Aerosolization of Microbial Pathogens and Indicator to Assess their Transport and Dispersion in Air

by

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ABSTRACT

"Airborne dispersal of microorganisms influences their biogeography, gene flow, atmospheric processes, human health and transmission of pathogens that affect humans, plants and animals" (Alsved et al., 2018). Many airborne pathogens cause diseases, such as Legionnaires disease, which is a type of pneumonia caused due to *Legionella*. Since the first report of a *Legionella* outbreak in 1976, or reports of Non – tuberculous Mycobacterium (NTM) outbreaks in hospital and healthcare settings by the CDC, it is significant to understand the behavior, occurrence and persistence of opportunistic pathogenic aerosols in the atmosphere. This study comprises a literature review and experimental work on airborne dispersion of 4 microorganisms – *E. coli*, *Legionella pneumophila*, *Mycobacterium phlei* and bacteriophage P22. The literature review summarizes their characteristics, their potential sources, disease outbreaks, collection and detection methodologies, environmental conditions for their growth and survival and few recommendations for reducing potential outbreaks. Aerosolization of each of these microorganisms was carried out separately in a closed environment using a spray gun and a nebulizer. The spraying time consisted of 1 sec, 5secs or 10secs, from one end of a chamber, and collecting air sample from the other end of the chamber, using a microbial air sampler. The air sample collection was performed to understand their transport, dispersion and reduction in air. *Legionella* showed a log reduction of ~4 using spray gun and ≤0.6 using nebulizer, whereas *Mycobacterium* showed a log reduction of ~4.5 using spray gun and ≤0.7 using nebulizer, respectively. Bacteriophage P22 on the other hand showed a 4 log reduction using spray gun and ≤1.4 using the nebulizer. This shows that aerosolization of microorganisms depends on its cell structure, size and survivability.

Legionella follows the air – to – water transmission route, and *Mycobacterium* is hydrophobic, due to which their aerosols are more stable and active, than *E. coli*. Other environmental properties such as relative humidity and temperature impact the transport and dispersion of microorganisms in air.

The experiments in this study validated the aerosolization and transport of *Legionella, Mycobacterium* and bacteriophage P22 in a closed environment over time. In general, microbial concentration collected in air increased with aerosolization time of the test water. On the other hand, their concentration significantly decreased as elapsed time progressed after aerosolization, due to settling effect of larger particles and potential reduction due to inactivation of bacterial and viruses in the air.

DEDICATION

I would like to dedicate this manuscript/thesis to my mother, father, brother, grandmother and grandfather. Without their unconditional love, support, encouragement and motivation, none of this would have been possible.

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CHAPTER 1

BACKGROUND

1.1 Introduction and Motivation

It is estimated that $\sim 10^{24}$ bacteria are emitted to the atmosphere each year on a global scale (Burrows et al., 2009). These bacteria remain suspended in air in form of aerosols, also known as bioaerosols. Bioaerosols are a subcategory of particles from the biological origin containing living and/or dead microorganisms. “Their study requires an intense interdisciplinary approach encompassing atmospheric chemistry, microbiology, aerosol microphysics, climate and medical sciences, and an understanding of diverse physical processes including human inhalation, ice nucleation, cloud formation and aerial dispersal” (Fernandez et al., 2018). Studies related to bioaerosols are being carried out since the 19th century but their research has increased in the past decades due to previous and current epidemics. To reduce the possibility of potential outbreaks of pathogens or opportunistic pathogens, it is important to study their survival strategies in air, use of proper methods for aerosolization, environmental conditions, methods of air sample collection and the detection methodology after aerosolization (Marthi 1990). It is thus, necessary to study the behavior of microorganisms in the air to further understand their impact on human health. For this project, *E. coli*, *Legionella pneumophila*, *Mycobacterium phlei* and bacteriophage P22 aerosolization and reduction were studied under laboratory conditions.

E. coli is a gram - negative, facultative anaerobic, rod - shaped coliform bacterium found in environment and the intestine of warm blooded organisms (Singleton 1999). Most *E. coli* strains are harmless but some can cause food poisoning and diarrhea

(CDC 2020). These bacteria can grow easily in a laboratory environment and are being studied for more than 60 years.

E. coli is used as an indicator organism to check for fecal contamination of drinking water (U.S. EPA 2012). Even though most *E. coli* strains do not cause diseases, some virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease. "*E. coli* is not usually known to spread diseases due to its bioaerosols, but studies have shown its transmission in air. "In general the survival of *E. coli* in air is higher at lower temperatures and higher humidity" (Wathens et al., 1986).

Legionella bacteria are found in natural aquatic environments, in fresh and marine waters, can occur in waters with various temperatures, pH, nutrient, and oxygen contents (Kozak et al., 2013). They are fastidious gram-negative aerobic bacilli (CDC 2005). *Legionella* in water may pose a health risk if aerosolized (i.e. in an air conditioning system or a shower) and then inhaled. Inhalation can result in a type of pneumonia known as Legionnaires disease (CDC 2005).

"Currently an estimated 10,000–15,000 cases of Legionnaires' disease are reported in the United States per year. The causative agent, *Legionella pneumophila*, is a vegetative Gram-negative bacterium that is ubiquitous in freshwater environments worldwide. It survives as an intercellular parasite of protozoa thereby resulting in protection for the bacterium while in the environment. Infective aerosols are generated from contaminated water spray devices such as showerheads, evaporative condensers, humidifiers, and fountains. Following the inhalation, *L. pneumophila* replicates in host macrophage resulting in severe pneumonia" (Stetzenbach 2009)

Legionella is a unique pathogen due to its water-to-air transmission route, transmission is more likely in - built environments that contain aerosol-generating features (Prussin II et al., 2017). These systems include cooling towers, evaporative condensers, plumbing equipment (i.e. faucets, showerheads, hot water tanks), humidifiers, respiratory-therapy equipment (i.e. nebulizers), and whirlpool baths (CDC 2018).

Non – tuberculous mycobacterium (NTM) are opportunistic pathogens with more than 160 different species of NTM (ATS 2007). NTM are found naturally in the environment including natural water sources (lakes, rivers streams). In municipal water (drinking water and showers) though, they can get aerosolized and transmitted to humans (CDC 2019). NTM can also form biofilms which could be present in moist environments like in premise plumbing (CDC 2019).

Most NTM disease cases involve the species known as *Mycobacterium avium complex* (MAC), *M. abscessus*, *M. fortuitum* and *M. kansasii*. *M. abscessus* is being seen with increasing frequency and is particularly difficult to treat (ATS 2007). The most common clinical manifestation of NTM disease is lung (pulmonary) disease, but lymphatic, skin/soft tissue, and disseminated disease are also important (ATS 2007).

Bacteriophage P22 of the *Podoviridae* family infects *Salmonella typhimurium* (The Bacteriophages 2005). It was the first generalized transducing phage to be discovered – a small fraction (~2%) of its virions carry a fragment of the host DNA instead of phage DNA, and this host DNA can be delivered into a host cell (Tsipis et al., 1972). P22 was used in this study as a surrogate for viruses and to understand and estimate their aerosol transport and dispersion.

1.2 Objectives

The main objective of this research is to study transmission of microorganisms through air. The specific objectives include:

- To document transport kinetics and microbial log reduction in air
- To measure suspension and transport of selected microorganisms using pressurized aerosolization
- To estimate the inactivation and reduction of microorganisms in air over time under laboratory conditions

CHAPTER 2

LITERATURE REVIEW

2.1 *Legionella*

In 1976, *L. pneumophila* was first noticed following a pneumonia outbreak at a Convention of the American Legion in Philadelphia. This outbreak caused a type of pneumonia called Legionnaires disease. Two hundred twenty - one people were infected with Legionnaires disease and 34 of those infected died (EPA 1999). *L. pneumophila* infects the lung tissue of humans. *Pneumophila* originates from the Greek word meaning "lung-loving" (Fang et al., 1989). The genus Legionella currently includes more than 50 bacterial species (abbreviated as "spp.") and approximately 70 distinct serogroups, many of which are considered pathogenic (DSMZ, 2014; LPSN, 2014; Pearce et al.,, 2012; WHO, 2007; Fields et al.,, 2002).

There are 53 known species of *Legionella*, and eighteen of the 53 species have been linked to pneumonia in humans (Lück et al., 2010). In addition, more than 40 isolates have been identified as *Legionella*-like organisms (LLOs) in the Centers for Disease Control and Prevention (CDC) strain collection. These LLOs resemble members of the *Legionellaceae* morphologically and require l-cysteine for growth. Seven of the 53 species are further divided into serogroups (Benson 1998). The bacterial strains within a species that can be divided by serotype are genetically homologous (based on DNA hybridization experiments), but can be differentiated by specific reactivity to antibodies (EPA 1985). Eighteen of the 53 species of *Legionella* have been linked to patients with pneumonia (Bangsborg 1997). The species *L. pneumophila* has sixteen serogroups, where serogroups one through six have been identified as the main cause of human

outbreaks of legionellosis. Legionellosis includes both Legionnaires disease and Pontiac fever. Legionnaires disease is a potentially fatal multi-system disease involving pneumonia and Pontiac fever is a self-limited influenza-like infection (Hoge and Brieman 1991). Pneumonia occurs in approximately 95 percent of *Legionella* infections (Nguyen et al., 1991). The second species of *Legionella*, *L. micdadei*, was discovered within two years of identifying *L. pneumophila* (Dowling et al., 1992). In the following years, advances in growth and enrichment media, combined with clinical and environmental studies, allowed for the discovery of numerous species of *Legionella* (Brenner 1987).

Legionella bacteria are fastidious gram-negative aerobic rods, which cause respiratory infections (EPA 1999). They are un-encapsulated, non-spore-forming, with physical dimensions from 0.3 to 0.9 micrometers (μm) in width and from 2 to 20 μm in length (Winn 1988). Most exhibit motility through one or more polar or lateral flagella (EPA 2001). The cell walls of *Legionella* contain significant amounts of both branched-chain cellular fatty acids and ubiquinones with side chains of more than 10 isoprene units, which make them unique (EPA 2001). These bacteria are aerobic, microaerophillic, and have a respirative metabolism that is non-fermentative and is based on the catabolism of amino acids for energy and carbon sources (Brenner et al., 1984).

Legionella are found everywhere in nature, but they exist primarily in aquatic environments (Fields 1996). *Legionella* can survive in varied water conditions, in temperatures of 0 to 63 °C, a pH range of 5.0 to 8.5, and a dissolved oxygen concentration in water of 0.2 to 15 ppm (Nguyen et al., 1991). Optimal temperatures for culturing are 35 to 37 °C (EPA 1985).

Experiments have demonstrated that *Legionella* in sterile tap water show long-term survival but do not multiply, whereas *Legionella* in non-sterile tap water survive and multiply (Surman et al., 1994). Furthermore, *Legionella* viability is maintained when they are combined with algae in culture, whereas *Legionella* viability decreases once the algae are removed (Winn 1988). The multiplication of *Legionella* depends on their relationships with other microorganisms.

It was first shown that *Legionella* shares a symbiotic relationship with other microorganisms when *L. pneumophila* was discovered co-existing in an algal mat from a thermally polluted lake (EPA 1999). In contrast, *Legionella* survive almost entirely as parasites of single-celled protozoa (Fields 1996). This relationship first became apparent to Rowbotham in 1980, with the demonstration of *L. pneumophila's* ability to infect two types of amoeba, *Acanthamoeba* and *Naegleria* (Rowbotham 1980). *Legionella* can infect a total of 13 species of amoebae and two species of ciliated protozoa (Fields 1996).

Legionella also can multiply intra-cellularly within protozoan hosts (Vandenesch et al., 1990). The strains that multiply in protozoa have been shown to be more virulent, possibly due to increased bacterial numbers (Kramer and Ford 1994). *Legionella* can infect and multiply within hosts because it provides them protection from harmful environmental conditions. This mean that they can survive in environments with a greater temperature range, are more resistant to water treatment with chlorine, biocides and other disinfectants, and survive in dry conditions if encapsulated in cysts (EPA 1999). Enhanced resistance to water treatment has major implications for disease transmittance and water treatment procedures (EPA 1999).

Legionella also grow symbiotically with aquatic bacteria attached to the surface of biofilms (Kramer and Ford 1994). Biofilms provide *Legionella* with protection from adverse environmental conditions (including during water disinfection) and nutrients for growth (Kramer and Ford 1994). The concentration of *Legionella* in biofilms depends on the temperature of the water (Kramer and Ford 1994). At higher temperatures, they can out compete other bacteria. *Legionella* have been found in biofilms in the absence of amoeba (Kramer and Ford 1994). Because biofilms colonize drinking water distribution systems, they provide a habitat suitable for *Legionella* growth in potable water, which can lead to human exposure (Kramer and Ford 1994).

Legionella is widely distributed in the aqueous environment in the United States and wherever they are sought (EPA 1985). Research has indicated that *Legionella* thrive in biofilms, and their interaction with other organisms in biofilms is essential for their survival and multiplication in aquatic environments (Kramer and Ford 1994, Yu 1997, Lin et al., 1998a). The survival of *Legionella* is greater when the bacteria form symbiotic relationships with other microorganisms. Sediment within biofilms stimulates the growth of these commensal microflora, which stimulate the growth of *Legionella* (EPA 1999). *Legionella* occurs in natural bodies of water, such as surface water and groundwater, and man-made waters, such as potable water, cooling towers, whirlpools, etc.

Natural Surface Waters

Legionella are present everywhere in the aqueous environment. Several studies clearly demonstrate the widespread occurrence of *Legionella* from natural surface freshwater sources (i.e. lakes and streams) in the United States (CDC 2005). More recent studies indicate that *Legionella* are also common in marine waters (Ortiz-Roque and

Hazen 1987, Palmer et al., 1993). Additionally, *Legionella* has been discovered in ocean water (Palmer et al., 1993). Their present in a nearby swimming area was due to surface runoff from a flood control channel and river. The channel and river were tested and it was determined that the water was contaminated with *Legionella* (Palmer et al., 1993).

Prior to 1985, there were no studies documenting the presence of *Legionella* in groundwater (EPA 1999). More recently, some studies have shown positive samples in water supply system wells for the presence of *L. pneumophila*; however, other studies have shown no positive samples (EPA 1999). More data and research needs to be conducted for *Legionella* in groundwater.

As noted previously, *Legionella* thrive in biofilms. Bacteria in biofilms are relatively resistant to standard water disinfection procedures; therefore, *Legionella* are able to enter and colonize potable water supplies (Kramer and Ford 1994, Lin et al., 1998a). Artificial aquatic habitats (i.e. components of water distribution systems and cooling towers) are believed to function as amplifiers or disseminators of *Legionella* present in potable water (CDC 2013). They can occur in a variety of man-made water sources, including components of internal plumbing systems (i.e. faucets, showerheads, hot water tanks, and water storage tanks), cooling towers, respiratory-therapy equipment, humidifiers, and whirlpools/spas (CDC 2013).

Whirlpools and spas are common because they are maintained at certain temperatures, which are ideal for *Legionella* growth (Hedges and Roser 1991). Additionally, organic nutrients suitable for bacterial growth often accumulate in these waters. Whirlpools and spas can produce water droplets of respirable size that have the potential to transmit *Legionella* to humans (Jernigan 1996). Other related sources of

Legionella include spa filters, spring water spas, and saunas. *Legionella* are found in wastewater, but they are not as common. It is difficult to isolate *Legionella* from wastewater because it contains so many other microorganisms. In a study conducted by Palmer et al., (1995), it was noted that researchers were not able to culture *Legionella* from reclaimed water samples suggesting that chlorine may injure *Legionella* and cause them to enter a viable but non-culturable state.

Additionally, they are present in water distribution systems of hospitals, hotels, clubs, public buildings, homes, and factories. Recent studies confirm that these systems continue to be a major source of *Legionella* exposure (CDC 2013).

The natural habitat for *Legionella* appears to be aquatic bodies and perhaps, for *L. longbeachae*, soil. However, *Legionella* can be found in air as part of aerosols. There are many methods of aerosolizing bacterial cells in a mist of liquid particles for a dispersing system. Spray bottles that contain a liquid undergo pressure and force and emerge as an aerosol or mist. Aerosolization is an important component of *Legionella* transmission from the aquatic environment to the human respiratory system. Aerosol-generating systems that had been linked to disease transmission included cooling towers, evaporative condensers, plumbing equipment (i.e. faucets, showerheads, hot water tanks), humidifiers, respiratory-therapy equipment (i.e. nebulizers), and whirlpool baths (CDC 2013). More recent studies published have confirmed the presence of *Legionella* in aerosols from several of these systems (Bollin et al., 1985, Seidel et al., 1987).

In most cases, disease outbreaks resulting from *Legionella* aerosolization have involved indoor exposure and outdoor exposure to within 200 meters. However, an outbreak that occurred in Wisconsin in which aerosolized *L. pneumophila* from an

industrial cooling tower was disseminated at least one mile (1.6 km) and perhaps up to two miles (3.2 km) (Addiss et al., 1989). Meteorological conditions that suppress vertical mixing and favor horizontal transport of aerosols (i.e. fog, high humidity, and cloud cover) occurred before and intermittently during the outbreak and presumably contributed to the lengthy transport (EPA 1999).

Legionella infection occurs in humans when the bacteria are inhaled or aspirated into lower respiratory tract and subsequently engulfed by enteric pulmonary macrophages (EPA 1999). The bacteria rapidly reproduce within the macrophages and are eventually released when the host cell lyses (EPA 1999). Recent research indicates that the ability of *Legionella* to infect certain strains of amoeba is a factor in their infection of human lung tissue, as the amoeba provides a habitat within the pulmonary system in which the bacteria can live and reproduce (EPA 1999). Resistance to *Legionella* infection is mainly cell-mediated, although humoral immune responses may also play a role (EPA 1999). Legionellosis in humans has typically been characterized as either an acute self-limiting, non-pneumonic condition known as Pontiac fever or a potentially fatal pneumonic condition known as Legionnaires disease (EPA 1999). Once diagnosed with Legionnaires disease, it is important for the patient to receive treatment immediately. Erythromycin has been used in the past to treat patients with Legionnaires disease; however, newer macrolides and quinolones are becoming accepted as the first choice for treatment. Additionally, risk factors for morbidity and/or mortality include: older age, male gender, African-American ethnicity, smoking, nosocomial acquisition of the disease, immunosuppression, end stage renal disease, and cancer (EPA 1999).

After *L. pneumophila* was first noticed at the American Legion Convention, investigations were conducted in order to determine whether previous undetected outbreaks had occurred. The investigations uncovered five additional outbreaks of legionellosis, which were attributed to *L. pneumophila* (EPA 1999). The first occurred in 1965 at St. Elizabeth's Hospital in Washington, D.C. 81 patients became ill with pneumonia, and 14 died (Lowry et al., 1993). The second pneumonia outbreak occurred in 1973 in Benidorm, Spain, and the third occurred in 1974 in the same hotel as the American Legion Convention outbreak of 1976. Additionally, two outbreaks of Pontiac fever occurred, one in Pontiac, Michigan, in 1968 and the other in 1973 in James River, Virginia. Aside from outbreaks, sporadic cases of legionellosis were detected in 1943, 1947, and 1959 (Brenner 1987).

In July 1994, at a hospital in Wilmington, Delaware, for those who lived, worked, or visited within 4 square miles of the hospital, the risk of illness decreased by 20% for each 0.10 mile from the hospital (Brown 1999). Additionally, it increased by 80% for each visit to the hospital, and it increased by 8% for each hour spent within 0.125 miles of the hospital (Brown 1999).

In November 2003, a community-wide outbreak of Legionnaire Disease occurred in Pas-de-Calais, France. There were 86 laboratory-confirmed cases (Nguyen et al., 2006). Eighteen (21%) of 86 cases led to fatality (Nguyen et al., 2006). A case-control study identified smoking, silicosis, and spending >100 min outdoors daily as risk factors. *Legionella* strains were isolated from cooling towers, wastewater and air samples from a petrochemical plant. The strains were assessed using pulsed-field gel electrophoresis subtyping. A model of atmospheric dispersion modeling of aerosols emitted was

conducted and it was determined that dispersion extended over a distance of at least 3.7 miles (6 km) (Nguyen et al., 2006). Additionally, people 7.4 miles (12 km) from the petrochemical plant in France were still infected (Nguyen et al., 2006).

Legionella have the ability to survive in a wide variety of temperatures. Growth has been observed at a water temperature as low as 16.5 °C (Bentham 1993). The highest water temperature of a sample cultivated by Botzenhart et al., (1986) was 64 °C, while Henke and Seidel (1986) claimed *Legionella* to be a "thermoresistant" organism, exhibiting survival in natural warm waters of up to 60 °C and artificially heated waters of 66.3 °C (EPA 1999). The optimal temperatures for the reproduction of *Legionella* are 32 to 45 °C (Vickers 1987, Kramer and Ford 1994).

Legionella growth is also increased by heat and high temperatures found in areas like whirlpools, hot springs, and blast zones (Henke and Seidel 1986, Lee and West 1991, Verissimo et al., 1991). Colbourne and Dennis (1989) contend that although *Legionella* are not thermophilic, they exhibit thermo-tolerance at temperatures between 40 and 60 °C, which gives them a survival advantage over other organisms competing in man-made warm water systems (EPA 1999). Although temperatures between 45 and 55 °C are not optimal for *Legionella*, these temperatures enable them to reach higher concentrations than other bacteria commonly found in drinking water, thus providing *Legionella* with a selective advantage over other microorganisms (Kramer and Ford 1994).

Routine culturing of *Legionella* from the environment is not a common practice; therefore, the occurrence of these bacteria is often indicated by outbreaks or sporadic cases of legionellosis. It would be prudent for routine culturing for *Legionella* to be a mandatory practice in order to prevent outbreaks and sporadic cases of legionellosis from

occurring. This would at least ensure that *Legionella* is identified before conditions worsen. Additionally, it is vital to avoid *Legionella* from transmitting long distances.

Legionellosis has been reported to occur in North and South America, Asia, Australia, New Zealand, Europe, and Africa (Edelstein 1988). Research suggests that Legionnaires disease is under reported to national surveillance systems (Marston et al., 1994, Edelstein 1988). Some physicians are unable to recognize the disease and do not have the resources available to diagnose it.

Although legionellosis has been reported throughout the world, most cases have been reported from industrialized countries. The environmental conditions that support *Legionella* growth (complex recirculating water systems and hot water at 35-55 °C) are not as common in developing countries, so the incidence of legionellosis may be comparatively low in these countries (Bhopal 1993). However, the geographical variation in the number of incidences of legionellosis is due to differences in definitions, diagnostic methods, surveillance systems, and data presentation (Bhopal 1993).

Since new cooling towers and cooling towers recently started up after shutdowns have an increased risk for the growth of the *Legionella* bacteria, it is recommended that the cooling towers be thoroughly cleaned before start-up and routinely cleaned during operation. Another recommendation is to possibly replace the air-intake vents of the air-handling system in the homes and buildings of the nearby communities of the cooling towers with double high-efficiency particulate air filters (Brown 1999). This could be an excellent preventative measure to avoid exposure and to minimize potential outbreaks in high-risk areas.

It is very important for management and control regimens to be established to a high standard. Additionally, cooling towers need to be cleaned routinely with high-pressure hot water every three to four weeks, and disinfection needs to be performed routinely. Pump and pipes need to be manually cleaned routinely because pipes can form layers of solid scale, which support biofilm formation. Biofilm formation enables the *Legionella* to form a symbiotic relationship, which makes the bacteria able to withstand harsh environmental conditions. New routines for cleaning and disinfection need to be implemented. Sampling and *Legionella* cultures to assess treatment need to be performed. New national regulations for aerosol producing installations that could facilitate *Legionella* growth and dispersion need to be established.

2.2 *Mycobacterium phlei*

Interest in NTMs and their associated diseases has varied over the last 4 decades (Donohue et al., 2015). Initial interest arose in the 1970s when state tuberculosis laboratories noticed that many respiratory specimens collected for tuberculosis testing were found to be positive for NTMs (Moulin 1985). A total of 20 species of NTM are currently considered to be of clinical interest: *Mycobacterium avium complex* (MAC), *Mycobacterium kansasii*, and *Mycobacterium abscessus* are the most common NTMs associated with pulmonary disease in the U.S. Human contact with potable water (showering, bathing, drinking, hand washing, and dish washing), soil (gardening), and food are all activities that can result in their exposure (Donohue et al., 2015).

NTM occurrence is usually found nosocomial in hospital and healthcare facilities. A study showed that ~75% of *Mycobacterium avium-intracellular* complex (MAC) isolates identified after successful treatment are the result of reinfection (Wallace Jr et al.,

2014). Little is known regarding the exact occurrence of NTM in such aerosolization systems – Showers, toilet bowls, swimming pools, hot tubs etc.

The term nontuberculous mycobacteria (NTM) generally refers to mycobacteria species other than the Mycobacterium tuberculosis complex and M. leprae (Haworth et al., 2017). NTM are widely distributed in human engineered and household environment. Water distribution systems are thought to be a main transmission route from natural surface water reservoirs to the household. NTM have been isolated from drinking water pipelines (Nishiuchi et al., 2017), and water tanks (Torvinen et al., 2004). NTM are readily aerosolized from natural water and soils due to its surface hydrophobicity (Parker et al., 1983). Naturally occurring aerosolization and subsequent inhalation are the major route of NTM lung diseases. Household and building plumbing systems provide a stable, nutrient-limited, disinfectant-containing habitat that is ideal for NTM growth and persistence. As NTM are relatively resistant to high temperature, these organisms can survive in water heaters and hot water pipes (Jeon 2018). NTM are also found in hospital environment and healthcare settings. Hospital water distribution system may serve as a reservoir of NTM. These organisms contaminate hospital materials and can cause nosocomial outbreaks and pseudo-outbreaks (Sood et al., 2017). Recently, several outbreaks due to M. chimaera have occurred from heater - cooler devices used during open-heart surgery (Williamson et al., 2017) . NTM species have been isolated from house dust and soils (Reznikov et al., 1971) (De Groote et al., 2006). NTM are found in soil and water, but factors influencing transmission from the environment to humans are mostly unknown (Halstrom et al., 2015). Mycobacteria were recovered from materials collected from water-damaged buildings, as well as from microorganisms normally

associated with building materials (Anderson et al., 1997). During reconstruction, those *mycobacteria* could be aerosolized in the dust. Although other microorganisms could be responsible for the respiratory problems, both saprophytic (e.g., *M. terrae*) and pathogenic (e.g., *M. avium*) strains isolated from moldy buildings were capable of inducing inflammatory responses in a mouse macrophage cell line (Hüttunen et al., 2000). Signs and symptoms of pulmonary lung disease caused due to NTM are vague and nonspecific and may include shortness of breath, cough, fatigue, malaise, and weight loss (CDC 2019).

Strategies that are effective in preventing NTM lung disease are still limited. Vaccines and prophylaxis medications are not available (Jeon 2018). Although, on the basis of several physiologic and ecologic characteristics of mycobacteria, several approaches to reduce the impact of Mycobacteria in these settings are possible. Because Mycobacteria are associated with particulates, their numbers in reservoirs can be reduced by removal of particular matter (e.g., filtration) (Falkinham, III 2001). UV light can be used to reduce mycobacterial numbers. Disinfection of Mycobacteria at high temperatures (e.g., 40°C) is more effective at reducing numbers, especially if cells were grown at lower temperatures (e.g., 30°C) (Falkinham III et al., 2001). Recent clinical studies have also revealed that reappearance of the same nontuberculous mycobacterium (NTM) infection is common after successful standard treatment (Wallace Jr. et al., 2014) (Koh et al., 2017). Using pulsed-field gel electrophoresis analysis, Wallace et al., found that ~75% of *Mycobacterium avium intracellulare* complex (MAC) isolates were identified after successful treatment are the result of reinfection.

A study showed that exposure to NTMs occurs primarily through human interactions with water (especially aerosolized). Aerosolization can result in >1,000-fold increase in numbers of viable mycobacterial cells per milliliter of water droplets ejected from water. Mycobacteria in natural aerosols are found in particles and droplets (i.e., <5 μm) that can enter the alveoli of the human lung. Cell surface hydrophobicity, net surface charge, is a major determinant of enrichment in ejected droplets (Parker et al., 1983). Inhalation of such aerosols appears to be the primary transmission route of NTM causing pulmonary disease. This usually occurs in artificial water environments such as hot-tubs and showers, but may involve garden soil and house dust. Mycobacteria may aerosolize more readily than other bacteria as they have highly hydrophobic cell walls (Halstrom et al., 2015). NTM have been isolated from natural water environments in which aerosolization increase the concentration of NTM in the air (Falkinham, III et al., 1996).

In a study conducted across the U.S., 68 taps were sampled 4 times over 2 years. The NTM species detected most frequently were: *Mycobacterium mucogenicum* (52%), *Mycobacterium avium* (30%), and *Mycobacterium gordonae* (25%). Of the taps that were repeatedly positive for NTMs, the species M. *avium*, *M. mucogenicum*, and *Mycobacterium abscessus* were found to persist most frequently. This study also observed statistically significant higher levels of NTM in chloraminated water than in chlorinated water (Donohue et al., 2015). *M. avium* is almost 500 times more resistant to chlorine than is *E. coli* (Taylor et al., 2000). *Mycobacteria*, including *M. avium* and *M. intracellulare*, can survive and grow in phagocytic amoebae and protozoa. *M.*

avium grown in amoebae or protozoa are more virulent (Cirillo et al., 1997) (Strahl et al., 2001).

In July 2015, the Pennsylvania Department of Health notified the Centers for Disease Control and Prevention (CDC) about a cluster of NTM infections among cardiothoracic surgical patients at 1 hospital. A controlled case study was conducted to identify exposures causing infection, examining 11 case-patients and 48 control-patients. Eight (73%) case-patients had a clinical specimen identified as *Mycobacterium avium* complex (MAC) (Lyman et al., 2017). CDC estimates that in hospitals where at least one infection has been identified, the risk of a patient getting an infection from any bacteria was between about 1 in 100 and 1 in 1,000. During spring 2015, investigators in Switzerland reported an outbreak of invasive infections with *Mycobacterium chimaera*, a distinct species within the NTM category *M. avium* complex (MAC), associated with contaminated heater–cooler devices (HCDs) used during cardiopulmonary bypass for cardiac surgery (Sax et al., 2015).

Mycobacterium phlei, which was considered for this study, was chosen due to its non- pathogenic properties and its ability to grow faster than *M. avium, M. mucogenicum,* and *Mycobacterium abscessus.* This non - tuberculosis mycobacterial species, was first described in 1898–1899 (Gordon et al., 1953). It is a saprophytic bacteria which is normally non – pathogenic. It is a rod – shaped bacteria 1.0 to 2.0 micrometers in length (Gordon et al., 1953). However reports first showed that it is pleiomorphic and can exist in a coccoid form under certain environmental conditions (Wyckoff et al., 1933). It is an acid – fast bacteria that can grow at any range from 28 °C to 52 °C (Gordon et al., 1953). *Mycobacterium* can be grown in less than 7 days at 52 °C. *Mycobacterium phlei* growth

in media culture has been particularly studied for the information concerning the growth requirements of the pathogenic acid-fast bacteria since this bacterium is not pathogenic it can easily studied in the laboratory (Paul E. et al., 1998). The specific mycobacteria – iron chelating compound was first discovered in *Mycobacterium* (Francis et al., 1953). It can form biofilms as other *Mycobacterium* species and can be easily aerosolized owing to their hydrophobicity due to lipid – rich outer membrane, which is also a key factor in their survival and proliferation (Bardouniotis et. Al 2001). As an effect of the lipid-rich outer membrane, these organisms are resistant to acid, antibiotics, disinfectants, and high temperature (Bodmer et al., 2000) (Robbecke et al., 1992) (Rastogi et al., 1981). It is non – pathogenic but can cause infections (Aguilar et al., 1989). Surprisingly it has also been known to have anti – cancer properties, in its cell wall DNA complex (MCC) due to which it has been added as an adjuvant in anti – cancer vaccines (Filion et al., 2001).

"The importance of this group of bacteria that includes both environmental and highly pathogenic species such as *Mycobacterium tuberculosis*, the causative agent of tuberculosis, provided incentive for a comparative genomic analysis of different *Mycobacterium* strains. This would expand our knowledge about the genomic content of one member of this group of bacteria and provide insight into its evolutionary path" (Das et al., 2016).

2.3 Bacteriophage P22

P22 is a temperate phage of *Salmonella* that is often described as lambdoid because of its temperate life cycle and similar genetic structure (Hyman et al., 2009). The generalized transducing phage P22 of *Salmonella typhimurium* was involved in the initial

discovery of transduction by Zinder and Lederberg in 1952 (Kutter 2009). The temperate phage vector, originally called PLT 22, is now commonly referred to as P22 and has continued to be the virus of choice for investigating the genetics of this bacterium (Byl et al., 2000). The development of *Salmonella* as a genetic system was made possible by discovery of the generalized transducing phage P22, which mediates genetic crosses between *Salmonella* strains (Roth 2017).

Morphologically P22 is a member of the virus family *Podoviridae*, which encompasses viruses with short, noncontractile tails (Ackermann 1998). Although a great deal is known about the life cycle of bacteriophage P22, the mechanism of phage DNA transport into *Salmonella* is poorly understood. Phage P22 is a temperate, icosahedral, "lambdoid" bacteriophage that is commonly used for generalized transduction in Salmonella and has a short, noncontractile tail that cannot penetrate both the outer and inner membranes of its host. If the ejection proteins facilitate transport of DNA across the cytoplasmic membrane, at least one of the ejection proteins must associate with the Salmonella cytoplasmic membrane (Gerardo 2009). In the case of herpesviruses and adenoviruses, as well as the double-stranded DNA bacteriophages such as P22, the initial product of the viral assembly pathway is not an infectious virion but a closed shell that does not contain DNA. These precursor shells, or procapsids, include proteins not found in the mature virion, but essential for their production. These proteins are termed "scaffolding proteins" (Green. et al., 1996). Though most proteins in tailed bacteriophages remain on the outside of the host cell during bacterial infection, these phages have evolved efficient mechanisms to ensure their genome is safely delivered to the bacterial cytoplasm. For phages such as P22 that infect Gram-negative hosts, the

genome must traverse the outer cell membrane, peptidoglycan layer, periplasmic space, and inner membrane to reach the cytoplasm. DNA delivery by short-tailed phages is poorly understood, and the roles of the ejection proteins remain unclear (McNulty et al., 2018). The double stranded DNA tailed phage virions such as bacteriophage P22 adsorb to specific features on the surface of target cells and then release their genomes through the cell membranes into the cytoplasm by a process called ‘injection’ or ‘ejection’ (Bohm et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Air Sampling

Experiments were conducted under laboratory conditions to measure transport and the dispersion of the selected microorganisms in air. To contain and prevent aerosolization of the opportunistic microorganisms in the laboratory, an airtight plastic container was used and air samples were collected using a PBI SAS-Super ISO Air Sampler (VWR International PBI S.r.L, Vio San Giusto, Milano, Italy) (Figure 1). Aerosolization was carried out using an air spray gun (20oz. HVLP Gravity feed air spray gun) and an aerosol compressor nebulizer (Invacare® Envoy). The air pressure of the gun was controlled using a pressure regulator. The air pressure was kept at 20 psi and 40 psi for initial runs, and then was limited to only 40 psi to maintain consistency in the result and simulate real time premise plumbing and municipality water distribution guidelines. Stock cultures of *E. coli* (ATCC strain 25922), *Legionella pneumophila* (ATCC strain 33152), *Mycobacterium phlei* (ATCC strain 12298) and bacteriophage P22 (ATCC strain 19585 – B1) were used. The host organism for P22 was selected as S*almonella enterica* subsp. enterica serovar Typhimurium (ATCC strain 19585), as recommended by ATCC. Buffered Charcoal Yeast Extract Agar (BCYE) agar media for *Legionella*, Brilliance agar media for *E. coli*, TSA media for both *Mycobacterium* and P22, respectively were prepared and used to culture the microorganisms. All experiments were conducted in a lab grade and safe environment as shown in Figure 2.

Figure 1A: Illustration of Bioscience International Microbial Air Sampler A) without Plate B) with BCYE Plate Inserted C) with TSA Plate Inserted and D) Assembled

The dimensions of the box/chamber used for aerosolization (Sterilite® ClearView Latch™; Townsend, MA, USA) were 87.9 cm, 47.6 cm, and 32.1 cm in length, width, and height, respectively. The volume of the box was 104 L (Figure 2).

Figure 1B: From left to right: Invacare® Envoy nebulizer, Spray gun setup with air pressure regulator and HVLP Gravity feed air spray gun

Figure 2A: Illustration of the chamber/box, Air Sampler, and Air spray gun

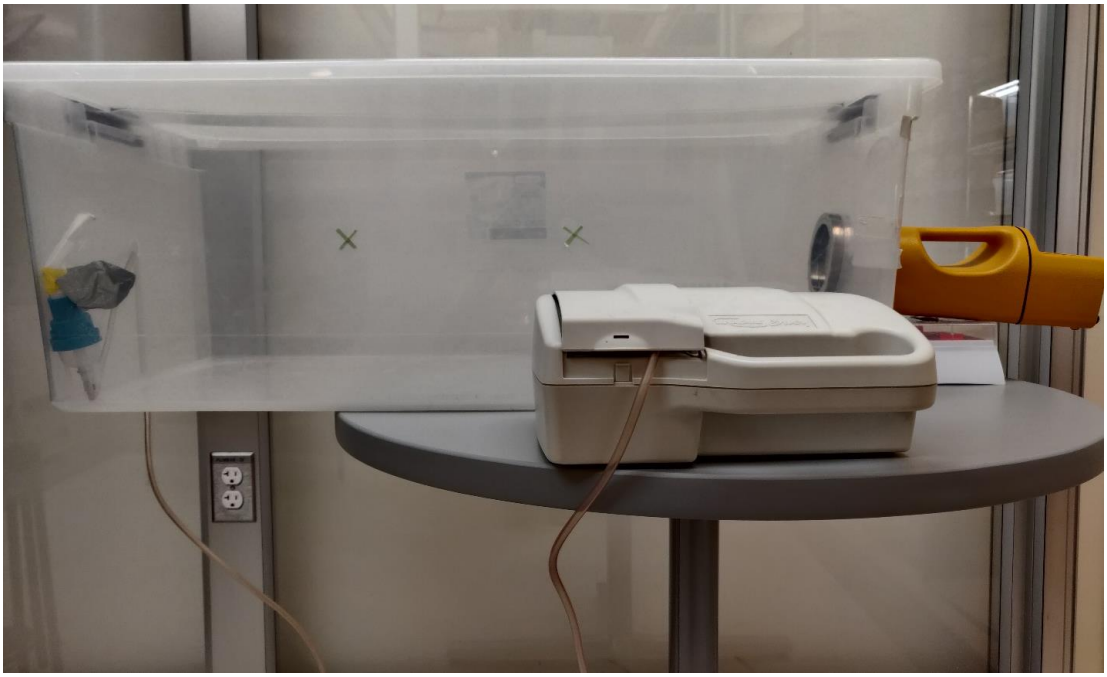


Figure 2B: Illustration of the chamber/box, Air Sampler, and nebulizer

On one side of the box, a 3 cm diameter opening was made for the spray gun and on the opposite side, a 12.5 cm diameter opening was made for the air sampler intake. Spray gun was filled with either 50 mL or 100 mL of Phosphate-Buffered Saline (PBS) containing one of the test microorganisms for the transport and dispersion experiments. Spray volume was determined to be 5mL, for the spray time of 5 seconds. The aerosol compressor had an operating flow rate of 6 lpm, with an operating aerosol compressor air pressure of 10 – 12 psi (Invacare® respiratory products manual). Also, the aerosol compressor gave out high number of particles below 5 microns (~80%). The Air pressure gun on the other hand, sprays out droplets of size of couple hundred microns.

The lid of the box was sealed to ensure that the box was airtight. The microbial sample was spiked in the box from one end and was collected on the other end using the air sampler. The volume of the air collected for each experiment was 100 L. To measure dispersion and exposure to aerosolized bacterial cells in a closed environment, air

samples were collected at different time intervals. After spraying and/or aerosolizing, a series of time elapsed samples were collected. After an initial spray into the box, a specific amount of time (i.e. 1 minute) was allowed to pass for any aerosolized particles to settle before turning on the air sampler. Elapsed time was incorporated into the experiments to allow large water droplets to inactivate and settle due to gravity.

These experiments were repeated with different variables, such as concentration of bacteria and virus (either *Legionella, E. coli, Mycobacterium* and P22) in the spray gun and nebulizer, elapsed time (1 minute, 3 minutes, 5 minutes) and aerosolization time (1second, 5 seconds, 10 seconds). The relative humidity in the chamber at a temperature of 20˚C was 74%, 72% and 70% for a spray time of 5 secs for an elapsed time of 1 min, 3 mins and 5 mins respectively. Similarly, for spray time 1 sec, the humidity was 61%, 60% and 59%. With the results from these various experiments, the behavior of the aerosols in the box were determined in air, which was used to estimate the log reduction of the microorganisms.

Preparation of Media for E. coli

Brilliance agar media (OXOID CM1046), a selective media, was prepared for the detection of *E. coli* and coliform bacteria. The colonies for *E. coli* are purple and red for other coliform bacteria. The first step was to boil 500 mL of DI water on a hot plate at 100 °C. The solution was continuously mixed using a magnetic bar (200 RPM or level 6-7). Next, 14.05 g of Brilliance agar base was weighed and added to the boiling water. After the media had boiled and thoroughly mixed, stirred and cooled. Brilliance agar media were poured into petri dishes. Each petri dish was filled with 15 mL – 20 mL of

Brilliance agar media. The media was cooled for at least 1 hour to let the media solidify, and then plates were ready to be used immediately or stored at 4 °C for later use.

Preparation of Media for Legionella

BCYE agar media (Becton Dickinson 212327) was prepared for the detection and enumeration of *Legionella* bacteria. The first step was to add 450 mL of nano-pure water in a beaker. The beaker was placed on a hot plate (Thermo Scientific Cimarec™ Digital Stirring Hotplates; USA or VWR® Hot Plate/Stirrer; Radnor, PA) at 100 °C until boiled. The solution was continuously mixed using a magnetic bar (200 RPM or level 6-7). Next, 19.15 g of BCYE agar base was added to the boiling water. After media was boiled and evenly mixed, pH was measured using a calibrated probe. An initial pH of 4.5-5 was adjusted to 6.8 – 6.9 by gradually adding pellets or 10N liquid solution of potassium hydroxide (KOH). The volume of water was then adjusted to 500 mL. The BCYE agar media was then autoclaved for 20 minutes at 121 °C with liquid setting. After the media was autoclaved, it was cooled to 50 °C prior to adding 0.4 g/L of L-cysteine. Antibiotics were omitted to enhance its recovery rate since *Legionella* was used in controlled environment. After adding L-cysteine, the media was thoroughly mixed and poured into petri dishes. Petri dishes were filled with 15 mL - 20 mL of media. A control plate was also made without L-cysteine to verify the integrity of *Legionella* stock as wild – type *Legionella* fails to grow in a media lacking L-cysteine. The media was cooled for at least an hour to let media solidify and dry, and then plates were ready to be used immediately or stored at 4 °C in the dark for later use.

Preparation of Media for Mycobacterium

Tryptic Soy Agar (Sigma Aldrich 22091) base was used for culturing *Mycobacterium.* The first step was to boil 500 mL of DI water in a beaker. The beaker was placed on hot plate at 100 °C until boiled. The solution was continuously mixed using a magnetic bar (200 RPM or level 6-7). Next, 20 g of Tryptic Soy Agar (Sigma Aldrich 22091) base was weighed and added to the boiling water. After the media had boiled, it was then autoclaved for 20 minutes at 121 °C with liquid setting. After the media was autoclaved, it was cooled to 50 °C and the poured into petri dishes. Each petri dish was filled with 15 mL – 20 mL of Tryptic Soy agar media. The media was cooled for at least 1 hour to let the media solidify and dry, and then plates were ready to be used immediately or stored at 4 °C for later use.

Preparation of Media for P22

Tryptic Soy Broth (Sigma Aldrich T8907) base was used for culturing P22. The first step was to boil 500 mL of DI water in a beaker. The beaker was placed on hot plate at 100 °C until boiled. The solution was continuously mixed using a magnetic bar (200 RPM or level 6-7). Next, 15 g of Tryptic Soy broth (Sigma Aldrich T8907) base was weighed and added to the boiling water. Next, 1.5 % Agar technical (Becton Dickinson 281230) was added to the mixture. After the media had boiled, it was then autoclaved for 20 minutes at 121 °C with liquid setting. After the media was autoclaved, it was cooled to 47.5 °C in a water bath for at least 90 minutes. It was then poured in the air sampled petri dishes of P22. Any remaining media was pipetted in a sterile petri dish. Each petri dish was filled with 15 mL – 20 mL of media. The media was cooled for at least 1 hour to let the media solidify and dry, and then plates were ready to be used immediately or stored at 4 °C for later use.

Preparation of Phosphate-Buffered Saline (PBS)

The following were dissolved in 800 mL of distilled water to make 0.5 M of PBS: 4 g of sodium chloride (NaCl), 0.1 g potassium chloride (KCl), 0.72 g disodium phosphate (Na_2HPO_4), and 0.12 g monopotassium phosphate (KH_2PO_4). The pH was adjusted to 7.4, and the volume was adjusted to 1 L of water. The solution was sterilized by autoclaving for 20 minutes at 121 °C with liquid setting. After autoclaving, the PBS was allowed to cool to room temperature and then was immediately used or stored at 4°C for later use.

Preparation of Stock Culture for E. coli

Pure culture of *E. coli*, strain 25922, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). An overnight culture was prepared by adding 1 mL of pure culture of *E. coli* to 9 mL of Tryptic Soy Broth (TSB). The 15mL centrifuge tube was incubated at 37°C for 24 hours before use to prepare an overnight culture.

A Nanodrop One/One Microvolume UV – Vis Spectrophotometer (Thermo Fisher Scientific®) was used to determine the concentration of *E. coli*. The nanodrop estimated 1 optical density unit (OD) at 600 nanometers (nM) of *E. coli* to reflect approximately $3x10^8$ colony forming units per milliliter (CFU/mL). The nanodrop required 1 - 2 μL of liquid for analysis, to obtain full-spectral data. The nanodrop was zeroed using 1 - 2 μL DI water. Then 1 - 2 μL of *E. coli* pure culture was placed on the nanodrop base to analyze the concentration.

Preparation of Stock Culture for Legionella

Pure culture of frozen *Legionella* (ATCC strain 33152) stock culture was streaked onto BCYE media and incubated at 37 °C for at least 48 hours allowing for mature colony formation. Colonies were isolated from the BCYE media and dissolved in 0.5mL sterilized Tryptic Soy Broth (Sigma Aldrich T8907) in a microcentrifuge tube (Thomas Scientific, 1.5mL). 0.1 mL of this broth was spread plated on BCYE media and incubated at 37°C for at least 48 hours to allow a monolayered growth of *Legionella* lawn over the media. This lawn was then dissolved in 10 mL PBS and stored in 15 mL centrifuge tubes for immediate use or stored at 4°C for later use.

A Nanodrop One/One Microvolume UV – Vis Spectrophotometer (Thermo Fisher Scientific®) was used to determine the concentration of *Legionella*. The nanodrop estimated 1 optical density unit (OD) at 600 nanometers (nM) of *Legionella* to reflect approximately $3x10^8$ colony forming units per milliliter (CFU/mL). The nanodrop required 1 - 2 μL of liquid for analysis, to obtain full-spectral data. The nanodrop was zeroed using 1 - 2 μL DI water. Then 1 - 2 μL of *Legionella* pure culture was placed on the nanodrop base to analyze the concentration.

Preparation of Stock Culture for Mycobacterium

Pure culture of frozen *Mycobacterium* (ATCC strain 12298) stock culture was streaked onto TSA media and incubated at 37 °C for at least 96 hours allowing for mature colony formation. Colonies were isolated from the TSA media and dissolved in 0.5mL sterilized Tryptic Soy Broth (Sigma Aldrich T8907) in a microcentrifuge tube (Thomas Scientific, 1.5mL). 0.1 mL of this broth was spread plated on TSA media and incubated at 37°C to allow a monolayered growth of *Mycobacterium* lawn over the media. A colony of

Mycobacterium was isolated from the plate and dissolved in 10 mL TSB. This was kept in the incubator for 96 hours. The stock was then immediately used or stored at 4°C for later use.

A Nanodrop One/One Microvolume UV – Vis Spectrophotometer (Thermo Fisher Scientific) was used to determine the concentration of *Mycobacterium*. The nanodrop estimated 1 optical density unit (OD) at 600 nanometers (nM) of *Mycobacterium* to reflect approximately $3x10^8$ colony forming units per milliliter (CFU/mL). The nanodrop required 1 - 2 μL of liquid for analysis, to obtain full-spectral data. The nanodrop was zeroed using 1 - 2 μL DI water. Then 1 - 2 μL of *Mycobacterium* pure culture was placed on the nanodrop base to analyze the concentration.

Preparation of Stock Culture for P22

A pure stock of P22 (ATCC strain 19585 – B1) was cultured using a double agar layer. A *Salmonella enterica* (ATCC strain 19585) overnight culture was prepared using it's pure stock. A *Salmonella* streak plate was prepared and incubated for 48 hours to grow mature colonies. A colony of *Salmonella* was isolated and dissolved in Tryptic Soy Broth (TSB) to prepare an overnight culture. This was used as a host for P22 and using double agar layer method P22 plaques were formed. The monolayer of these plaques were dissolved in PBS, to form a stock culture for P22 and then further diluted to desired concentrations. The stock culture was kept at 4°C to store for later use.

3.2 Microbial Aerosolization and Transport in Closed Environment

A set of experiments was performed to measure *E. coli*, *Legionella*, *Mycobacterium* and P22 aerosolization and transport, respectively in a closed

environment. The experiments were repeated twice for all microorganisms, to obtain duplicate assays.

Preparation of Spiked Samples for Aerosolization and Transport for bacteria

First the concentration of each prepared stock culture was estimated by optical density (O.D.) measurement using a Nanodrop One/One Microvolume UV – Vis Spectrophotometer (Thermo Fisher Scientific®). The OD 600 measurement was approximately $3x10^8$ colony forming units per milliliter (CFU/mL); this number tallied with the spread plates prepared for verification.

Dilutions were conducted in order to achieve the concentration levels necessary to run the air sample collection. These dilutions were mathematically calculated using the equation:

c1V1 = c2V2, where

c1 = Initial concentration or molarity (pure/stock culture of bacteria)

V1 = Initial volume (pure/stock culture of bacteria)

c2 = Final concentration or molarity (Spray and/or test liquid to be aerosolized)

V2 = Final volume (Spray and/or test liquid to be aerosolized)

Concentrations were initially varied from 10^6 CFU/mL to 10^5 CFU/mL to finally 10^4 CFU/mL, after many experimental runs of aerosolization on *E. coli*. The plates incubated after an initial microbial concentration of 10^6 CFU/mL and 10^5 CFU/mL, had innumerable bacterial colonies, which were too numerous to count. To achieve an initial concentration of 10^4 CFU/mL for aerosolization, using the equation above:

c1 = 10^8 CFU/mL (pure/stock culture of bacteria)

V1 = Initial volume (pure/stock culture of bacteria)

c2 = 10^4 CFU/mL (Spray and/or test liquid to be aerosolized)

V2 = 100mL (Spray and/or test liquid to be aerosolized)

$$V1 = 10^4 \frac{CFU}{mL} * \frac{1}{10^8} \frac{mL}{CFUmL} * 100\ mL = \ 0.01\ mL = 10\ \mu L$$

For a spray concentration 10^4 CFU/mL, 10 µL stock solution of the bacteria was mixed with 100mL buffer solution (PBS) in the spray gun container for aerosolization.

For final concentration of test solution as 10^6 CFU/mL, using the same equation:

c1 = 10^8 CFU/mL (pure/stock culture of bacteria)

V1 = Initial volume (pure/stock culture of bacteria)

c2 = 10^5 CFU/mL (Spray and/or test liquid to be aerosolized)

V2 = 100mL (Spray and/or test liquid to be aerosolized)

$$V1 = 10^5 \frac{CFU}{mL} * \frac{1}{10^8} \frac{mL}{CFUmL} * 100\ mL = \ 0.1\ mL = 100\ \mu L$$

For a spray concentration 10^5 CFU/mL, 100 µL stock solution of the bacteria was mixed with 100mL buffer solution (PBS) in the spray gun container for aerosolization.

Similarly, for final concentration of test solution as 10^6 CFU/mL, using the same equation:

c1 = 10^8 CFU/mL (pure/stock culture of bacteria)

V1 = Initial volume (pure/stock culture of bacteria)

c2 = 10^6 CFU/mL (Spray and/or test liquid to be aerosolized)

V2 = 50mL (Spray and/or test liquid to be aerosolized)

$$V1 = 10^6 \frac{CFU}{mL} * \frac{1}{10^8} \frac{mL}{CFUmL} * 50\ mL = \ 0.5\ mL$$

For a spray concentration 10^6 CFU/mL, 0.5 mL stock solution of the bacteria was mixed with 100mL buffer solution (PBS) in the spray gun container for aerosolization.

The final concentration of the test solution was adjusted to 10^4 CFU/mL. Five milliliters of this test solution was added to the nebulizer cup for its aerosolization using the nebulizer.

To verify these concentrations, spread plates of Brilliance media, BCYE media and TSA media were prepared for *E. coli*, *Legionella* and *Mycobacterium* to check their viability.

Aerosolization experiment of bacteria (E. coli, Legionella and Mycobacterium)

A Brilliance agar plate for *E. coli*, BCYE plate for *Legionella* and TSA plate for *Mycobacterium* (as shown in Figure 1) was placed directly onto the air sampler. Testing was conducted spraying a set for 1 second and 5 seconds using the spray gun and after specific elapsed times (i.e. 1 minute, 3 minutes and 5 minutes) the air sampler was turned on and 100 L of air from the box/chamber was collected. Aerosolization time using the nebulizer was 5 seconds and 10 seconds. After specific elapsed times (i.e. 1 minute, 3 minutes and 5 minutes) the air sampler was turned on and 100 L of air from the box was collected. After testing was complete, the agar plates were removed from the air sampler and incubated at 37 °C for at least 96 hours for *Mycobacterium* and at least for 48 hours for the remaining bacteria. The parameters for testing were decided after the procedure was repeated for various experimental variables such as bacterial (*E. coli*) concentrations, different spray time, and elapsed times, which provided negative results. These test runs are mentioned in Appendix A.

Preparation of Spiked Samples for Aerosolization and Transport for P22

First the concentration of P22 culture was estimated by optical density (O.D.) measurement using a Nanodrop One/One Microvolume UV – Vis Spectrophotometer

(Thermo Fisher Scientific®). The OD 600 measurement was approximately $3x10^8$ plaque forming units per milliliter (PFU/mL); this number tallied with the double agar layer plates prepared for verification.

The pure stock of P22 was diluted to a concentration to 10^6 plaque forming units per milliliter (PFU/mL). This dilution was performed using the equation:

c1V1 = c2V2, where

c1 = Initial concentration or molarity (pure/stock culture of P22)

V1 = Initial volume (pure/stock culture of P22)

c2 = Final concentration or molarity (Spray and/or test liquid to be aerosolized)

V2 = Final volume (Spray and/or test liquid to be aerosolized)

Using the given formula:

$$V1 = 10^6 \frac{CFU}{mL} * \frac{1}{10^8} \frac{mL}{CFUmL} * 100\,mL = \ 1\,mL$$

For a spray concentration 10^6 CFU/mL, 1 mL stock solution of P22 was mixed with 100mL buffer solution (PBS) in the spray gun container for aerosolization.

To achieve an initial concentration of 10^4 CFU/mL for aerosolization, using the equation above:

c1 = 10^6 CFU/mL (pure/stock culture of bacteria)

V1 = Initial volume (pure/stock culture of bacteria)

c2 = 10^4 CFU/mL (Spray and/or test liquid to be aerosolized)

V2 = 100mL (Spray and/or test liquid to be aerosolized)

$$V1 = 10^4 \frac{CFU}{mL} * \frac{1}{10^6} \frac{mL}{CFUmL} * 100\,mL = \ 1\,mL$$

The initial concentration of the test liquid containing P22 was fixed 10^4 CFU/mL and was used for the aerosolization experiments. For a spray concentration 10^4 CFU/mL, 1 mL stock solution of P22 was mixed with 100mL buffer solution (PBS) in the spray gun container for aerosolization. Five milliliters of this test solution was added to the nebulizer cup for its aerosolization.

Aerosolization experiment of P22

~ 0.1mL glycerol (Mallinckrodt 5092) was added to an empty petri dish. This was spread evenly using Kimtech® delicate task wipes throughout the petri dish forming an even thin layer. This petri dish was then placed directly onto the air sampler. Testing was conducted spraying a set for 1 second and 5 seconds using the spray gun and after specific elapsed times (i.e. 1 minute, 3 minutes and 5 minutes) the air sampler was turned on and 100 L of air from the box/chamber was collected. Similarly, aerosolization time using the nebulizer was 5 seconds and 10 seconds. After specific elapsed times (i.e. 1 minute, 3 minutes and 5 minutes) the air sampler was turned on and 100 L of air from the box was collected. After testing was complete, the plates were removed from the air sampler and 1mL *Salmonella* stock culture in its log phase (Log phase is reached when 1mL of the overnight *Salmonella* culture is added to 10mL of sterile TSB and incubated for 3 hours) was added to the plate. Media prepared for P22 in the water bath (as mentioned in section 3.1) at 47.5°C was poured in the petri dishes. The plates were shaken a little to let the media and its contents to evenly mix and distribute throughout the plate. The media was allowed to harden and care was taken that the plates were not moved during the hardening process. After cooling down to room temperature, they were incubated at 37 °C for 12 hours – 16 hours. This particular method to sample P22

aerosols was decided after numerous experiments which provided negative results. These methods are explained in Appendix C. The above mentioned parameters were decided after the procedure was repeated for various experimental variables such as P22 concentrations, different spray time, and elapsed times, to get consistent and viable results.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Air Sampling

The experiments were initially conducted with *E. coli* bacteria; *E. coli* was chosen as a surrogate for bacterial pathogens due to its comparable size and features and ease of use in the experimental work (Mui 2014).

E. coli was initially considered at concentration of 10^5 and 10^6 CFU/mL. Concentration of 10^5 CFU/mL and 10^6 CFU/mL for *E. coli* in sprayed test water was too high to be counted in an air sample. Whereas, cells were separately detected in the air sample after spraying at the concentration of 10^4 CFU/mL in the sprayed test water. Based on the initial results, it was determined that for future experiments, the concentration would be decreased to 10^4 CFU/mL. Also, these experiments were carried out at various elapsed times and air pressures. Data from the initial experiments are listed in Appendix A. P22 was considered at a concentration of 10^4 PFU/mL, from its initial experiments, with a spray time of 1 second and 5 seconds when using the spray gun and 5 seconds and 10 seconds using the nebulizer.

TSA media was first used for culturing *E. coli.* This yielded false positive results due to presence of other bacterial cells in the air, thus selective media using Brilliance agar was selected for *E. coli* experiments. Supplemental data on TSA media is also included in Appendix A.

Table 1: Summary of Parameters used for Microbial Aerosolization Experiments

Aerosolization device	Aerosolization time (sec)	Elapsed Time (min)	Initial concentration of microorganisms (CFU/mL or PFU/mL)	Air pressure (psi)
Gravity feed air spray gun	1, 5	1, 3, 5	10^4 CFU/mL	40
Aerosol Compressor	5, 10	1, 3, 5		N/a*

N/a: Not applicable

4.1.1 Log reduction calculation

The Log reduction for all microorganisms in Tables 2, 3, 4, 5 are theoretically calculated. Using the following formula,

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right)$$

or,

$$Log\ reduction = log_{10}(A) - log_{10}(B)$$

Where, A = number of microorganisms before aerosolization

B = number of microorganisms after aerosolization

The sample volume aerosolized using the spray gun was 5 mL for spray time of 5 seconds and 1 mL for spray time of 1 second, respectively. The initial concentration for all the microorganisms was adjusted to 10^4 CFU/mL. Thus, the total concentration of the test solution aerosolized was 5 x 10^4 CFU/mL for spray time of 5 seconds and 10^4 CFU/mL for spray time of 1 second, respectively. This solution was assumed to be evenly aerosolized in the chamber with air volume ~100 L.

For 1 second spray time: The concentration of *E. coli* in the solution aerosolized in equivalent amount of air was 10^4 CFU/100 L air.

∴ A = 10^4 CFU/100 L air.

B = 20 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{10^4\ \text{CFU/100 L air}}{20\ \text{CFU/100 L air}}\right) = 2.699$$

For 5 second spray time: The concentration of *E. coli* in the solution aerosolized in equivalent amount of air was 5 x 10^4 CFU/100 L air.

∴ A = 5 x 10^4 CFU/100 L air.

B = 26 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{5\ \text{x}\ 10^4\ \text{CFU/100 L air}}{26\ \text{CFU/100 L air}}\right) = 3.284$$

Similar calculations were carried out for other values of *E. coli* concentrations and other microorganisms, after specific elapsed times.

The relationship between the elapsed time and the number of *E. coli* cells detected in the air samples is displayed in Figures 3A and 3B. In general, as elapsed time increases, the number of detected bacterial cells decreases. Additionally, the relationship between the spray time and the number of bacterial cells detected is shown in Figure 3. As the spray time increases, the number of bacterial cells detected also increases. Figure 4 shows the Incubated plates after air sampling for aerosolizing using the spray gun, for 1 sec and 5 secs respectively.

4.2 *E. coli* aerosolization

Table 2A: Transport of *E. coli* after aerosolization using spray gun at different air pressures

Aerosolization time (sec)	Air pressure (psi)	*E. coli* concentration (CFU/100 L air) after elapsed time (min)			Cumulative Log_{10} reduction of aerosolized *E. coli* after elapsed time (min)		
		1	3	5	1	3	5
1	20	2	0	0	3.7	≥4.0	≥4.0
	30	6	1	0	3.2	4.0	≥4.0
	40	20	2	1	2.7	3.7	4.0
5	20	2	0	0	4.4	≥4.7	≥4.7
	30	12	0	0	3.6	≥4.7	≥4.7
	40	26	4	1	3.3	4.1	4.7

Note: The values are the average for duplicate assays

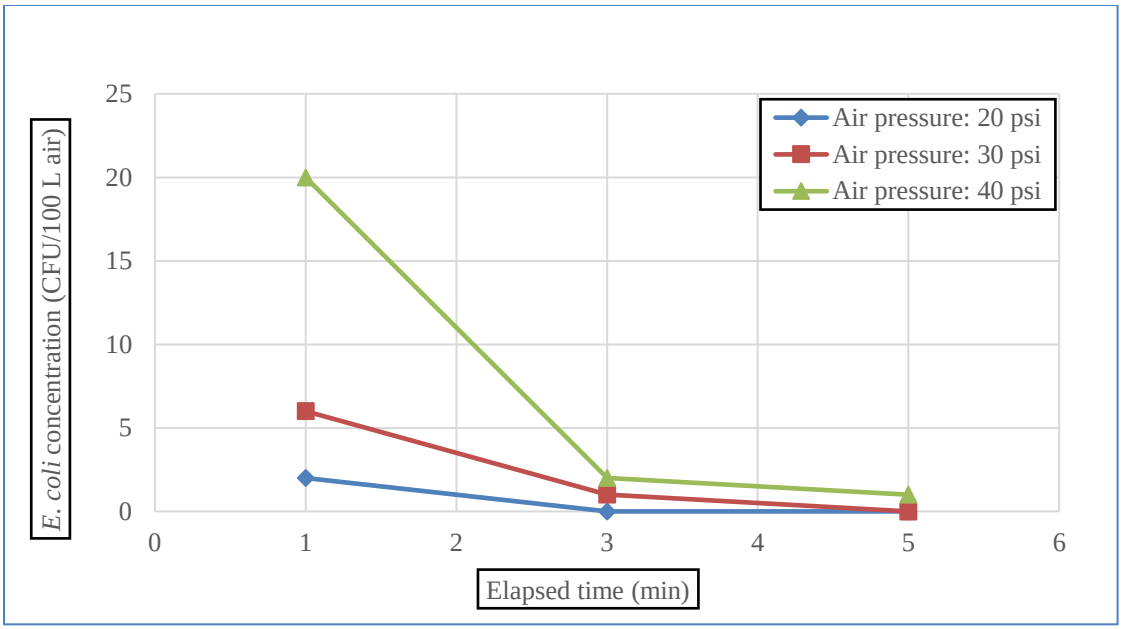


Figure 3A: *E. coli* aerosolization for 1 sec at different air pressures using spray gun

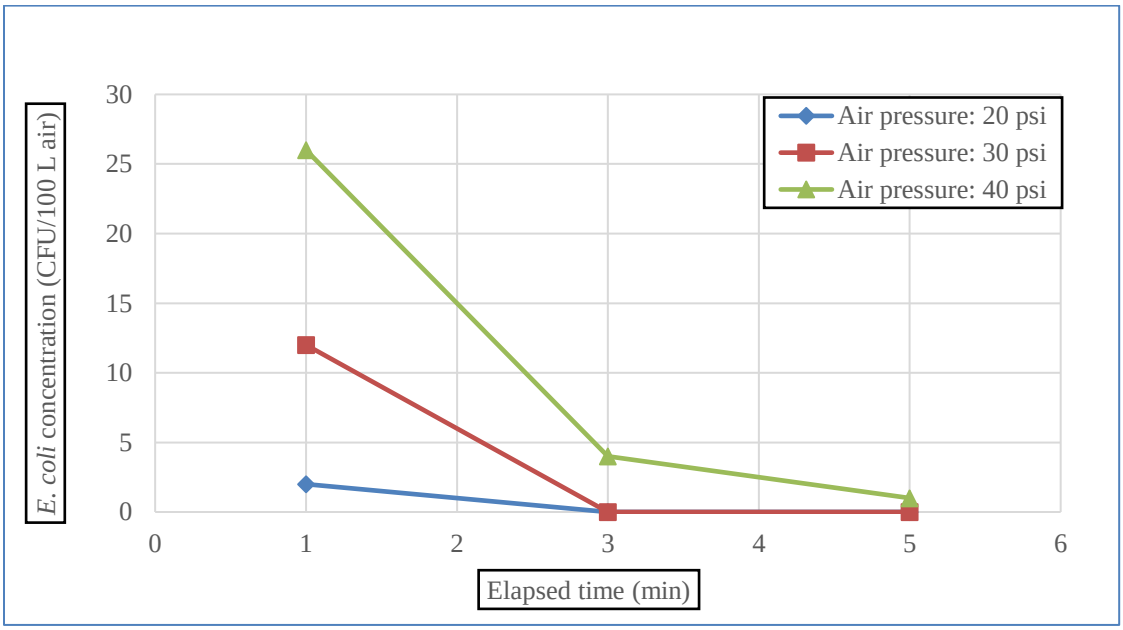


Figure 3B: *E. coli* aerosolization for 5 secs at different air pressures using spray gun

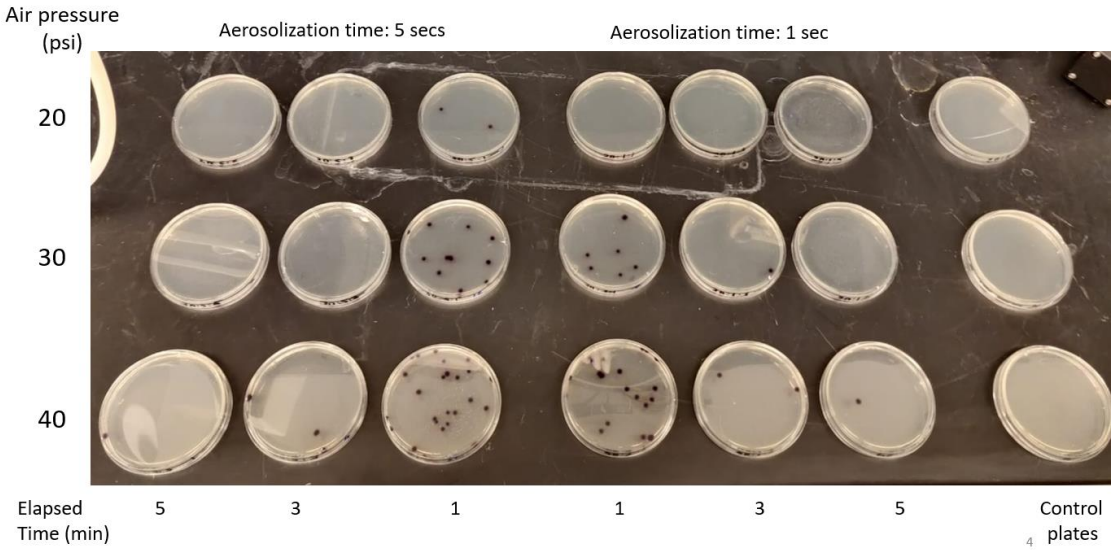


Figure 4A: Brilliance agar plates showing *E. coli* colonies after aerosolization using spray gun

Experiments were conducted by aerosolizing *E. coli* for 1 second and 5 seconds respectively and collecting air samples after each elapsed time (1 minute, 3 minutes, 5 minutes). Experiments were conducted as such that after each aerosolization (using the spray gun), samples were collected, i.e. after spraying for 1 second, a sample was collected after 1 minute. The test solution was sprayed again, and a sample was collected after 3 minutes. The test solution was sprayed yet again, and a sample was collected after 5 minutes. This data is available in Appendix B. This experiment was conducted to observe changes in the microbial concentration due to different spray/aerosolization patterns. There wasn't much difference observed in consecutive aerosolizing and collecting *E. coli* v/s one aerosolization and consecutive air sample collection. Thus, it was decided to spray/aerosolize once and collect consecutive air samples after specific elapsed times (1 minute, 3 minutes, 5 minutes). This would also help in replicating real time situations during a bioaerosol outbreak.

Table 2B: Transport and dispersion of *E. coli* after aerosolization using spray gun at different air pressures and locations in the chamber

Aerosolization time (sec)	Air Pressure (psi)	*E. coli* concentration (CFU/ 100 L air) after aerosolization time 1 min									
		Locations in the chamber									
		WR1	WR2	WL1	WL2	F1	F2	F3	F4	SR	SL
1	20	0	2	0	3	0	5	17	28	13	20
	40	0	0	15	24	0	3	21	34	19	29
5	20	1	2	0	5	2	3	20	31	36	41
	40	0	4	31	43	0	8	38	48	54	62

Note: Initial *E. coli* concentration: 10^6 CFU/ mL
See Figure 4B for the abbreviations

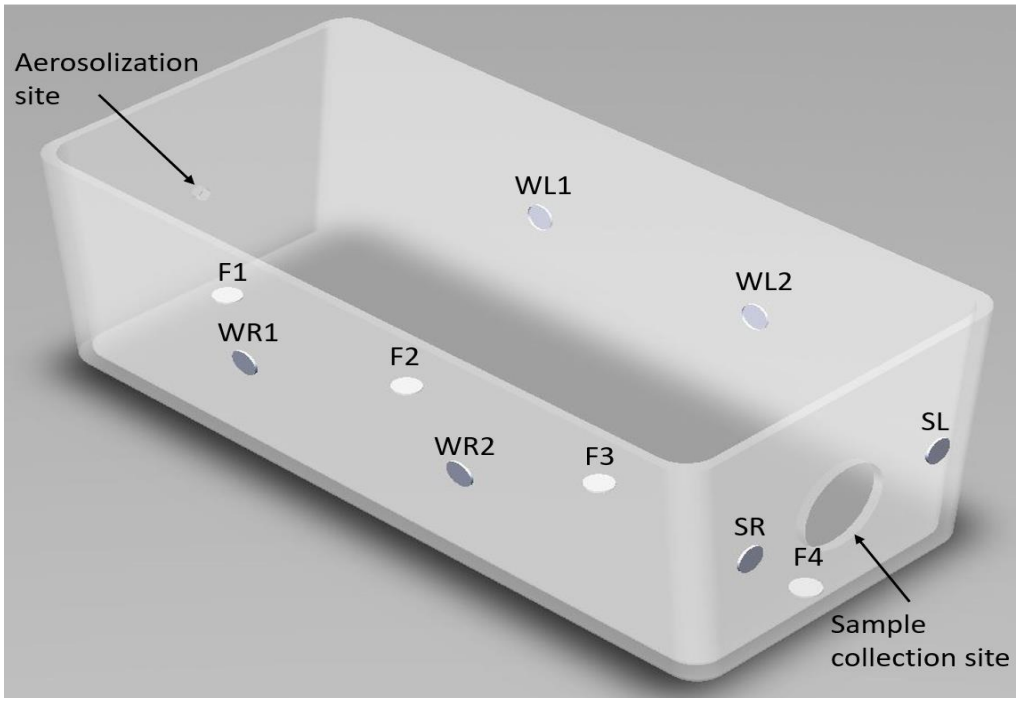


Figure 4B: Illustration of locations for collection of *E. coli* for their dispersion determination

Membrane filters (0.45 μm, 25 mm, Thomas Scientific Inc.) were placed equidistant from one another for collection of the aerosolized and dispersed *E. coli* (Figure 4B).

E. coli was sprayed in the chamber and once it was dispersed, they were collected on the membranes previously placed in the chamber/box. This particular experiment provided data on the aerosolizing pattern generated by the spray gun. It showed a plume formation of dispersed *E. coli* (Table 2B, Figure 4B) where most number of *E. coli* colonies were found at the location nearest to the collection site (F4). A low concentration of *E. coli* colonies was detected on the walls of the chamber (WL1, WL2, WR1, WR2), whereas a high concentration was seen on the walls opposite to the spray site (SR, SL). *E. coli* colonies showed an increase in count with increase in pressure and aerosolization time, for all locations inside the chamber.

4.3 *Legionella* aerosolization

The results from the air sample collection for a concentration of 10^4 CFU/mL with aerosolization time of 1 sec and 5 secs for the spray gun and 5 secs and 10 secs using nebulizer are shown in Tables 3A and 3B, respectively. An elapsed time of 1 minute, 3 minutes and 5 minutes is shown from left to right, respectively. It is seen that the number of cells transported decreases as the elapsed time increases. At higher concentrations, the number of cells transported also begins to overlap. This can sometimes lead to an underestimation of colony forming units collected in 100 L air. Thus, a concentration of 10^4 CFU/mL was fixed to avoid these false negative results.

The Log reduction for *Legionella* was calculated similarly as for *E. coli*:

For 1 second spray time: The concentration of *Legionella* in the solution aerosolized in equivalent amount of air was 10^4 CFU/100 L air.

$\therefore$ A = 10^4 CFU/100 L air.

B = 91 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{10^4\ \text{CFU/100 L air}}{91\ \text{CFU/100 L air}}\right) = 2.041$$

For 5 second spray time: The concentration of *Legionella* in the solution aerosolized in equivalent amount of air was 5 x 10^4 CFU/100 L air.

∴ A = 5 x 10^4 CFU/100 L air.

B = 238 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{5\ \text{x}\ 10^4\ \text{CFU/100 L air}}{238\ \text{CFU/100 L air}}\right) = 2.322$$

Similar calculations were carried out for other values of *Legionella* concentrations formed by aerosolization using spray gun, after specific elapsed times. It was observed that log reduction increases with elapsed time after aerosolization, as shown in Table 3A.

Table 3A: Transport of *Legionella* after aerosolization using spray gun

Aerosolization time (sec)	***Legionella* concentration (CFU/100 L air) after elapsed time (min)**			**Cumulative Log_{10} reduction of aerosolized *Legionella* after elapsed time (min)**		
	1	**3**	**5**	**1**	**3**	**5**
1	91	31	9	2.0	2.5	3.0
5	238	49	8	2.3	3.0	3.8

Note: Experiment was conducted at air pressure of 40 psi.
The values are the average for duplicate assays.

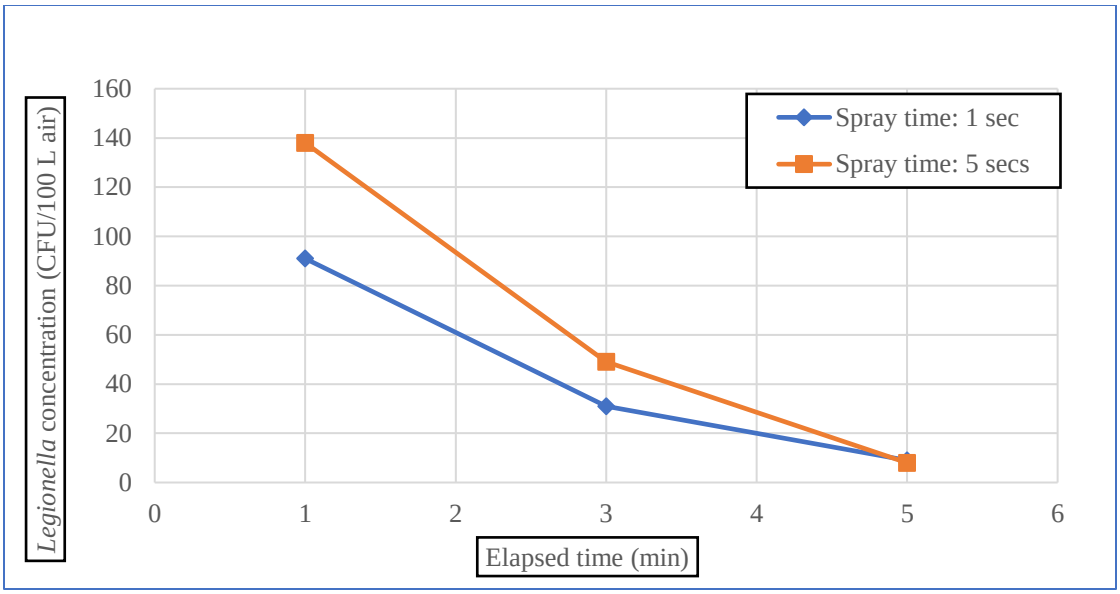


Figure 5A: *Legionella* aerosolization using spray gun

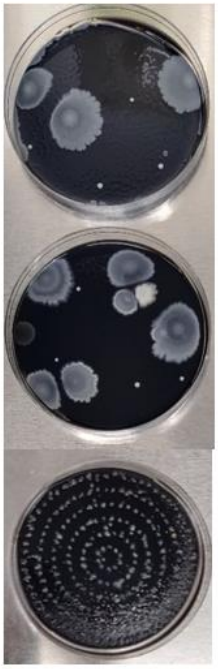
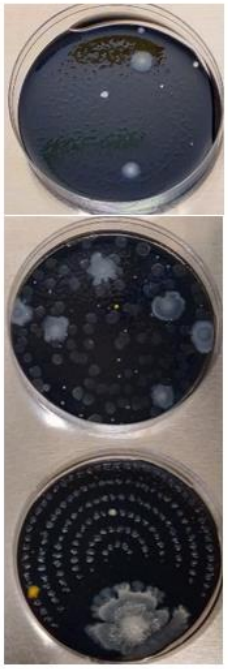
Elapsed time (min)	Aerosolization time (sec)	
	1	5
5		
3		
1		

Figure 5B: BCYE media plates showing *Legionella* colonies after aerosolization using spray gun

The Log reduction for *Legionella* after aerosolization using nebulizer was also calculated using the formula,

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right)$$

or,

$$Log\ reduction = log_{10}(A) - log_{10}(B)$$

Where, A = number of microorganisms before aerosolization

B = number of microorganisms after aerosolization

As mentioned, the initial concentration for *Legionella* was fixed to 10^4 CFU/mL. The sample volume aerosolized using the nebulizer was calculated by spiking the nebulizer cup with specific volume of water and observing the time required for its entire aerosolization. Thus, the nebulizer required ~4 minutes to completely aerosolize 2 mL of test liquid. Thus, it aerosolizes approximately 50 µL (0.05 mL) in 5 seconds and 100 µL (0.1 mL) in 10 seconds. This is considered as the volume of test liquid aerosolized evenly in the box/chamber with air volume ~100 µL. Concentration of this liquid is 500 CFU/ 100 L air for 5 seconds aerosolization and 1,000 CFU/ 100 L air for 10 seconds aerosolization, respectively.

For 5 second aerosolization time: The concentration of *Legionella* in the solution aerosolized in equivalent amount of air was 500 CFU/ 100 L air

∴ A = 500 CFU/ 100 L air

B = 365 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{500\ \text{CFU}/100\ \text{L air}}{365\ \text{CFU}/100\ \text{L air}}\right) = 0.136$$

For 10 second aerosolization time: The concentration of *Legionella* in the solution aerosolized in equivalent amount of air was 1,000 CFU/100 L air.

∴ A = 1,000 CFU/100 L air.

B = 572 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{1{,}000\ \text{CFU/100 L air}}{572\ \text{CFU/100 L air}}\right) = 0.242$$

Similar caculations were carried out for other values of *Legionella* concentrations formed by aerosolization using nebulizer, after each elapsed time. It was observed that log reduction increases with elapsed time after aerosolization, as shown in Table 3B.

Table 3B: Transport of *Legionella* after aerosolization using nebulizer

Aerosolization time (sec)	***Legionella* concentration (CFU/100 L air) after elapsed time (min)**			**Cumulative Log_{10} reduction of aerosolized *Legionella* after elapsed time (min)**		
	1	**3**	**5**	**1**	**3**	**5**
5	365	284	212	0.1	0.2	0.3
10	572	297	258	0.2	0.5	0.6

Note: The values are average of duplicate assays.

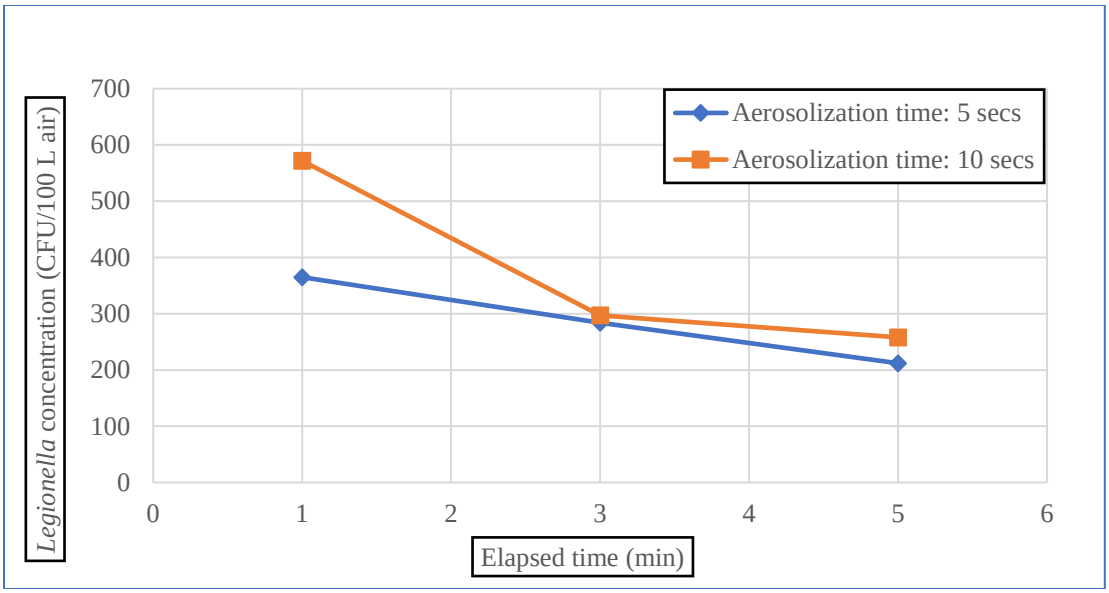


Figure 6A: *Legionella* aerosolization using nebulizer

Elapsed time (min)	Aerosolization time (sec)	
	5	10
5		
3		
1		

Figure 6B: BCYE media showing *Legionella* colonies after aerosolization using nebulizer

As seen from Figures 6A and 5A, there is decrease in the *Legionella* colonies with increase in elapsed time. Moreover, the log reduction also increases with elapsed time. The log reduction is higher for aerosolization using spray gun than the nebulizer. This could be as the nebulizer aerosolizes particles to ~5 microns in size which are easily captured by the air sampler, via its indentations. Moreover, since the overall surface area of smaller particles is higher, more number of viable microorganisms could sustain as bioaerosols. Thus, large number of colonies were formed using the nebulizer unlike the spray gun. Figure 7 depicts a comparison between aerosolization capacities of the spray gun and nebulizer by showing the colony forming units formed for the same aerosolization time (5 seconds). It is clearly seen that the nebulizer has higher aerosolization efficiency which help microorganisms to sustain longer as aerosols.

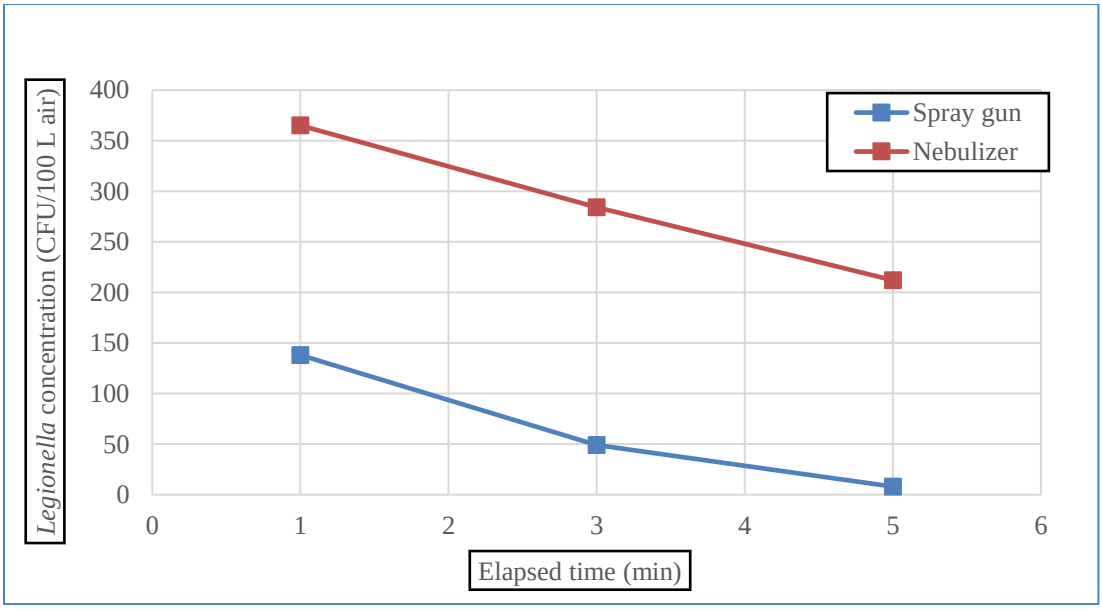


Figure 7: Legionella aerosolization using spray gun and nebulizer
Note: Aerosolization time: 5 secs

4.4 *Mycobacterium* aerosolization

The results from the air sample collection for concentration of 10^4 CFU/mL with aerosolization time of 1 sec and 5 secs for the spray gun and 5 secs and 10 secs using nebulizer are shown in Table 4A and 4B, respectively. An elapsed time of 1 minute, 3 minutes and 5 minutes is shown from left to right, respectively. It is seen that the number of cells transported decreases as the elapsed time increases. At higher concentrations, the number of cells transported also begins to overlap. This can sometimes lead to an underestimation of colony forming units collected in 100 L air. Thus, a concentration of 10^4 CFU/mL was fixed to avoid such false negative results.

The Log reduction for *Mycobacterium* was calculated similarly as for *E. coli*, and *Legionella*:

For 1 second spray time: The concentration of *Mycobacterium* in the solution aerosolized in equivalent amount of air was 10^4 CFU/100 L air.

∴ A = 10^4 CFU/100 L air.

B = 24 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{10^4\ \text{CFU/100 L air}}{24\ \text{CFU/100 L air}}\right) = 2.619$$

For 5 second spray time: The concentration of *Mycobacterium* in the solution aerosolized in equivalent amount of air was 5 x 10^4 CFU/100 L air.

∴ A = 5 x 10^4 CFU/100 L air.

B = 30 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{5\ \text{x}\ 10^4\ \text{CFU/100 L air}}{30\ \text{CFU/100 L air}}\right) = 3.221$$

Similar caculations were carried out for other values of *Mycobacterium* concentrations formed by aerosolization using spray gun, after each elapsed time. It was observed that log reduction increases with elapsed time after aerosolization, as shown in Table 4A.

Table 4A: Transport of *Mycobacterium* after aerosolization using spray gun

Aerosolization time (sec)	***Mycobacterium* concentration (CFU/100 L air) after elapsed time (min)**			**Cumulative Log_{10} reduction of aerosolized *Mycobacterium* after elapsed time (min)**		
	1	**3**	**5**	**1**	**3**	**5**
1	24	2	1	2.6	3.7	4.0
5	30	3	2	3.2	4.2	4.4

Note: Experiment was conducted at air pressure of 40 psi.
The values are average of duplicate assays.

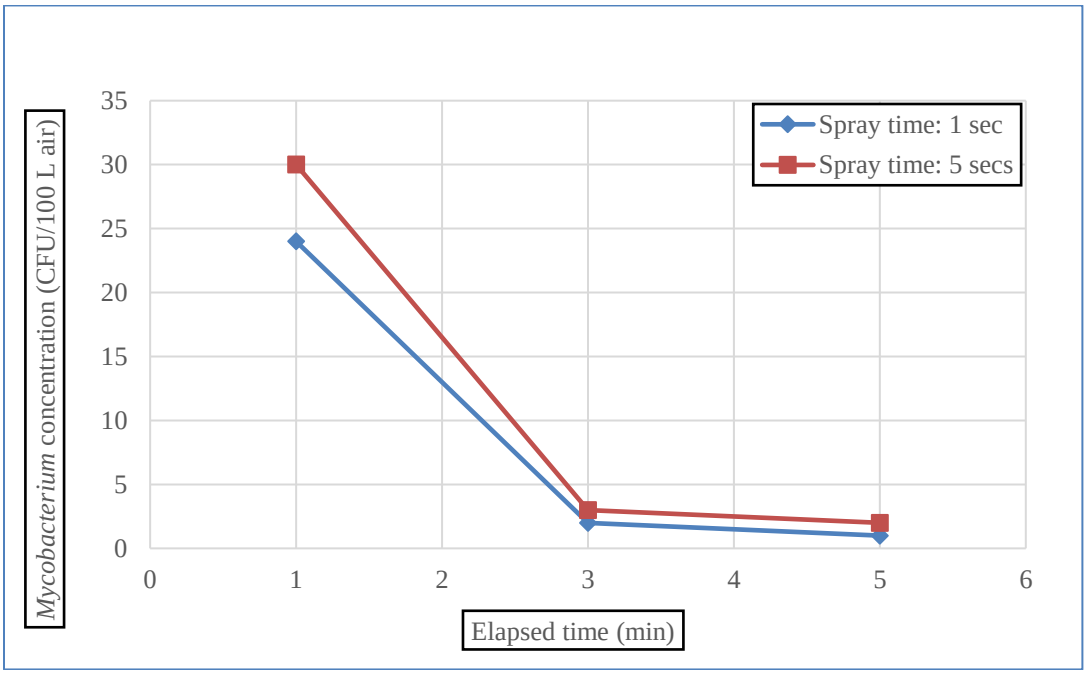


Figure 8A: *Mycobacterium* aerosolization using spray gun

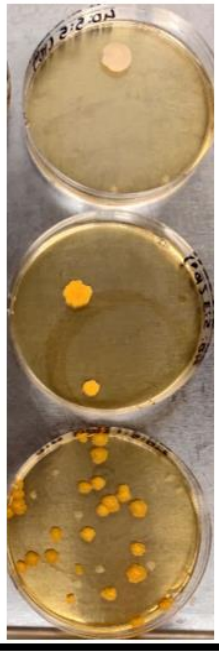
Elapsed time (min)	Aerosolization time (sec)	
	1	5
5		
3		
1		

Figure 8B: TSA media plates showing *Mycobacterium* colonies after aerosolization using spray gun

The Log reduction for *Mycobacterium* after aerosolization using nebulizer is calculated as:

For 5 second aerosolization time: The concentration of *Mycobacterium* in the solution aerosolized in equivalent amount of air was 500 CFU/ 100 L air

∴ A = 500 CFU/ 100 L air

B = 212 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{500\ \text{CFU/100 L air}}{212\ \text{CFU/100 L air}}\right) = 0.372$$

For 10 second aerosolization time: The concentration of *Mycobacterium* in the solution aerosolized in equivalent amount of air was 1,000 CFU/100 L air.

∴ A = 1,000 CFU/100 L air.

B = TNTC (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{1{,}000\ \text{CFU}/100\ \text{L air}}{\text{TNTC CFU}/100\ \text{L air}}\right) = \frac{N}{a} = Not\ applicable$$

Similar caculations were carried out for other values of *Mycobacterium* concentrations formed by aerosolization using spray gun, after each elapsed time. It was observed that log reduction increases with elapsed time after aerosolization, as shown in Table 4B.

Table 4B: Transport of *Mycobacterium* after aerosolization using nebulizer

Aerosolization time (sec)	***Mycobacterium* concentration (CFU/100 L air) after aerosolization time (min)**			**Cumulative Log_{10} reduction of aerosolized *Mycobacterium* after elapsed time (min)**		
	1	**3**	**5**	**1**	**3**	**5**
5	212	206	190	0.37	0.38	0.42
10	TNTC	226	206	N/a	0.64	0.68

Note: The values are average of duplicate assays

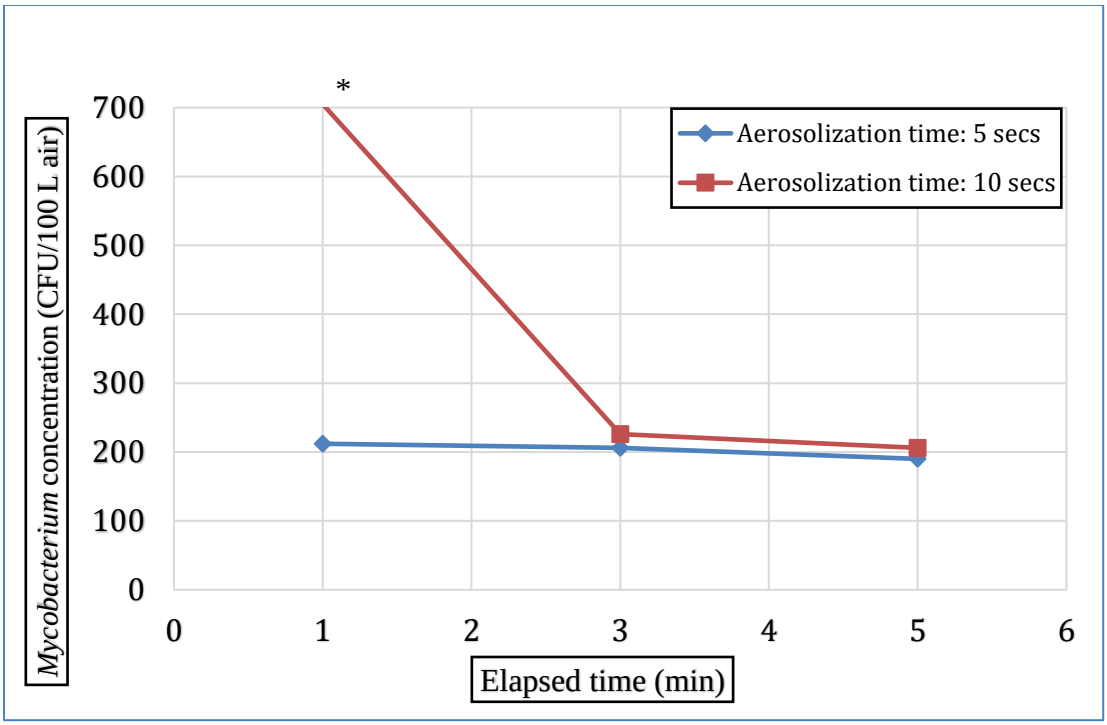


Figure 9A: *Mycobacterium* aerosolization using nebulizer
*The value of CFU/mL were too numerous to count (TNTC)

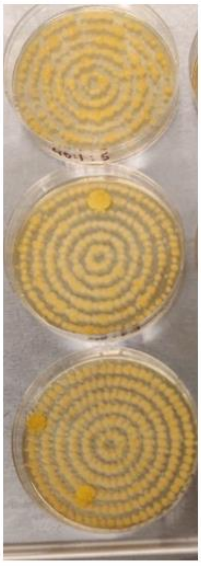
Elapsed time (min)	Aerosolization time (sec)	
	5	10
5		
3		
1		

Figure 9B: TSA media plates showing *Mycobacterium* colonies after aerosolization using nebulizer

As seen from Figures 8A and 9A, there is decrease in the *Mycobacterium* colonies with increase in elapsed time. Moreover, the log reduction also increases with elapsed time. The log reduction is higher for aerosolization using spray gun than the nebulizer. This could be as the nebulizer aerosolizes particles to ~5 microns in size which are easily captured by the air sampler, via its indentations. Moreover, since the overall surface area of smaller particles is higher, more number of viable microorganisms could sustain as bioaerosols. Thus, large number of colonies were formed using the nebulizer unlike the spray gun. Figure 7 depicts a comparison between aerosolization capacities of the spray gun and nebulizer by showing the colony forming units formed for the same aerosolization time (5 seconds). It is clearly seen that the nebulizer has higher aerosolization efficiency which help microorganisms to sustain longer as aerosols.

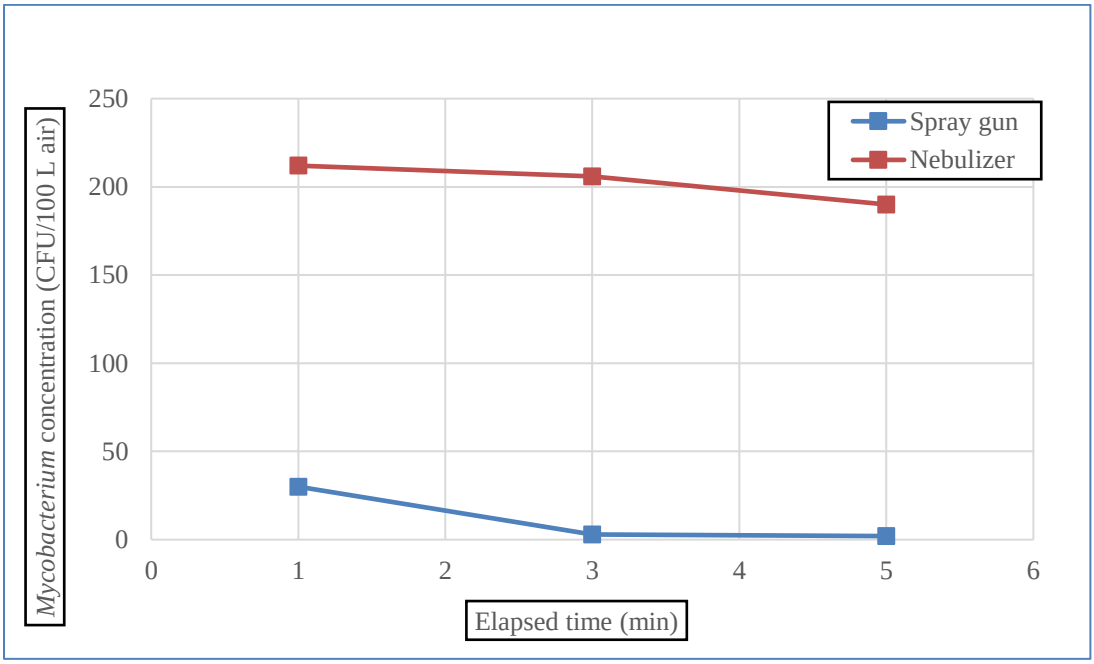


Figure 10: *Mycobacterium* aerosolization using spray gun and nebulizer
Note: Aerosolization time: 5 secs

4.5 Bacteriophage P22 aerosolization

The results from the air sample collection for concentration of 10^4 PFU/mL with aerosolization time of 1 sec and 5 secs for the spray gun and 5 secs and 10 secs using nebulizer are shown in table 5A and 5B, respectively. An elapsed time of 1 minute, 3 minutes and 5 minutes is shown from left to right, respectively. It is seen that the number of cells transported decreases as the elapsed time increases. At higher concentrations, the number of cells transported also begins to overlap. This can sometimes lead to an underestimation of colony forming units collected in 100 L air. Thus, a concentration of 10^4 PFU/mL was fixed to avoid such false negative results.

The Log reduction for P22 was calculated similarly as for *E. coli, Legionella* and *Mycobacterium*:

For 1 second spray time: The concentration of P22 in the solution aerosolized in equivalent amount of air was 10^4 PFU/100 L air.

∴ A = 10^4 PFU/100 L air.

B = 187 PFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{10^4\ \text{PFU/100 L air}}{187\ \text{PFU/100 L air}}\right) = 1.728$$

For 5 second spray time: The concentration of P22 in the solution aerosolized in equivalent amount of air was 5 x 10^4 PFU/100 L air.

∴ A = 5 x 10^4 PFU/100 L air.

B = 369 PFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{5 \times 10^4\ \text{PFU/100 L air}}{369\ \text{PFU/100 L air}}\right) = 2.131$$

Similar caculations were carried out for other values of P22 concentrations formed by aerosolization using spray gun, after each elapsed time. It was observed that log reduction increases with elapsed time after aerosolization, as shown in Table 5A.

Table 5A: Transport of P22 after aerosolization using spray gun

Aerosolization time (sec)	**P22 concentration (PFU/ 100 L air) after elapsed time (min)**			**Cumulative Log_{10} reduction of aerosolized P22 after elapsed time (min)**		
	1	**3**	**5**	**1**	**3**	**5**
1	187	17	4	1.7	2.7	3.4
5	369	74	5	2.1	2.8	4.0

Note: Experiment was conducted at air pressure of 40 psi.
The values are the average from duplicate assays.

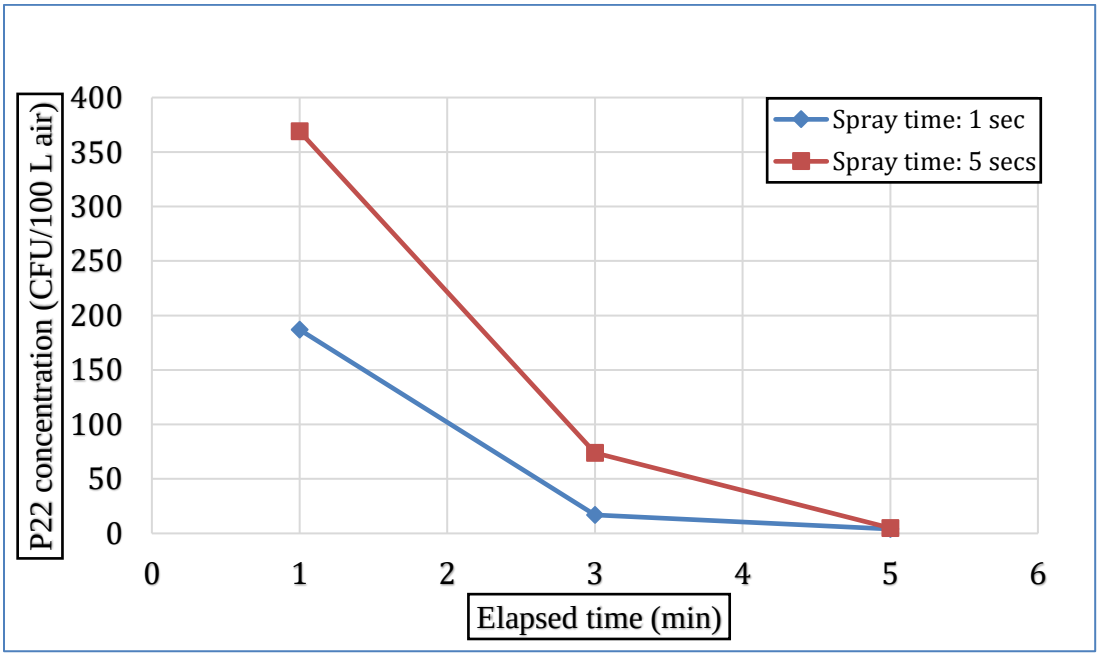


Figure 11A: P22 aerosolization using spray gun

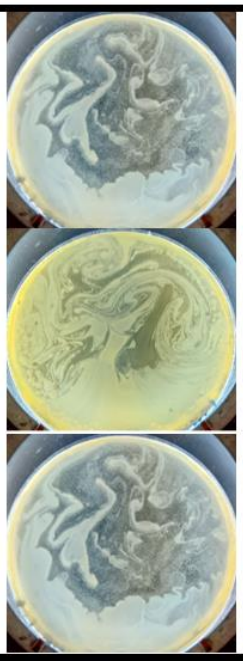
Elapsed time (min)	Aerosolization time (sec)	
	1	5
5		
3		
1		

Figure 11B: Plates showing P22 plaques after aerosolization using spray gun

The Log reduction for P22 after aerosolization using nebulizer was calculated as:

For 5 second aerosolization time: The concentration of P22 in the solution aerosolized in equivalent amount of air was 500 CFU/ 100 L air

∴ A = 500 CFU/ 100 L air

B = 488 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{500\ \text{CFU/100 L air}}{488\ \text{CFU/100 L air}}\right) = 0.010$$

For 10 second aerosolization time: The concentration of P22 in the solution aerosolized in equivalent amount of air was 1,000 CFU/100 L air.

∴ A = 1,000 CFU/100 L air.

B = TNTC (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{1{,}000\ \text{CFU}/100\ \text{L air}}{\text{TNTC CFU}/100\ \text{L air}}\right) = \frac{N}{a} = Not\ applicable$$

Similar caculations were carried out for other P22 concentrations formed by aerosolization using spray gun, after each elapsed time. It was observed that log reduction increases with elapsed time after aerosolization, as shown in Table 5B.

Table 5B: Transport of P22 after aerosolization using nebulizer

Aerosolization time (sec)	**P22 concentration (CFU/100 L air) after aerosolization time (min)**			**Cumulative Log_{10} reduction of aerosolized P22 after elapsed time (min)**		
	1	**3**	**5**	**1**	**3**	**5**
5	488	158	20	0.01	0.5	1.4
10	TNTC	872	56	N/a	0.06	1.25

Note: The values are the average from duplicate assays.

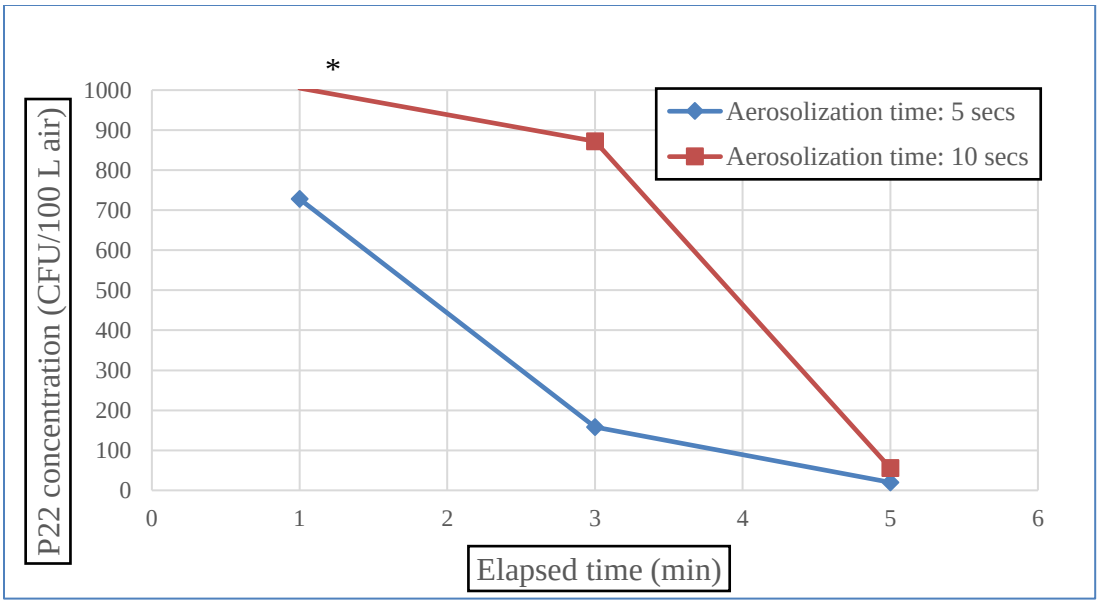


Figure 12A: P22 aerosolization using nebulizer.
*The value of PFU/mL were too numerous to count (TNTC)

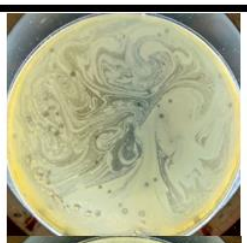
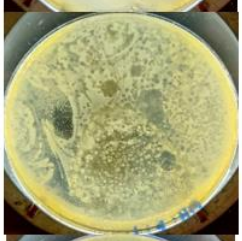
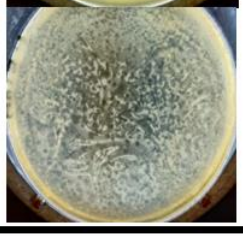
Elapsed time (min)	Aerosolization time (sec)	
	5	10
5		
3		
1		

Figure 12B: Plates showing P22 plaques after aerosolization using nebulizer

As seen from Figures 11A and 12A, there is a decrease in P22 with increase in elapsed time. Moreover, the log reduction also increases with elapsed time. The log reduction is higher for aerosolization using spray gun than the nebulizer. This could be as the nebulizer aerosolizes particles to ~5 microns in size which are easily captured by the air sampler, via its indentations. Moreover, since the overall surface area of smaller particles is higher, more number of viable microorganisms could sustain as bioaerosols. Thus, large number of colonies were formed using the nebulizer unlike the spray gun. Figure 7 depicts a comparison between aerosolization capacities of the spray gun and nebulizer by showing the colony forming units formed for the same aerosolization time (5 seconds). It is clearly seen that the nebulizer has higher aerosolization efficiency which help microorganisms to sustain longer as aerosols.

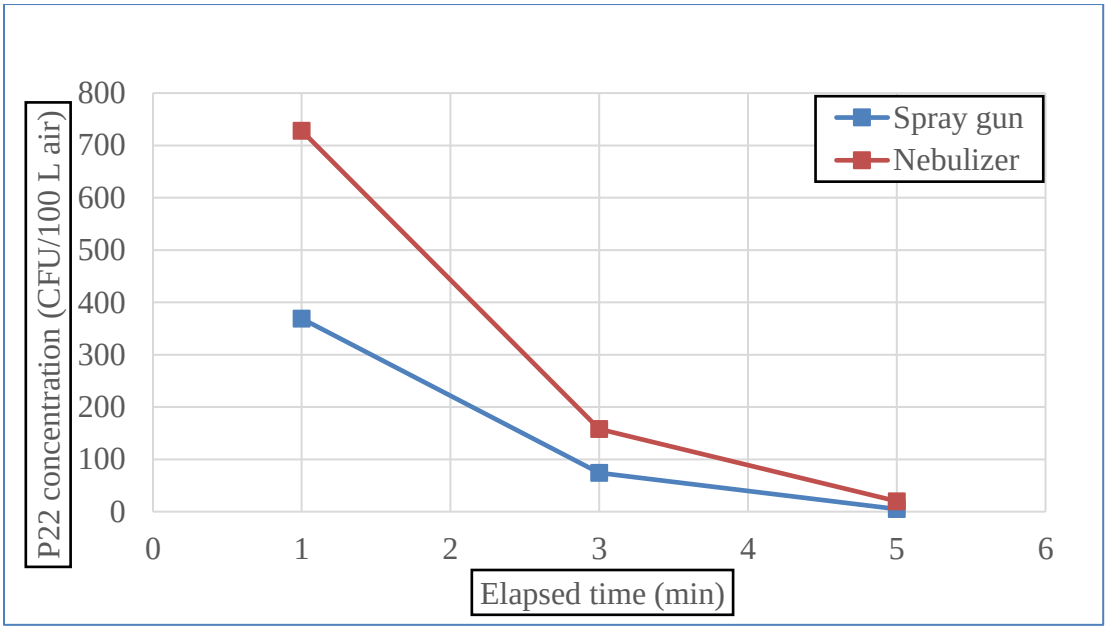


Figure 13: P22 aerosolization comparison between spray gun and nebulizer
Note: Aerosolization time: 5 secs

CHAPTER 5

CONCLUSIONS

The experiments in this study validated the aerosolization and transport of *Legionella*, *Mycobacterium* and bacteriophage P22 in a closed environment over time. In general, microbial concentration collected in air increased with aerosolization time of the test water. On the other hand, their concentration significantly decreased as elapsed time progressed due to settling effect of larger particles and potential reduction due to inactivation of bacterial and viruses in the air.

The log removal for *E. coli* after aerosolization is high i.e., on the order of 4 Log reduction due to physical removal and higher inactivation rate. This could suggest that *E. coli* is unstable in aerosols, due to the impact of dehydration, shrinking, and also dispersing in the air causing it to settle on the walls and floor of the chamber. Based on the limited dispersion measurements, most bacterial cells were transferred to the opposite sides directly across the spray site, and with the highest settling of bacterial cells on the floor. This could be true for all microorganisms under consideration, as all underwent some form of inactivation, causing their colonies to decrease in number with increase in elapsed times. *Legionella* aerosol reduction was the lowest within the bacterial groups. This may support higher potential to *Legionella* from drinking water systems, water distribution lines and most importantly cooling towers (CDC 2019). Log reduction in *Legionella* <4.0, with the lowest being just 2.0 using the spray gun. Aerosolization using the nebulizer yielded a much lower Log reduction of $\leq$0.6. *Mycobacterium* showed a similar trend with its Log reduction varying from 2.6 – 4.4 for the spray gun and $\leq$0.68

using the nebulizer. P22, surprisingly showed high number of plaque counts with its Log reduction ranging from 1.7 – 4.0 for the spray gun and ≤1.25 using the nebulizer.

5.1 Recommendations for controlling airborne pathogens

The key to preventing legionellosis and lung diseases from water, is proper maintenance of the water systems in which *Legionella* and *Mycobacterium* may grow, including drinking water systems, hot tubs, decorative fountains, cooling towers, household premise plumbing (CDC 2019). It is important to follow the appropriate guidelines for temperatures and chemical treatment of water for legionellosis prevention (CDC 2019). Required maintenance and routine procedures should be increased during extreme conditions. Additionally, proper cleaning and installation procedures of these water systems are vital. There are no vaccines that can prevent legionellosis and/or pulmonary diseases and people are at an increased risk of infection should avoid high-risk exposures, such as being in or near a hot tub (CDC 2013). Proper practices will help to reduce the number of bacteria in the systems resulting in less exposure to pathogens such as *Legionella*. These are all important preventative measures to decrease bacterial disease outbreaks from occurring. Since, there no risk of illness from bacteriophage P22 via aerosolization, the results of this study could be used as a base to understand viral survival and transport mechanism in air. As documented, P22 does aerosolize and remain in air for a period of time.

5.2 Future Work

Based on the lessons learned from these experiments, improvements can be made to enhance accuracy and reliability. Some factors that should be incorporated into future experimental plans include adjustment of humidity, air temperature, water temperature,

and wind speed inside the closed environment. Additionally, it is essential to evaluate and improve the procedural steps including aerosolization by different types of sprayer, air sample collection, and microbial quantification.

REFERENCES

Addiss DG, Davis JP, LaVenture M, Wand PJ, Hutchinson MA, McKinney RM. 1989. Community- acquired Legionnaires' Disease Associated with a Cooling Tower: Evidence for Longer-Distance Transport of *Legionella pneumophila*. Am J Epidemiol. 130(3):557-568.

Bangsborg JM. 1997. Antigenic and genetic characterization of *Legionella* proteins: contributions to taxonomy, diagnosis and pathogenesis. APMIS Supplementum: 70(105):1-53.

Barbaree JM, Fields BS, Feeley JC, Gorman GW, Martin WT. 1986. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. Appl Environ Microbiol. 51(2):422-424.

Barbaree JM, Gorman GW, Martin W, Fields BS, Morrill WE. 1987. Protocol for sampling environmental sites for Legionellae. Appl Environ Microbiol. 53(7):1454-1458.

Bentham RH. 1993. Environmental factors affecting the colonization of cooling towers by *Legionella* spp. in South Australia. Int Biodeterior Biodegrad. 31(1):55-63.

Benson RF, Fields BS. 1998. Classification of the genus *Legionella*. Semin Respir Infect. 13(2):90-9.

Bhopal RS. 1993. Geographical Variation of Legionnaires' Disease: a Critique and Guide to Future Research. Int J Epidemiol. 22(6):1127-1136.

Bollin GE, Plouffe JF, Para MF, Hackman B. 1985. Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets. Appl. Environ. Microbiol. 50(5):1128-1131.

Brenner DJ. 1987. Classification of *Legionellae*. Seminars in Respiratory Infections. 2(4):190-205.

Brenner DJ, Feeley JC, Weaver RE. 1984. Family VIII *Legionellaceae*. In Bergey's Manual of Systematic Bacteriology. Krieg NR, Holt JG (Eds). Williams and Wilkins, Baltimore, MD. (1):279.

Brown C, Nuorti P, Breiman R, Hathcock A, Fields B, Lipman H, Llewellyn G, Hofmann J, Cetron, M. 1999. A community outbreak of Legionnaires' disease linked to hospital cooling towers: an epidemiological method to calculate dose of exposure. *Int. J. Epidemiol.* 28 (2), 353-359; DOI 10.1093/ije/28.2.353.

CDC. 1997. Guidelines for Prevention of Nosocomial Pneumonia. MMWR. 46(Rr-1):1-79.

CDC 2005. Procedures for the Recovery of *Legionella* from the Environment. U.S. Department of Health and Human Services.

CDC. 2013. Causes and Transmission of *Legionella* (Legionnaires Disease and Pontiac Fever). http://www.cdc.gov/legionella/about/causes-transmission.html.

Colbourne JS, Dennis PJ. 1989. The ecology and survival of *Legionella pneumophila*. Thames Water Authority Journal of the Institution of Water and Environmental Management. 3(4):345-350.

Dowling JN, Saha AK, Glew RH. 1992. Virulence Factors of the Family *Legionellaceae*. Microbiol Rev. 56(1):32-60.

Edelstein PH. 1988. Nosocomial Legionnaires' disease: a global perspective. J Hosp Infect. Suppl A:182-188.

Edelstein PH. 1987. Laboratory Diagnosis of Infections Caused by *Legionellae*. Eur J Clin Microbiol. 6(1):4-10.

EPA. 2014. Lesson 6: Plume Dispersion and Air Quality Modeling. United States Environmental Protection Agency. http://yosemite.epa.gov/oaqps/eogtrain.nsf/b81bacb527b016d785256e4a004c0393/c9862a32b0eb4f9885256b6d0064ce2b/$FILE/Lesson%206.pdf.

EPA. 2001. *Legionella*: Drinking Water Health Advisory. United States Environmental Protection Agency, Office of Water. Washington, DC.

EPA. 1999. *Legionella*: Human Health Criteria Document. United States Environmental Protection Agency, Office of Water. Washington, DC.

EPA. 1985. *Legionella* Criteria Document. United States Environmental Protection Agency, Office of Water. Washington, DC.

Fang GD, Yu VL, Vickers RM. 1989. Disease due to the *Legionellaceae* (other than *Legionella pneumophila*): Historical, microbiological, clinical, and epidemiological review. Medicine (Baltimore). 68(2):116-132.

Fields BS. 1996. The molecular ecology of *Legionellae*. Trends Microbiol. 4(7):286-90.

Grimont PA. 1986. Rapid methods for identification of *Legionella*--a review. Isr J Med Sci. 22(10):697-702.

Hedges LJ, Roser DJ. 1991. Incidence of *Legionella* in the urban environment in Australia. Water Research. 25(4):393-399.

Helms CM, Massanari RM, Wenzel RP, Pfaller MA, Moyer NP, Hall N. 1988. Legionnaires' Disease Associated with a Hospital Water System: A five-year progress report on continuous hyperchlorination. JAMA. 259(16):2423-2427.

Henke M, Seidel KM. 1986. Association between *Legionella pneumophila* and amoebae in water. Isr J Med Sci. 22(9):690-695.

Hoge CW, Brieman RF. 1991. Advances in the epidemiology and control of *Legionella* infections. Epidemiol Rev. 13:329-40.

Jernigan DB, Hofmann J, Cetron MS, Genese CA, Nuorti JP, Fields BS, Benson RF, Carter RJ, Edelstein PH, Guerrero IC, Paul SM, Lipman HB, Breiman RF. 1996. Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. Lancet (North American Edition). 347(9000):494-499.

Kozak NA, Lucas CE, Winchell JM. 2013. Identification of *Legionella* in the environment. *Methods Mol Biol*. 954:3-25.

Kramer MH, Ford TE. 1994. Legionellosis: ecological factors of an environmentally 'new' disease. Zentralbl Hyg Umweltmed. 195(5-6):470-482.

Kusnetsov JM, Martikainen PJ, Jousimies-Somer HR, Vaisanen M, Tulkki AI, Ahonen HE, Nevalainen AI. 1993. Physical, chemical and microbiological water characteristics associated with the occurrence of *Legionella* in cooling tower systems. Water Research. 27(1):85.

Lee JV, West AA. 1991. Survival and growth of *Legionella* species in the environment. Soc Appl Bacteriol Symp Ser. 20:121S-129S.

Lin YE, Stout JE, Yu YL, Vidic RD. 1998a. Disinfection of water distribution systems for *Legionella*. Seminarsin Respiratory Infections. 13(2):147-159.

Lin YE, Vidic RD, Stout JE, Yu VL. 1998b. *Legionella* in Water Distribution Systems: Regular culturing of distribution system samples is the key to successful disinfection. J American Water Works Assoc. 90:112-121.

Lowry PW, Tompkins LS. 1993. Nosocomial legionellosis: a review of pulmonary and extrapulmonary syndromes. Am J Infect Control. 21(1):21-27.

Lück PC, Jacobs E, Röske I, Schröter-Bobsin U, Dumke R, Gronow S. 2010. *Legionella dresdenensis sp. nov.,* isolated from river water. Int J Syst Evol Microbiol 60, 2557–2562.

Marston BJ, Lipman HB, Breiman RF. 1994. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. Arch Intern Med. 154(21):2417-2422.

Mui KW, Wong LT, Yu HC. 2014. Determine the aerodynamic properties of *Legionella pneumophila* for a drag force expression. http://www.sisconev.com.br/Uploads/CIB2014/Trab01280000151920140705_000000.PDF.

Nguyen T, Llefe D, Jarraud S, Rouils, L, Campese, C, Chel, D, Haeghebaert S, Ganiayre F, Marcel F, Etienne J, Desenclos J. 2006. A Community-Wide Outbreak of Legionnaires Disease Linked to Industrial Cooling Towers—How Far Can Contaminated Aerosols Spread. *J Infect Dis*. 193 (1), 102-111; DOI 10.1086/498575.

Nguyen MH, Stout JE, Yu VL. 1991. Legionellosis. Infectious Disease Clinics of North America. 5(3):561-584.

Ortiz-Roque CM, Hazen TC. 1987. Abundance and distribution of *Legionellaceae* in Puerto Rican waters. Appl Environ Microbiol. 53(9):2231-2236.

Palmer CJ, Tsai Y-L, Paszko-Kolva C, Mayer C, Sangermano LR. 1993. Detection of *Legionella* species in sewage and ocean water by polymerase chain reaction, direct fluorescent-antibody, and plate culture methods. Applied and Environmental Microbiology. 59(11):3618-3624.

Paszko-Kolva C, Shahamat M, Colwell RR. 1993. Effect of temperature on survival of *Legionella pneumophila* in the aquatic environment. Microb Releases. 2(2):73-79.

Seidel K, Baez G, Boernert W, Seeber E, Seifert B, Esdorn H, Fischer M, Rueden H, Wegner J(Eds.). 1987. *Legionellae* in aerosols and splashwaters in different habitats. Conference Title: INDOOR AIR '87: 4th international conference on indoor air quality and climate. Berlin, F.R. Germany. (1):690-693.

States SJ, Conley LF, Knezevich CR, Keleti G, Sykora JL, Wadowsky RM, Yee RB. 1989. Free- Living Amoebae in PublicWater Supplies: Implications for *Legionella, Giardia,* and *Cryptosporidia*. Proceedings Water Quality TechnologyConference Advances in Water Analysis and Treatment. St. Louis, Missouri, November 13-17, 1988. p 109-125.

States SJ, Conley LF, Kuchta JM, Oleck BM, Lipovich MJ, Wolford RS, Wadowsky RM, McNamara AM, Sykora JL, Keleti G, Yee RB. 1987. Survival and Multiplication of *Legionella pneumophila* in Municipal Drinking Water Systems. Appl Environ Microbiol. 53(5): 979-986.

Stout JE, Yu VL. 1997. Current Concepts (Review Article): Legionellosis. N Engl J Med. 337:682- 687.

Stout JE, Yu VL, Best MG. 1985. Ecology of *Legionella pneumophila* within Water Distribution Systems. Appl. Environ. Microbiol. 49(1):221-228.

Stout JE, Yu VL, Yee YC, Vaccarello S, Diven W, Lee TC. 1992. *Legionella pneumophila* in residential water supplies: environmental surveillance with clinical assessment for Legionnaires' disease. Epidemiol Infect. 109(1):49-57.

Surman SB, Morton LHG, Keevil CW. 1994. The dependence of *Legionella pneumophila* on other aquatic bacteria for survival on R2A medium. International Biodeterioration & Biodegradation. 33(3):223-236.

Ta AC, Stout JE, Yu VL, Wagener MM. 1995. Comparison of Culture Methods for Monitoring *Legionella* Species in Hospital Potable Water Systems and Recommendations for Standardization of Such Methods. Journal of Clinical Microbiology. 33(8):2118-2123.

The Local. 2013. Legionnaires bacteria found at leading brewery. *The Local News.* http://www.thelocal.de/20130912/51877.

UT. 2003. Gaussian Plume Modeling. *University of Texas at Austin.* http://www.utexas.edu/research/ceer/che357/PDF/Lectures/gaussian_plume_modeling.pdf.

Vandenesch F, Surgot M, Bornstein N, Paucod JC, Marmet D, Isoard P, Fleurette J. 1990. Relationship between free amoeba and *Legionella:* studies in vitro and in vivo. Zentralbl Bakteriol. 272(3):265-275.

Verissimo A, Marrao G, Gomes da Silva F, da Costa MS. 1991. Distribution of Legionella spp. in hydrothermal areas in continental Portugal and the island of Sao Miguel, Azores. Applied and Environmental Microbiology. 57(10):2921-2927.

Vickers RM, Yu VL, Hanna SS, Muraca P, Diven W, Carmen N, Taylor FB. 1987. Determinants of *Legionella pneumophila* contamination of water distribution systems: 15-hospital prospective study. Infect Control . 8(9):357-363.

Winn WC Jr. 1993. *Legionella* and the clinical microbiologist. Infect Dis Clin North Am. 7(2):377- 92.

Winn WC Jr. 1988. Legionnaires disease: Historical Perspective. Clin Microbiol Rev. 1(1):60-81.

Yu VL. 1997. Prevention and control of *Legionella*: An idea whose time has come [editorial]. Infect. Dis. Clin. Pract. 6(7):420-421.

APPENDIX A

TEST RUNS FOR *E. COLI* AT VARIOUS CONCENTRATIONS AND ELAPSED TIMES

Table 6: *E. coli* aerosolization by spray gun cultured on different media

Media Used	Air Pressure (psi)	*E. coli* concentration (CFU/mL) after elapsed time (secs)		
		0	**5**	**60**
Tryptic Soy Agar (TSA)	20	>1500	950	780
	40	TNTC	>1,000	900
Brilliance	20	TNTC	1,000	690
	40	TNTC	>1,000	890

Note: Aerosolization time: 5 secs

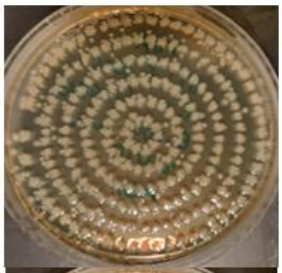
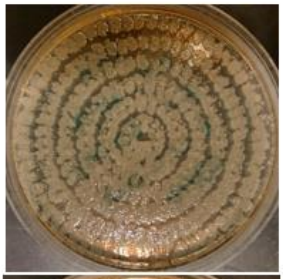
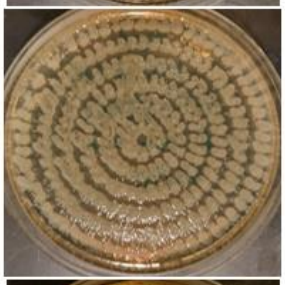
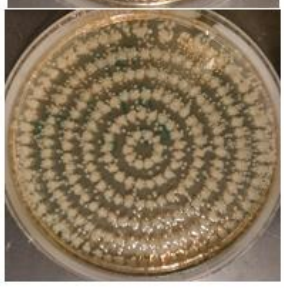
Elapsed time (secs)	Air pressure (psi)	
	20	40
60		
5		
0		

Figure 14A: TSA plates showing *E. coli* colonies after aerosolization using spray gun

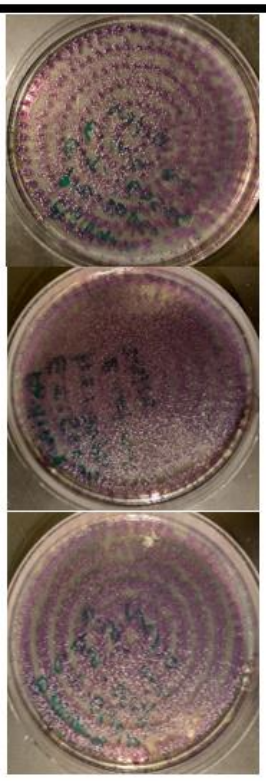
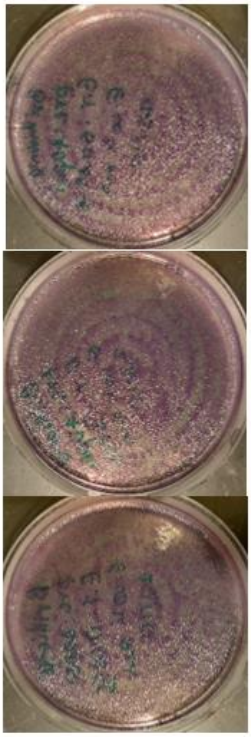
Elapsed time (secs)	Air pressure (psi)	
	20	40
60		
5		
0		

Figure 14B: Brilliance plates showing *E. coli* colonies after aerosolization using spray gun

Table 7A: *E. coli* aerosolization using spray gun at different air pressures and elapsed time (1 min, 5 mins, 10 mins), cultured on TSA

Aerosolization time (secs)	Air Pressure (psi)	*E. coli* concentration (CFU/ml) after Elapsed time (mins)		
		1	5	10
1	20	77	2	2
	40	54	2	2
5	20	345	49	8
	40	296	18	2

Table 7B: *E. coli* aerosolization using spray gun at different air pressures and elapsed time (1 min, 3 mins, 5 mins), cultured on TSA

Aerosolization time (secs)	**Air Pressure (psi)**	***E. coli* concentration (CFU/ml) after Elapsed time (mins)**		
		1	3	5
1	20	134	6	1
	40	531	98	11
5	20	364	112	8
	40	653	175	53

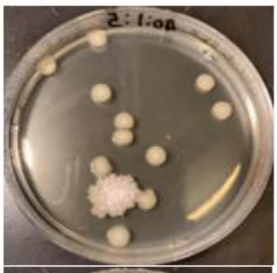
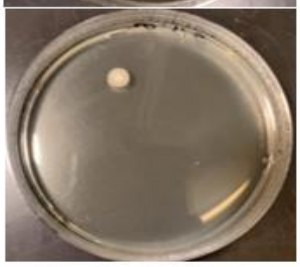
Elapsed time (min)	Air pressure (psi)	
	20	40
5		
3		
1		

Figure 15A: TSA plates showing *E. coli* colonies after aerosolization for 1 sec using spray gun for different elapsed times (1 min, 3 mins, 5 mins) (Table 7A)

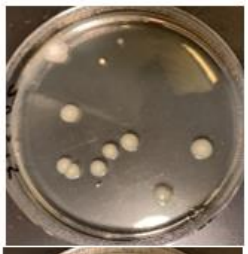
Elapsed time (min)	Air pressure (psi)	
	20	40
5		
3		
1		

Figure 15B: TSA plates showing *E. coli* colonies after aerosolization for 5 secs using spray gun for different elapsed times (1 min, 3 mins, 5 mins) (Table 7A)

Elapsed time (min)	Air pressure (psi)	
	20	40
10		
5		
1		

Figure 15C: TSA plates showing *E. coli* colonies after aerosolization for 1 sec using spray gun for different elapsed times (1 min, 5 mins, 10 mins) (Table 7B)

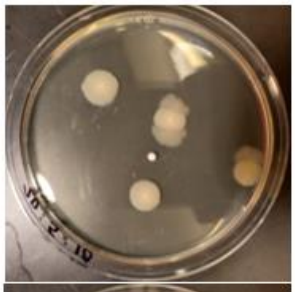
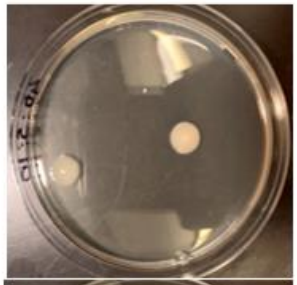
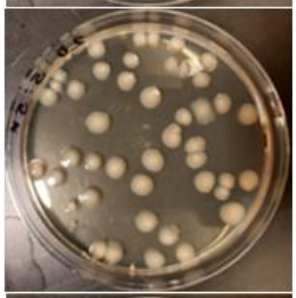
Elapsed time (min)	Air pressure (psi)	
	20	40
10		
5		
1		

Figure 15D: TSA plates showing *E. coli* colonies after aerosolization for 5 secs using spray gun for different elapsed times (1 min, 5 mins, 10 mins) (Table 7B)

APPENDIX B

TRANSPORT OF *E. COLI* AFTER AEROSOLIZATION USING SPRAY GUN

Table 8: Transport of *E. coli* after aerosolization using spray gun

Aerosolization time (sec)	*E. coli* concentration (CFU/100 L air) after elapsed time (min)			Log reduction of aerosolized *E. coli* after elapsed time (min)		
	1	**3**	**5**	**1**	**3**	**5**
1	28	6	2	2.5	3.2	3.7
5	33	9	4	3.2	3.7	4.1

Note: Experiment was conducted at air pressure of 40 psi.
The values are the average from duplicate assays.

The sample volume aerosolized using the spray gun was 5 mL for spray time of 5 seconds and 1 mL for spray time of 1 second, respectively. The initial concentration for *E. coli* was adjusted to 10^4 CFU/mL. Thus, the total concentration of the test solution aerosolized was 5 x 10^4 CFU/mL for spray time of 5 seconds and 10^4 CFU/mL for spray time of 1 second, respectively. This solution was assumed to be evenly aerosolized in the chamber of air volume ~100 L.

For 1 second spray time: The concentration of *E. coli* in the solution aerosolized in equivalent amount of air was 10^4 CFU/100 L air.

∴ A = 10^4 CFU/100 L air.

B = 28 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{10^4\ \text{CFU/100 L air}}{28\ \text{CFU/100 L air}}\right) = 2.553$$

For 5 second spray time: The concentration of *E. coli* in the solution aerosolized in equivalent amount of air was 5 x 10^4 CFU/100 L air.

∴ A = 5 x 10^4 CFU/100 L air.

B = 33 CFU/100 L air (after elapsed time of 1 sec at air pressure of 40 psi)

$$Log\ reduction = log_{10}\left(\frac{A}{B}\right) = log_{10}\left(\frac{5 \text{ x } 10^4 \text{ CFU/100 L air}}{33 \text{ CFU/100 L air}}\right) = 3.180$$

APPENDIX C

FAILED ATTEMPTS TO CULTURE P22 MEDIA USING DIFFERENT AGAR METHODS

1. Double Agar method

This method was normally carried out, except while adding *Salmonella* and P22 together in the top (soft) agar, only *Salmonella* was added and the top agar was poured in a TSA plate and left to solidify. This plate was then inserted in the air sampler to collect bioaerosols of P22.

Figure 16A: Double agar layer plate method attempt to collect P22

2. Pour plate method

In this method, TSA media was prepared as described in section 3.1, and it was left in the water bath for at least 90 minutes at 47.5°C. 1 mL of *Salmonella* was added per 15mL of agar, and poured in the petri dish. This was allowed to cool at room temperature and then inserted in the air sampler to collect P22 aerosols.

Figure 16B: Pour plate method attempt to collect P22

3. Spread plate method

An overnight culture of *Salmonella* was prepared to its Log phase and 0.1 L of this culture was spread plated over TSA media. This plate was then incubated at 37˚C for 48 hours to allow an even monolayer growth of *Salmonella.* After incubation, the plates were ready to use in the air sampler for P22 collection.

Figure 16C: Spread plate method attempt to collect P22