below a pH of 4.5. A study in 1990 by Husmark and Ronner examined the adherence of spores of *Bacillus cereus* to both hydrophobic and hydrophilic surfaces under different pH and medium polarities. Results showed the spores to be most adherent when the pH of the media resulted in an uncharged cell surface. Another study showed *Escherichia coli* cells in stationary phase to adhere in higher numbers than those in mid-log phase (Walker, *et al*., 2005).

Proposed studies. There have been many studies on NTM biofilm formation, while few studies have examined the effects of typical surfaces found in household plumbing. Previous studies have also failed to account for the separation of adherence, and growth and biofilm formation as independent processes. This study proposes that mycobacteria will more readily adhere to certain surfaces. This information would be critical in identifying higher-risk

household plumbing systems and employing potential prevention measures through the use of surfaces with lower affinity for biofilm formation. Most significantly, the proposed association between NTM biofilm formation ability and virulence as well as drug-resistance would argue that biofilm formation increases the probability of household plumbing-associated infection (Falkinham, 2007).

Many techniques have been employed previously to measure both adherence and biofilm formation in mycobacteria and other microorganisms, including the rotating disc reactor (Zelver *et al.,* 1999) and the annular reactor (Camper *et al.,* 1996). In 2002, Donlan *et al.* developed the CDC biofilm reactor, or CBR, which employs paddles containing 24 removable coupons, which serve as biofilm growth surfaces (Figure 1). These coupons come in a variety of materials including, polyvinyl chloride (PVC), glass, copper, stainless steel, and galvanized (zinc). Previous studies have shown the placement of the 8 rods and 24 coupons within the rod to have no effect on overall density of biofilms (Goeres *et al.,* 2005). The use of the CDC biofilm reactor (CBR) allows for easy manipulation of surface type, temperature, shear, and addition of other components such as organic matter or cations. However, the main focus of this study is to examine the effects of various surfaces, and their properties, on NTM adherence and subsequent growth and biofilm formation.

Hypotheses

(1) Nontuberculous mycobacteria (NTM) readily adhere to and form biofilm on surfaces commonly found in household plumbing

(2) Nontuberculous mycobacteria (NTM) adherence and biofilm formation is influenced by surface type.

Objectives

1. Measure adherence of *Mycobacterium abscessus* strain AAy-P-1, *Mycobacterium avium* strain A5, and *Mycobacterium intracellulare* strain TMC 1406^T, to surfaces found in household plumbing.

2. Measure growth and biofilm formation of *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T, and *M. abscessus* strain AAy-P-1, on surfaces found in household plumbing.

Materials and Methods

Mycobacterial Strains. *Mycobacterium avium* strain A5, is a plasmid-free strain and was obtained from McClellan Veterans Hospital in Little Rock, Arkansas and originally isolated from an AIDS patient. (Beggs, *et al.*, 1995) *Mycobacterium intracellulare* strain TMC 1406^T was obtained from the Trudeau Memorial Collection (Mayer and Falkinham, 1986). *Mycobacterium abscessus* strain AAy-P-1 was isolated as part of a study by Falkinham, 2011.

Biofilm device. The CDC biofilm reactor (CBR) was employed (Donlan *et al.* 2002) and used with glass, steel, copper, PVC and galvanized steel coupons. Each coupon was 0.3 cm thick with a 1.27 cm diameter yielding a 2.54 cm^2 surface area available for colonization. The glass vessel allowed for approximately 350 mL of operational fluid capacity and contained eight, removable, polypropylene rods, each capable of holding three coupons, which allowed for 24 sampling opportunities.

Growth of mycobacteria. Strains were grown to log phase in 50 ml M7H9 broth containing 0.5% glycerol and 10% (v/v) oleic acid albumin in 500 mL nephalometer flasks with agitation (60 rpm). Absorbance (540 nm) was recorded twice daily until culture reached mid-log phase.

Water acclimation of mycobacteria. Cells previously grown to log phase were collected by centrifugation (5,000 x g) for 20 min. The supernatant was discarded

and cells suspended in 100 ml of sterile Blacksburg tap water (BTW) and centrifuged for 20 min. This process was repeated and washed cell suspensions were incubated in 250 mL plastic centrifuge bottles for 1 week at room temperature to allow for water acclimation of cells. Following acclimation, colony counts were measured using serial dilutions in sterile BTW on M7H10 agar containing 0.5% glycerol and 10% (v/v) oleic acid albumin. Cell suspensions were aliquoted and frozen at -80°C.

Measurement of adherence. CDC biofilm reactor (CBR) was filled with 350 mL of sterile BTW to fully cover coupons. CBR was inoculated with a sufficient volume of mycobacterial cells to reach a final density of approximately 10,000 cfu/ml.Immediately, and after 1, 2, 3, and 6 hours incubation at room temperature, a paddle was removed and washed by dipping in 50 mL screw capped tube containing 30 mL sterile BTW. Individual coupons were removed aseptically and placed in separate sterile 50 ml screw cap centrifuge tubes containing 2 ml of sterile BTW. Each tube was vortexed for 1 min at the highest setting, plated on M7H10 agar and incubated at 37°C for 7-14 days. Refer to Figure 1. for stepwise breakdown of experimental design. Following incubation, colony counts were performed and CFU/cm^2 were calculated for each coupon. CFU/cm^2 was determined using the amount of liquid used for suspension of adherent cells and surface available for adhesion. The shortest time required for maximal adherence for each strain was determined based upon colony counts and CFU/cm^2 (Figure 2).

Measurement of growth and biofilm formation. Mycobacterial cells were incubated in CBR for previously determined time required for maximal adherence. The entire paddle support system (8 paddles) was removed and washed by dipping in sterile BTW. Mycobacterial suspension was discarded and CBR rinsed thoroughly in sterile BTW. CBR was filled to required volume of 350 mL with sterile BTW, containing 3 mg humic acid/L, and paddle support with adherent mycobacteria was replaced. At 1, 7, 14, and 21 days incubation at room temperature, a paddle was removed, washed by dipping in 30 mL of sterile BTW, vortexed for 2 min at the highest setting in 10 mL Butterfield Buffer (0.8 g KH_2PO_4, 2 g peptone, 20 mL Tween 80/ per liter) and processed for measurement and calculation of mycobacterial CFU/cm^2.

Statistical Analysis. CFU/cm^2 data collected from adherence and biofilm experiments was compiled and analyzed with Student's T test using JMP 9® Version 9.0.0

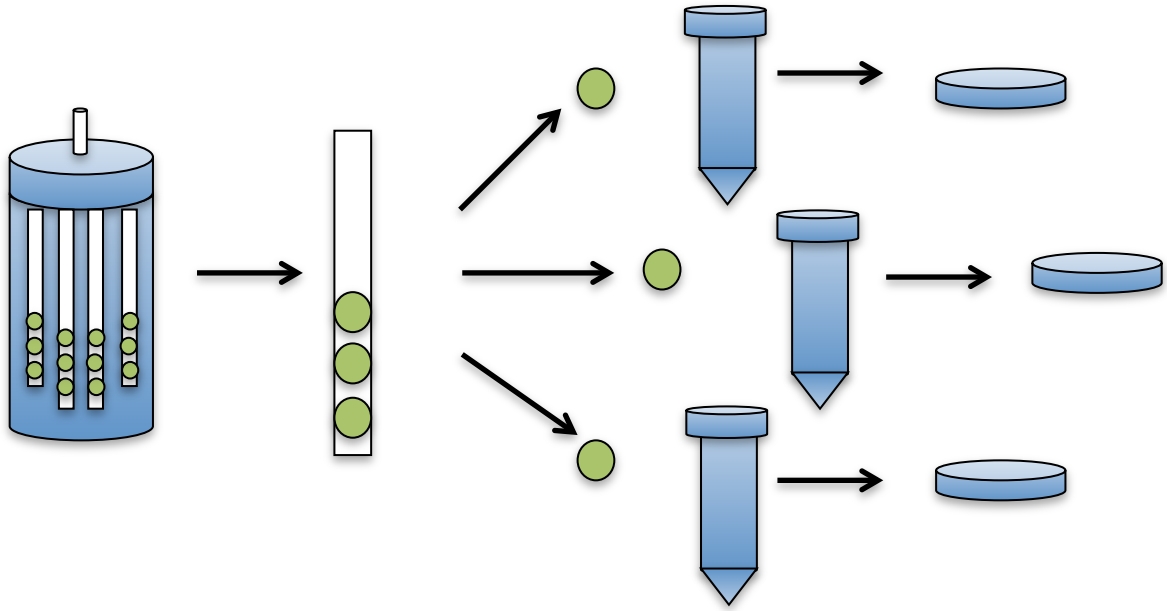

Figure 1. CDC biofilm reactor (CBR) and three basic steps of experimental design. First, wash coupons by dipping. Secondly, vortex to suspend adherent cells. Last, plate to count and calculate CFU/cm^2

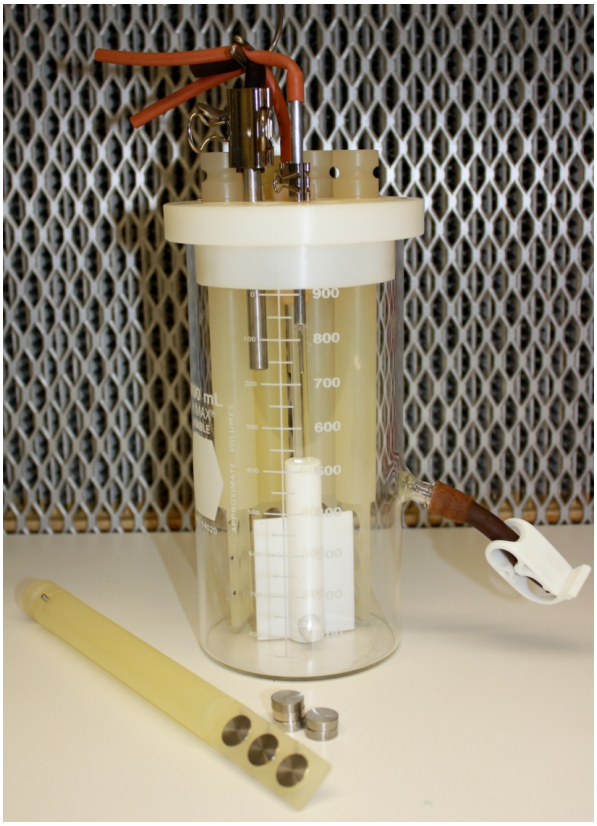

Figure 2. CDC biofilm reactor (CBR) with front rods removed to display magnetic stirring apparatus. Photograph by Summer Mullis (2011).

Results

Reproducibility of data. The variation and large standard deviations seen in the data can be explained by the characteristics of NTM. The hydrophobic surface causes the cells to clump. This clumping may cause skewed colony counts when plated, yielding high standard deviations. In addition, the shear forces applied to the coupon surface upon vortexing may cause portions of biofilm of varying sizes to dislodge, which may not have completely dissociated prior to plating. There was also variation, as expected, across the three NTM species. However *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T adhered similarly to galvanized and PVC surfaces. Data was collected using culture-based methods and has been shown in other biofilm studies to produce significantly lower numbers of *M. avium* cells than other molecular methods, such as fluorescence *in situ* hybridization (FISH) (Lehtola, *et al*., 2007). Therefore, these results may be an underestimate of actual biofilm.

Measurement of adherence. The results of the adherence experiments are shown in Tables 1-5. *M. avium* strain A5 adherence was the highest with 15,100 CFU/cm^2 to galvanized (zinc) surfaces at 6 hours incubation (Table 3). Strain A5 adherence to galvanized surfaces was also the highest overall adherence at each time sampled (Table 3). *M. intracellulare* strain TMC 1406^T, adherence was also highest, approximately 6,800 CFU/cm^2 on galvanized (zinc) surfaces at 6 hours (Table 3). In contrast, *M. abscessus* strain AAy-P-1 showed the highest adherence to glass and stainless steel surfaces at 6 hours incubation (Table 1;Table 2).

In contrast, *M. avium* strain A5 adherence was lowest on stainless steel coupons, with 430 CFU/cm^2 at 6 hours incubation. *M. intracellulare* strain TMC 1406^T was lowest on glass with 700 CFU/cm^2 after 6 hours incubation. *M. abscessus* strain AAy-P-1 adherence was lowest on polyvinyl chloride (PVC) with 40 CFU/cm^2 at 6 hours incubation.

Both *M. avium* strain A5 and *M. intracellulare* strain TMC 1406T adherence was highest on galvanized and PVC surfaces, while *M. abscessus* strain AAy-P-1 adherence was highest on glass and stainless steel. This implies strain similarities between A5 and TMC 1406^T, as well as identifies galvanized, PVC, glass and stainless steel surfaces as high risk for adherence of NTM in household plumbing. Refer to Table 6. for a complete ranking of surface type by adherence.

Table 1. CFU/cm^2 values for *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T and *M. abscessus* strain AAy-P-1 for glass surface

	Strain		
Duration of exposure (hr.)	**A5**	**TMC 1406^T**	**AAy-P-1**
0	1,600 ±780	700 ±500	70 ±80
1	1,300 ±990	700 ±400	400 ±320
2	650 ±440	500 ±300	670 ±520
3	1,500 ±1,300	600 ±300	590 ±340
6	1,600 ±650	700 ±700	900 ±710

Table 2. CFU/cm^2 values for M. avium strain A5, M. intracellulare strain TMC 1406^T and M. abscessus strain AAy-P-1 for stainless steel surface

	Strain		
Duration of exposure (hr.)	**A5**	**TMC 1406^T**	**AAy-P-1**
0	280 ±80	700 ±500	40 ±50
1	160 ±40	900 ±700	220 ±110
2	470 ±150	1,200 ±700	710 ±340
3	660 ±370	800 ±400	570 ±200
6	430 ± 280	1,300 ±800	760 ±360

Table 3. CFU/cm^2 values for *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T and *M. abscessus* strain AAy-P-1 for galvanized (zinc) surface

	Strain		
Duration of exposure (hr.)	**A5**	**TMC 1406^T**	**AAy-P-1**
0	930 ±630	1,200 ±200	3 ±4
1	3,800 ±1,500	2,000 ±300	30 ±20
2	7,300 ±390	2,600 ±1,300	60 ±80
3	9,900 ±4,700	4,900 ±2,100	70 ±50
6	15,100 ±2,400	6,800 ±1,000	140 ±80

Table 4. CFU/cm^2 values for *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T and *M. abscessus* strain AAy-P-1 for copper surface

	Strain		
Duration of exposure (hr.)	**A5**	**TMC 1406^T**	**AAy-P-1**
0	300 ±110	1,100 ±600	60 ±90
1	590 ±210	800 ±900	270 ±340
2	240 ±80	900 ±600	230 ±210
3	860 ±350	1000 ±700	160 ±80
6	1,200 ±310	1,800 ±1,200	330±230

Table 5. CFU/cm^2 values for *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T and *M. abscessus* strain AAy-P-1 for polyvinyl chloride surface

	Strain		
Duration of exposure (hr.)	**A5**	**TMC 1406^T**	**AAy-P-1**
0	1,600 ±980	900 ±500	50 ±30
1	2,900 ±2,200	2,300 ±600	20 ±10
2	1,100 ±870	700 ±400	50 ±30
3	730 ±320	2,000 ±1,700	150 ±70
6	2,700 ±1,000	2,500 ±1,800	40 ±30

Table 6. Surface types by adherence (highest to lowest)

Strain	Ranking of Adherence
M. avium strain A5	galvanized, PVC, glass, copper, stainless steel
M. intracellulare strain TMC 1406^{T}	galvanized, PVC, copper, stainless steel, glass
M. abscessus strain AAy-P-1	glass, stainless steel, copper, galvanized, PVC

Measurement of biofilm formation. The results of the biofilm experiments are shown in Tables 7-9. These experiments separated adherence from biofilm formation, and as a result, the increase in cell numbers can be entirely attributed to growth and biofilm formation, and not continued accumulation of cells.

M. avium strain A5 biofilm formation was highest on galvanized surfaces, 1.4 $x10^7$ CFU/$cm^{2,}$ after 21 days incubation (Table 7). Biofilm formation of A5 was also high on PVC and glass surfaces with 6.7 $x10^6$ CFU/cm^2 and 2.8 $x10^6$ CFU/cm^2 after 21 days. *M. intracellulare* strain TMC 1406^T biofilm formation was highest on stainless steel surfaces with 3.3 $x10^5$ CFU/cm^2 after 21 days (Table 8). *M. abscessus* strain AAy-P-1 biofilm formation was highest on galvanized surfaces with 1.3 $x10^5$ CFU/cm^2 after 21 days (Table 9).

In contrast, *M. avium* strain A5 biofilm formation was lowest on copper surfaces, with 0.55 $x10^4$ CFU/cm^2 at 21 days incubation. *M. intracellulare* strain TMC 1406^T was biofilm formation was lowest on galvanized surfaces, with .19 $x10^4$ CFU/cm2 at 21 days incubation. *M. abscessus* strain AAy-P-1 biofilm formation was lowest on copper surfaces with .46 $x10^4$ CFU/cm^2 at 21 days. Refer to Table 10 for a complete ranking of surface type by amount of biofilm formation.

Samples of cell suspension were taken from the reactor at 1, 7, 14 and 21 days incubation to ensure absence of contamination and to observe cell numbers throughout the duration of the experiment. For *M. avium* strain A5, plating of water samples yielded approximately 10,000 CFU/mL for every sample taken over the 21-day period. During other experiments, such as those measuring *M.*

intracellulare strain TMC 1406^T biofilm formation on galvanized surfaces, differences of 5-fold or less were observed in the reactor suspension during the 21-day experiment. Refer to Table 11 for CFU/mL in reactor suspension.

Separation of adherence and biofilm formation is key in these experiments. A failure to differentiate between the two processes would prevent an accurate estimate of growth and biofilm formation, as all increases in countable cell numbers might be only further accumulation of adherent cells.

Table 7. *M. avium* strain A5 biofilm formation (CFU/cm^2) over time by surface type

	CFU/cm^2 surface				
Post-Adherence (days)	**Copper**	**Glass**	**PVC**	**Stainless Steel**	**Galvanized**
1	1.5 x10^4 ±0.76	.0023 x10^6 ±0.42	.002 x10^6 ±0.51	3.0 x10^4 ±2.7	9.7 x10^3 ±2.9
7	1.5 x10^4 ±1.15	1.7 x10^6 ±0.66	1.6 x10^6 ±1.1	9.7 x10^4 ±4.7	2.9 x10^3 ±1.1
14	1.2 x10^4 ±0.63	3.6 x10^6 ±1.1	6.4 x10^6 ±2.3	4.1 x10^4 ±2.9	1.5 x10^6 ±.1.0
21	.55 x10^4 ±3.8	2.8 x10^6 ±2.1	6.7 x10^6 ±2.4	2.2 x10^4 ±2.3	1.4 x10^7 ± .53

Table 8. *M. intracellulare* strain TMC 1406^T biofilm formation (CFU/cm^2) over time by surface type

	CFU/cm^2 surface				
Post-Adherence (days)	**Copper**	**Glass**	**PVC**	**Stainless Steel**	**Galvanized**
1	.70 x10^4 ±.24	3.6 x10^3 ±.79	5.5 x10^3 ±.68	.079 x10^5 ±.028	6.1 x10^4 ±3.6
7	.94 x10^4 ±. 55	3.7 x10^3 ±.82	3.2 x10^3 ±1.6	1.2 x10^5 ± .26	1.3 x10^4 ± .31
14	3.1 x10^4 ±.61	4.1 x10^3 ±1.9	10 x10^3 ±.22	6.0 x10^5 ±.45	.08 x10^4 ±.03
21	8.7 x10^4 ±5.5	5.9 x10^3 ±.83	7.6 x10^3 ±1.3	3.3 x10^5 ± 1.2	.19 x10^4± .03

Table 9. *M. abscessus* strain AAy-P-1 biofilm formation (CFU/cm^2) over time by surface type

	CFU/cm^2 surface				
Post-Adherence (days)	**Copper**	**Glass**	**PVC**	**Stainless Steel**	**Galvanized**
1	.022 x10^4 ±.012	.032 x10^4 ±.074	.45 x10^4 ±.95	.36 x10^4 ±. 26	.0018 x10^5 ±.0020
7	.15 x10^4±.33	4.9 x10^4 ±6.8	4.6 x10^4 ±4.6	.76 x10^4 ±. 36	1.4 x10^5 ±1.4
14	2.1 x10^4 ±2.1	2.1 x10^4 ±3.8	1.1 x10^4 ±.38	2.7 x10^4 ±9.7	1.2 x10^5 ± .64
21	.46 x10^4 ±.40	1.9 x10^4 ±2.8	1.1 x10^4 ±.76	4.1 x10^4 ±3.6	1.3 x10^5 ±1.8

Table 10. Surface types by biofilm formation from highest to lowest

Strain	Ranking of Biofilm Formation
M. avium strain A5	galvanized, PVC, glass, stainless steel, copper
M. intracellulare strain TMC 1406^T	stainless steel, copper, PVC, glass, galvanized
M. abscessus strain AAy-P-1	galvanized, stainless steel, glass, PVC, copper

Table 11. Average CFU/mL of *M. intracellulare* strain TMC 1406^{T} in reactor suspension during biofilm experiment with galvanized surfaces

Post-adherence (days)	CFU/mL
1	3,300 ±400
7	800 ±200
14	700 ±70
21	700 ±100

Discussion

These experiments are significant in that they have measured adherence and biofilm formation separately. Studies that do not separate the two cannot distinguish growth and biofilm formation from continued accumulation of cells. After allowing for an initial adherence period of 6 hours, the cell suspension was discarded and new sterile BTW was employed for the remainder of the experiment. Thus any increase in cell numbers was primarily due to growth of adherent cells and not additional accumulation of cells from the surrounding media. To provide a better estimate of adherence and biofilm formation, the cells used in these experiments were not taken directly from nutrient-rich laboratory media. Rather, cells were adapted to sterile Blacksburg tap water by 7 days incubation prior to beginning the experiments. A study by Carter, *et al.* (2003) showed strains of *M. avium*, including A5 to form significantly more biofilm when incubated in water compared with nutrient-rich M7H9 media. The separation of adherence from growth and biofilm formation as well as the use of water-adapted cells simulated natural conditions for biofilms found in household plumbing systems.

The data obtained in these experiments was highly reproducible using the CDC Biofilm reactor and corresponding coupons. Temperature and flow velocity were kept constant for the duration of each experiment and throughout all experiments. Water was obtained from one of two taps in the laboratory connected to chloraminated water from the New River regulated through the Blacksburg-Christiansburg-VPI Water Authority and sterilized prior to use.

Although, the water supply is monitored monthly for hardness and other factors, minor variation is seen on a month-to-month basis. In 2008, water hardness ranged from 42 to 51 mg/L, which is classified by the U.S. Geological Survey as soft water. Regions in which water hardness is increased may yield different results based on ionic conditions. These studies also only examined adherence and biofilm formation in the absence of the normal microbial flora present in Blacksburg tap water. Studies with the normal microbial flora may yield different results, as other microorganisms found in tap water may help or hinder the ability of NTM to adhere to and colonize a surface. Finally, only one strain of each of the three NTM species was employed for these studies. Strain differences may play a role in biofilm formation, as previous studies have linked the ability to form biofilm to virulence (Yamazaki, *et al.*, 2006). For *M. avium* strain A5, galvanized surfaces had a significantly higher overall number of adherent cells after the 6 hour incubation period (6,360 CFU/cm^2) ($p<.0001$) than the other surfaces. PVC (1,830 CFU/cm^2) was also significantly higher than copper (650 CFU/cm^2) ($P=0.0239$) or stainless steel (400 CFU/cm^2) ($p=0.0070$). For *M. intracellulare* strain TMC 1406^T, adherence was also highest on galvanized surfaces over the 6 hour incubation period (3480 CFU/cm2) ($p<.0001$). PVC (1680 CFU/cm^2) was also significantly higher than copper ($p=0.0245$), stainless steel ($p=0.0053$) and glass ($p<.0001$). However, for *M. abscessus* stain AAy-P-1, glass and stainless steel showed the highest average cell adherence over the 6 hour incubation period with 550 CFU/cm^2 and 460 CFU/cm^2 respectively, which was significantly higher than copper ($p<.0014$), galvanized ($p<.0001$), and PVC ($p<.0001$)

surfaces. Of all the 3 strains and 5 surface types tested, strain A5 had the highest adherence on galvanized surfaces. In another study examining different strains of *M. avium*, A5 was observed to form the most biofilm after 14 days incubation in water on PVC surface (Carter, *et al.*, 2003).

Based on the high rate of adherence of A5 to galvanized coupons, high growth and biofilm formation was expected for the 21-day biofilm experiment. Of all of the strains and surface types tested, A5 had the highest biofilm formation on galvanized surfaces with 1.4 $x10^7$ CFU/cm^2. Overall biofilm formation on galvanized surfaces was significantly higher than glass ($p=0.0187$), stainless steel ($p<.0001$) and copper ($p<.0001$). Biofilm formation of A5 was also high on PVC surfaces with 6.7 $x10^6$ CFU/cm^2 at 3 weeks incubation. Overall biofilm formation of *M. intracellulare* strain TMC 1406^T was significantly higher on stainless steel surfaces than all other surfaces ($p<.0001$). Finally, overall biofilm formation of *M. abscessus* strain AAy-P-1 was significantly higher on galvanized surfaces than all other surfaces ($p<.0001$). For NTM patients and those at risk for developing NTM disease, household plumbing systems employing these piping materials should be avoided. Household plumbing systems do not employ glass pipes, however, many water heaters are made of stainless steel and lined with glass. Storage tank water heaters are the most common type of water heater. The tanks are cylindrical and approximately 5 ft in height and 2 ft in diameter. Given that the tank is entirely glass-lined, this would provide approximately 35,000 cm^2 surface area for colonization. For *M. avium* strain A5, this could yield as many as 9.8 $x10^{10}$ CFU, as well as 2.1 $x10^8$ CFU of *M. intracellulare* strain

TMC 1406^T and 6.7 x10^8 CFU of *M. abscessus* strain AAy-P-1 per household water heater. These calculations were based on biofilm data obtained at 3 weeks incubation. One way of reducing the risk of survival of NTM in a household water heater is to raise the water temperature to 50° C (122° F). In a study by Falkinham (2011), households with water heater temperatures below 50° C were significantly more likely to contain NTM.

The high adherence and biofilm formation of *M. avium* strain A5 on galvanized surfaces could be attributed in part to surface roughness. The galvanized surfaces used in these experiments were significantly rougher than the glass, copper, stainless steel and PVC surfaces. The rougher texture of the surface creates a more suitable environment for microorganisms as it provides larger surface area for adherence as well as protects microorganisms from shear forces. High adherence on galvanized steel surfaces has also been observed in other microorganisms such as *Pseudomonas* and *Aeromonas* spp. (Dogruoz *et al.*, 2009). However, the same study also found that initial colonizers of galvanized surfaces such as *Pseudomonas aeruginosa* were not part of the biofilm after 19 days. This could be in part due to physicochemical properties of the galvanized surface. Past research has shown zinc and copper to be toxic to microorganisms. (Cohen, *et al*., 1991; Utgikar, *et al*., 2003) Galvanized pipes were common in houses built before the 1960's and are also used in cooling towers and storage tanks. The incidence of NTM disease is higher in the elderly, who may also have a higher likelihood of residing in an older home.

In contrast, biofilm formation by A5 was significantly lower on copper and stainless steel surfaces. TMC 1406^T biofilm formation was lowest on glass and PVC and AAy-P-1 biofilm formation was lowest on copper. These surfaces may provide safer alternatives for the household plumbing systems of NTM patients and those at risk for developing NTM disease in the future. The average household plumbing system contains approximately 15,000 cm^2 surface area, which is readily available for colonization by NTM. This includes approximately 9,000 cm^2 of ¾" diameter piping and 6,000 cm^2 of ½" diameter piping. Thus, a household containing all galvanized pipes may contain up to 2.1 x 10^{11} CFU of *M. avium*. In contrast, a household containing all copper pipes could contain 8.3 x 10^7 CFU of *M. avium*. Refer to Table 11. for a complete breakdown of potential mycobacterial CFU based on household piping surface type.

This data is important as it allows consumers to choose household plumbing systems that would harbor less NTM and decrease their risk for developing NTM disease in the future. This data also gives current patients insight on how large a role their homes and plumbing systems are playing in their health. Future studies would expand the number of strains tested as well as the variety of surface types. As surface roughness may play a role in the high adherence and biofilm formation seen in the data, further study examining rough and smooth surfaces are warranted.

Table 12. Potential mycobacterial CFU in a home, based on strain and household plumbing surface type available. Values calculated based on biofilm data collected at 3 weeks incubation.

Strain	Household plumbing type	Total mycobacterial CFU
M. avium strain A5	Copper	8.3×10^{7}
	Stainless Steel	3.3×10^{8}
	PVC	1.0×10^{11}
	Galvanized	2.1×10^{11}
M. intracellulare strain TMC 1406^{T}	Copper	1.3×10^{9}
	Stainless Steel	5.0×10^{9}
	PVC	1.1×10^{8}
	Galvanized	2.9×10^{7}
M. abscessus strain AAy-P-1	Copper	6.9×10^{7}
	Stainless Steel	6.2×10^{8}
	PVC	1.7×10^{8}
	Galvanized	2.0×10^{9}

References

Arnold JW, GW Bailey. 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning, electron and atomic force microscopy study. Poult Sci. 79: 1839-1845.

Beggs, ML, JT Crawford, and KD Eisenach. 1995. Isolation and sequencing of the replication region of Mycobacterium avium plasmid pLR7. J. Bacteriol. 177:4836-*4840*

Bendinger B, HHM Rijnaarts, K Altendorf, AJB Zehnder. 1993. Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain length of mycolic acids. Appl Environ Microbiol. 59:3973–7.

Brennan PJ and H Nikaido. 1995. The envelope of mycobacteria. Annu Rev Biochem. 64: 29-61.

Camper AK, WL Jones, and JT Hayes. 1996. Effect of growth conditions and substratum composition on the persistence of coliforms in mixed-population biofilms. 4014-4018.

Carter GM, M Wu, DC Drummond, LE Bermudez. 2003. Characterization of biofilm formation by clinical isolates of Mycobacterium avium. J Med Microbiol 52: 747-752.

Chatterjee, D. 1997. The mycobacterial cell wall: structure, biosynthesis and sites of drug action Curr. Opin. Chem. Bioll.

Chin DP, PC Hopewell. 1992. Mycobacterium avium complex in the respiratory

or gastrointestinal tract precedes MAC bacteremia. *Front Mycobacteria*. 15.

Cole, ST, R Brosch, J Parkhill, T Garnier, C Churcher, D Harris, SV Gordon, K Eiglmeier, S Gas, CE Barry 3rd, F Tekaia, K Badcock, D Basham, D Brown, T Chillingworth, R Connor, R Davies, K Devlin, T Feitwell, S Gentles, N Hamlin, S Holroyd, T Hornsby,K Jagels, A Krogh, J McLean, S Moule, L Murphy, K Oliver, J Osborne, MA Quail, MA Rajandream, J Rogers, S Rutter, K Seeger, J Skelton, R Squares, S Squares, JE Sulston, K Taylor, S Whitehead, and BG Barrell. 1998. Deciphering the biology of M. tuberculosis from the complete genome sequence , Nature.

Covert, TC, MR Rodgers, AL Reyes and GN Jr. Selma.1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl Environ Microbiol 65, 2492-2496

Dai X, J Boll, ME Hayes, DE Aston. 2004. Adhesion of Cryptosporidium parvum and Giardia lamblia to solid surfaces: the role of surface charge and hydrophobicity. Colloids Surf B Biointerfaces. 34: 259-263.

De Groote MA and G Huitt. 2006. Infections due to rapidly growing mycobacteria. Clinical Infectious Diseases. 42 (12): 1756-1763

Dogruoz, N, D Goksay, E Ilhan-Sungur, A Cotuk. 2009. Pioneer colonizer microorganisms in biofilm formation on galvanized steel in a simulated recirculating cooling-water system. Journal of Basic Microbiology. 40, S5-S12.

Dogruoz, N, B Minnos, E Ilhan-Sungur, A Cotuk. 2009. Biofilm Formation on Copper and Galvanized Steel Surfaces in a Cooling-Water System. IUFS Journal of Biology. 68(2): 105-111.

Donlan RM, R Murga, J Carpenter, E Brown, R Besser and B Fields. 2002. Monochloramine disinfection of biofilm-associated Legionella pneumophila in a potable water model system. Legionella. 406-410.

Donlan, R M. 2002. Biofilms: Microbial Life on Surfaces.Emerging Infectious Diseases. 8 (9).

Ebihara T, H Sasaki. 2002. Image in clinical medicine. Bronchiectasis with *Mycobacterium avium* complex infection. *N Engl J Med*. 346: 1372–1378.

Falkinham, J.O. III., B.C., Parker and H. Gruft. 1980. Epidemiology of infection by nontuberculous mycobacteria. I. Geographic distribution in the eastern United States. *Am. Rev. Respir. Dis.* 121: 931-7.

Falkinham JO III, CD Norton, MW LeChevalier. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other mycobacteria in drinking water distribution systems. Appl Environ Microbiol. 67: 1225-1231.

Falkinham JO III, MD Iseman, P de Haas, D van Soolingen. 2008. Mycobacterium avium in a shower linked to pulmonary disease. J Water Health. 6:209-213.

Falkinham JO III. 2007. Growth in catheter biofilms and antibiotic resistance of Mycobacterium avium. J Med Microbiol. 56: 250-254.

Falkinham JO III. 2011. Nontuberculous mycobacteria from household plumbing of patients with Nontuberculous mycobacter disease. Emerging Infectious Disease. 3: 419-424.

Fang, HHP, L Xu, and K Chan. 2002. Effects of toxic metals and chemicals on biofilm and biocorrosion. Water Research 36: 4709-4716.

Fletcher M. 1988. Attachment of Pseudomonas fluorescens to glass and influence of electrolytes on bacterium-substratum separation distance. J Bacteriol. 170: 2027-2030.

Fry, KL, PS Meissner, and JO Falkinham III. 1986. Epidemiology of infection by nontuberculous mycobacteria. VI.Identification and use of epidemiologic markers for studies of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Am. Rev. Respir. Dis. 134:39-43

Geesey GG, RJ Gillis, R Avci, D Daly, WA Hamilton, P Shope and G Harkin. 1996. The influence of surface features on bacterial colonization and subsequent substratum chemical changes of 316l stainless steel. Corros. Sci 38: 73-95

George, KL, AT Pringle and JO Falkinham III. 1986. The cell surface of Mycobacterium avium-intracellulare and M. scrofulaceum: effect of specific chemical modifications on cell surface charge*. Microbios*. 45: 199-207.

Goeres DM, LR Loetterle, MA Hamilton, R Murga, DW Kirby, RM Donlan. 2005. Statistical assessment of a laboratory method for growing biofilms. Microbiology. 151: 757-762.

Ichiyama, S, K Shimokata, and M Tsukamura. 1988. The isolation of

Mycobacterium avium complex from soil, water, and dust. Microbiol. Immunol. 32:733-739.

Kennedy TP and DJ Weber. 1994. Nontuberculous mycobacteria. An underappreciated cause of geriatric lung disease. Am J Respir Crit Care Med. 149: 1654-1658.

Kim KY, JF Frank. 1994. Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. J Food Prot. 58: 246-251.

Lawrence, JR, PJ Delaquis, DR Korber and DE Caldwell. 1987. Behavior of Pseudomonas fluorescens Within the Hydrodynamic Boundary Layers of Surface Microenvironments. Microb Ecol. 14: 1-14.

Lehtola MJ, J Talis, IM Miettinen, T Vartiainen, and PJ Martikainen. 2004. Formation of biofilms in drinking water distribution networks, a case study in two cities in Finland and Latvia. J Ind. Microbiol. Biotechnol. 31:489-494.

Lehtola, MJ, M Laxander, IT Miettinen, TV Hirvonen, and P Martikainen. 2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. *Wat. Res*. 40, 2151-2160.

Lehtola MJ, E Torvinen, J Kusnetsov, T Pitkanen, L Maunula, CH von Bonsdorff, PJ Martikainen, SA Wilks, CW Keevil, IT Miettinen. 2007. Survival of Mycobacterium avium, Legionella pneumophila, Escherichia coli, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Appl Environ Microbiol 73:2854–2859.

Liu Y, S Yang, Y Li, H Xu, L Qin, J Tay. 2004. The influence of cell and substratum surface hydrophobicities on microbial attachment. J Bacteriol. 110: 251-256.

Marras, TK and CL Daley. 2002. Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin Chest Med 23, 553-567

Mayer, BK, and JO Falkinham III. 1986. Catalase activity and its heat Methods Enzymol. 310: 608-628.

Minnikin, DE. 1991. Chemical principles in the organization of lipid components in the mycobacterial cell envelope. *Res Microbiol* 142**:** 423–427.

Minnikin, DE. 1982. Lipids: Complex lipids, their chemistry, biosynthesis and roles. In The Biology of the Mycobacteria: Physiology, Identification and Classification. Ratledge, C., and Stanford, J. (eds). London: Academic Press, pp. 95– 184

Mueller, RF, WG Characklis, WL Jones, and JT Sears. 1992. Characterization of Initial Events in Bacterial Surface colonization by two Pseudomonas species using image analysis. Biotechnology and Bioengineering. 39: 1161-1170.

Norton CD, MW LeChevallier, JO Falkinham III. 2004. Survival of Mycobacterium avium in a model distribution system. Water Research 38: 1457-1466.

Percival SL, JS Knapp, DS Wales, R Edyvean. 1998. Biofilm, stainless steel and mains water. Wat Res 32: 2187-2201

Pittner, GA, and G Bertler. "Point-of-use Contamination Control of High Purity

Water Through Continuous Ozonation, *Ultrapure Water 5(4)*, pp. 16-22. May/June 1988

Potera C. 1999. Forging a link between biofilms and disease. Science. 283: 1837-1839

Rijnaarts HHM, W Norde, J Lyklema, AJB Zehnder. 1999. DLVO and steric contributions to bacterial deposition in media of different ionic strengths. J Colloid Interface Sci. 14: 179-195.

Stamm, LM, **JH Morisaki, LY Gao, RL Jeng, KL McDonald, R Roth, S Takeshita, J Heuser, MD Welch, and EJ Brown.** 2003. *Mycobacterium marinum* Escapes from Phagosomes and Is Propelled by Actin-based Motility. JEM *vol. 198 no. 9 1361-1368*

Steed KA, and JO Falkinham III. 2006. Effect of growth in biofilms on chlorine susceptibility of Mycobacterium avium and Mycobacterium intracellulare. Appl Environ Microbiol 72:4007-4100.

Tebbs SE, A Sawyer and TS Elliot. 1994. Influence of surface morphology on in vitro bacterial adherence to central venous catheters. Br J Anaesth 72: 587-591.

Torriani FJ, CA Behling, JA McCutchan, RH Haubrich, and DV Havlir. 1996 . Disseminated *Mycobacterium avium* complex: correlation between blood and tissue burden. J Infectious Disease. 173: 942-949.

Torvinen E, MJ Lehtola, PJ Martikainen, and IT Miettinen. 2007. Survival of M. avium in drinking water biofilms as affected by water flow velocity,

availability of phosphorus, and temperature. Applied Environ Microbiol. 6201-6207

Verran J, and KA Whitehead. 2006. The effect of surface topography on the retention of microorganisms. Food Bioprod Proc. 84(C4): 253-259.

Walker SL, JE Hill, JA Redman, M Elimelech. 2005. Influence of the growth phase on adhesion kinetics of Escherichia coli D12g. Appl Environ Microbiol. 71: 3093-3099.

Williams, MM., and RM Donlan. 2005. Mycobacterium fortuitum biofilm formation on water distribution pipe materials. In Distribution system bio-film control. Proceedings of the American Water Works Association Water Quality Technology Conference and Exposition, Quebec City, Canada, 6 to 10 November 2005.

Witty LA, VF Tapson, CA Piantadosi. 1994. Isolation of mycobacteria in patients with pulmonary alveolar proteinosis. Medicine (Baltimore) ;73:103–9.

Wolinsky E. 1995. Mycobacterial lymphadenitis in children: a prospective study of 105 nontuberculous cases with long-term follow-up. Clin Infect Dis. 20: 954-963.

Yamazaki Y, L Danelishvili, M Wu, E Hidaka, T Katsuyama, B Stang, M Petrofsky, R Bildfell, L Bermudez. 2006. The ability to form biofilm influences Mycobacterium avium invasion and translocation of bronchial epithelial cells. Cellular Microbiology. 8(5): 806-814.

Zelver N, M Hamilton, B Pitts, D Goeres, D Walker, P Sturman and J Heersink. 1999. Measuring antimicrobial effects on biofilm bacteria: from laboratory to field.

Zhi XY, WJ Li, and E Stackebrandt. 2009. An update of the structure and16S rRNA gene sequence-based definition of higher ranks of the class Actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. Int. J. Syst. Evol. Microbiol. 59:589 – 608