OCCURRENCE OF PATHOGENIC *LEGIONELLA* AND AMOEBAE SPP. FROM SOURCE (GROUNDWATER) TO EXPOSURE (TAPS AND COOLING TOWERS) IN A COMPLEX WATER SYSTEM

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Microbiology and Molecular Genetics – Doctor of Philosophy

2020

ABSTRACT

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Legionella species are gram-negative bacteria that are known to cause a severe lung infection, which is known as Legionnaires' Disease and a less severe illness called Pontiac Fever. *Legionella* species can be aerosolized from showers, faucets, cooling towers, and decorative fountains. Once aerosolized, individuals can contract both diseases via inhalation of these pathogenic bacteria. Legionnaires Disease is of particular concern because the incidence of Legionnaires Disease is rising in the United States. Chapter one will review known information about specific *Legionella* species associated with human disease. The discussion will focus on the history of Legionnaires Disease, the taxonomy of *Legionella* species, specific pathogens associated with the disease, epidemiology cases of the disease, and environmental occurrence in premise plumbing systems. Chapter two will confirm that *Legionella* species associated with human disease are present in different types of buildings from the influent to the taps, all utilizing the same water system. I will also compare physiochemical parameters (water temperature, turbidity, pH, conductivity, and residual chlorine) that affect the concentrations and species of *Legionella* in cold and hot water taps. Chapter three will demonstrate that water age plays a role in the occurrence and concentration of *Legionella* species in the water distribution and premise plumbing system. In this chapter, I present data on the concentration of *Legionella* spp. in the influent and effluent of the reservoir, two buildings, and cooling towers. Examining *Legionella* species throughout the MSU campus from the water source to the taps, and the

cooling towers provide a wholistic view of the MSU water system. Chapter four will confirm the co-occurrence of pathogenic *Legionella* species and *Acanthamoeba* spp. *Naegleria fowleri* in the drinking water supply system on the MSU campus. I show that *Naegleria fowleri* co-occurs with *Legionella bozemanii* and *Legionella longbeachae* in two buildings (F and ERC) on the MSU campus. In chapter five, I conclude by addressing future research trajectories needed to better understand how to manage the risk from the various other pathogenic *Legionella* species besides the primary water-related bacterium, *L. pneumophila*. In addition, there is a critical need to develop better methods for the detection of *Legionella* species in water systems to improve primary prevention strategies instead of reactive approaches. A proactive approach of monitoring for specific *Legionella* species in a building water system is the best approach to control Legionnaires' disease outbreaks in large buildings.

To my husband and four children

ACKNOWLEDGMENTS

It is nearly impossible to properly thank each person that has contributed to my success at Michigan State University in a reasonable amount of space—that means I would have to write another dissertation to fully thank everyone. This great accomplishment was not achieved alone; thus, I have to acknowledge with great gratitude a few key people that were particularly helpful to me on this daunting, well worth it journey.

First and foremost, to my advisor, Dr. Joan B. Rose. I am extremely thankful for the opportunity that you gave me to do research in your laboratory; I absolutely love investigating *Legionella* in drinking water supply systems. I truly thank you for accepting me for me, having patience with me, and meeting me where I was. When I came to your lab, I was broken into pieces, and your unwavering guidance, insight, and encouragement motivated and helped me reach my goal. Your feedback and suggestions made me rise to the challenge, and I thank you. I thank you for all the feedback and will always remember all the suggestions to become a better writer and scientist. You believed in me, and I honestly cannot thank you enough. Also, thank you for giving me the opportunity to go to the Water Institute summer school in Waterloo, Canada; I now have a passport because of this trip. Lastly, many thanks for reading over my emails and for your letters of recommendation. I could not have asked for a better mentor; you are truly the definition of what a mentor should be. This would not have been possible without you! Thank you, Joan, for a second chance!

I would like to express my deepest appreciation to Drs. Edward Walker, Ashley Shade, and Bjoern Hamberger for also giving me a second chance by agreeing to be on my committee.

Thank you all for your knowledge and helpful suggestions. I could not have asked for a better set of people to help me through this tough journey.

I'd also like to thank the Microbiology and Molecular Genetics Department. Thanks, Drs. Christopher Waters, Claire Vielle, and Victor DiRita for your guidance and support throughout my time here at MSU. A very special thanks go to Donna Koslowsky for rooting for me from the very start of my graduate career. Thanks so much for listening, supporting, and encouraging me at my weakest points.

I would like to acknowledge the financial support from the United States Environmental Protection Agency— Right Sizing Tomorrow's Water Systems for Efficiency, Sustainability, and Public Health. I also would like to thank the Dissertation Completion Award, MSU Alliance for Graduate Education and the Professoriate (AGEP), and the Charles Drew Science Scholars for their financial support; these awards allowed me to have a social life here in East Lansing.

To the Rose gang, past and present, thank you. You all have taught me so much and challenged me to improve the quality of my research. I feel fortunate to share lab and office space with such wonderful people. I finally have a sense of inclusion at MSU.

To my church family, Lansing Church of God in Christ, thank you all for the prayers and emotional support.

To my ma and granny. Thank you both for your financial and emotional support. Thanks for supporting my children, my travel to Canada, and much more. I am lucky to have you both. I love you!

To Denae, Daniel, Dalan, and Daniil. I went through so much so that I can provide a better life for you all. I set the foundation so that you all can have a promising future. I am your trailblazer. I did this for you all. I love you, my children!

And finally, I would be nowhere without my husband, Danny! Thank you for supporting me throughout my entire "school journey." You have been by my side since 2008, when I was just a "little girl" getting a bachelor's degree. And now we have created a large family. I thank you for taking care of our children when I had to go to school (early or late), deadlines to meet, and meetings to attend. You are the definition of a super-husband. It was you that got me through each milestone. You were definitely rooting for me from the very start. You always pushed me to keep going in the midst of my trying times. You saw it all, the tears, the frustration, and my mental breakdowns. I really went through a lot to obtain a quality education, and you never gave up on me. We weathered the storm together. Thank you for it all! I love you so much!

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CHAPTER ONE

LEGIONELLA SPECIES IN A DRINKING WATER SUPPLY SYSTEM

1.1 Introduction

In 1968, an epidemic of an unknown etiology occurred in a health department located in Pontiac, Michigan (Glick et al., 1978). Health care workers experienced fever, headaches, muscle ache, and sometimes asthenia (Glick et al., 1978). It was not until eight years later that Pontiac Fever (PF) was traced back to the newly established bacterium *Legionella pneumophila* (*L. pneumophila)* (described below) (McDade et al., 1979).

Legionella pneumophila was established after a large outbreak (~221 infected) in 1976 among members of the American Legion and in individuals who were near the defective cooling tower (Brenner et al., 1979; Fraser et al., 1977). The people who were ill suffered from a lower respiratory infection. In 1977, this pneumonia became known as Legionnaires Disease (LD) (McDade et al., 1977). *Legionella pneumophila* was the etiological agent responsible for LD (Frasser et al., 1977).

In 1978, LD and PF were both referred to as legionellosis (Fraser et al., 1977). Legionellosis presents with two clinical features: Legionnaires' Disease (LD), a severe lung infection, while PF is a mild self-limiting disease (McDade et al., 1979; Fraser et al., 1977). Thus, the rest of the dissertation will soley focus on LD.

Legionella species are gram-negative bacteria. Currently, at least 61 species and 70 serogroups of *Legionella* have been characterized, and approximately half of these species are associated with human disease (Cunha and Cunha, 2016). Five species, such as *L. pneumophila, L. anisa, L. bozemanii, L. micdadei,* and *L. longbeachae,* are responsible for the majority of the LD cases (Yu et al., 2002). However, *L. pneumophila* is the most common pathogenic species responsible for the majority (~90) of LD (Yu et al., 2002). *Legionella pneumophila* is subcategorized into 16 serogroups (Brenner et al., 1979). Of the 16 serogroups, *L. pneumophila*

serogroups 1 (sg1) is associated with 88.2% of the cases confirmed by culture (Yu et al., 2002; Beauté, 2017). Given that the urinary antigen test only detects *L. pneumophila* sg1, the percentage of confirmed cases could simply be due to a diagnostic bias (Hackman et al., 1996; Fields et al., 2002; Jarraud et al., 2013; Byrne et al., 2018).

Legionnaires Disease cases are rising in the United States (US [MacIntyre et al., 2018]). In one year alone (2018), there were ~10,000 cases of LD reported to health officials (CDC, 2018). Scientific data indicates that the majority (<95%) of the LD cases are sporadic—with regional differences, and outbreak cases account for ~5 of the cases (Farnham et al., 2014; MacIntyre et al., 2018; Sopena et al., 2007; Hicks et al., 2011). Although outbreak cases only account for a small percentage, these cases receive most of the attention because the cases are associated with hotels, resorts, cruise ships, hospitals, and nursing facilities (Hlavsa et al., 2018; Beaute et al., 2019; Papadakis et al., 2018). It is also interesting to note that the majority of outbreak cases of LD usually presents with a seasonal pattern (summer and fall) due to the use of cooling towers, (Smith et al., 2019; Fitzhenry et al., 2017; Lucas et al., 2018; Quinn et al., 2015; Watkins et al., 2015; Smith et al., 2015) but cases can occur year-round (Schumacher et al., 2020). Nevertheless, the exposure sites for sporadic cases are rarely ever identified (Stout et al., 1992). Sporadic cases are underreported to public health officials (Benin et al., 2002), and this is because the milder cases go undetected and occur in a non-outbreak setting (two or more individuals exposed to *Legionella* in the same place and get the LD around the same time). Usually, sporadic cases are identified retrospectively, and these cases can occur in residential homes or hospital water systems (Sopena et al., 2007; Stout et al., 1992).

Legionella species are widespread in the aquatic environment. More specifically, *Legionella* species survive in a variety of natural environments such as surface water (Lesnik et al., 2016),

groundwater (Brooks et al., 2004; Costa et al., 2005), rivers (Huang et al., 2017), and lakes (Zhang et al., 2018). Apart from natural environments, *Legionella* can survive, colonize and proliferate within engineered water systems such as taps (Donohue et al., 2014; Donohue et al., 2019; Barna et al., 2016; Byrne et al., 2018), air conditioners (Al-Matawah et al., 2015; Nakanishi et al., 2019; Zeng et al., 2019; Gong et al., 2017; Llewellyn et al., 2017; Lin et al., 2009), showers (Collins et al., 2017; Donohue et al., 2019; Barton et al., 2017; Rhoads et al., 2016; Farhat et al., 2012), and cooling towers (Llewellyn et al., 2017; Lucas et al., 2018; Quinn et al., 2015; Watkins et al., 2015; Smith et al., 2015).

Legionella species survive and multiply in biofilms and free-living amoebae within engineered water systems (Valster et al., 2009; Barker et al., 1993; Shaheen et al., 2019), such as commercial buildings, air conditioners, and cooling towers (Scheikl et al., 2016; Tsao et al., 2019; Liu et al., 2019). Very specific free-living amoebae-, such as *Acanthamoeba* spp, and *Naegleria fowleri* (Marciano-Cabral et al., 2010; Valster et al., 2009; Barker et al., 1993) serve as protection to *L. pneumophila* against disinfection residual in drinking water systems (Casini et al., 2018; Liu et al., 2019; Cervero-Aragó et al., 2015). The amplification of *L. pneumophila* in aquatic environments (listed above) depends on the availability of amoebae and the environmental conditions (Buse and Ashbolt 2011; Shaheen et al., 2019). For example, *Acanthamoeba* spp. interact with *L. pneumophila* at lower water temperatures (<20), while *Naegleria fowleri* interaction occurs at higher water temperatures (>20) (Marciano-Cabral et al., 2010; Valster et al., 2009; Buse and Ashbolt 2011). Nevertheless, *Naegleria fowleri* and *Acanthamoeba* spp. have been shown to alter *L. pneumophila* biological properties. For example, once *L. pneumophila* is free in its environment from amoebae, there is a higher capacity of virulence, and antibiotic resistance as a result of horizontal gene transfer from

amoebae to *Legionella* (Marciano-Cabral et al., 2010; Kuiper et al., 2004; Barker et al. 1993; Cirillo et al. 1994).

Factors that are associated with the growth *Legionella* species in building water systems are warm water temperature, inadequate disinfectant levels, water stagnation, and increased water age (Shaheen et al., 2019; Rhoads et al., 2016; Besic et al., 2017; Ambrose et al., 2020; Totaro et al., 2018). The amplification of *Legionella* is mostly detected in the hot-water pipe (Donohue et al., 2019; Valcina et al., 2019; Bollin et al., 1985; Hrubá et al., 2009; Borella et al., 2004; Bédard et al., 2019; Totaro et al., 2017; Rhoads et al., 2016; Brazeau and Edwards, 2013; Baron et al., 2014; Ditommaso et al., 2010), while the cold-water pipe serves as a survival site for these species (de Moulin et al., 1988; Donohue et al., 2014; Donohue et al., 2019; Lesnik et al., 2016; Valcina et al., 2019). The optimal temperature for *Legionella*'s growth ranges from 25 to 45°C (Bedard et al., 2015; Konishi et al., 2006; Katz and Hammel), hence why the occurrence and the detection of *Legionella* are usually higher in the hot-water taps (Donohue et al., 2014; Peter and Routledge et al., 2018; Totaro et al., 2017; Rhoads et al., 2016; Toyosada et al., 2017; Proctor et al., 2017).

The mere detection of *Legionella* species in the building water system does not constitute a risk. *Legionella* occurs in bulk-water (Lesnik et al., 2016, Whiley et al., 2017; Farhat et al., 2012; Donohue et al., 2014; De Giglio et al., 2019; Shen et al., 2015; Ishizaki et al., 2016), biofilms (Deines et al., 2010; Yu et al., 2010; Shaheen et al., 2019; Falkinham et al., 2015; Buse et al., 2019; Buse et al., 2017; Lu et al., 2014), and in aerosols (Bollin et al., 1985a; Ishimatsu et al., 2001; Nguyen et al., 2006; Dutil et al., 2007; Zmirou-Navier et al., 2007; Chang et al., 2010). However, the risk of infection is by inhaling aerosols containing *Legionella* species (Davis et al., 1982; Breiman and Hrwitz, 1987; Wright et al., 2003; Baskerville et al., 1983; Kishimoto et al.,

1979). Furthermore, the risk of infection depends on the infectious dose, strain infectivity, and the host (Armstrong and Haas, 2007; Ashbolt et al., 2015; Bollin et al., 1985). A quantitative microbial risk assessment estimated that *Legionella* containing aerosols requires 10_6 CFU/L viable cells to be infectious (Ashbolt et al., 2015). Once aerosolized, there has to be a disseminator. Cooling towers, showerheads, faucets, and decorative fountains disperse *Legionella* containing aerosols (Bollin et al., 1985a; Ishimatsu et al., 2001; Nguyen et al., 2006; Dutil et al., 2007; Zmirou-Navier et al., 2007; Chang et al., 2010). Thus, the aerosolization of *Legionella* is a public health concern.

The current consensus is that LD cases are underdiagnosed and under-reported. There needs to be an improved diagnostic test for the detection of pathogenic *Legionella* species that are known to cause disease in all patients who presents with pneumonia, regardless of the degree of the case (mild or severe). Also, there needs to be an improved understanding of the epidemiology of LD— this will enhance the primary source of infection of LD and improve risk evaluation. A monitoring scheme is needed to understand the ecology of pathogenic *Legionella* species. Understanding the ecology will give knowledge about the risks of disease from pathogenic *Legionella* species.

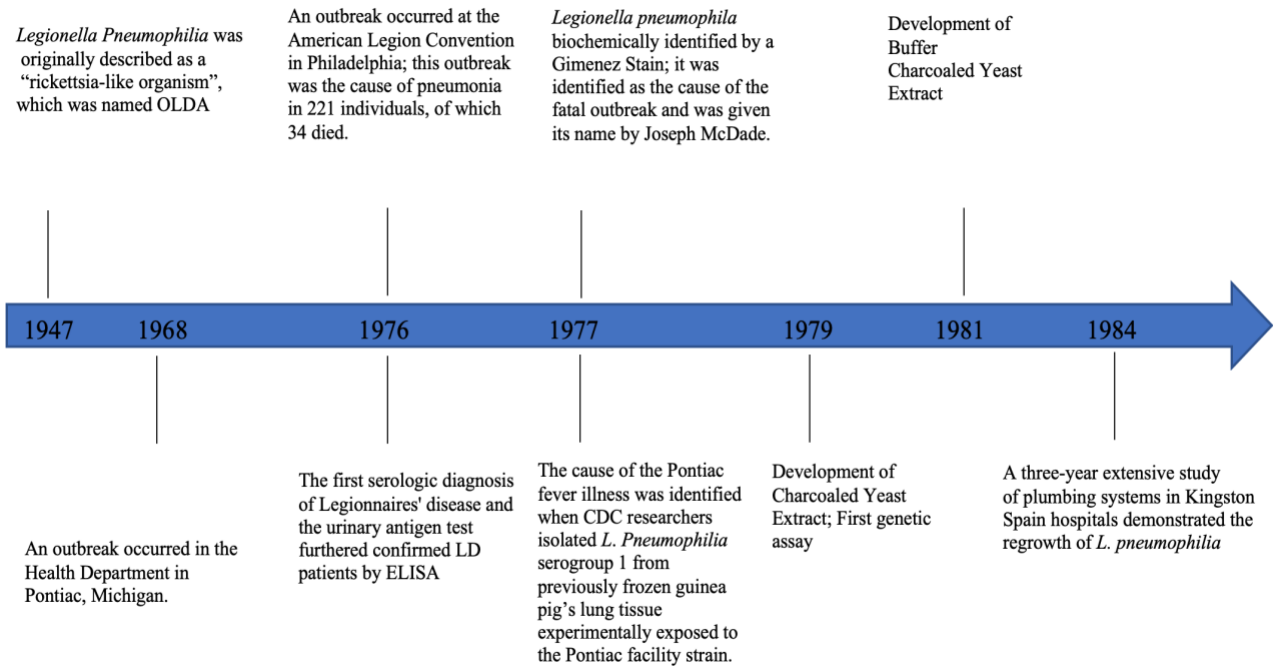
1.2 History of *Legionella pneumophila*

In 1947 *L. Pneumophilia* was initially described as a "rickettsia-like organism," which was named OLDA (McDade et al., 1979). This organism was isolated from a sick guinea pig that had been inoculated with the blood of a patient with a respiratory illness. During this time, *L. pneumophila* did not grow on bacteriological media; thus, the microbiological and clinical aspects of the LD and PF were unknown.

An outbreak occurred in 1968, affecting several people working in the Health Department in Pontiac, Michigan, who experienced flu-like symptoms and a fever (Glick et al., 1978). The Michigan Health Department analyzed blood samples from these workers. They found that ~144 people experienced fever, headache, muscle ache, and malaise (Glick et al., 1978). However, during this time, they did not know the cause until after the identification of *L. pneumophila.* The disease was later linked to *L. pneumophila* (described below) and named PF. The name PF was coined after an upper respiratory illness affected many people in Pontiac, Michigan (Glick et al., 1978).

The beginning of the discovery of bacteria, *Legionella,* did not surface until there was a pneumonia outbreak that occurred twenty-nine years later. In 1976, an outbreak occurred at an American Veterans' convention in Philadelphia (Fraser et al., 1977) this disease was later named LD. Legionnaires' disease is a pneumonic (lower) respiratory infection. *Legionella,* the bacterium, was given its name by Joseph McDade, at the time a CDC microbiologist, after this fatal outbreak with 221 individuals ill, of which 34 died (Fraser et al., 1977). *Legionella pneumophila* is the first typed bacterial species of this genus, named for presence in the lung, where it was identified as the cause of pneumonia. The source of this pathogen was traced back to the hotel environment – more specifically, the cooling tower. Figure 1.2 shows the history of this disease, the genus, and the culture technology that ensued to isolate and study the bacteria.

Figure 1.2. *Legionella pneumophila* History Timeline



In the late summer of 1976 (August and September), Joseph McDade investigated the clinical aspects of LD. Guinea pigs were experimentally inoculated with lung tissue from infected humans to determine the gram stain of the tissue, but the process to classify the bacteria was challenging (McDade et al., 1979; Katz and Hammel, 1982). After many trials and errors, *Legionella* was identified with Gimenez stain, which was developed for Rickettsiae (Janssen and Kedlund, 1981). Following the isolation procedure, a sera-antibody analysis was performed. An antibody response to what was referred to as the "newly isolated bacterium" *L. pneumophila* was found in the sera of the Philadelphia patients by using the indirect immunofluorescence technique (Hindahl et al., 1986; Gabay et al., 1985).

The cause of the PF illness was now identified in 1977 when CDC researchers isolated *L. pneumophilia* sg1 from previously frozen guinea pig's lung tissue experimentally exposed to human blood samples from the outbreak in Pontiac, Michigan (Kaufmann et al., 1981; Glick et al., 1978). A faulty air-conditioning system was implicated as the source (Glick et al., 1978; Friedman et al., 1987). Moreover, in 1977, the sporadic case of LD was identified as the same species and serogroup as the PF bacterium (McDade et al., 1979). A year later, LD and PF were both referred to as legionellosis (Fraser et al., 1977).

Legionella pneumophilia was named as the pathogenic gram-negative bacterium in 1979 (McDade et al., 1977). DNA hybridization determined the DNA relatedness of the OLDA isolate (named a "rickettsia-like" organism) to the Philadelphia strain; McDade demonstrated that these two strains were 90% related (McDade et al., 1977). These two organisms reacted similarly in serologic tests. Both organisms were confirmed by culturing with charcoal-yeast extract agar, enriched Mueller-Hinton agar, and F-G agar (McDade et al., 1977). The charcoal-yeast extract was developed in 1979 (Feeley et al., 1979); this extract was a primary isolation medium for *L.*

pneumophila for two years. In 1981, a new selective media was developed; buffered charcoal-yeast extract, which remains the medium of choice in research and clinical laboratories (Pasculle, et al. 1980; Edelstein et al., 1981).

In 1981, *L. pneumophila* was first discovered in surface water (Fliermans et al., 1981) and groundwater in 1989 (Bornstein et al., 1989). Fliermans et al., 1981 analyzed 23 lakes for the presence of *Legionella;* specifically, *L. pneumophila* sg1 was detected in pond waters at concentrations ranging from 2.7×10^5 to 9.6×10^6 CFU/L by colonies grown on the charcoal-yeast extract. Additionally, *L. pneumophila* was detected in a hot spring spa by culturing the water sample onto buffered charcoal yeast extract agar; *L. pneumophila* sg1 concentrations ranged from 10^3 to 10^5 CFU/L (Bornstein et al., 1989).

Following the first LD outbreak in 1976, a three-year extensive study of plumbing systems in Kingston Spain hospitals was described. In 1984, *L. pneumophilia* was isolated from rubber washers in shower fittings, and experimentally the growth of *L. pneumophilia* in water in contact with the same rubber material was demonstrated (Colbourne et al., 1984). This research coined the association of *L. pneumophilia* within premise plumbing (Colbourne et al., 1984). A year later, the first plumbing-related outbreak associated with *L. pneumophilia* was described in the United States (Parry et al., 1985).

Other *Legionella* species have been identified and implicated with legionellosis, such as *L. micdadei,* causing Lochgoilhead fever (Pasculle et al., 1979, Goldberg et al., 1989), and *L. bozemanae* (Brenner et al., 1980), and *L. longbeachae* (McKinney et al., 1981). An additional species of *Legionella* associated with pneumonia, *L. anisa,* was isolated from drinking water and a cooling tower in 1985 (Gorman et al., 1985). However *L. pneumophila* covers the majority of LD. *Legionella pneumophila* currently comprises 16 serogroups, of which sg1 is the most

frequently encountered (Aurell et a., 2003; Yu et al., 2002; Helbig et al., 2002; Beer et al., 2015; Shachor-Meyouhas et al., 2010; Cheng et al., 2012; Haupt et al., 2012).

Legionnaires Disease has been on the rise in the United States (US) since the early 2000s. During 2001- 2008, *Legionella* represented 33% of all water-related outbreak etiologies (Craun, 2012). From 2011- 2014, *Legionella* was responsible for 57% of the waterborne outbreaks in the US (Dooling et al., 2015; Benedict et al., 2017).

Legionella remains a paramount public health concern and common source of waterborne disease (Bartlett et al., 2011). For 2016 data, more than 6100 cases of LD were reported to the CDC (CDC, 2016). US insurers estimate their annual payouts for LD hospitalizations alone are at approximately $433 million per year (Collier et al., 2012). *Legionella pneumophila* sg1 is the primary cause of diagnosed LD in the US, accounting for 90% of LD pneumonia cases and deaths (Beer et al., 2015; Lai et al., 2010; Shachor-Meyouhas et al. 2010; Cheng et al. 2012; Haupt et al. 2012; Jarraud et al., 2013; Yu et al., 2002). The fatality rate of *L. pneumophilia* sg1 ranges from 5% to 30% (Dominguez et al., 2009).

The mode of transmission for *Legionella* is mainly through contaminated water supplies that, when aerosolized, can result in infection of the lower respiratory tract of humans, most commonly those who are more susceptible to disease. Susceptible individuals include those who are 65 years or older, current or former smokers, and pre-existing illnesses. (Parry et al., 1985; Costa et al., 2010; Haupt et al., 2012; Montagna et al., 2016).

Although endemic to many freshwater environments, such as lakes and streams, these sources of exposure are not considered to be a public health-related issue. In natural aquatic habitats, *Legionella* species are likely in insufficient quantities ($1.1 \times 10_3$ cells liter $_1$) and/or not aerosolized (Brooks et al., 2004; Wullings et al., 2011) to cause disease. The major risk of

exposure to humans relates to human-made water distribution systems capable of producing aerosolized droplets (Ashbolt et al., 2015; Craun et al., 2010). Exposure sites, such as sinks, showers, hot tubs, plumbing systems, and cooling towers are sources of where contaminated water can be aerosolized (Bollin et al., 1985; Demirjian et al., 2015; Farhat et al., 2012; Donohue et al., 2014). Contaminated cooling towers have been implicated in major outbreaks (Fitzhenry et al., 2017; Thornley et al., 2017; Quinn et al., 2015), including the seminal finding of LD as a water-related disease at the 1976 annual convention of the American Legion, that resulted in more than 200 people infected, of whom 34 died (Fraser et al., 1977). Cooling towers, which were first patented in 1918 by Frederik van Iterson and Gerard Kuypers, (F.K.T. Van and Kuypers, G. August 1916.) absorb and remove heat through water evaporation, but both the warmer temperature of water in cooling towers, especially during summer and the resulting mist from these towers' present risks for *Legionella* growth and dispersal respectively.

The abundance of *Legionella* species in the natural environment has been reported to range from 1.1×10^3 cells liter^{-1} in groundwater to 7.8×10^5 cells liter^{-1} in surface water in the Netherlands (Wullings et al., 2006). The detection of *Legionella* spp. in the source (groundwater or surface water) shapes the detection of *Legionella* in the drinking water supply system (Wullings et al., 2006; Barna et al., 2016). The maximum concentration of *L. pneumophila* found in a groundwater sample was 8×10^2 CFU/L in North America (Brooks et al., 2004) while it was 1.9×10^6 CFU/L in surface water in a comparative study in Norway (Olsen et al., 2010). Another study analyzed the presence of *Legionella* species in groundwater and examined 374 isolates over seven years (Costa et al., 2005). *Legionella pneumophila, L. oakridgensis, L. sainthelensi,* and *L. londiniensis* were identified by fatty acid methyl ester, and concentrations ranged from $3 \times 10^2 - 2.4 \times 10^4$ CFU/L (Costa et al., 2005). Using phylogenetic analysis of 16S rRNA

sequences, *L. lytica, L. londiniensis, L. rowbothamii, L. drozanski, L. fallonii,* have been detected in surface water, while *L. steigerwaltii, L, micdadei, L. fairfieldensis, L. adelaidensis* were identified in groundwater (Wullings et al., 2006). Five years later, Wullings et al., 2011 analyzed the diversity of *Legionella* species in two drinking water distribution systems that were supplied with groundwater and detected *L. bozemanae, L. pneumophila, L. donaldsonii, L. yabuuchiae, L. anisa, L. lytica, L. worsleinsis, and L. adelaidensis* by cloning and sequencing. The concentrations of *Legionella* species in unchlorinated drinking water supplies (raw water, and distribution system) ranged from 2.9 x 10^2 to 2.5 x 10^3 cells $liter^{-1}$ (Wullings et al., 2011). These studies suggest that the water source is a contributor to the presence of *Legionella* species in the drinking water distribution and plumbing systems.

1.3 Taxonomy of *Legionella*

Legionella belongs to the Phylum Proteobacteria, the Class Gammaproteobacteria, and the order Legionellales (Graells et al., 2018; Garrity et al. 2005). Gammaproteobacteria have more identified genera and the number of species (181/755, respectively) than any other proteobacterial class (Kersters et al., 2006). There is not any obvious characteristic that distinguishes Gammaproteobacteria from these other classes; the majority of Gammaproteobacteria are either symbiotic or parasitic bacteria of vertebrate and invertebrate species (Kersters et al., 2006). The order, Legionellales, comprises two families, including *Legionella* and *Coxiella* (Graells et al., 2018; Garrity et al. 2005). The genus, *Legionella*, has ~61 species and three subspecies and 70 serogroups (Wilkinson et al., 1987; Jaeger et al., 1988; Khodr et al., 2016; Vaccaro et al., 2016). And half the species of *Legionella* are associated with human disease (Burillo et al., 2017; Muder and Victor, 2002; Fields et al., 2002).

There are 16 serogroups of *Legionella pneumophila*, and two in *L. bozemanii*, and *L. longbeachae,* and one serogroup in each of the remaining species (Thacker et al., 1985; Totaro et al., 2017; Li et al., 2015; McDade et al., 1977; Lindsay et al., 2012; Fragou et al., 2012; Leoni et al., 2005; Negrón-Alvíra et al., 1988; Doebbeling et al., 1989; Gorman, 1985 Dilger et al., 2017; Koide et al., 1991; Sanchez et al., 2013) (Table 1.3). Of these *Legionella* species and serogroups that have been characterized there are five species (Table 1.3) that make up most of the incidence of LD (Yu et al., 2002). And *L. pneumophila* sg1 appears to be the most virulent strain causing the majority of disease (described below).

The rest of the taxonomy discussion will focus on *L. pneumophila*. *Legionella pneumophila* is divided into 16 serogroups, which are based on the structure of its lipopolysaccharide (outer membrane). The serogroups of *L. pneumophila* were first discovered in 1979 (Wilkinson et al., 1979). Wilkinson et al., 1979, identified four serogroups (1-4) by indirect immunofluorescent-antibody test. Serogroups 5 (England et al., 1980) and 6 (McKinney et al., 1980) were identified by a single indirect fluorescent antibody test. Serogroup 7 was later determined by an immunoglobin test (Bibb et al., 1983). Serogroup 8 was identified by direct fluorescent antibody, and immune-electrophoresis tests (Bissett and Lindquist, 1983) and sg9 was determined by a slide agglutination test (Edelstein et al., 1984). Serogroup 10 and 11 were identified by a direct immunofluorescence assay, and a slide agglutination test (Meenhorst et al., 1985; Thacker et al., 1986). Serogroup 12 was identified by indirect fluorescent-antibody and tube agglutination test (Thacker et al., 1987). Serogroup 13 and 14 were determined by slide agglutination test (Lindquist et al., 1988; Benson et al., 1988). Serogroup 15 was determined by direct fluorescent antibody test (Brenner et al., 1988). Serogroup 16 was identified by targeting the *mip* gene and later confirmed by DNA sequencing (Ratcliff et al., 1988). Different

serological tests identified *L. pneumophila* serogroups 1-15, and a genetic analysis identified serogroup 16 (Table 1.3.1).

Legionella pneumophila sg1 is the leading cause of diagnosed water-related disease, and it is the only serogroup that is clinically detected by the urinary antigen test (Beer et al., 2015; Mercante and Winchell, 2015; Fields et al., 2002; Jarraud et al., 2013; Byrne et al., 2018). The fact that there are no specific DNA molecular or urinary antigen tests to detect serogroups 2-16, (Benitez and Winchell, 2013; Hackman et al., 1996; Kazandjian et al., 1997) this underestimates the true burden of LD.

Sequence-based typing (SBT) is used to determine the genetic diversity within or between strains of *Legionella* (Galli et al. 2008; Fragou et al., 2012; Zhan and Zhu, 2018; Qin et al., 2014; Lévesque et al., 2016). In particular, sequence-based typing distinguishes between the different sequence types, which is defined by the DNA sequence of seven genomic loci. These seven chromosomal alleles are *flaA, pile, asd, mip, mompS, proA, and neuA.* The combination and the concentration of these virulence genes (listed above) are the major factor causing pathogenicity of *L. pneumophila* sg1 (Byrne et al., 2018).

Legionella pneumophila sg1 has been shown to have different characteristics than serogroups 2-16. *Legionella pneumophila* sg1 may be more pathogenic, due to its genetic diversity (Cazalet et al., 2008; Merault et al., 2011; David et al., 2017), and this serogroup could be better adapted in humans than the other serogroups. In addition to its genetic diversity, the composition of *L. pneumophila* sg1 lipopolysaccharide O-antigen is hydrophobic (Zähringer et al., 1995); this may enable this serogroup to resist killing of the alternate complement pathway (an innate component of the human immune system [Khan et al., 2013]). For example, the hydrophobicity in the lipopolysaccharide O-antigen could help *L. pneumophila* survival in the

lungs by evading toxic lysosomes (Fernandez-Moreira et al., 2006). Such characteristics make *L. pneumophila* sg1 a major public health concern.

DNA relatedness is increasingly becoming the basis of classification for bacterial taxonomy (Gupta and Sharma, 2015). For example, a recent report showed that *L. pneumophila* sg1 isolates from puddles and soil were genetically close compared to clinical isolates (Kanatani et al., 2013).

Table 1.3. Taxonomy of Disease Relevant *Legionella* Species

***Legionella* Species**	**Genome Size Mb**	**No. of Serogroups**	**Serogroup mostly associates with disease (% of disease)**	**Specific Diagnostic Test**	**Exposure Sites and Site of Isolation in humans**	**Ref**
L. pneumophila	3.4	16	1 (90)	Yes (Urinary Antigen Test)	Hot water system; Cooling towers; Lung tissue from Human	Totaro et al., 2017; Li et et al., 2015; McDade and R. E. Weaver, 1977
L. longbeachae	4.1	2	Unknown (3.9)	No	Soil; Tap water and cooling tower; Transtracheal aspirate	Lindsay et al., 2012; Fragou et al., 2012; R. Porrschen, 1980
L micdadei	3.3	1	Unknown (0.9)	No	Tap water; Cooling Tower; Lung Tissue	Leoni et al., 2005; Negrón-Alvíra et al., 1988; Doebbeling et al., 1989
L. bozemanii	4.1	2	Unknown (2.4)	No	Tap water; Cooling tower; Lung tissue	Fragou et al., 2012; Negrón-Alvíra et al., 1988
L. anisa	4.5	1	Unknown (0.9)	No	Hot water; Cooling towers; Lung Tissue	G.W. Gorman, 1985 Dilger et al., 2017; Koide et al., 1991; Sanchez et al., 2013

Table 1.3.1. *Legionella pneumophila* Identification of Serogroups

Serogroups	Identification	Health Risk	Ref
1	Serological Test	Yes	Wilkinson et al., 1979
2-15	Serological Test	Unknown	Wilkinson et al., 1979; England et al., 1980; McKinney et al., 1980; Bibb et al., 1983; Bissett et al., 1983; Edelstein et al., 1984; Meenhorst et al., 1985; Thacker et al., 1986; Lindquist et al., 1988; Benson et al., 1988; Brenner et al., 1988
16	Genetic Test	Unknown	Ratcliff et al., 1988

1.4 Epidemiology

Legionnaires' Disease incident rate has increased significantly in the United States since the early 2000s (Garrison et al., 2016; Falconi et al., 2018; Adams et al., 2016). According to the Morbidity and Mortality Weekly Report, the incidence of reported LD cases are higher in the warmer seasons (summer and early fall) comparative to the colder seasons (winter and spring) (Falconi et al., 2018; Simmering et al., 2017; Beaute et al., 2017; Garrison et al., 2016; Gleason et al., 2016; Dooling et al., 2015; McClung et al., 2017; Soda et al., 2017; Adams et al., 2016). Legionnaires' Disease also shows geographical and demographical patterns. Geographically, California, New York, and Ohio states had higher reported LD cases reported in 2014-2015 (Garrison et al., 2016; Dooling et al., 2015; McClung et al., 2017; Soda et al., 2017; Adams et al., 2016).

Demographically, the incidence of LD increases with individuals older than 50 years of age, and among males (Garrison et al., 2016; Dooling et al., 2015; McClung et al., 2017; Soda et al., 2017). Underlying conditions such as smoking, diabetes, immune-compromised, chronic obstructive pulmonary disease, and chemotherapy increases a person's risk for acquiring LD (Costa et al., 2010; Haupt et al., 2012; Montagna et al., 2016). Unfortunately, there is no real

"control" of LD, it is, however, preventable by maintaining the building water systems to reduce the risk of *Legionella* growth.

Legionnaires' Disease is a type of pneumonia associated with engineered water systems (taps, cooling towers, hot tubs) and has four classifications: nosocomial, travel-related, community-acquired or sporadic (Fitzhenry et al., 2017; Garrison et al., 2016; Dooling et al., 2015; McClung et al., 2017; Soda et al., 2017; Adams et al., 2016; Fields et al., 2002). In 2015, the case fatality rate increased (25%) among patients with healthcare-associated LD (Soda et al., 2017), whereas the case-fatality in community-acquired infection was lower (10%) (Soda et al., 2017). Although the focus has been on nosocomial, and travel-associated outbreaks, it is thought that majority of cases are sporadic, community-acquired (Chen et al., 2012; Che et al., 2008; Adams et al., 2016; McClung et al., 2017; Shah et al., 2018). The sporadic cases are rarely ever identified.

1.4.1 Disease Incident Rate of *Legionella pneumophila* in the 21st Century

In the United States, there is an increasing trend of LD (Shah et al., 2018). The National Notifiable Diseases Surveillance System received LD cases reported from 52 jurisdictions from 2000 to 2015, and there were 49,930 confirmed cases (Shah et al., 2018). During those years, the incidence of legionellosis increased from 0.42 to 1.89 cases per 100,000 individuals (Shah et al., 2018). In 2014, there were 5,166 confirmed LD cases and increased to 6,079 cases in 2015 (Shah et al., 2018). Cases identified during 2014-2015 through Active Bacterial Core surveillance showed that the frequency was in persons 50 years or older, and 65% of patients were male (Dooling et al., 2015). The primary underlying condition was a consequence of smoking (38%) (Che et al., 2018; Straus et al., 1996; Bagaitkar et al., 2008; Marston et al., 1994; Farnham et al., 2014). The secondary reason was diabetic individuals (30%), followed by chronic obstructive

pulmonary disease (16%), immune compromise (14%) and former smokers (14%) (Shah et al., 2018; Farnham et al., 2014; Campese et al., 2011). Legionnaires' Disease continues to increase significantly. For example, in the years 2017 and 2018, there were approximately 7500 and 10,000 cases reported in the United States, respectively (CDC, 2018). However, these estimated number (~7500 and 10,000) does not represent the actual incidence rate since LD is underdiagnosed (Spiegelman et al., 2020; Cassell et al., 2019; Priest et al., 2019; Spiegelman et al., 2020; Kleinberg and Antony, 2019).

1.4.2 Mode of Transmission of *Legionella*

The mode of transmission for *Legionella* spp. occurs from inhalation of aerosols carrying the bacteria or aspiration of water with the bacteria (Armstrong and Haas, 2007; Hines et al., 2014; Davis et al., 1982; Blatt et al., 1993; Venezia et al., 1994). Inhalation of *Legionella*-contained aerosols is the primary mode of transmission (Hines et al., 2014; Azuma et al., 2013; Davis et al., 1982; Schoen and Ashbolt, 2011; Breiman et al., 1990). After *Legionella* spp. grows in the water systems, it then must be aerosolized and inhaled to cause disease (Perkins et al., 2009; Gargano et al., 2017; Falkinham et al., 2015; Kwaik et al., 1998; Schoen and Ashbolt, 2011).

Legionella pneumophila grows in devices and locations in a building's water system that can cause a mist (Perkins et al., 2009; Schoen and Ashbolt, 2011). These include showers, humidifiers, mist machines, air conditioning systems, wastewater treatment plant, thermal springs faucets, hot tubs, water fountains, and cooling towers (Tyndall et al., 1989; Tyndall et al., 1995; Woo et al., 1992; Donohue et al., 2014; Hamilton et al., 2018; Llewellyn et al., 2017; Al-Matawah et al., 2015; Collins et al., 2017; Sharaby et al., 2019). Cooling towers are notorious for the spread of aerosolized water droplets containing *L. pneumophila* (Walser et al., 2014; Weiss et

al., 2017; Fitzhenry et al., 2017). A Gaussian dispersion model estimated that the cooling system disperses aerosols over long distances—at least six kilometers (Nguyen et al., 2006; Ferre et al., 2009; Marrão et al., 1993); this explains the community-acquired outbreaks that are increasing in the United States.

Other modes of transmission can occur from contaminated environmental sources through the aspiration of fluids. Franzin et al., 2001 reported the first nosocomial case of contracting *L. pneumophila* sg1 via aspiration in a neonate from a water birth in the hospital. In 2014, there was another case of a neonatal infection from *L. pneumophila* via aspiration from a water birth (Fritschel et al., 2015). In this particular case, however, the infant was delivered at a midwife facility, and the water came directly from a private borehole well (Fritschel et al., 2015). Unfortunately, the baby died after 19 days of suffering in the hospital (Fritschel et al., 2015). There are many other ways to contract *Legionella* via aspiration, such as nasogastric tube use, water fountains, ice and ice machines, and pools (Blatt et al., 1993; Hosein et al., 2005; Squier et al., 2000; Venezia et al., 1994; Marrie et al., 1991). While there has been extensive research on the transmission of *Legionella* via aspiration, the devices that harbor these bacteria are not routinely monitored so as to examine ways to prevent its growth in the built environment.

Soil is another alternative form of transmitting *Legionella* species, which have been shown to persist in soil (Wallis and Robinson, 2005; Steele et al., 1990; Schalk et al., 2014; van Heijnsbergen et al., 2016; Travis et al., 2012; Whiley and Bentham, 2011; Casati et al., 2009). Joung et al., 2017 suggested that the mode of exposure of *Legionella* from soil could be aerosolized after a rainfall event. Another mode of exposure could be through ingestion (eating or drinking) after gardening before cleaning of the hands (Plouffe et al., 1986; O'connor et al., 2007). Although potting soil is a confirmed source of *Legionella* amplification, it is not well-

understood if *Legionella* in natural soil can lead to disease (Wallis and Robinson, 2005; Heijnsbergen et al., 2014).

1.4.3 Waterborne Outbreaks of Legionnaires' Disease

The most recent LD outbreak information from CDC was published in 2017, and the surveillance data was for 2013-2014 (Benedict et al., 2017). During 2013-2014, 24 outbreaks were reported, accounting for 130 cases, 109 hospitalizations, and 13 deaths (Benedict et al., 2017). Because national data is lacking beyond the year of 2014, the following paragraph will focus on LD cases from 2015 to present that has been reported in the literature.

Major outbreaks have been associated with large buildings and exposure to showers, fountains, plumbing systems, and cooling towers (Petzold et al. 2017; Beer et al., 2015; Weiss et al., 2017; Walser et al., 2014; Lucas et al., 2018; Smith et al., 2019; Sánchez-Busó et al., 2015; Francois Watkins et al., 2017; Smith et al., 2015; Fitzhenry et al., 2017; Garrison et al., 2016; Hampton et al., 2016; Quinn et al., 2015). Table 1.4.3 shows the number of cases of LD that occurred in the United States. In August 2015, a large outbreak occurred at a California State Prison and there were fourteen cases and no deaths. The source of exposure was a cooling tower (Lucas et al., 2018). In the summer of 2015, there were a total of four separate LD outbreaks in two different states, Ohio and New York (Fitzhenry et al., 2017; Quinn et al., 2015). In Ohio, there was one outbreak with 39 cases and six deaths. In New York, there were three separate outbreaks, with a total of 169 cases and 17 deaths in two cities (Bronx and Queens) (Fitzhenry et al., 2017).

The cooling towers were the site of exposure for Ohio and New York (Quinn et al., 2015; Fitzhenry et al., 2017). Potable water within buildings is another common exposure site. Legionnaires' Disease outbreak has occurred in hospitals and hotels (Francois Watkins et al.,

2017; Smith et al., 2015). A well-known-LD outbreak occurred in Flint, Michigan (Smith et al., 2019). During this (Flint, MI) outbreak, there were 87 cases and 12 deaths. The exposure sites were from cooling towers, the potable water source in residents and hospitals (Smith et al., 2019) (Table 1.4.3).

Table 1.4.3. Legionnaires' Disease Outbreak in the United States

Cases	Deaths	Location	Month of occurrence	Environmental Source of exposure	Ref
64	0	California	August	Cooling towers	Lucas et al., 2018
39	6	Ohio	July	Cooling towers	Quinn et al., 2015
138	16	New York	July-Aug	Cooling towers	Fitzhenry et al., 2017
15	1	New York	Sep-Oct	Cooling towers	Fitzhenry et al., 2017
16	0	New York	April-Jun	Housing complex potable water system	Fitzhenry et al., 2017
10	0	Alabama	March-June	Hospital potable water system	Francois Watkins et al., 2017
87	12	Michigan	July	Hospital and residential potable water system, and cooling towers	Smith et al., 2019
11	3	Illinois	August	Hotel fountain	Smith et al., 2015

Legionnaires' Disease outbreaks have been associated with water system deficiencies at the treatment level, such as inadequate disinfection, improper maintenance (Benedict et al., 2017; Garrison et al., 2016; Brunkard et al., 2011; Beer et al., 2015; Craun et al., 2010). Other issues can occur in the water main, such as breaks in the piping system (Garrison et al., 2016; Waak et al., 2018; Rhoads et al., 2017; Craun et al., 2010) and in the premise plumbing, which can have a lower disinfectant residual (Craun et al., 2010; Lu et al., 2014; Hull et al., 2017).

1.4.4 Diagnosis

Isolation of *L. pneumophila* from lung tissue and pleural fluid is a necessary procedure for identifying LD (Maiwald et al., 1998; Oliverio et al., 1991). In many outbreaks, the goal is to identify and compare the clinical and environmental isolate (Kozak-Muiznieks et al., 2014; Byrne et al., 2018; Kozak et al., 2009). The primary diagnostic method used to detect LD is the urinary antigen test (UAT) (Couturier et al., 2014; Souche et al., 2020; Muyldermans et al., 2020; Como et al., 2019; Badoux et al., 2020). The UAT is a rapid test, which detects outbreaks and sporadic cases of LD caused by *L. pneumophila* sg1 (Mojtahedi et al., 2019; Hicks and Garrison, 2012; Chen et al., 2015; Garrison et al., 2014). The UAT sensitivity and specificity for *L. pneumophila* sg1 range from 70-90% and 95-100%, respectively, by using an ELISA test and a rapid immunochromatographic test (Domínguez et al., 1999; Mercante and Winchell, 2015; Badoux et al., 2020; Helbig et al., 2001; Diederen and Peeters, 2007). However, the reported incidence is likely to be underestimated because the UAT only detects *L. pneumophila* sg1 (Hicks and Garrison, 2012; Spiegelman et al., 2020; Cassell et al., 2019; Priest et al., 2019; Spiegelman et al., 2020; St-Martin et al., 2013; Garrison et al., 2014).

The culture technique is referred to as the 'gold standard' method; this can be used for environmental and clinical samples (Chen et al., 2015; Morrill et al., 1990; Lee et al., 1993). The

culture of sputum specimens from LD patients is vital to confirm the case (Mizrahi et al., 2017; Botelho-Nevers, 2016). The medium used is buffered charcoal-yeast extract agar enriched with α-ketoglutarate to determine if the genus (*Legionella*) is present regardless of the species or serogroup (Edelstein, 1981). This media was first established to support the growth of *L. pneumophila* (Warren and Miller, 1979). *Legionella pneumophila* collected from sputum samples takes three to five days to grow (Warren and Miller, 1979; Zahran et al., 2018). But it may take longer to grow other *Legionella* species (Lee et al., 1993; Lucas et al., 2011). The sensitivity of the culture method is 81%, and the specificity is 99% (Vickers et al., 1994; Lindsay et al., 2004).

Direct fluorescent antibody staining is a rapid test (2-4 hours) to determine *Legionella* spp. in cultures, respiratory secretions, patient tissue and environmental samples (Finkelstein et al., 1993; Edelstein et al., 1987; Bahl et al., 1997; Ramirez and Summersgill, 1994; Yamamoto et al., 1993; Hayden et al., 2001; Bibb et al., 1984). The genus-specific monoclonal antibody, immunoglobulin G2a (2125), recognizes the 60-kDa heat shock protein of all *Legionella* spp. (Steinmetz et al., 1992). Monoclonal antibodies (MAB2 and MAB3/1) detect *L. pneumophila* sg1 (Dournon et al., 1988; Kozak et al., 2009; Francois-Watkins et al., 2017). Monoclonal antibody 2 and 3/1 recognizes the lipopolysaccharide epitope (encoded by the lag1-gene) in *L. pneumophila* sg1 (Helbig et al., 1995; Lück et al., 2001; Gosselin et al., 2011; Kozak et al., 2009). Specific polyclonal antibodies detect other human-associated *Legionella* species (Lebrun et al., 1986; Aurell et al., 2004; Edelstein and Edelstein, 1989). Fluorescein isothiocyanate (FITC)-labeled *Legionella* polyclonal antibodies were used for the selective detection of *L. pneumophila* sg 1 to 6, *L. bozemanii, L. micdadei,* and *L. longbeachae* in clinical and environmental samples (Vickers et al., 1990; Palmer et al., 1993; Edelstein et al., 1981; Tang et

al., 1984; Cercenado et al., 1987). For example, anti-*L. pneumophila* and anti-*L. bozemanii* (FITC) conjugated polyclonal IgG rabbit antibodies were used to detect *L. pneumophila* in tap water (Keserue et al., 2012; Füchslin et al., 2010) and *L. bozemanii* in lung aspirate of a patient with pneumonia (Tang et al., 1984). Two polyclonal conjugates were utilized to detect *L. pneumophila* in hot and cold storage tank water (Vickers et al., 1990).

The detection of *L. pneumophila* sg1 using monoclonal antibodies has a specificity that ranges from ~94% and sensitivity that ranges from 11 - 40% (Vickers et al., 1990; Lindsay et al., 2004; Hayden et al., 2001; She et al., 2007; Edelstein et al., 1980). There is some cross-reactivity with other species using polyclonal antibodies (Vickers et al., 1990; Palmer et al., 1993; Edelstein and Edelstein, 1989). The sensitivity of monoclonal antibody and the cross-reactivity using polyclonal antibodies are the reasons the direct fluorescent antibody staining method is rarely used to diagnose LD (She et al., 2007; Reller et al., 2003; Mercante and Winchell, 2015).

The serological test detects antibodies (IgA, IgG, and IgM) against *Legionella* spp. in patient serum. However, the disadvantage of serological testing is the inability to distinguish between *Legionella* species and serogroups (Wilkinson et al., 1979a; Wilkinson et al., 1983; Harrison et al., 1987; Helbig et al., 1997; Ditommaso et al., 2008). Serology testing has declined because this test cross-react with other bacteria (Boswell et al., 1996; Musso and Raoult, 1997) and this takes time to indicate whether a patient has LD (Monforte et al., 1988; Benz-Lemoine et al., 1991; Reller et al., 2003; Simonsen et al., 2015).

Polymerase Chain Reaction (PCR) is a molecular diagnostic tool with the possibility to detect all known species of *Legionella* in respiratory and environmental samples. The most common gene to identify *Legionella* at the genus level is 16S or 23S, conserved gene (Templeton et al., 2003; Cloud et al., 2000; Hayden et al., 2001; Jonas et al., 1995; Rantakokko-Jalava and

Jalava, 2001; Reischl et al., 2002; Wellinghausen et al., 2001; Yong et al., 2010; Nazarian et al., 2008; Nazarian et al., 2008; Herpers et al., 2003; Templeton et al., 2003; van der Zee et al., 2002). On the species level, *Legionella pneumophila* is determined by a macrophage inhibitor protein (*mip*), which is a virulent gene, which enables entry of the bacterium into various amoebae species and human macrophages (Ratcliff et al., 1998; Phin et al., 2014; Ballard et al., 2000; Nazarian et al., 2008; Hayden et al., 2001; Kessler et al., 1993; Koide et al., 1995; Lindsay et al., 1994; Murdoch et al., 1996; Ratcliff et al., 1998; Wellinghausen et al., 2001). The *mip* gene specifically detects *L. pneumophila* (Nazarian et al., 2008; Templeton et al., 2003; Wilson et al., 2003). The *wzm* gene encodes for the lipopolysaccharide biosynthesis (Cazalet et al., 2008; Mérault et al., 2011). The *wzm* gene specifically detects *L. pneumophila* sg1 (Cazalet et al., 2008; Mérault et al., 2011; Benitez and Winchell, 2013; Mentasti et al., 2015; Mentasti et al., 2017; Collins et al., 2015; Toplitsch et al., 2018). However, there are no primer sets to distinguish among *L. pneumophila* serogroups 2-14 (Katsiaflaka et al., 2016); thus, when utilizing a PCR based method, these serogroups (2-14) are categorized into one group (Benitez and Winchell, 2013; Merault et al., 2011).

Currently, there is a new PCR multiplex assay that targets other key *Legionella* spp., that are associated with LD, such as *L. longbeachae, L. micdadei, L. anisa, and L. bozemanii* (Cross et al., 2016). This assay targets the 23S-5S intergenic spacer region for all *Legionella* species, (Cross et al., 2016; Grattard et al., 2006; Robinson et al., 1996; Campocasso et al., 2012) and five uniquely 5′ hydrolysis probes were designed within this region for the specific detection of *L. pneumophila, L. longbeachae, L. micdadei, L. anisa, and L. bozemanii* by qPCR (Cross et al., 2016). The sensitivity and specificity for multiplexing all five species ranged from 92-100%

(Cross et al., 2016). This assay has been shown to accurately identify *L. longbeachae, L. micdadei, L. anisa, and L. bozemanii* in clinical and environmental samples (Cross et al., 2016).

Referral and research laboratories take advantage of PCR based methods such as Real-Time PCR and LightCycler PCR for the detection of *Legionella* in environmental (water from cooling towers, rivers, and hot tubs) and clinical samples (sputum, BAL fluid, serum, and urine) (Merault et al., 2011; Hayden et al., 2011; Cross et al.,2016; Botelho-Nevers et al., 2016; Ricci et al., 2018). Although referral laboratories use PCR-based methods, digital droplet PCR has not been used for epidemiology purposes to compare *Legionella* strains in the suspected environment to clinical specimens.

One academic laboratory quantified *Legionella* DNA certified reference material utilizing digital droplet PCR (Baume et al., 2019). Baume et al., 2019 suggested that digital droplet PCR can be useful for reference material as it estimated a value (9,089,730 GU/5uL) that was consistent with a slight degradation of the *Legionella* certified reference material DNA. The previously reported value of the *Legionella* certified reference material DNA from 2009 was 10,627,646 GU/5uL by qPCR (Baume et al., 2019). Digital droplet PCR is a technique that is accurate and precise.

Digital droplet PCR (ddPCR™) is an absolute quantification and is more specific than qPCR methods (Gobert et al., 2018). To utilize qPCR, a standard curve must be constructed for the PCR threshold (Green and Sambrook, 2018; Kuypers and Jerome, 2017; Conte et al., 2018). Digital droplet PCR forfeits this need and is more effective in detecting rare DNA targets in environmental and clinical samples (Xue et al., 2018; Doi et al., 2015; Li et al., 2018; Wood et al., 2019; Yang et al., 2017).

1.4.5 Treatment and Antibiotic Resistance of *Legionella*

Unfortunately, the treatment of LD occurs before diagnosis. The antibiotic therapy includes treatment with fluoroquinolones (ciprofloxacin, moxifloxacin, levofloxacin) and macrolides (doxycycline, rifampicin, trimethoprim, sulfamethoxazole, and azithromycin) (Postma et al., 2015; Wunderink and Yin, 2016; Grau et al., 2006; Epping et al., 2010; Sabrià et al., 2005; Vandewalle-Capo et al., 2017; Fraser et al., 1978; Garcia-Vidal et al., 2017; Griffin et al., 2010). In some cases, treatment may be necessary for up to 3 weeks (Amsden, 2005).

Internationally, there has been research on the presence of antibiotic resistance genes in the drinking water system (Xi et al., 2009; Bergeron et al., 2015; Bergeron et al., 2017; Subbarram et al., 2017; Al Sulami et al., 2013; Sharaby et al., 2019; Sikora et al., 2017; Graells et al., 2018; Rahimi and Vesal, 2017; Xiong et al., 2016; Jia et al., 2019), but the antibiotic-resistant profile of *L. pneumophila* in the US has yet to be investigated.

A study reported the antibiotic profile (azithromycin, clarithromycin, erythromycin, doxycycline, moxifloxacin, cefuroxime, cephalexin, amoxicillin, vancomycin, and clavulanate) on *Legionella* spp. from several different water sources such as lakes, ponds, rivers, and water tanks (Subbarram et al., 2017). Out of four hundred water samples, *L. pneumophila* (320/400), *L. micdadei* (24/400), *L. bozemanii* (20/400), *L. feeleii* (20/400), and *L. dumoffii* (8/400) was isolated from 80, 7, 6, 5 and 2%, respectively (Subbarram et al., 2017). The isolation rate of antibiotic resistance was not clearly described, but out of five *Legionella* species, two *Legionella* species were resistant to antibiotics (Subbarram et al., 2017). The disc diffusion test revealed that *L. pneumophila* was resistant to azithromycin, clarithromycin, erythromycin, doxycycline, moxifloxacin, cefuroxime, cephalexin; while amoxicillin, vancomycin, and clavulanate inhibited the growth of *L. pneumophila* (Subbarram et al., 2017). *Legionella feeleii* was only resistant to

amoxicillin and sensitive to azithromycin, clarithromycin, erythromycin, doxycycline, moxifloxacin, cefuroxime, cephalexin, vancomycin, and clavulanate (Subbarram et al., 2017) (Table 1.4.5).

Al Sulami et al., 2013 investigated the antibiotic susceptibility of *L. pneumophila* isolated from a drinking water supply system in Iraq. Al Sulami et al., 2013 collected 49 water samples from different phases of the water treatment plant, influent (13), precipitation tanks (13), filtration tanks (10) and effluents (13), 127 tap water from 18 districts, and 46 samples from five reverse osmosis plant suppliers (water tanks). Ten isolates (eight isolates were sg1, and 2 were sg 2-15) of *L pneumophila* were tested for antibiotic sensitivity to doxycycline, erythromycin, streptomycin, gentamicin, chloramphenicol, and ampicillin by stoke disk diffusion method. *Legionella pneumophila* sg1 isolates were 83% (6/8) resistance to ampicillin, 37.5% (3/8) to erythromycin, and 50% (4/8) to chloramphenicol and gentamicin; all isolates were 100% (8/8) sensitive to doxycycline. *Legionella pneumophila* sg2-15 isolates were 75% (1.5/2) resistant to ampicillin, 100% (2/2) sensitive to doxycycline, erythromycin, and streptomycin, 50% (1/2) sensitive to chloramphenicol and gentamicin (Al Sulami et al., 2013) (Table 1.4.5).

Twenty-eight isolates in Poland were evaluated for antibiotic resistance; the water samples were collected from a drinking water system in several large buildings, such as hospitals, sanatoriums, hotels, and other public buildings (Sikora et al., 2017). *Legionella pneumophila* isolates were sg1-14, which 16 of them were from serogroup 1. Sikora et al., 2017 showed that *L. pneumophila* sg2-14 isolated from a sanatorium showed resistance to azithromycin. The susceptibility E-test indicated that all *L. pneumophila* isolates (sg1-14) were susceptible to ciprofloxacin and rifampicin (Sikora et al., 2017). A study a year later isolated *L. pneumophila* sg2 -14 from hot water systems (taps, showers, and water heaters) and from a

cooling tower in a Barcelona hospital (Graells et al., 2018). The environmental isolates were pulsotyped, by pulse-field gel electrophoresis, and these isolates were grouped into five pulsotypes (Graells et al., 2018). Disk diffusion and E-test showed the antibiotic profile (Graells et al., 2018). Levofloxacin and azithromycin were the two antibiotics that inhibited the growth of all five pulsotyped environmental isolates of *L. pneumophila* (Graells et al., 2018) (Table 1.4.5). Though both papers discussed the susceptibility of *L. pneumophila* sg2-14, the results were different. One study indicated that *L. pneumophila* was resistant to azithromycin, while azithromycin inhibited its growth in another study. Thus, this suggests that ecological factors play a role in the development and spread of antibiotic resistance of *L. pneumophila*.

If environmental factors truly shape the antibiotic resistance (ABR) of *L. pneumophila*, the environmental and clinical isolates should have a similar ABR profile. Rahimi and Vesal, 2017 received respiratory samples from hospitalized patients suffering a respiratory tract infection. The respiratory samples were cultured, and out of the 250 samples collected, 11% (27/250) of the isolates were positive for *L. pneumophila* (Rahimi and Vesal., 2017). *Legionella pneumophila* positive samples were confirmed by lepA gene-specific PCR testing (Rahimi and Vesal, 2017). Disk diffusion susceptibility test showed that 81.48 (22/27), 77.77 (21/27), 51.85 (14/27), 48.14% (13/27) of the *L. pneumophila* isolates were resistant to ciprofloxacin, erythromycin, clarithromycin, and moxifloxacin, respectively (Rahimi and Vesal, 2017). Sharaby et al., 2019, evaluated 12 clinical samples for antibiotic resistance to ciprofloxacin, moxifloxacin levofloxacin, tigecycline, doxycycline, azithromycin, erythromycin, clarithromycin, sulfamethoxazole, and rifampicin. Clinical strains of *L. pneumophila* isolated from sputum samples from hospitalized pneumonia patients were more resistant to doxycycline (Sharaby et al., 2019). The isolation percentage of antibiotic resistance was not described (Sharaby et al.,

2019) (Table 1.4.5). The antibiotic resistance of *Legionella* strains depends on the geographical location, and this means that the the environmental and clinical isolates may have a different resistance profile. Thus, knowing the antibiotic resistance profile by geographical location is imperative for antibiotic therapy.

Table 1.4.5. Antibiotic Resistance of *Legionella* Species in Various Water Types

Location	Water Types	No. of isolates	*Legionella* species	Antibiotic Resistance	Ref
N/A	Lakes, ponds, rivers and water tanks	N/A	*L. pneumophila, L. feelei*	azithromycin, clarithromycin, erythromycin, doxycycline, moxifloxacin, cefuroxime, cephalexin (amoxicillin,)	Subbarram et al., 2017
Iraq	Drinking water supply system	10	*L. pneumophila* sg1, sg 2-14	ampicillin, erythromycin chloramphenicol and gentamicin (ampicillin)	Al Sulami et al., 2013
Israel	Drinking water distribution system	12	*L. pneumophila*	ciprofloxacin, tigecycline, clarithromycin, rifampicin and sulfamethoxazole	Sharaby et al., 2019
Poland	Drinking water system	28	*L. pneumophila* sg1, sg2-14	azithromycin	Sikora et al., 2017
Barcelona	hot water systems (taps, showers, and water heaters) and from a cooling tower	42	*L. pneumophila* sg2 -14	-	Graells et al., 2018
Macau and Guangzhou	Water fountains and cooling towers of public facilities (hotels, schools, and shopping malls)	60	*L. pneumophila, L. rubrilucens, L. gormanii, L. shakespearei, L. feeleii, L. wadsworthii, L. quateirensis*	-	Xiong et al., 2016
China	hot springs, cooling tower, and from pipeline water	37	*Legionella pneumophila* sg1	azithromycin	Jia et al., 2019

Dashed line (-) means no antibiotic resistance found.

Barker et al., 1995 studied the antimicrobial susceptibility of *L. pneumophila* grown in human macrophages and *Acanthamoeba polyphaga*. Barker et al., 1995 grew *L. pneumophila* on buffered charcoal-yeast extract agar and then inoculated isolate in yeast extract broth. For intracellular amoebic and human macrophage growth, they separately co-cultured *Acanthamoeba polyphaga* and U937 cells with *L. pneumophila* (Barker et al., 1995). After both co-culture assays, they released intracellularly *Legionella pneumophila* cells by vortexing for one minute and harvested from the suspension by centrifugation (Barker et al., 1995). They determined if intracellular grown *L. pneumophila* was more resistant to erythromycin, ciprofloxacin, and rifampin (Barker et al., 1995). *Legionella pneumophila* grown in vitro were sensitive to ciprofloxacin and rifampicin but showed resistance after growing inside of U937 (macrophage cell line) and *Acanthamoeba polyphaga* (amoeba) (Barker et al., 1995). From these results, Barker et al., 1995 hypothesized that intraphagocytic (macrophages and amoeba) growth induces the antibiotic-resistant phenotype of *L. pneumophila*. Amoeba and macrophage-grown *L. pneumophila* cells alter the surface composition (proteins, lipopolysaccharides, and fatty acid content) of the organism, and this phenotype is not seen in-vitro grown *L. pneumophila* cells (Barker et al., 1993; Barker et al., 1995; Anwar et al., 1990; Abu Kwaik et al., 1993). The changes in the surface properties of *L. pneumophila* causes alterations in the ability of antibiotic molecules to cross the cell surface (Anwar et al., 1990; Brown et al., 1990; Barker et al., 1995). Overall, the data suggest that *L. pneumophila* has become resistant to antibiotics as a consequence of residing inside macrophages and amoeba. An environmental study of *L. pneumophila* antibiotic profile could be beneficial for understanding the distribution of resistance and susceptibility of various strains geographically in the US.

1.4.6 Prevention

Unfortunately, there is not a vaccine for legionellosis, and currently, there are no EPA regulations specifically for monitoring or controlling *Legionella* in drinking water supplies (Lu et al., 2015). The key to preventing LD is through educating building owners and managers to maintain the building water systems to reduce the risk of the growth and spread of *Legionella* (Ambrose et al., 2020; Lu et al., 2015; Pierre et al., 2019; Valcina et al., 2019; David et al., 2018). At the federal level, there are two federal agencies with different guidelines and recommendations to prevent the accumulation of *Legionella* in the building water systems. The Center for Disease Control and Prevention (CDC, 2018) recommends that all health care facilities maintain a high suspicion (which includes routine sampling) for health-care-associated LD. The CDC also suggests that buildings such as retirement homes, hotels, apartments, or buildings with ten stories or more have a water management plan for hot and cold-water systems. The Environmental Protection Agency has set the maximum contaminant level goal for *Legionella* at zero, but the *Safe Drinking Water Act* does not have an explicit control for this bacterium (EPA, 2018).

The state-level also lacks guidelines and recommendations for monitoring and controlling *L. pneumophila* in the drinking water system. In the state of Michigan, LD increased in 2014 and 2015 in Genesee County (Shah et al., 2018). The Michigan Department of Human and Health Services, the Genesee County Health Department, and Genesee County Medical Society are collaborating to build a better surveillance program to detect and track LD cases (Michigan Department of Health and Human Services, 2018). The Michigan Health Department of Human and Health Services distributed a toolkit from CDC to building managers and local health departments statewide (Michigan Department of Health and Human Services, 2018). The CDC

toolkit does the following i. Helps building leaders develop a water management plan, ii. Identifies areas in the premises that contribute to *Legionella* regrowth, iii. Addresses how to decrease that risk of potential *Legionella* growth, like areas where water stagnation may occur, or changes in water quality (lower disinfectant residuals, increase turbidity [CDC, 2018]).

Genesee County has also provided its residents with additional Legionellosis preventative measures (Michigan Department of Health and Human Services, 2018). They recommend that the showerheads are cleaned regularly and soaked with bleached and water for at least two hours regularly. Genesee County also suggests that the water in humidifiers, nebulizers, or water heaters are to be maintained in the resident by manually cleaning the inside of humidifier per the manufacturer's instructions (Michigan Department of Health and Human Services, 2018).

At the local level, ASHRAE standard 188-2018 Legionellosis: Risk Management for Building Water Systems is the only North American accredited standard. ASHRAE 188-2018 is a voluntary standard, which means this consensus guides without regulatory authority unless incorporated into local building codes (ASHRAE, 2018). This standard was created by a committee of people from different backgrounds such as, "water treatment practitioners, plumbing specialists, hospital and health-care officials, CDC staff, filtration providers, and regulatory experts" (Root, 2018). This standard applies to a wide range of building types and adapted by the CDC into the CDC toolkit (CDC, 2018). Both ASHRAE and CDC have seven recommendations within the water management plan for *Legionella* control; create a water safety management team, describe the water system and cooling tower, identify risk, decide where to apply control limit, corrective actions, verification and validation, and documentation. It may or may not include the monitoring for *Legionella* in the water systems (CDC, 2018; ASHRAE, 2018).

1.5 Ecology of *Legionella*

Legionella ecology is relatively complex. *Legionella's* natural niche are rivers, streams, groundwater, and thermally polluted waters (Lesnik et al., 2016, Whiley et al., 2017; Farhat et al., 2012; Donohue et al., 2014; De Giglio et al., 2019; Shen et al., 2015; Ishizaki et al., 2016) as well as engineered systems including cooling towers, air conditioners, (Nakanishi et al., 2019; Zeng et al., 2019; Gong et al., 2017; Llewellyn et al., 2017; Lin et al., 2009) hot tubs, hot tap water lines and showers (Donohue et al., 2019; Barton et al., 2017; Collins et al., 2017; Rhoads et al., 2016; Farhat et al., 2012). In the US, *Legionella* has been isolated from 67 bodies of water, with temperatures ranging from 5.7°C to 63°C (Fliermans., 1996). Experimentally, the optimal temperature for *Legionella* growth is between 25-45°C (Bedard et al., 2015; Konishi et al., 2006; Katz and Hammel, 1987) but will die off at 70°C (Cervero-Aragó et al., 2019).

1.5.1 *Legionella* and *Legionella pneumophila* occurrence in water systems

Groundwater serves as one of the natural reservoirs for *Legionella* (Mapili et al., 2020; De Giglio et al., 2019; Costa et al., 2005; Blackburn et al., 2004; Brooks et al., 2004). *Legionella pneumophila* has been detected in groundwater samples tested in the US(King et al., 2016; Mapili et al., 2020), and internationally (Wullings and van der Kooij, 2006; Wullings et al., 2011; Brooks et al., 2004; Costa et al., 2005; Riffard et al., 2001). In the US, the detection of *L. pneumophila* ranges from 1.1 MPN and 4.74 MPN per mL. In Canada, *Legionella* ranged in density (Riffard et al., 2001). Twelve samples from several different groundwaters were between 1×10^2–8.4×10^4 CFU/L (Riffard et al., 2001). Another study in Canada showed that the occurrence of *Legionella* in both warm and cold groundwater samples varied in concentration from 10^2 to 10^5 CFU/L (Brooks et al., 2004). The frequency and density of *L. pneumophila* in groundwater wells were dependent on the location, whether the well was treated or untreated

(Lieberman et al. 1994 & Craun et al., 2010). Twenty-one percent (6/29) of the sampling sites were positive for *L. pneumophila* in untreated wells (Lieberman et al. 1994). In contrast, *L. pneumophila* was undetected in a treatment plant, using groundwater as the water source (Wullings et al., 2011).

Legionella has been shown to multiply on filters inside the drinking water treatment plants (Li et al., 2017; Lin et al., 2014; Hou et al., 2018; Zanacic et al., 2017; Gomes et al., 2020). During the treatment process, *Legionella* species accumulated on the biological activated (BAC) filtration, increasing in BAC effluents. After the BAC step, chlorine disinfection eliminated *Legionella* species from the drinking water before being distributed to the public (Li et al., 2017). Another study examined the concentrations of *Legionella* species in the biofilm, and the water samples throughout the different stages of treatment (Lin et al., 2014). *Legionella* levels decreased to 4.45 log copies g^{-1} in the biofilm sample but increased to 6.37 log copies g^{-1} after chlorine disinfection in the water sample (Lin et al., 2014). After secondary treatment in drinking water treatment plants (DWTP), *Legionella* spp. have been present in water samples (Lin et al., 2014; Hou et al., 2018; Gomes et al., 2020; Wang et al., 2019; Hull et al., 2017). King et al., 2016 collected 25 samples from several drinking water treatment plants in the US, and *L. pneumophila* was found in 4% (1/25) of treated water samples by qPCR. Ji et al., 2015 collected 60, 54, 59, 60, and 59 water samples from five drinking water treatment plants. *Legionella* spp. was also detected in four out of five water treatment plants in the eastern part of the U.S. by Illumina sequencing (Ji et al., 2015). The percentage of *Legionella* spp. detected in A, B, C, and E drinking water treatment plants were 1.7, 7.4, 78.0, and 13.6%, respectively (Ji et al., 2015).

Water distribution systems are also sites where *L. pneumophila* may grow and persist (Jjemba et al., 2010; Perrin et al., 2019; Chen et al., 2019; Waak et al., 2018; Ji et al., 2015; Stout et al., 1985). *Legionella pneumophila* was detected at a low frequency in six different locations at the same distribution system in a metropolitan area in the U.S. (Lu et al., 2016). A higher concentration of *Legionella* species was detected at distal sites relative to the point of entry of the drinking water distribution system (Lu et al., 2016). *Legionella* spp. were detected in 57% (23/41) of the samples at a concentration of 85 CE/ L^{-1} by qPCR analysis (Lu et al., 2016). In Virginia, 69% (20/29) of *Legionella* species and less than 13.7% (4/29) of *L. pneumophila* occurrence was observed in a drinking water distribution system (Wang et al., 2012). In a drinking water distribution system in Florida, the occurrence of *Legionella* spp. and *L. pneumophila* was 100% (15/15) and 20% (3/15), respectively (Wang et al., 2012). In Virginia, the concentrations of *Legionella* spp. were 2.3 X 10^3 GC/ mL, while in Florida, the concentration was lower, 759.6 GC/ mL (Wang et al., 2012). The concentration of *L. pneumophila* was 13.7 and 219.4 GC/ mL in Virginia and Florida, respectively (Wang et al., 2012). These concentrations were quantified by qPCR (Wang et al., 2012). Though the concentration of *Legionella* diminishes after treatment (chlorine), some species (including *L. pneumophila*) persist (Wang et al., 2014). The type of disinfectant was not mentioned in Lu et al., 2015 study, but chlorine was utilized in Virginia and Florida with concentrations of 2.21 and 2.15 mg/L, respectively (Wang et al., 2012). Although *L. pneumophila* has occurred in the drinking water supply system, the concentrations found are relatively low; this implies that the treatment is effective to some degree. For example, tap water under a chlorinated distribution system in Minneapolis U.S. showed no detection of *Legionella pneumophila* (Waak et al., 2018).

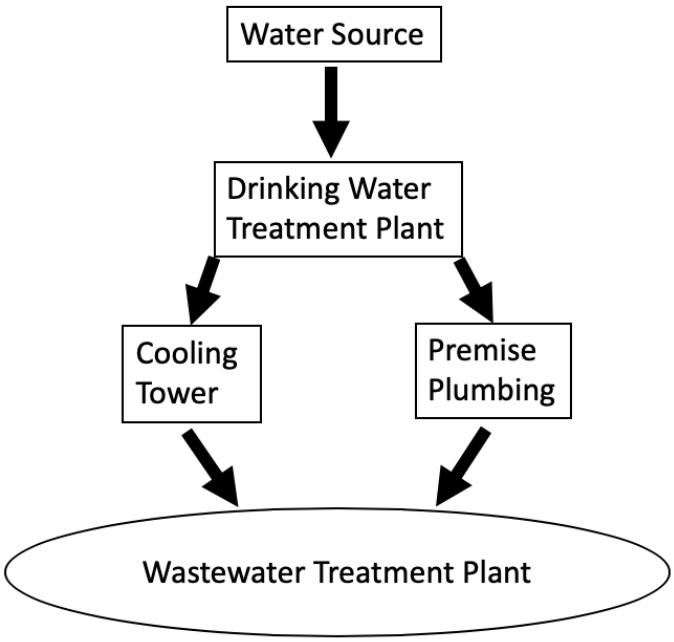
Legionella pneumophila can grow in the influent water pipe (water entering into the building). So far, there has only been one study to examine the influent water pipe in a building (Rhoads et al., 2016). *Legionella pneumophila* was below the quantification limit (qPCR) in the influent in a healthcare facility (Rhoads et al., 2016), which used monochloramine as their type of disinfection (Rhoads et al., 2016).

Concerning points of use, *Legionella* spp. are present in faucets (cold and hot), showers, decorative fountains, and whirlpool spas (Valcina et al., 2019; Tachibana et al., 2013; Haupt et al., 2012; Thomas et al., 2014; Collins et al., 2017). The detection frequency and concentration of *Legionella* spp. in cold water samples suggest that this environment is only conducive for its survival (Donohue et al., 2014; Donohue et al., 2019; Lesnik et al., 2016; Valcina et al., 2019). In contrast, *Legionella* spp. are frequently detected in hot-water samples, and the concentrations are higher, this suggests that this water temperature serves as a site for the amplification of these species (Valcina et al., 2019; Bollin et al., 1985; Hrubá et al., 2009; Borella et al., 2004; Bédard et al., 2019; Totaro et al., 2017; Rhoads et al., 2016; Brazeau and Edwards, 2013; Baron et al., 2014; Ditommaso et al., 2010; Donohue et al., 2019). Thus, the rest of the paragraph will focus on the characterization of *Legionella* in hot water samples (hot-water heaters, taps, etc.). Surveying hot water systems, *L. pneumophila* increases in concentration in water temperature around 30-35°C but decreases at 50-60°C (Hrubá et al., 2009). *Legionella* species were detected and quantified at the bottom of the water heater at a healthcare facility at high concentrations (>1500 gene copies per mL) (Rhoads et al., 2016). Recirculation tanks increase *Legionella* growth by having lower levels of disinfectant residual and water temperatures generally set at 49°C (Brazeau et al., 2013). *Legionella pneumophila* was positive in 37% (116/299) of samples tested in the recirculation loop, and 12% (27/299) were positive at distal sites of the loop

(Ditommaso et al., 2010). Baron et al., 2014 surveyed and collected hot water from five new faucet filters and five sinks at a cancer center in Northwestern Pennsylvania. The presence of *L. pneumophila* sg1 in the faucet filter was sporadic and was recovered from 1 site. In contrast, *L. pneumophila* was seen at every sink tested, and the quantification ranged from 1-10 CFU/mL to 1,150 CFU/mL (Baron et al., 2014). A study investigated the prevalence of *Legionella* spp. by culture and qPCR in household showers (Collins et al., 2017). *Legionella* spp. and *L. pneumophila* sg1 were detected in 8% (8/99) and 2% (2/99) of showers, respectively (Collins et al., 2017).

Other hot water exposure sites, such as cooling towers, serves as a major source for the amplification of *Legionella* species (Paranjape et al., 2019; García-Fulgueiras et al., 2003; Bentham, 1993; Llewellyn et al., 2017; Farhat et al., 2018; Li et al., 2015; Baudart et al., 2015; Scheikl et al., 2016; Gong et al., 2017; Canals et al., 2015). Li et al., 2015 tested 22 industrial cooling towers, and 36% (91/255) of 255 water samples tested were *Legionella*-positive. The concentrations ranged from 100 to 88,000 CFU/L (Li et al., 2015). From the identified/isolated colonies, there was a range of serogroups present, Sg1, Sg6, Sg5, Sg8, Sg3, and Sg9, with sg1 being the most prevalent and sg9 being the least abundant (Li et al., 2015). Figure 1.5.1 shows the fate of *Legionella* in the environment. As described above, *Legionella* species are present in the natural environment and enters the drinking water treatment plant by the surviving chemical disinfectants and can enter the premise plumbing and cooling tower systems. *Legionella* enters the wastewater treatment plant from daily use (toilet flushing, shower, handwashing, etc.) in the building, and the cycle repeats itself.

Figure 1.5.1. The Ecology of *Legionella* spp.



1.5.2 *Legionella* Biofilms in Distribution and Premise Plumbing Systems

Biofilms are ubiquitous within drinking water distribution, and premise plumbing (Deines et al., 2010; Yu et al., 2010; Shaheen et al., 2019; Falkinham et al., 2015; Buse et al., 2019; Buse et al., 2017; Lu et al., 2014). The water distribution and the premise plumbing systems have a wide range of physiochemical parameters that impacts the proliferation of *Legionella* (Garner et al., 2018; Shaheen et al., 2019, Van der Kooij et al., 2016). Lower hot (warm) water temperatures are a contributing factor to the formation of biofilms (Rhoades et al., 2016). The nutrients, pipe material, and optimal water temperatures (25- 45°C) in both systems (distribution and premise) provide a favorable condition for biofilms (Buse et al., 2019; Shaheen et al., 2019; Lu et al., 2014; Wang et al., 2014; Liu et al., 2014; van der Kooij et al., 2005; Schwartz et al., 2003; Waak et al., 2018; De Filippis et al., 2018). *Legionella* growth in the premise depends on their association with biofilms (Shaheen et al., 2019). An experimental

approach showed that inoculation of *L. pneumophila* onto biofilm reactors, which were managed at 20°C, *L. pneumophila* can persist for up to 4 months in uPVC-grown drinking water biofilms (Buse et al., 2013b). *Legionella pneumophila* has also been shown to persist on the walls of a water tank (Lin et al., 2014). Though, biofilms developed in the distribution and premise plumbing systems, the bacterial biofilm composition differ between pipe material (Moritz et al., 2010, Valster et al., 2010, Messi et al., 2011). In drinking water systems, copper and uPVC are commonly used; this influences the biofilm growth in chlorinated systems, reaching up to log 6–7 cm^{-2} cells of bacterial growth (Morvay et al., 2011). Thus, it is crucial to understand how the pipe material can influence the re-growth of *L. pneumophila* in the premise, as it has been shown experimentally to colonize copper surfaces more effectively and shed from the biofilm at a higher frequency and duration compared to uPVC surfaces (Buse et al., 2013).

1.5.3 *Legionella* and Other Bacteria in the Biofilm

Several different bacteria shape the microbial community in premise plumbing. The most abundant bacterial classes of biofilms in the drinking water distribution are *Alpha-*, *Beta-*, and *Gamma-proteobacteria* (De Sotto et al., 2020; Garner et al., 2019; Fish et al., 2017). The most abundant species prevalent in the bacterial biofilm are *Acinetobacter, Pseudomonas aeruginosa, Mycobacterium avium complex, E.coli, staphylococcus aureus, Aeromonas, Enterococcus, and Klebsiella* (September et al., 2004; Mahapatra et al., 2015). The occurrence of these biofilms increases the resistance of the bacteria, including *Legionella* spp. to disinfection in the whole drinking water supply system (Fish et al., 2018; Hollinger et al., 2014).

1.5.4 *Legionella* and Amoebae

Legionella growth in the premise depends on their association with amoeba hosts (Kuiper et al., 2004; Lau and Ashbolt, 2009; Valster et al., 2011; Buse and Ashbolt, 2011; Shaheen et al.,

2019). Naturally-occurring amoeba facilitates the growth and transportation of *Legionella* in their aquatic environments (Kuiper et al., 2004; Swanson and Hammer, 2010). The density of *Legionella* supposedly depends on the concentration and the composition of amoebae present (Buse and Ashbolt, 2011). *Legionella pneumophila* grows inside of amoebae and can survive unfavorable aquatic environment conditions such as the presence of chemical disinfectants, regularly used to treat water in engineered water systems (Hsu et al., 2015; Kwaik et al. 1998; Marciano-Cabral 2004; Declerck 2010; Valster et al., 2009). Besides surviving inside of amoebae, *Legionella* also replicates before being released into its aquatic environment (Muchesa et al., 2017; Barker et al. 1993; Cirillo et al. 1994). Once *L.pneumophila* is free in its environment from amoebae, there is altered properties, a higher capacity of virulence, and antibiotic resistance as a result of horizontal gene transfer (Marciano-Cabral et al., 2010; Barker et al. 1993; Cirillo et al. 1994).

Legionella and *Acanthamoeba* spp*, Naegleria fowleri,* and *Hartmannella vermiformis* are present in the same aquatic environments (Wang et al., 2012; Marciano-Cabral et al., 2010; Jjemba et al., 2015; Liu et al., 2019; Delafont et al., 2013). Lasheras et al., 2006 surveyed a hospital water distribution system and detected amoebae in 69% (73/106) of samples assessed and 87% (58/67) were *Legionella* species-positive samples. *Legionella pneumophila, Acanthamoeba* spp*. and Hartmanella vermiformis* have been isolated from taps, distribution water (Thomas and Ashbolt, 2011; Wang et al., 2012; Delafont et al., 2013; Lu et al., 2016) reservoirs and water treatment plants (Garcia et al., 2013; Valcina et al., 2019). In an experimental system, Anand et al., 1983 showed that *Legionella* is engulfed into amebae cells at 20°C, which is a suitable condition for *Legionella* multiplication in *Acanthamoeba palesinensis*, but at 35°C *Legionella* lyses the amoeba cell, releasing *Legionella* into the environment. An

environmental study many years later, surveyed two chlormianted drinking water distribution systems in Virginia and Florida during the spring and fall and demonstrated that *Hartmanella vermiformis* was more abundant than *Acanthamoeba* spp. in both samples, indicating that its abundance is relative to lower ambient temperature (Wang et al., 2012). Wang et al., 2012 and Marciano-Cabral et al., 2010 found a seasonal trend of the co-occurrence between amoebae and *Legionella* species. *Acanthamoeba* spp. was detected in hotter climates (summer season), and the concentration was relatively low (Marciano-Cabral et al., 2010; Wang et al., 2012), while *Hartmanella vermiformis* (Wang et al., 2012) and *Naegleria fowleri* were frequently detected and the concentrations were more abundant (Marciano-Cabral et al., 2010). Marciano-Cabral et al., 2010 suggested that there is a seasonal relationship driving the interaction of *Legionella* specific amoebae. *Legionella* species co-exist with *Acanthamoeba castellanii, Acanthamoeba polyphaga, Hartmannella vermiformis,* and *Naegleria fowleri* (Dobrowsky et al., 2016 & Coşkun et al., 2013) in the drinking water supply system (Valcina et al., 2019; Muchesa et al., 2017; Liu et al., 2019).

1.6 Effect of Residual Chlorine, Temperature, Water Stagnation, and Pipe Material on *Legionella pneumophila* in Premise Plumbing Systems

Drinking water treatment plants use chlorine as one of the secondary disinfectants to inhibit the growth of microbial material (EPA, 2017). However, disinfectant residuals decay in the premise relative to the drinking water treatment plant and the water distribution systems (Bédard et al., 2018; Charron et al., 2015; Prévost et al., 1997). Water age (water residence time) is another favorable growth condition for *L. pneumophila* (Rhoads et al., 2016; Masters et al., 2015; Ambrose et al., 2020; Wang et al., 2012; Hull et al., 2017, Wang et al., 2014, Wang et al., 2015, Rhoads et al., 2020). In the case of increased water age, when the water reaches the tap,

the water temperature increases in the cold-water tap and decreases in the hot-water taps (Zlatanovic et al., 2017; Moerman et al., 2014; Agudelo-Vera et al., 2020; Bédard et al., 2019). Water stagnation (water sitting in the pipes—as a direct result of low water usage patterns) significantly influence the growth of *L. pneumophila* in premise plumbing (Buse et al., 2019; Totaro et al., 2017; Ji et al., 2015; Rhoads et al., 2015; Huang et al., 2020). Pipe materials also influences the proliferation of *L. pneumophila* in premise plumbing (Proctor et al., 2017; Ji et al., 2015; Learbuch et al., 2019; Völker et al., 2016; Paniagua et al., 2020). Collectively, chlorine residual, water age, water stagnation, and pipe material are factors favoring growth of *L. pneumophila* in premise plumbing systems.

1.6.1 Effect of Residual Chlorine on *Legionella pneumophila* Growth in Premise Plumbing Systems

Chlorine is a common practice to control and prevent *L. pneumophila* amplification in drinking water systems of large buildings (EPA, 2017). The residual goal for free chlorine in the distribution systems should be between 0.2 to 0.5 mg/L (Zahran et al., 2018; Daley et al., 2017). Although free chlorine at 0.5 mg/L inactivates *L. pneumophila* in a laboratory setting (Cervero-Arago et al., 2015), this organism can still survive within the premise despite chlorine residuals (Buse et al., 2019; Donohue et al., 2019; Cervero-Arago et al., 2015; Pierre et al., 2019; Totaro et al., 2017; Lu et al., 2017; Marchesi et al., 2016). For example, *Legionella* spp. were detected in 29.8% (60/201) of hot water samples with a concentration of 3.0×10^2 CFU liter^{-1} (Marchesi et al., 2016). *Legionella pneumophila* colonized 85% of the hot water samples with residual chlorine ranging from 0.3 to 0.5 mg/L (Marchesi et al., 2016). The concentrations of *L. pneumophila* ranged from 10^2 to 10^3 (Marchesi et al., 2016).

The rising number of LD outbreaks causes concern that chlorine is not very effective in removing *L. pneumophila* from premise plumbing systems (Berjeaud et al., 2016; Declerck, 2010; Zahran et al., 2018; Rafiee et al., 2014; Hlavsa et al., 2018). Considering amoebae and biofilms serve as protection to *L. pneumophila* against chlorine disinfection in water system (Hsu et al., 2015; Kwaik et al. 1998; Marciano-Cabral 2004; Declerck 2010; Valster et al., 2009), another alternative disinfectant method should be used to eradicate its colonization throughout the whole drinking water system.

1.6.2 Effect of Temperature on *Legionella pneumophila* Growth in Premise Plumbing Systems

Water temperature is a critical factor that can increase the risk of *L. pneumophila* growth in the premise. *Legionella pneumophila* can survive in cold water temperatures for extended periods (Rogers et al., 1994; Donohue et al., 2014; Donohue et al., 2019; Lesnik et al., 2016; Valcina et al., 2019), by entering a low metabolic state (Söderberg and Cianciotto, 2010). And when environmental conditions become favorable, *L. pneumophila* will replicate (Valcina et al., 2019; Bollin et al., 1985; Hrubá et al., 2009; Borella et al., 2004; Bédard et al., 2019; Totaro et al., 2017; Rhoads et al., 2016; Brazeau and Edwards, 2013; Baron et al., 2014; Ditommaso et al., 2010; Donohue et al., 2019). As stated above, the optimal water temperature for *L.pneumophila* ranges between 25–45°C (Falkinham et al., 2015; Hrubá et al., 2009).

Water temperatures set higher than 60°C is an essential procedure for disinfecting *L. pneumophila* in engineered water systems (Stout et al., 1998; Chang et al., 2007). In a controlled experiment, Proctor et al., 2017 demonstrated how various temperatures in simulated water heaters affect the growth of *L. pneumophila*. As the temperature increased from 41°C to 45, 49, and 53°C, *L. pneumophila* gene copies decreased (Proctor et al., 2017). A surveillance

study was conducted on houses with hot water storage tanks and recirculation of hot water (Mathys et al., 2008). The range of the water heaters was 50-70°C, and the points of use (taps, etc.) water temperature ranged from 47-51°C (Mathys et al., 2008). In the recirculating hot-water tap, the concentration of *Legionella* ranged from 387-100,000 CFU/100 mL (Mathys et al., 2008). *Legionella pneumophila* positive samples accounted for 93.9% (375/400), and 71.8% (287/400) belonged to *L. pneumophila* sg1, but the exact concentrations of the species and serogroup were not disclosed (Mathys et al., 2008). Because water temperatures are lower in distal sites (Rhoads et al., 2015; Rhoads et al., 2016), it is a good practice to set the water heater temperature above 60°C to ensure water temperatures are higher than 55°C by the time the water reaches the points of use [Arvand et al., 2011; Blanc et al., 2005]). Setting the temperature in the water heater at 60°C can be effective in reducing the percentage of *Legionella* positive samples (Arvand et al., 2011; Blanc et al., 2005).

1.6.3 Effect of Water Stagnation and Water Age on *Legionella pneumophila* in Premise Plumbing Systems

Water stagnation is defined as water that sits for a long duration of time, which could be several hours to some days (Buse et al., 2019; Totaro et al., 2017; Ji et al., 2015; Rhoads et al., 2015; Huang et al., 2020; Rhoads et al., 2016; Bédard et al., 2018; Lipphaus et al., 2014). Stagnant water supports the growth of *Legionella pneumophila* (Fisher-Hoch et al., 1982; Tobin et al., 1981; Ciesielski et al., 1984; Rhoads et al., 2016). As water sits in a piping system, the disinfectant residual will dissipate over time, this allows *Legionella* to build up in the premise plumbing (Rhoads et al., 2016; Jjemba et al., 2010; Buse et al., 2019). No flow or low flow of water in pipes create dead legs, are ecological niches for *Legionella* growth (Liu et al., 2006; Totaro et al., 2017; Darelid et al., 2002). For example, stagnated water for 8hrs a day

influenced *Legionella* spp. (Ji et al., 2015). Areas with dead-legs and low use taps played a role in the colonization of *Legionella* spp. in a large hospital building (Gavaldà et al., 2019). The hot water system was sampled monthly for over eight years (Gavaldà et al., 2019). When water was infrequently used the percentage of positive samples and the concentration of *Legionella* spp. was more significant than the areas with frequently used water (Gavaldà et al., 2019). For example, when water was used daily, the percentage of *Legionella* positive samples was 54% (34/62), with 74% (46/62) of the water samples with a concentration below 10^3 CFU/L (Gavaldà et al., 2019). When water was not used daily, the percentage of *Legionella* positive samples was 75% (65/86), with 40% of the water samples with a concentration greater than 10^3 CFU/L (Gavaldà et al., 2019). This same trend was also seen when the hospital was renovated, creating dead legs (Gavaldà et al., 2019). Forty-six percent (15/32) of *Legionella* positive samples reached a concentration greater than 10^3 CFU/L. In contrast, when parts of the hospital were not renewed, 68% (80/116) of the *Legionella* positive samples was lower than 10^3 CFU/L (Gavaldà et al., 2019).

Water age (hydraulic retention time) is defined by the distance in which the water travels from the treatment plant to the points of use (Tinker et al., 2013), and this can vary from building to building. For example, the hydraulic retention time for a conventional house was ~one day, and for an office suite, it was ~1-6 months (30 to 180 days) (Rhoads et al., 2016). The concentration of *Legionella* spp. in the office was 4.58 Log_{10} GC/ mL, whereas, in the conventional house, it was not detected (Rhoads et al., 2016). Thus, there is evidence that increases water age influences *Legionella pneumophila* regrowth at the building level (premise-plumbing), which is downstream of the municipal level (distribution system water age).

1.6.4 Effect of Pipe Material on *Legionella Pneumophila* Growth in Premise Plumbing Systems

Different pipe material can influence the build-up of *Legionella* in premise plumbing (Proctor et al., 2017; Niedeveld et al., 1986; Rogers et al., 1994; Buse et al., 2014; Gião et al., 2015; van der Kooij et al., 2002; Learbuch et al., 2019; Schoenen et al., 1988). For example, *L. pneumophila* can significantly proliferate in cast iron pipes (van der Lugt et al., 2017), while the growth is intermediate in plastic piping (Buse et al., 2014; Gião et al., 2015; van der Kooij et al., 2002). In contrast, copper piping is the least favorable pipe condition for its regrowth (Rogers et al., 1994; Learbuch et al., 2019; Schoenen et al., 1988). Proctor et al., 2017 showed that copper reduces the growth of *L. pneumophila,* and cross-linked polyethylene (PEX) pipes (plastic material) significantly supported its proliferation. When copper was experimentally used, the relative abundance of *L. pneumophila* on the first day was 3.0 Log_{10} GC/ mL. By the end of the experiment (300 days), it was no longer detected (Proctor et al., 2017). When PEX was used, however, the concentration of *L. pneumophila* was 4.5 Log_{10} GC/ mL, and this remained the same throughout the experiment (Proctor et al., 2017). Copper has also resulted in less biofilm growth than plastic pipe materials (Proctor et al., 2018; van der Kooij et al., 2017). Overall, *L. pneumophila* growth is diminished when copper is used.

1.6.5 A Combined Effect of Chlorine, Temperature, Water Age, Water Stagnation and Pipe Material on *Legionella pneumophila*

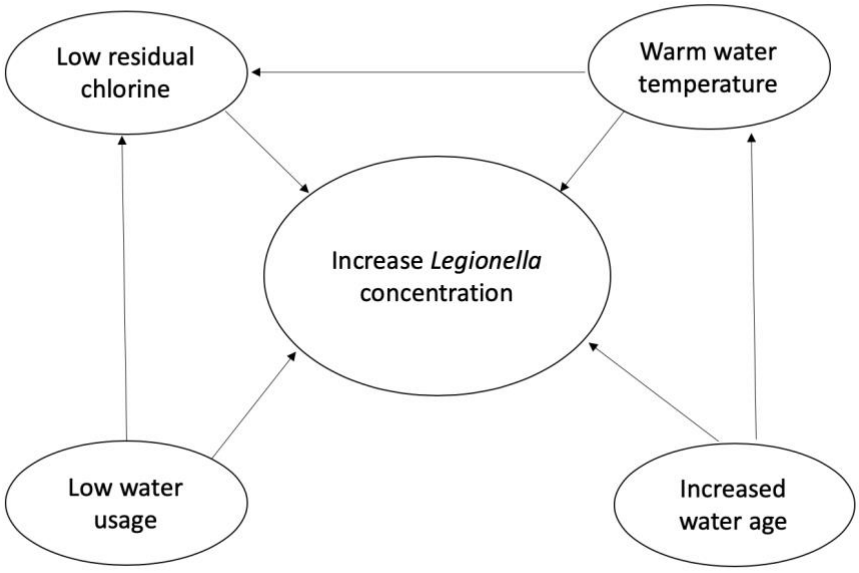
A combination of environmental factors influences the regrowth of *Legionella pneumophila* in a water system (Proctor et al., 2017; Rhoads et al., 2016; Lehtola et al., 2005). Water age, pipe material, and disinfectant residuals interact with each other to produce different physiochemical conditions and ecological niches. Thus, the following paragraphs will focus on

the combined effects of chlorine, temperature, water age, water stagnation, and pipe material, and the combination affects *Legionella pneumophila*.

Chlorine decay is affected by many factors such as water temperature, water stagnation, water age, and pipe materials (metal or plastic) (Rhoads et al., 2016; Zheng et al., 2015; Lu et al., 2014; Zhang et al., 2008; Zhang and Edwards, 2009; Liu et al., 2013; Nguyen et al., 2012). Rhoads et al., 2016 showed that the rapid rate of residual chlorine in premise plumbing was due to increased water age. Wang et al., 2012 also examined how water age influenced the residual chlorine. As the water aged, the chlorinated simulated distribution system decayed (Wang et al., 2012). Another effect on residual chlorine is copper. For example, copper interacts with dissolved organic matter in water and from this interaction, copper lowers the disinfection residual (Lehtola et al., 2005; Zheng et al., 2015; Lu et al., 2014; Zhang et al., 2008; Zhang and Edwards, 2009; Liu et al., 2013; Nguyen et al., 2012). The combined effect of increased water age and copper pipe material are factors that decrease residual chlorine, and this combined effect leads to an increased *Legionella pneumophila* in water (Rhoads et al., 2016; Wang et al., 2012) (Figure 1.6.5).

Proctor et al., 2017 showed that temperature and pipe material influenced *L. pneumophila.* At a constant temperature (32°C), copper pipe material decreased *Legionella pneumophila* gene copies, while PEX pipe material increased its concentration (Proctor et al., 2017). *Legionella pneumophila* gene copies significantly increased when the water temperature was near its optimal range (41°C) with PEX pipes (Proctor et al., 2017). Thus, temperature and pipe material were the most effective factors affecting the growth of *L. pneumophila* (Proctor et al., 2017).

Figure 1.6.5. Combined Effects on the Amplification of *Legionella* spp.



1.7 Risk Assessment of *Legionella*

Legionnaires' Disease is significantly rising in the United States (Shah et al., 2018), and this is of significant concern to public health authorities and building owners, particularly hospitals. The rise in outbreaks has led to *Legionella* control guidelines from four federal agencies; CDC, EPA, OSHA, and VHA (Parr et al., 2015). These agencies provide guidance and recommendations to prevent the accumulation of *Legionella* in the building water systems but are not federally regulated. The American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE) standard 188-2018 Legionellosis: Risk Management for Building Water Systems is the only North American accredited standard (Root, 2018). However, ASHRAE is voluntary, which means a consensus approach guides implementation without regulatory authority unless incorporated into local building codes (Cotruvo et al., 2019). Yet the success of these management strategies to decrease the growth of *Legionella* has not been well documented to date.

Risk assessment models are now developing for *Legionella (*Armstrong and Haas, 2008; Ahmed et al., 2010; Azuma et al., 2013; Hamilton et al., 2018; Hamilton et al., 2019; Sharaby et al., 2019; Prasad et al., 2017; Volker et al., 2016; Perinel et al., 2018; Storey et al., 2004; Pourchez et al., 2017). The focus has been on hazard identification assessment, but the exposure to address the public health risk from these bacteria has come with the most uncertainties.

1.7.1 Hazard Identification

Legionella bacteria are free-living organisms that grow in aquatic sources and also exist as intracellular organisms of freshwater amoebae (Richards et al., 2013). Legionnaires Disease is a type of pneumonia that can be severe. As describe in the introduction, LD is caused primarily by *L. pneumophila,* but other pathogenic *Legionella* species also can cause this disease. This disease affects susceptible individuals after water-related environmental aerosol exposures. Various issues are surrounding community-acquired LD. For example, the other *Legionella* species associated with human disease are not as well studied (Table 1.7.1.1 to 1.7.1.4) (Vaccro et al., 2016, Potts et al., 2013; Waldron et al., 2015; Siegel et al., 2010; Neiderud et al., 2013; Gobin et al., 2009; Murdoch and Chambers, 2000; Buchbinder et al., 2004; Muder and Yu, 2002). Another issue is the variability in the virulence characteristics within individual species; *L. pneumophila* sg1 is responsible for 70-90% of the documented cases of human disease (Brady and Sundareshan, 2018), while *Legionella anisa* infectivity is seen as rare (Mee-Marquet et al., 2006, Fallon and Stack, 1990). Clinical laboratories are limited in the diagnosis of non-pneumophila infections; this creates a diagnostic bias that exists in the reported cases because of the widely used urinary antigen test, which only recognizes *L. pneumophila* sg1 (Mercante and Winchell, 2015).

Tables 1.7.1.1 to 1.7.1.4 describe the documented cases *for Legionella anisa, Legionella longbeachae, Legionella micdadei,* and *Legionella bozemanii.*

The first case of LD caused by *Legionella anisa* in the US occurred in 1986 (Thacker et al., 1990). These documented cases are likely underestimated as *L. anisa* has been isolated from potable waters and a cooling tower since 1980 (Gorman et al., 1985). During the summer of 1986, there was a sporadic case of LD caused by *Legionella anisa*; this bacterium was detected from a diabetic patient. *Legionella anisa* was identified by a slide agglutination test (Table 1.7.1.1). Since the first documented case, *Legionella anisa* has since accounted for several LD cases (McNally et al., 2000; Yu et al., 2002; Sanchez et al., 2013). During these instances, the source of infection was unknown.

Table 1.7.1.1. *Legionella anisa.* Isolated from Humans Samples in the United States Since the Identification of the Genus, *Legionella*

Year	Peak Month	Case	Host Risk Factors	Isolation Type	Detection Method	Ref
1986	July	Sporadic	Unknown; Diabetes	Bronchoalveolar lavage specimen of a non-insulin dependent diabetes mellitus patient	Slide Agglutinin Test	Thacker et al., 1990
1992 - 1993	March	Retrospective Serological Survey	Unknown; 4 cases were detected from patients with community acquired patients	Acute and convalescent sera collected from patients	Indirect immunoflu o- rescence Antibody Assay	McNally et al., 2000
1980 - 2001	Not Specified	Retrospective Survey of Sporadic Cases	Unknown; cases were detected from patients with community acquired patients	Respiratory Tract Specimens	Culture	Yu et al., 2002
2013	June	Retrospective Detection of a Sporadic Case	Stage IV angioimmunoblastic T-cell lymphoma	Lung Tissue	16S rRNA Gene Sequencing and Culture	Sanchez et al., 2013

In the United States, there have been documented cases of *Legionella longbeachae* infections since the early 1980s (Table 1.7.1.2). Four patients in different geographical locations were diagnosed during a similar time frame (McKinney et al., 1981), and later that year, there was another sporadic case from a pneumonia patient (Bibb et al., 1981). To date, the most recent report of *L. longbeachae* infection occurred in 2000 (Duchin et al., 2000). The cases listed in Table 1.7.1.2 are likely to be underestimated — factors explaining this is a lack of surveillance and diagnostic tools.

Table 1.7.1.2. *Legionella longbeachae* Isolated from Humans Samples in the United States Since the Identification of the Genus, *Legionella*

Year	Peak Month	Case	Host Risk Factors	Isolation Type	Detection Method	Ref
1981	Not Specified	Sporadic Case	Unknown	Respiratory Tract Specimens	Culture Method	McKinney et al., 1981
1981	June-July	Sporadic Case	Diabetes Mellitus; Heart Disease, Failure	Postmortem Lung Tissue of a Pneumonia Patient	Culture Method	Bibb et al., 1981
2000	May-June	Sporadic Case	Unknown	Patients Sputum	Amplified Fragment Length Polymorphism Typing	Duchin et al., 2000

One year after the first outbreak of *L. pneumophila, L. micdadei* was identified by causing infections (Doebbeling et al., 1989). Table 1.7.1.3 details the hospital-acquired outbreaks of LD caused by *L. micdadei* (Pasculle et al., 1979; Myerowitz et al., 1979; Cordes et al., 1981; Harrington et al., 1996; Knirsch et al., 2000). *Legionella micdadei* also causes sporadic infections, and these cases (Table 1.7.1.3) have been documented since 1980 (Pasculle et al., 1980; Schwebke et al., 1990; Halberstam et al., 1992; Abernathy-Carver et al., 1994; Koch et al., 1997; Johnson and Huseby, 1997; Ernest 1998; Nzerue and Gowda, 2001; Fukuta et al., 2012; Waldron et al., 2015; Lachant and Prasad, 2015). *Legionella micdadei* is most commonly isolated from human specimens (Table 1.7.1.3). The most recent documented sporadic cases occurred in 2015 (Waldron et al., 2015; Lachant and Prasad, 2015), and its identity is still falsely identified (Waldron et al., 2015).

Table 1.7.1.3. *Legionella micdadei* Isolated from Humans Samples in the United States Since the Identification of the Genus, *Legionella*

Year	Peak Month	Case	Host Risk Factors	Isolation Type	Detection Method	Ref
1977-1988	June-January	Outbreak	10/16 Cases were renal transplant recipients. Other cases were cancer patients (lung, breast and bladder)	Lung Tissue or Pleural Fluid	Retrospectively identified by Culture Method and DFA	Doebbeling et al., 1989
1978	October – December	Outbreak	Renal Transplant	6 Samples of Lung Tissues	Retrospectively identified by Direct Fluorescent-Antibody (DFA) Presumptively identified by DFA First studied by histologic sections of lung-biopsy	Cordes et al., 1981; Thomason et al., 1980 & Rogers et al., 1979
1979	Not Specified	Outbreak	8 Renal Transplant Patients	Lung Tissues	Culture Method & DNA Hybridization Serologic studies with an indirect fluorescent-antibody technique	Pasculle et al., 1979 & Myerowitz et al., 1979

Table 1.7.1.3. (cont'd)

1980	Not Specified	Sporadic	Not Specified; Associated with human disease	Lung Tissues	Culture Method, Cellular Fatty Acid Composition, Antigenic reactivity, & Genetic Homology	Pasculle et al., 1980
1990	June-August	Sporadic	Acute lymphocytic leukemia & Chronic myelogenous leukemia	Lung Tissue	DFA	Schwebke et al., 1990
1992	Not Specified	Sporadic	Hepatosplenomegaly	Chest Tube Specimen Collection	DFA	Halberstam et al., 1992
1994	Not Specified	Sporadic	Asthma	bronchoscopy specimen collection	Culture Method	Abernathy-Carver et al., 1994
1995	June	Nosocomial Outbreak	Renal and Cardiac Transplant Patients, Lacked Environmental Surveillance	Bronchoscopy	Retrospectively identified by serologic testing or Culture Method	Knirsch et al., 2000
1996	Not Specified	Outbreak at a bone marrow transplant center	Different Underlying Disease	Not Specified	DFA	Harrington et al., 1996
1997	Not Specified	Sporadic	Lupus Erythematosus AIDS	bronchoalveolar lavage	Culture Method & DFA	Koch et al., 1997 & Johnson and Huseby, 1997

Table 1.7.1.3. (cont'd)

1998	Not Specified	Sporadic	type II diabetes mellitus	Surgical and bronchoscopy specimens	Culture Method & Serogrouping	Ernest 1998
2001	Not Specified	Sporadic	HIV	Broncho alveolar lavage specimen cultures	Culture Method	Nzerue and Gowda, 2001
2012	Not Specified	Sporadic	Systemic lupus erythematosus and antiphospholipid syndrome	Valve Tissue from Brain abscess	Polymerase Chain Reaction of 16S rRNA & Silver Stain	Fukuta et al., 2012
2015	Not Specified	Sporadic	Kaposi's sarcoma (KS) and precursor T cell acute lymphoblastic leukemia with concurrent myelodysplastic syndrome; Hepatitis, ulcerative colitis, primary sclerosing cholangitis, and cirrhosis Patient	Lung Biopsy Tissue Collected from the patient; CT Guided Biopsy	Polymerase Chain Reaction of16S rRNA and Culture Method	Waldron et al., 2015; Lachant and Prasad, 2015

Legionella bozemanii is known to cause pneumonia and lung abscesses. In the documented cases detailed below (Table 1.7.1.4), *L. bozemanii* was described in nine sporadic cases (Bozeman et al., 1968; Thomason et al., 1979; Sober et al., 1983, Parker et al., 1983; Strampfer et al.,1986 & Brettman et al., 1986; Jaeger et al., 1988; Taylor and Albrecht 1995; Harris et al., 1998 & Miller et al., 2007) and one nosocomial outbreak (Parry et al.,1985). An environmental-water source was the cause of infection in 4 cases, three occurred from being in direct contact with *L. bozemanii* in its natural environment, such as freshwater or swamp water (Bozeman et al., 1968; Thomason et al., 1979; Sobel et al., 1983), and one case from an engineered water system, such as drinking water (Parry et al., 1985). The three environmental exposure cases mentioned above were from an individual's scuba diving or submerging in water. Two of the individuals did not have any underlying conditions before being infected with *L. bozemanii* (Bozeman et al., 1968; Sobel et al., 1983). Unfortunately, one of the healthy hosts was unable to fight off the disease due to the bacterium being unidentified and treated improperly (Bozeman et al., 1968). The outcome of the healthy patient, mentioned previously, later died (Bozeman et al., 1968). Prompt treatment with the correct antibiotics (macrolides and quinolones) cures LD if diagnosed accurately.

Table 1.7.1.4. *Legionella bozemanii* Isolated from Humans Samples in the United States Since the Identification of the Genus, *Legionella*

Year	Peak Month	Case	Host Risk Factors	Isolation Type	Detection Method	Ref
1968	Summer	Sporadic	No underlying condition	Lung Tissue	Unidentified	Bozeman et al., 1968
1979	Not Specified	Sporadic	Submersion Leukemia	Lung Tissue	Culture Method	Thomason et al., 1979
1983	June	Sporadic	No Underlying Condition	Lung Tissue	Culture Method & Indirect FA	Sobel et al., 1983
1983	Not Specified	Sporadic	Systemic lupus erythematosus	Sputum specimens	DFA	Parker et al., 1983
1983-1984	October-February	Noscomial Outbreak	Lymphoma & Uremia	Sputum specimens	Culture Method	Parry et al., 1985
1986	Not Specified	Sporadic	Leukopenia; Lymphoma	Not Specified	Culture Method	Strampfer et al.,1986 & Brettman et al., 1986
1985	July-August	Sporadic	Diabetes	Bronchoalveolar lavage fluid	DFA & Culture Method	Jaeger et al., 1988
1995	Not Specified	Sporadic	Hepatitis	Broncho alveolar lavage fluid and pleural fluid	Culture Method & DFA	Taylor and Albrecht 1995
1997	Not Specified	Sporadic	AIDS	Broncho alveolar lavage fluid	Culture Method	Harris et al., 1998
2007	Not Specified	Sporadic	cavitary pulmonary disease	Pulmonary Abscess Fluid	Culture Method, PCR & DNA Sequencing	Miller et al., 2007

1.7.2 Exposure Assessment

As described above in section 1.6, many factors (physiochemical) promote the growth of *Legionella,* it is critical to understand the ecological impacts on *Legionella* growth within complex premise plumbing, and how the growth of *Legionella* from the water storage tank is transported to exposure sites. The piping systems leading from the water storage tank to the distribution system, then to the taps, may allow the growth of the bacteria to certain concentrations associated with the risk of infection after it is released to aerosols. A better-described *Legionella* exposure assessment is needed, and the information could be developed into a useful tool to assess the potential exposure of all *Legionella* species, either by inhalation of aerosolized *Legionella* water droplets or aspiration of drinking *Legionella* contaminated water, from common water uses.

While there is information on percent positives of *L. bozemanii,* and *L. micdadei* in a groundwater source, water treatment plant, distribution system, and exposure sites there is no concentration data on these species (De Giglio et al., 2019; Wullings et al., 2011; Wullings and van der Kooij, 2006; Dobrowsky et al., 2016; Patterson et al., 1997; Dilger et al., 2017; Chochlakis et al., 2013; Collins et al., 2017; Gorman et al., 1985; Marrie et al., 1994; Guan et al., 2012; Thornley et al., 2017). For example, *L. bozemanii,* and *L. micdadei* were nested together, and the concentration was reported as one entity (De Giglio et al., 2019; Wullings et al., 2011; Wullings and van der Kooij, 2006; Dobrowsky et al., 2016; Patterson et al., 1997; Dilger et al., 2017; Chochlakis et al., 2013; Collins et al., 2017; Gorman et al., 1985; Marrie et al., 1994). Various studies used various methods such as sequencing, agglutination test, and qPCR to identify *L. bozemanii,* and *L. micdadei* (De Giglio et al., 2019; Wullings et al., 2011; Wullings and van der Kooij, 2006; Dobrowsky et al., 2016; Patterson et al., 1997; Dilger et al., 2017;

Chochlakis et al., 2013; Collins et al., 2017; Gorman et al., 1985; Marrie et al., 1994; Guan et al., 2012). *Legionella longbeachae* was identified from an "aquatic environment," but the exact environment was not disclosed (Saint and Hot, 1999). Nevertheless, the concentration was 4000 CFU/L (Saint and Hot, 1999). *Legionella anisa* was isolated from a hospital in France, and the concentration was 170-180 CFU/mL (Mee-Marquet et al., 2006) (Table 1.7.2). Overall, *Legionella* species that cause LD have not been characterized by the occurrence and concentration.

Table 1.7.2. Examples of Pathogenic *Legionella* Species Distributed in the Water Source and the Built Environment

Organism in Each Water System	Concentration CFU/L	Country Species Identified	Reference
Groundwater Source			
Legionella spp.	300-50000 CFU/L	Italy	De Giglio et al., 2019;
L. pneumophila	100 CFU/L	United States	Brooks et al., 2004; De Giglio et al., 2019
Treated water (Post water treatment plant)			
Legionella spp.	1100-17000 CE/L	Netherlands	Wullings et al., 2006
L. pneumophila	290 CE/L	Netherlands	Wullings et al., 2011
Distribution System			
Legionella spp.	78,000 copies cm−2	United States and Norway	Waak et al., 2018
L. pneumophila	3000 GC/L	Paris	Perrin et al., 2019
Exposure Site (Shower, Taps, or Cooling Tower)			
Legionella spp.	100,000 CFU/L	Greece	Papadakis et al. 2018
L. anisa	4000 CFU/L	France	Mee-Marquet et al., 2006
L. pneumophila	13,000 CFU/L	Netherlands	Walraven et al., 2016
L. longbeachae sg1	170-180 CFU/mL	New Zealand	Saint and Hot, 1999

Each species could vary in occurrence and concentration within a drinking water system, and their virulence characteristics could also differ within individual species, but there are very little data that have addressed this. There is emerging information that suggests that the internalization of *L. pneumophila* into *Acanthamoeba castellanii* enhances virulence (Barker et al. 1993; Cirillo et al. 1994). *Legionella* species also are able to persist in drinking water facilities, distribution systems, and within the different premise plumbing systems because of the intracellular forms in protozoan symbionts and ability to colonize biofilms (Bertelli et al., 2018; Rasch et al., 2016; Shaheen et al., 2019; Lu et al., 2015; Dupuy et al., 2011; Buse et al., 2017. Resistance to chlorine disinfection has also been noted (Buse et al., 2019; Canals et al., 2015; Cooper and Hanlon, 2010; Storey et al., 2004; Thomas et al., 2004). Chlorine disinfection residual, as a secondary water treatment standard, may become ineffective downstream in the premise plumbing system (Vargas et al., 2019; Wang et a., 2014; Zheng et al., 2015; Sheikhi et al., 2014; Al-Jasser, 2007). Once the water reaches downstream into the premise plumbing system, biofilms can also travel with the bulk water due to hydraulic pressures (Tsvetanova, 2019; Chan et al., 2019; Fish et al., 2017; Zhang et al., 2018). Hydraulic pressures dislodge the biofilms from the pipe surface upstream (distribution system), causing a release of *Legionella spp.* into the drinking water (Jjemba et al., 2015; Makris et al., 2013; Ingerson-Mahar et al., 2013; Douterelo et al., 2013; Pinto et al., 2012).

1.7.3 Ecological Factors Impacting *Legionella* Growth

As mentioned above, at least two critical environmental factors that increase the risk of LD, are the biofilm and free-living amoebae associated-*Legionella* species. *Legionella* spp. can colonize the distribution systems, faucets, showerheads, and solid surfaces of cooling towers, and in these environments form biofilms. The biofilm is composed of *Legionella* spp., *Pseudomonas*

spp., (Moritz et al., 2010; Stewart et al., 2012; Mampel et al., 2006; Vervaeren et al., 2006) *Mycobacterium, E. coli, Klebsiella* spp., and other organisms such as free-living protozoa (amoebae) (Bagh et al., 2004, Wingender and Flemming, 2004, Payment and Robertson, 2004, Rakic et al., 2012). The specific species of amoeba and their relative abundance also help to determine the growth of *Legionella* spp. within the biofilm (Abu Khweek and Amer, 2018, Stewart et al., 2012; Mampel et al., 2006; Vervaeren et al., 2006; Guerrieri et al., 2008). The rate of biofilm growth also correlates with the physicochemical properties of the water, such as temperature, pH, hardness, organic materials, nutrients, residual disinfection concentrations, and heavy metals (Rakic et al., 2012; Oder et al., 2015; Buse et al., 2017; Lu et al., 2017; Gião et al., 2010; Abdel-Nour et al., 2013).

Water temperatures (20-45C), increased water age, the presence of sediments, and declined water usage promote the growth of *Legionella* species in the water supply system (Qin et al., 2017; Flemming et al., 2013; Wang et al., 2014; Lu et al., 2015; Stout et al., 1985; Stout et al., 1985). Water flow velocity, corrosion of distribution system pipes, and pipe fittings also promote the growth of *Legionella* (Rakic et al., 2012; Lu et al., 2014; Stewart et al., 2012; Liu et al., 2006; Rhoads et al., 2017; Ward et al., 2010; Halabi et al., 2001).

1.7.4 Water Stagnation

Within commercial buildings, the water becomes stagnant in some pipes (faucet and floor dependent) throughout the day, depending on use (Rhoads et al., 2016). When water sits, the stagnated areas in the piping system support biofilm growth, and the water temperature increase (Buse et al., 2019; Sing and Coogan, 2005; Liu et al., 2006; Stout et al., 1985) thus affecting the bacterial growth. Also, as water sits in a piping system, the disinfectant residual will dissipate

over time, this also allows *Legionella* to grow in the premise plumbing (Tesauro et al., 2010; Lu et al., 2014).

1.7.5 Water Age

Stagnation is also related to water age, which is the time the water spends in pipes (distribution and premise plumbing) post-treatment. The most significant contributors to water age are the size of the drinking water system and the storage design. Drinking water systems are designed in such a way to accommodate future water needs; thus, these systems are made with large pipes and storage facilities (Lu et al., 2014; Peter and Routledge, 2018; Lu et al., 2015; Qin et al., 201). Water storage tanks have significant impacts on water quality, such as chemical and biological issues (Lu et al., 2014; Peter and Routledge, 2018; Lu et al., 2015; Qin et al., 201) with oversized storage facilities resulting in longer detention times, loss of chlorine residual, and other water quality concerns (Peter and Routledge, 2018; Lu et al., 2014 and Lu et al., 2015). Water age contributes to water quality deterioration by the interaction of pipe and/or tank material and water/chemical reactions (natural organic material and chlorine) (EPA, 2002). Rhoads et al., 2016 showed that water age impacts the chemical (corrosion of pipe materials) and bacteriological (microbial growth) quality of drinking water in building plumbing systems. A water system with a high-water age significantly decays disinfectant residuals in premise plumbing systems (Rhoads et al., 2016). The concentration of *Legionella* spp. was higher in buildings with a high-water age (Rhoads et al., 2016).

1.7.6 Risk Estimates via Various Routes of Exposure

Quantitative Microbial Risk Assessment (QMRA) has been used to estimate risks of LD, where the computed calculation has corresponded to the actual risk associated with exposure to *Legionella*-contained-aerosols from contaminated whirlpools and spas (Armstrong and Haas,

2007). Armstrong and Haas, 2007 used guinea pig models to represent the human dose-response models for *L. pneumophila* exposure from concentrations found in spas and showed that the mortality projections from the exponential model indicated one in 10,000 risks at a retained dose of approximately one CFU.

Another QMRA predicted LD risk associated with exposure to *Legionella* aerosols from contaminated residential bathrooms (Azuma et al., 2013). QMRA was adequate to calculate a LD outbreak that sporadically occurred in bathrooms in condominiums (Azuma et al., 2013). Based on the inhalation exposure model, the infectivity and mortality risk levels yearly were 1 in 100 and 1 in 100,000, respectively (Azuma et al., 2013) (Table 1.7.6).

Several studies have aimed to develop a *Legionella* quantitative risk assessment analysis for reclaimed water aerosols (Hamilton et al., 2018), potable and non-potable uses of roof-harvested rainwater (Hamilton et al., 2017; Ahmed et al., 2010), spa outbreaks (Armstrong and Haas, 2007; Armstrong et al., 2007), distribution water systems (Storey et al., 2004), indoor residential water uses (Hamilton et al., 2019), recreational and garden areas of hotels (Papadakis et al., 2018), greywater reuse (Blanky et al., 2017), municipal drinking water (Kool et al., 1999) and a drinking water supply system (Sharaby et al., 2019).

Hamilton et al., 2018 presented a Legionnaires' Disease QMRA model to explore the exposure risks of *Legionella* in water systems that use reclaimed water for toilet flushing and cooling towers. Multiple toilet types were analyzed, and the toilet flushing annual infection risks ranged from 4.08×10^{-4} to 2.72×10^{-2} CFU/L for culture-based and 2.23×10^{-2} to 7.41×10^{-1} GC/L for qPCR (Hamilton et al., 2018), but the clinical severity infection (CSI) risks for either technique did not exceed those values. The CSI risks were estimated at 4.60×10^{-7} to 2.58×10^{-5} CFU/L for culture and 2.70×10^{-5} to 1.38×10^{-3} GC/L for qPCR. The second factor

that affected the outcomes of the infection risk models was the cooling tower circulating water flow rate, followed by the dose-response parameter, and cooling tower drift efficiency (Hamilton et al., 2018). Hamilton et al., 2018 suggested that the annual infection risk level should be set to 1 in 10,000 people for cooling towers (Table 1.7.6).

Another study surveyed three toilet faucets and two showerheads in Israel for three years to detect the prevalence of *Legionella* (Sharaby et al., 2019). Sharaby et al., 2019 utilized a disease burden measurement (Disability-Adjusted Life Years index) to express the data. Their results revealed that the annual risk levels for toilet faucets were 5.52×10^{-4} DALY'S per person per year, and the showers were 2.37×10^{-3} DALY'S per person per year. The risk levels increased from June to December and decreased towards the end of December to March due to the changes in *Legionella* concentrations. QMRA results revealed that the summer months were the highest seasonal infection risk values for both faucets, 8.09×10^{-4} DALY'S per person per year and showers, 2.75×10^{-3} DALY'S per person per year (Table 1.7.6). Overall, Sharaby et al., 2019 concluded that faucets and showers associated with *Legionella* contamination possess a higher infection risk in the summer and autumn months.

Storey et al., 2004 has assessed the risk of *L. erythya* and *L. pneumophila* from a laboratory-scale distribution system. Storey et al., 2004 evaluated the efficacy of disinfection methods (chlorine and thermal) against *Legionella* spp. Total chlorine residuals of 2 mg/L reduced the risk of infection by 2.5 logs relative to 5 mg/L of free chlorine. Thermal disinfection at 80°C was the most effective means of reducing (8-log reduction) risk from *L. erythya* and *L. pneumophila* (Storey et al., 2004). Although risk models have been developed for *Legionella,* a model that accurately describes *Legionella* within premise plumbing is currently lacking

Table 1.7.6. Example Studies Focusing on Risk Assessment at Point of Use

Aerosol Exposure Pathway and Exposure Duration	Exposed Population	Measurement Method	Infection Risk	Ref
Toilet Flushing; 1-5 Min/Flush	Residential	Culture EMA-qPCR qPCR	4.08 x 10^{-4} CFU/L 5.69 x 10^{-2} GC/L 2.23 x 10^{-2} GC/L	Hamilton et al., 2018
Bathtub; 21 min	Residential	Culture	2.0 x 10^{-3} CFU/100mL	Azuma et al., 2013
Shower; 18.3 exposure events/yr	Residential	qPCR	3.0 x 10^{-2} to 8.6 x 10^{-2} GC/L	Ahmed et al., 2010
Hosing; 5.2 exposure events/yr	Residential	qPCR	1.8 x 10^{-2} to 5.1 x 10^{-2} GC/L	Ahmed et al., 2010
Toilets; 2 min 48 sec/wk Showers; 49 min/wk	College campus area	Culture	8.09 x 10^{-4} 2.75 x 10^{-3} CFU/L	Sharaby et al., 2019

1.7.7 Human Exposure via Cooling Towers

The largest LD outbreaks have been associated with cooling towers with 213 to ~800 cases (Fitzhenry et al., 2017; Garcia et al., 2003; Shivaji et al., 2014; Levesque et al., 2012; Bennett et al., 2014; Castilla et al., 2008; Weiss et al., 2017). In the United States, 62% of LD cases occur during the summer, when commercial air conditioning systems associated with cooling towers are most needed for the warmer weather (MMWR, 2011). An estimated 28% of sporadic LD documented cases are caused by emissions from cooling towers (Fitzhenry et al., 2017; Bhopal et al., 1991). In a cooling tower associated with an outbreak, the reported numbers of *Legionella* exceed 1000 CFU/mL (Fitzhenry et al., 2017).

There are various guidelines and regulations for the control of microorganisms in cooling tower systems. For example, in 2016, New York City and the State of New York created an ordinance (10 New York Codes of Rules and Regulations Part 4 “Protection against Legionella”) to decrease the contamination of *Legionella* in cooling towers (New York State Department of Health). These regulations require the building owner to register the cooling tower, routine sample (every three months) for *Legionella*, monitor physiochemical and microbial parameters three times per week and follow the ASHRAE 188 standard. Concerning routine sampling, a sample result above 10^5 to 10^6 CFU/L from cooling towers is indicative of amplification; in this case, remediation needs to take place (American Industrial Hygiene Association, 2015). Cooling towers are widely used devices, which produce large volumes of aerosols that can disseminate over long distances (Ferré et al., 2009; Türetgen et al., 2005). Therefore, it is critical to identify the cooling towers that are contaminated with *Legionella*; this will help mitigate the exposure pathway and the number of people exposed.

1.7.8 Detection Methods

The sample collection depends on the environmental source. For drinking water systems and cooling towers, water samples are collected using sodium thiosulfate (1 ml of 10% sodium thiosulfate per 1L) to neutralize chlorine residuals. For drinking water, 1-1L of water should be collected from the faucet into a sterile bottle, and the sample should be processed from the concentrate. One of the sites of collection for the cooling towers are from the make-up water. One 1L of bulk water should be collected into a sterile bottle, and the sample can be processed directly after sample collection (CDC, 2019).

The requirement for sample processing depends on the properties of the environmental source. The microbial composition in drinking water systems is less complex than cooling

towers. Thus, the sample techniques are different (Joly et al., 2006; Steele et al., 1990). For drinking water systems, the samples have to be ultra-filtered to concentrate the water to ensure that the microbial material will be detected. In comparison, samples from cooling towers require heat treatment (50°C) to reduce the microbial composition and abundance (Bopp et al., 1981; Leoni and Legnani 2001; Robers et al., 1987).

The culture technique is the 'gold standard' for the detection of *Legionella* spp. The most commonly used media is the Buffered Charcoal Yeast Extract (BCYE) Agar for the growth of *Legionella* spp. and it is made up of many supplements, amino acids, and trace elements (Pine et al., 1979; Reeves et al., 1981; Warren and Miller, 1979; Feeley et al.; 1979; Pendland et al., 1997; Roberts et al., 1987; Ta et al., 1995). The specific additions are L-Cysteine (essential amino acid for *Legionella* spp.), activated charcoal (decompose the toxic hydrogen periodxide), Ferric Pyrophosphate (iron source), 0.1% a-ketoglutarate monopotassium salt (stimulate *Legionella* growth), vancomycin, polymyxin B and cycloheximide (antimicrobial molecules to reduce the growth of competing bacteria). However, buffered charcoal yeast extract was designed to detect *L. pneumophila* samples; for that reason, this occasionally gives false-negative results for other *Legionella* species, for ex: *L. micdadei* and *L. longbeachae* (de Bruin et al., 2018). But the addition of albumin (1%) enhances the recovery and growth of some species (Morrill et al., 1990).

For the detection of *Legionella* spp., the environmental water samples go through an acid shock to reduce the interference of microbial flora before culturing (Bopp et al., 1981). After processing of the sample, (as described above), it is then diluted into an HCl-KCl buffer (pH 2.2) and incubated for 15 minutes; it is then placed into a 5% CO_2 incubator at 35°C for three to five days.

Cultivation is the primary approach for environmental surveillance; however, the rates of recovery of *Legionella* spp. using culture methods are low (Villaria et al., 1998). The presence of the viable but non-culturable *Legionella* spp., and the growth of other microorganisms, which inhibits the growth of *Legionella* spp. (Bopp et al., 1981; Shih and Lin, 2006), create barriers to the enumeration of *Legionella*. spp. When culturable, the slow growth rate of *Legionella* leads to plate overgrowth by competing organisms (with a rapid generation time). These challenges (described previously) creates a consequence of reporting false negatives for sporadic, or an outbreak case/s. Furthermore, the reduction of *Legionella* spp. (by competing bacteria) can lead to underreporting of the concentration of these species (Alary and Joly, 1992; Bopp et al., 1981; Steele, 1990).

Polymerase Chain Reaction methods (qPCR and ddPCR) are rapid, sensitive and specific for all known strains of *Legionella* when using 5S, 16S 18S, or 23S rRNA primers in environmental and clinical samples (Joly et al., 2006; Behets et al., 2007; Dusserre et al., 2008; Wellinghausen et al., 2001; Yaradou et al., 2007; Gruas et al., 2014; Merault et al., 2011; Benitez et al., 2013). PCR -based methods can detect *Legionella* cells in a viable non-culturable state, low levels of target DNA, and cells living within amoebae (Ng et al., 1997; Bates et al., 2000; Ditommaso et al., 2014).

Concerning PCR-based methods, researchers' have developed ways to detect viable cells using intercalating dyes: propidium monoazide (PMA) and ethidium monazide (EMA) (Chang et al., 2010; Ditommaso et al., 2014; Ditommaso et al., 2015; Ditommaso et al., 2016). Pre-treatment with PMA and EMA enables amplification of viable cells, and when exposed to light, these dyes bind to DNA that is not protected by a cell membrane; thereby preventing the amplification of this form (dead cells) of DNA (Chang et al., 2010; Delgado et al., 2009; Nocker

et al., 2006; Chiao et al., 2014; Fittipaldi et al., 2011). Thus, the enumeration of DNA is from intact, viable DNA (Flekna et al., 2007; Kobayashi et al., 2009).

Several researchers have utilized the viability PCR technique. Propidium monoazide-qPCR using the 5S rRNA gene of total *Legionella* spp. detected this genus in 100% (86/86) of the dental unit water line and tap samples, while the culture method only detected 7% (6/86) of the samples (Ditommaso et al., 2016). The concentration of the positive samples using PCR ranged from 10^2 to 10^6 GU/L, whereas the concentration of the culture-positive samples was mostly 10^2 CFU/L, but one water sample was 10^3 CFU/L (Ditommaso et al., 2016). Studies mentioned above demonstrated that viability PCR eliminated the background noise of dead cells and VBNC just by including PMA and EMA treatment.

The rate of recovery *Legionella* in previous studies varied depending on the analytical method used. When using the culture method, the detection of *Legionella* is generally always lower (Ditommaso et al., 2016). Whereas, when utilizing a PCR-based method, there is a 100% detection rate of the samples for *Legionella* spp. (Ditommaso et al., 2014; Ditommaso et al., 2016).

1.8 Current Understanding of *Legionella*

Since the discovery of *Legionella pneumophila* in 1977, the majority of LD cases have been found outside North America (Beaute et al., 2017; Garrison et al., 2016). However, the primary source of outbreaks of the waterborne disease of *Legionella* is not well understood. Recent reports suggest there to be sources other than cooling towers. In 2015, in New Jersey, there was an outbreak of *Legionella*, but there were not any cooling towers to explain the in potable water (Cohn et al., 2015). A *Legionella* outbreak in Flint, Michigan was also linked to

drinking water, as this outbreak resulted from corrosion in the distribution system (Schkwake et al., 2016).

There has been limited research done on the occurrence of *Legionella* in a drinking water supply system served by groundwater (Valcina et al., 2019). There is an assumption that systems served by groundwaters will have fewer *Legionella* species. Table 1.8 shows the current research that has been done on *Legionella* spp. (genus level) or specifically *L. pneumophila* from a groundwater source, yet, there is still a critical need to investigate various pathogenic *Legionella* species such as *L. bozemanii, L. anisa, L. micdadei,* and *L. longbeachae*—as they also cause LD. Thus, we aim to investigate the species the covers the most incidence of LD by exploring the microbial content in a complete drinking water supply system served by a groundwater source in the US.

Table 1.8 Research on Groundwater and *Legionella*. Abbreviations: GW, Groundwater; SW. Surface Water; DWDS, Drinking Water Distribution System; SG1. Serogroup 1; DW, Drinking Water

Research Results	Survey	Year	Location	Reference
Compared GW to Municipal and GW contains pathogenic bacteria	Survey	2018	USA	Richards et al., 2018
Microbial communities in water samples are different among service areas within DWDS	Survey	2018	USA	Gomez-Alvarez et al., 2018
Found a new automated system to reduce variability over long periods of time when comparing SW, GW and Cooling towers	Survey	2012	USA	Leskinen et al., 2012
Causes of DW outbreaks are from untreated or improperly treated GW & *Legionella*	Survey	2010	USA	Craun et al., 2010
Saw an association of WB outbreaks associated with GW from private non-community wells between 2001-2002	Survey	2004	USA/Canada	Blackbum ett al., 2004
Showed that *Legionella* was found in cold and hot GW samples around the United States	Survey	2004	USA	Brooks et al., 2004

CHAPTER TWO

ENUMERATION AND CHARACTERIZATION OF FIVE PATHOGENIC *LEGIONELLA* SPECIES FROM LARGE RESEARCH AND EDUCATIONAL BUILDINGS

2.1 Abstract

A study on the occurrence of five *Legionella* species in five different large buildings (BPS, ERC, F, FH, and M) was undertaken during two seasons (late summer (August-September) and early winter (January)). A total of 37 large-volume samples (influents to the buildings and exposure sites (taps)) were collected and analyzed using droplet digital™ PCR (ddPCR™). *Legionella* spp. (23S rRNA) were present in all water samples during both seasons. The majority (66%) of the exposure sites (bathroom taps) over the two seasons were positive for at least one target *Legionella* species (*L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* or *L. longbeachae*). During the summer season, the percent positives for the target *Legionella* species, found in the influents of the ERC and BPS buildings were 80 and 40%, respectively, while 20% of the pathogenic species were positive at exposure sites in three buildings (F, FH, and ERC). During the winter season, the percent positives for any one of the pathogenic *Legionella* species at the hot-water taps was 80% in building F and 40% in BPS, M, FH, and ERC. In the cold-water taps, the percentage of pathogenic *Legionella* positive samples were 40% in F, BPS, M, and 20% in FH. In the hot-water taps, the percentage of pathogenic *Legionella* positive samples were 40% in all five buildings. *Legionella pneumophila* and *L. longbeachae* were found in the highest concentrations (2.0 Log_{10} Gene Copies (GC)/100 mL) at the hot-water taps in buildings F and ERC, respectively. General *Legionella* spp. concentrations increased in the winter season suggested that lower water usage (lower occupancy and no use of cooling towers, lead to more water stagnation or time in the system) played a role in the occurrence of *Legionella* spp. in the various buildings. Exposure to *Legionella* spp. at the tap (cold and hot) warrants further exploration through a quantitative microbial risk assessment for different pathogenic *Legionella* species.

2.2 Significance of Importance

Legionella pneumophila is the causative agent responsible for Legionnaires' disease (LD) —a severe life-threatening respiratory infection. Reports of LD cases and outbreaks have been linked to drinking water systems of large, complex buildings. Drinking water systems are an important amplifier source for general *Legionella* species—some key factors that affect the amplification in the water column in a building are low disinfectant residuals, low water use, and increased water age. However, due to the Centers for Disease Control and Prevention (CDC, 2019) sampling strategy (collecting 1-L of sample and using the culture method), low levels of *Legionella* from building water systems may go undetected. A large volume sample (10-L) was collected from the buildings' influents and at the points of use (e.g., taps) from five large buildings; this study evaluated five pathogenic *Legionella* species: *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae* along with a general *Legionella* spp. target (23s rRNA) using ddPCR. *Legionella pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae* were identified in the buildings' water system (taps) at concentrations that ranged from 1.4 to 2.0 Log_{10} GC/100 mL. These results demonstrate that non-*pneumophila Legionella* spp. are present in the water column of the buildings' water systems and can be detected at low concentrations (10^1 Log_{10} GC/100 mL) using ddPCR. Overall, examining the building's points of use for each floor (collecting 10-L composite sample) instead of individual points of use (collecting 1-L per tap) allows for the detection of pathogenic *Legionella* spp. in buildings' water systems.

2.3 Introduction

Legionella spp. are gram-negative, opportunistic waterborne pathogens that reside in premise plumbing (i.e., building) as well as other engineered water systems. *Legionella*

pneumophila is the etiologic agent responsible for most LD with other species identified less frequently causing severe pneumonia and the less-studied Pontiac Fever (an acute, but generally milder set of cold-like signs and symptoms (as reviewed by Pierre et al., 2017). *Legionella* naturally colonizes freshwater and groundwater environments, as well as engineered systems including cooling towers, air conditioners, hot tubs, taps, and showers (Farhat et al., 2012; Donohue et al., 2014). *Legionella* infections are acquired via inhalation of aerosols and air droplets generated from these structures containing the bacteria (as reviewed by Prussin et al., 2017). The first recognized outbreak of LD, caused by *Legionella pneumophila*, occurred in 1976 (Fraser et al., 1977). In the United States (US), LD prevalence has increased significantly since 2000, and in 2018 there were approximately 10,000 reported cases (CDC, 2018). *Legionella* species are difficult to assess and control in the drinking water system because they survive in the biofilm on the surface of the pipes and within amoebae hosts (Nishida et al., 2019; Gomes et al., 2020). The difficulty in assessing and controlling *Legionella* species makes these bacteria and their associated diseases a paramount public health concern.

Many outbreaks of LD occur at the community level, as was the case in Flint, Michigan between 2014 and 2015 when Michigan saw a 375% increase in cases, most of which were part of the Flint outbreak (Smith et al., 2019; Michigan Department of Health and Human Services, 2018). During the 2014-2015 Flint outbreak, it was suggested that there were multiple sources of exposure, including the hospital water system, water at home (showers or taps), and residential proximity to cooling towers (Smith et al., 2019). Although the LD outbreak during the water crisis in Flint, Michigan, was the largest in the state, there has been an increased number of cases statewide from 2000 to 2016 (Michigan Department of Health and Human Services, 2018).

Legionella pneumophila, serogroup 1 is the most often diagnosed agent accounting for 90% of identified LD pneumonia cases, perhaps due to the restriction of the urinary antigen test (Beer et al., 2015; Shachor-Meyouhas et al., 2010; Cheng et al., 2012; Haupt et al., 2012; Jarraud et al., 2013). In recent years, other *Legionella* species found in drinking water have also been identified in about 10% of cases (Foissac et al., 2019; Chakeri et al., 2019; Dilger et al., 2017; Stallworth et al., 2012; Svarrer and Uldam, 2012; Cameron et al., 2016; Isenman et al., 2016; Vaccaro et al., 2016). *Legionella micdadei, L. bozmanii, L. longbeachae,* and *L. anisa* have been isolated from human patients (Yu et al., 2002; Beaute et al., 2013). There have been five drinking water outbreaks caused by *L. micdadei,* (Craun et al., 2010; Best et al., 1983; Knirsch et al., 2000; Doebbeling et al., 1989; Harrington et al., 1996; Pasculle et al., 1980; Myerowitz et al., 1979; Rogers et al., 1979), one by *L. bozemanii* (Parry et al., 1985) and two by *L. anisa* (Craun et al., 2010) in the US. To date, there have not been any reports of *L. longbeachae* related infections associated with building water systems in the US, but there have been outbreaks in Australia (Broadbent, 1996; Grove et al., 2002). In Australia, *Legionella* infections are commonly caused by *L. longbeachae,* and one of the exposure pathways is from potting mixes and compost (Steele et al., 1990).

The majority of reported LD outbreaks have occurred in large complex plumbing systems, which are used in hospitals and healthcare facilities (Garrison et al., 2016). However, 97% of LD cases are sporadic infections (Hilborn et al., 2013), for which the environmental source of exposure is usually unknown. The National Academies report on "Management of *Legionella* in Water Systems" (2019) stated that for every one outbreak case, there are nine more sporadic cases (National Academies of Sciences, Engineering, and Medicine, 2020). Despite a substantial amount of research on the molecular virulence mechanisms and ecology of

Legionella, annual incidence rates of the disease continue to rise along with great uncertainty on how to control the colonization of water systems.

Currently, only a few studies have simultaneously characterized multiple pathogenic *Legionella* species (*L. anisa, L.micdadei, L. bozemanii, and L. longbeachae*) in utility drinking water systems (Wullings et al., 2011, Wullings and van der Kooij, 2006) and in drinking water systems (Fleres et al., 2018; Dilger et al., 2017; Fiume et al., 2005; Lesnik et al., 2016). *Legionella bozemani, L. dumoffii, L. longbeachae, L. anisa, L. moravica, L. parisiensis, L.brunensis, L. londinensis,* and *L. hackeliae,* among many others, have been detected in water samples collected from hospitals in Italy (Fiume et al., 2005), warm water systems in Germany (Dilger et al., 2017), and in utility drinking water systems in the Netherlands (Wullings et al., 2011, Wullings and van der Kooij, 2006). In 2016, Lesnik et al. found *L. pneumophila, L. longbeachae, L. worsleiensis, L. anisa,* and *L. dumoffii,* (among many others) from source water to the cold- and hot-water taps in Germany using genus-specific PCR amplicons (16S rRNA) and single-strand conformation polymorphism fingerprint analyses. *Legionella anisa* was detected in the Netherlands in three of four dental care units (75%) at a concentration of $1 \times 10_2$ CFU/mL using the Dutch *Legionella* standard culture technique and was identified by whole-genome sequencing (MALDI-TOF) (Fleres et al., 2018).

The goals of this study were to quantify the concentrations of general *Legionella* spp., *L. pneumophila, L. anisa, L. micdadei, L. bozemanii, and L. longbeachae* in a drinking water system in the premise plumbing system of five large research, classroom, and office buildings (all utilizing the same water source) at the influent and taps. Utilizing droplet digital PCR, this study addressed the following objectives (i) characterization and quantification of *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae* in the drinking water

along with changes in chemical/physical water quality parameters in the influent and points of use of each building, (ii) exploration of the associations of *Legionella* species with respect to temperature, chlorine, conductivity, pH, and heterotrophic plate count (HPC), and (iii) assessment of whether there were differences between two sampling periods of the year (August and September, and January) for five pathogenic *Legionella* species. The quantitative data presented in this study should improve quantitative risk assessment of various specific pathogenic *Legionella* species within a drinking water system.

2.4 Materials and Methods

2.4.1 Site Location and Sampling

Water samples were collected during two seasons (Summer 2018 and Winter 2019) from five buildings (F, BPS, M, FH, and ERC) on a large research institution of higher education. Sample collection was conducted on August 13th and 27th, September 4th, 2018, and January 7th-9th, 14th, and 15th, 2019. This included research buildings F, ERC, and BPS, as well as buildings FH and M containing offices and classrooms. Building age, water use, and distance from the reservoir are shown in Table 2.4.1. The buildings are listed based on its pipe mileage from the effluent reservoir. Each influent sample was collected at the most accessible sampling port on each building's influent pipe with the exception of ERC. The ERC influent sampling port was inaccessible; thus, it was decided to sample the nearest valve to the influent pipe, which was an eye-wash station in the mechanical room where the influent pipe entered the building. Point of use sampling locations for each building included cold- and hot-water taps (sink faucets and showerheads) located in bathrooms, locker rooms, and breakrooms. All sinks described below were used for sample collection. Building F had two floors, with two sinks on the first floor and three sinks on the second floor; BPS had six floors, with 20 sinks on the first floor and four sinks

on the sixth floor; M had two floors, with four sinks on the first floor and two sinks on the second floor; FH had two floors, with 17 sinks on the first floor and ten sinks on the second floor; ERC had one floor, with 11 sinks and two showers.

For influent samples, 10 L were collected from each building's influent sampling location. For tap samples, a large-volume (10 L) composite sample was collected to evaluate the water quality of each building's taps rather than the quality of individual taps. The first flush with equal total volumes from each tap was collected and composited into 10 L for the first floor and top floor, respectively. For the summer approach, a cold- and hot-water composite sample was collected to evaluate and compare the water quality on the first and top floors, separately. During the summer sampling, a total of three 10-L samples were collected from each building (influent, first floor taps, and top floor taps). For the winter approach, the cold- and hot-water taps were collected as separate samples to evaluate and compare the water quality of the cold-water taps and the hot-water taps on the first and top floor, respectively. For the winter sampling event, a total of five 10-L samples were collected per building, one influent, one cold- and one hot-water sample from the first and top floor, respectively. The one exception was building ERC, which only had one floor, where three 10-L samples were collected. The volume collected from each tap that was composited was determined by the number of taps on each floor. Table 2.4.1 shows the number of taps and the volume collected to construct the composite samples during the summer and winter. All samples were collected in carboys (influent and tap samples) with 10% sodium thiosulfate to neutralize residual chlorine. Temperature and chlorine were recorded from each tap to examine the variation by tap and by floor.

Table 2.4.1. Building and Sampling Site Information for Summer (August 13th and 27th and September 4th, 2018) and Winter (January 7th, 8th ,9th ,14th and 15th, 2019). The Buildings are Listed Based on Their Increasing of Distance from the Water Source

Building[a] (Construction Year) and Pipe Material	**Volume of Water Used Geomean per Month (5-year Average) Consumption (KGAL)**	**Distance from Reservoir (Km)**	**Building Size (m^2)**	**Floors Sampled (# of Floors)**	**# of Taps**	**Volume[b] of Water Collected from each Tap (L) (Summer) Winter[c]**
F (1948) 75% Galvanized 25% Copper	45.7	4.7	7,118	First Second	4 6	(2.5) 5 (1.67) 3.33
BPS (2001) 50% Galvanized 50 % Copper	1,634	6.8	35,045	First Sixth	40 8	(0.5) 0.25 (2.5)1.2
M (1940) 90% Galvanized 10% Copper	75.3	9.6	5,926	First Second	8 4	(2.5) 1.25 (5) 2.5
FH (1964) 50% Galvanized 50% Copper	86.5	10.2	36,057	First Second	34 20	(0.5) 0.29 (1) 0.5
ERC (1986) 50% Galvanized 50% Copper	195.1	19.4	11,896	First	26	(0.77) 0.38

[a]10-L composite sample were collected from all buildings; the volume of water from each tap was dependent upon the number of taps per floor per building. [b] For the summer event, hot and cold composite samples were collected as one 10-L sample from each building per floor. [c] Single faucet fixtures with two taps (½ were cold and the other ½ were hot water pipes). For the winter event, hot and cold composite samples were separated so that two 10-L samples were collected per floor.

2.4.2 Chemical-Physical Analysis

A 100-mL sample was collected for conductivity, pH, and turbidity analyses. Temperature and residual chlorine (total and free) were measured onsite. Chlorine was measured using the Test Kit Pocket Colorimeter II (HACH®, CO, USA) according to the manufacturer's instructions. Conductivity, pH, and turbidity were measured offsite at the laboratory according to the manufacturers' instructions using a Russell RL060C Portable Conductivity Meter (Thermo Scientific, MA, USA), UltraBasic pH meter (Denver Instrument, NY, USA), and a Turbidity Meter code 1970-EPA (LaMottee Company, MD, USA).

2.4.3 Microbiological Analysis

All samples were transported on ice to the laboratory and preserved at 4ºC until processed. While the on-campus water utility tests for coliform bacteria on a routine basis, all samples collected for this study were tested according to the standard methods for coliform bacteria and *E. coli* using Colilert (IDEXX Laboratories, ME, USA) as well as with heterotrophic plate count (HPC) analyses using membrane filters (47 mm diameter, 0.45 µm pore size) (PALL Corporation, NY, USA) on m-HPC agar (Becton, Dickinson and Company, Difco™, MI, USA), incubated for 48 ± 2 h at 35-37ºC, then enumerated for colony-forming units (CFU) (Clescerl et al., 1998). Total coliforms were assayed for the summer and winter sampling events, while the HPC analyses were performed only for the summer.

2.4.4 Water Sample Processing and DNA Extraction

The 10-L water samples were processed using a single-use Asahi REXEED-25S dialysis filter (Dial Medical Supply, PA, USA), which was pretreated with 0.01% of sodium hexametaphosphate and utilized in a dead-end mode. A high-pressure single-use elution fluid canister (Innovaprep LLC, MO, USA) was used to concentrate the 10 L to ~50 mL.

2.4.5 Molecular Analysis

Each ultrafiltration concentrate was split into 10-mL subsamples. One 10-mL subsample was further filtered through a 47-mm, 0.45-µm polycarbonate filter (Whatman, Kent, UK) for DNA extraction and analyzed by ddPCR. The remaining subsamples were stored at -80°C.

2.4.6 DNA Extraction and Quantitative Detection of *Legionella* Droplet Digital™ PCR

Each 10-mL subsample was filtered on a polycarbonate filter (described above) using a sterilized 0.47-mm magnetic filter funnel (PALL Corporation, NY, USA). Immediately afterward, the polycarbonate filter was folded into a 1/8 shape with contents of filter folded to the inside. The filter was then transferred to a 2.0-mL polypropylene screw cap tube (VWR, PA, USA) containing 0.3 g of 212-300 µm acid-washed glass beads (Sigma, MO, USA). DNA extraction was performed by adding 590 µL of AE buffer (Qiagen, Hilden, EUR) to the samples then bead milling using a FastPrep-24™ 5G Instrument MP Biomedicals (VWR, PA, USA). Samples were milled at 6,000 rpm for one minute, followed by centrifugation at 12,000 x g for one minute. The supernatant (~400 µL) was transferred to a new clean microcentrifuge tube and centrifuged at 12,000 x g for an additional three minutes to pellet any remaining debris. Extracted nucleic acid was eluted (~ 350 µL) into a final clean microcentrifuge tube. The eluted volume was then aliquoted (~60 µL) into several microcentrifuge tubes for storage at -80°C— to reduce the need for several freeze/thaw cycles. One aliquot per water sample was later used for PCR analysis.

Droplet digital PCR (Bio-Rad Laboratories CA, USA) technology was performed according to the manufacturer's instructions to analyze each sample for general *Legionella* spp. (23S rRNA), *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae.* The primers and probes used in this study are listed in Table 2.4.6. Duplex reactions were performed

for three separate assays: the first assay consisted of *Legionella* spp. and *L. pneumophila*, the second assay comprised of *L. micdadei,* and *L. anisa,* and the third assay consisted of *L. bozemanii,* and *L. longbeachae* (Table 2.4.6).

For each reaction mixture, 2X supermix (no dUTP) (Bio-Rad Laboratories CA, USA) was mixed with a final concentration of 900nM of forward and reverse primers, 250nM for each probe (Eurofins Genomics Co., AL, USA), and DNA template (up to 330 ng) to a final volume of 20 µL, as recommended by Bio-Rad. The 20 µL samples were loaded into a DG8 cartridge (Bio-Rad Laboratories, CA, USA), followed by 70 µl of droplet generator oil (Bio-Rad Laboratories, CA, USA). The samples were then loaded into the QX200 Droplet Generator, and droplets were generated. The droplet emulsion (~40 µl) was then transferred into a 96-well plate using a multichannel pipet. The plate was then heat sealed with pierceable foil heat seals using a PX1™ PCR Plate Sealer (Bio-Rad, Laboratories, CA USA). The sample reaction mixture was amplified using a Benchmark TC9639 thermal cycler (Benchmark Scientific Inc, NJ, USA) with the following thermocycling parameters: 95°C for 10 min, followed by 40 cycles of 94°C for 30 sec and 57°C for 1 min, with a final 10 min cycle at 98°C for 10 min. Droplets were then read using a QX200 droplet reader (Bio-Rad QX200™ Droplet Digital™ PCR System, CA, USA).

Two negative controls, a filtration blank (phosphate-buffered water) and a non-template control (molecular grade water) were run with each ddPCR plate. Positive controls using DNA from *L. pneumophila, L. micdadei, L. anisa, L. bozemanii,* and *L. longbeachae* for each assay target were run with each ddPCR plate. Sample results were only considered for analysis when the reader accepted 10,000 or more droplets as part of the quality control. Sample reactions with three or more positive droplets per well were identified as positive for their assay target. Three

technical replicates were run for each sample to determine the reproducibility of the assay results.

Table 2.4.6. Primers and Probes for Target *Legionella* Species

Target Species	Primer/Probe Name	Primer/Probe Sequence	Reference
Legionella species	23SF 23SR 23SP probe	5'-CCCATGAAGCCCGTTGAA-3' 5'-ACAATCAGCCAATTAGTACGAGTTAGC-3' 5'-HEX[a]-TCCACACCTCGCCTATCAACGTCGTAGT-BHQ1[b]-3'	Nazarian et al., 2008
L. pneumophila (*mip* gene)	mipF mipR LmipP	5'-AAAGGCATGCAAGACGCTATG-3' 5'-GAAACTTGTTAAGAACGTCTTTCATTTG-3' 5'-FAM[a]-TGGCGCTCAATTGGCTTTAACCGA-BHQ1-3'	
L. micdadei *L. anisa* *L. bozemanii* *L. longbeachae*	Pan-*Legionella* F Pan-*Legionella* R LmicdadeiP LanisaP LbozemaniiP LlongbeachaeP	5'- GTACTAATTGGCTGATTGTCTTG-3' 5'- TTCACTTCTGAGTTCGAGATGG-3' 5'-FAM-AGCTGATTGGTTAATAGCCCAATCGG-BHQ1-3' 5'-HEX-CTCAACCTACGCAGAACTACTTGAGG-BHQ1-3' 5'-FAM-TACGCCCATTCATCATGCAAACCAGnT-BHQ1-3' 5'-HEX-CTGAGTATCATGCCAATAATGCGCGC-BHQ1-3'	Cross et al., 2016

[a]Hexachlorofluorescein (HEX), and Fluorescein amidites (FAM), reporter dyes that are added to the 5' end of an oligonucleotide. [b]Black Hole Quencher (BHQ1), dark quenchers that does not absorb or emit light and are added to the 3' end of an oligonucleotide

2.4.7 Statistical Analysis

Descriptive statistics were conducted in GraphPad Prism 8 software (GraphPad Software, CA, USA). Sample concentrations were transformed from gene copies (GC)/100 mL into Log_{10} GC/100 mL for statistical analysis. A geometric mean for each sample was calculated using only the technical replicates that had ≥ three positive droplets. If one technical replicate was positive, only that value was used. The biological data were expressed as geometric means with standard deviations (SD). Correlation analysis was performed between the concentrations of *Legionella* species (23S rRNA) present in samples and water quality parameters tested (temperature, chlorine, turbidity, pH, and conductivity). One-way analysis of variance (ANOVA) was also performed to compare each variable (building influents, taps on the first and top floor (if any), cold- and hot-water taps, and among both sampling events). Statistical results were interpreted at the level of significance $p<0.05$.

2.5 Results

2.5.1 Characterization and Concentrations of *Legionella* 23S rRNA and Five Pathogenic *Legionella* Species

Overall, a total of 37 samples were analyzed during this study: 14 from the summer sampling event, and 23 from the winter sampling period (Table 2.5.1). *Legionella* species (23S rRNA) were found in all water samples at concentrations ranging from 1.4 to 4.5 Log_{10} GC/100 mL (Table 2.5.1), and 54% of the samples were positive for at least one of the target species: *L. pneumophila* (2/37), *L. anisa* (5/37), *L. micdadei,* (1/37), *L. bozemanii,* (16/37), and *L. longbeachae* (11/37) at average geomean concentrations of 1.7, 1.6, 1.7, 1.6, and 1.6 Log_{10} GC/100 mL, respectively.

Table 2.5.1. Presence and Concentrations of Five Pathogenic *Legionella* Strains in 37 Water Samples Collected in Five Buildings During Summer (August 13 & 27 and September 04, 2018) and Winter (January 7th, 8th ,9th ,14th and 15th, 2019)

Sample Location	**General *Legionella*[a] spp. (Log_{10} GC/100 ml)**	***Legionella*[b] Species (Log_{10} GC/100 ml)**	**General[a] *Legionella* spp. (Log_{10} GC/100 ml)**	***Legionella*[b] Species (Log_{10} GC/100 ml)**
	Summer		**Winter**	
Building F				
Influent	4.0	ND[c]	3.6	ND
1st Floor	3.5	*L. anisa* 1.5	**Cold:** 1.7 **Hot:** 1.6	**Cold**: ND **Hot**: *L. pneumophila* 2.0, *L. anisa* 1.7
2nd Floor	1.7	ND	**Cold**: 1.7 **Hot:** 1.5	**Cold**: *L. anisa* 1.7, *L. bozemanii* 1.8 **Hot**: *L. bozemanii* 1.5, *L. longbeachae* 1.8
Building BPS				
Influent	2.3	*L. bozemanii* 1.6, *L. longbeachae* 1.5	4.5	ND
1st Floor	4.5	ND	**Cold:** 1.7 **Hot:** 1.6	**Cold**: *L. bozemanii* 1.7, *L. longbeachae* 1.7 **Hot**: *L. bozemanii* 1.8, *L. longbeachae* 1.9
6th Floor	2.4	ND	**Cold:** 1.4 **Hot:** 1.8	**Cold**: *L. bozemanii* 1.4 **Hot**: *L. bozemanii* 1.8, *L. longbeachae* 1.7

Table 2.5.1. (cont'd)

Building M				
Influent	2.4	ND	3.7	ND
1st Floor	2.6	ND	**Cold:** 3.6 **Hot:** 2.5	**Cold:** ND **Hot**: *L. bozemanii* 1.7, *L. longbeachae* 1.5
2nd Floor	3.2	ND	**Cold**: 3.2 **Hot:** 2.9	**Cold:** *L. bozemanii* 1.7, *L. longbeachae* 1.7 **Hot**: *L. bozemanii* 1.4, *L. longbeachae* 1.4
Building FH				
Influent	1.9	ND	2.1	ND
1st Floor	3.5	*L. anisa* 1.6	**Cold:** 3.7 **Hot:** 2.9	**Cold**: *L. longbeachae* 1.4 **Hot**: *L. bozemanii* 1.7
2nd Floor	3.1	ND	**Cold:** 3.3 **Hot:** 2.8	**Cold**: *L. bozemanii* 1.4 **Hot**: *L. anisa* 1.7, *L. bozemanii* 1.7
Building ERC				
Influent	4.1	*L. pneumophila*, 1.5, *L. micdadei* 1.7, *L. bozemanii* 1.8, *L. longbeachae* 1.5	1.4	ND
1st Floor	4.1	*L. bozemanii* 1.5	**Cold:** 4.2 **Hot:** 4.1	**Cold**: ND **Hot**: *L. bozemanii* 1.8, *L. longbeachae* 2.0

[a] Value applies to General *Legionella* species (23S rRNA) detected in both tap (cold- and hot-water) listed. [b] Specific *Legionella* species. [c] Non-Detect (ND).

2.5.2 Five *Legionella* Species Detected in the Influent and Tap Water Samples in Five Different Buildings

Pathogenic *Legionella* species were detected in influent pipes only during the summer sampling event. *Legionella bozemanii* and *L. longbeachae* were detected in the influent of the BPS building at a concentration of 1.6 and 1.5 Log_{10} GC/100 mL, respectively. *Legionella pneumophila, L. micdadei, L. bozemanii,* and *L. longbeachae* were detected in the influent (eyewash site) of the ERC building at concentrations of 1.5, 1.7, 1.8, 1.5 Log_{10} GC/100 mL, respectively (Table 2.5.1).

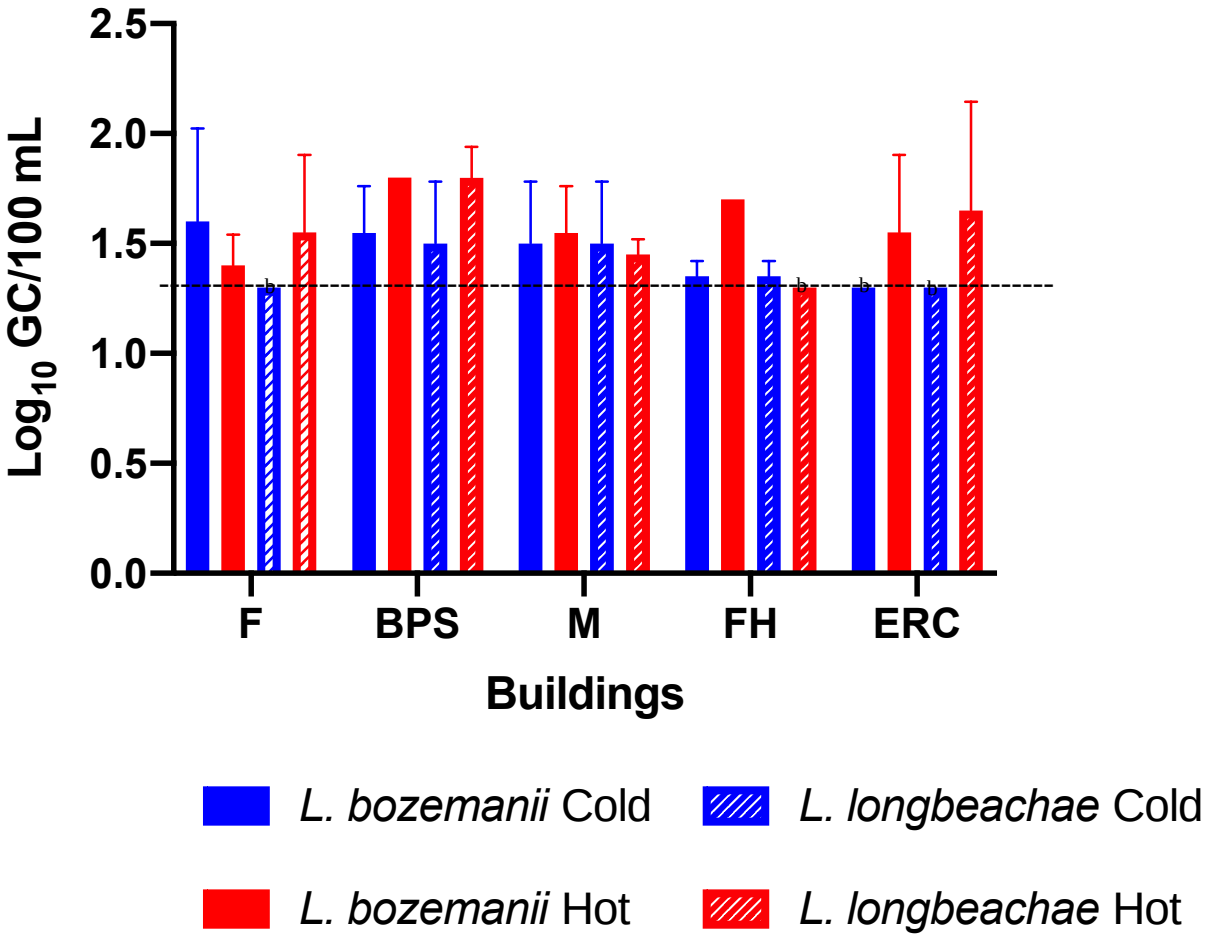
Below specific *Legionella* species detected in the taps during the summer and winter season in all five buildings are presented. *Legionella pneumophila* was detected in the hot water tap in the F building (winter sample) at a concentration of 2.0 Log_{10} GC/100 mL. *Legionella anisa* was present in the composite sample (cold- and hot-water sample) on the first floor in the F and FH buildings (summer samples) at concentrations of 1.5 and 1.6 Log_{10} GC/100 mL, respectively. *Legionella anisa* was also present in both the cold- and hot-water taps in the building F and hot water tap in the FH building (winter samples) at concentrations ranging from 1.4 to 1.7 Log_{10} GC/100 mL. During the winter sampling event, *L. bozemanii* and *L. longbeachae* were found in 56.5% (13/23) and 39.1% (9/23) of cold- and hot-water taps of all five buildings, respectively with concentrations ranging from 1.4 to 2.0 Log_{10} GC/100 mL (Table 2.5.1).

Overall, the results showed that building M contained the least number of samples (n=3) with detectable pathogenic *Legionella* species, and building BPS contained the most number of samples (n=5) with detectable pathogenic *Legionella* species. Interestingly, four out of five *Legionella* species tested for (except for *L. anisa*) were found in the eyewash influent sample of the ERC building (Table 2.5.1).

2.5.3 *Legionella* Species in Cold Compared to Hot Taps

Figure 2.5.3 compares the Log_{10} gene copies of the most prevalent species, *L. bozemanii* and *L. longbeachae* in the cold- and hot-water taps. *Legionella bozemanii* concentrations were higher in the hot-water samples (geomean of 1.7) than in the cold-water samples in BPS, M, FH, and ERC. *Legionella longbeachae* concentrations were higher in the hot-water samples (geomean of 1.8) than in the cold-water samples in F, BPS, and ERC buildings. Overall, the five target *Legionella* species were more prevalent in hot-water samples (39% positive, 9/23) compared to the cold-water samples (26% positive, 6/23) (Table 2.5.1). Within the hot-water samples, there appeared to be more diversity of the target *Legionella* species (*L. pneumophila, L. anisa L. bozemanii, or L. longbeachae*) present, compared to the cold-water tap samples, where *L. pneumophila* was not detected (Table 2.5.1).

Figure 2.5.3. Presence of *L. bozemanii* and *L. longbeachae* in Cold- and Hot-water Taps in the Five Buildings in the Winter Samples. Bars Reflect All Measurements Collected at Each Tap. Dashed Line Represents the Detection Limit (1.3 Log_{10} GC/100 mL). Samples With No Signal are Reported as the Detection Limit.



[a] F building, positive for *L. pneumophila* in the hot tap; F building, positive for *L. anisa* in the cold- and hot-water taps. FH building, positive for *L. anisa* in the hot tap. *L. micdadei* was not detected in the winter.[b] Results without a standard deviation correspond to samples with no signal.

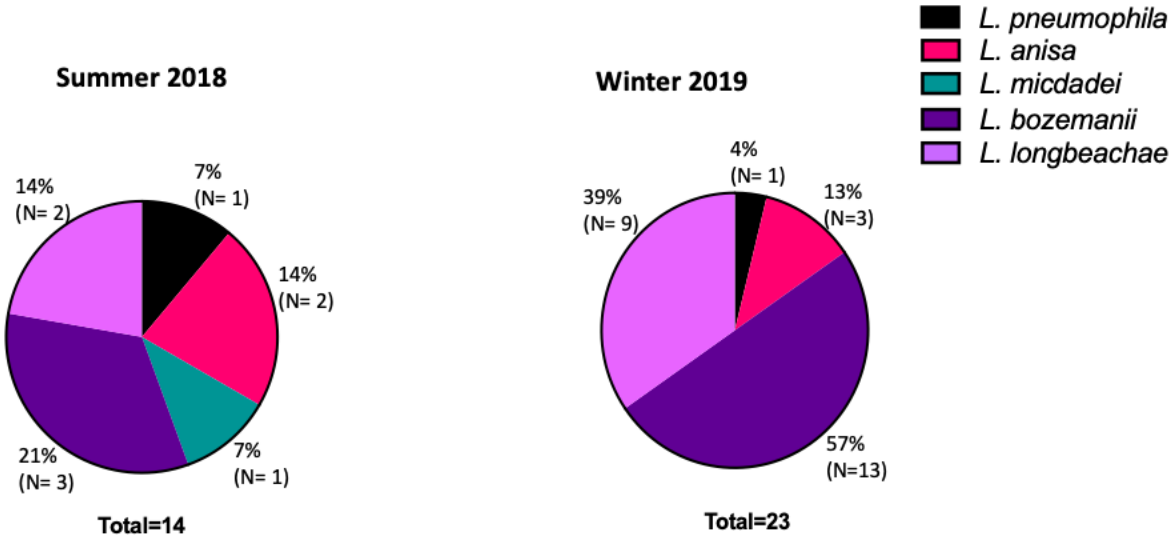
2.5.4 Comparison of Five Target *Legionella* Species in Summer and Winter

In the summer, there was a greater diversity of *Legionella* species but with lower concentrations relative to the winter. For example, all five specific *Legionella* spp. (*L.*

pneumophila, L. anisa, L. micdadei, L. bozemanii and *L. longbeachae*) were detected in the summer, but *L. micdadei* was not positive in any water sample in the winter. Interestingly, the detection rates for *L. bozemanii* and *L. longbeachae* increased two-fold in the winter samples compared to the summer. Overall, *L. bozemanii* (43%, 16/37) and *L. longbeachae* (29.7%, 11/37) accounted for the majority of the *Legionella* positive samples detected in both seasons (Table 2.5.1 and Figure 2.5.4).

Table 2.5.1 and Figure 2.5.4 compares the presence and absence of the pathogens in tap water samples from the summer and winter seasons. *Legionella pneumophila, L. anisa, L. micdadei, L. bozemanii, and L. longbeachae* were present in low quantities (near the detection limit, 1.3 Log_{10} GC/100 mL) throughout the buildings drinking water system (Table 2.5.1). The concentrations ranged from 1.5 to 1.8 Log_{10} GC/100 mL in the summer samples, and from 1.4 to 2.0 Log_{10} GC/100 mL in the winter samples. More specifically, during the summer, 7% (1/14) of the samples were positive for *L. pneumophila* and *L. micdadei,* 14% (2/14) for *L. anisa,* and *L. longbeachae,* and 21% (3/14) for *L. bozemanii* (Figure 2.5.4). Collectively, five target *Legionella* species were detected in 36% (5/14) of the summer samples. During the winter, 65% (15/23) samples were positive with one or more of the target *Legionella* species. *Legionella bozemanii* had the highest occurrence at 57% (13/23), followed by *L. longbeachae* at 39% (9/23), *L. anisa* at 13% (3/23), and *L. pneumophila* at 4% (1/23) (Figure 2.5.4). *Legionella micdadei* was not detected in any of the winter samples (Table 2.5.1 and Figure 2.5.4).

Figure 2.5.4. *Legionella* Species in All Water Samples Collected During Summer and Winter Sampling Events. For the Summer and Winter, the N Values are the Number of Samples in Which the Species were Detected.



2.5.5 Chemical-Physical Water Quality

For the summer event, the cold- and hot-water taps were composite samples; thus, the chemical-physical parameters are reflective of this as the interest was determining the difference of water quality by floors. In the summer, water temperatures in the influent of all buildings ranged from 12.6 to 20.2◦C, with an average of 16.5◦C (Table 2.5.2). The temperatures of the composite cold- and hot-water samples were similar in range among both floors but slightly different across buildings. The water temperature across the buildings ranged from 25.8 to 34.2◦C and 27.1 to 36.7◦C on the first floor and top floor, respectively. Free chlorine ranged from 0.04 mg/L to 0.52 mg/L, with an average of 0.3 mg/L in the influents. The buildings' average free chlorine on the first floor was 0.09 mg/L and increased on the top floor to 0.21 mg/ (Table 2.5.2). The conductivity ranged from 750 to 867 μS/cm, with an average of 802 μS/cm in the buildings' influent. The buildings' average conductivity was 915.8 μS/cm on the first floor and then decreased on the top floor (827.8 μS/cm). Turbidity ranged from 1.3 to 66.2 NTU, with an

average of 19.5 NTU in the buildings' influent. The buildings' average turbidity dropped to 3.2 NTU on the first floor and slightly increased on the top floor (5.9 NTU). The mean pH was 7.4 in the influents, first floors, and the top floors.

Table 2.5.2. Chemical-Physical and Microbial Data for Influents and Composite Tap Water Samples August 13th, 27th and September 4th, 2018.

Composite Tap						
	F	**BPS**	**M**	**FH**	**ERC[a]**	**Building Average**
Influent						
Temperature (°C)	15.7	12.6	18.3	15.9	20.2	16.5
Conductivity (µS)	750	867	755	780	858	802
Turbidity (NTU)	24.5	3.58	1.3	1.89	66.2	19.5
pH	7.3	7.5	7.5	7.5	7.6	7.48
Total (Free) Chlorine Residual (mg/L)	0.58 (0.52)	0.52 (0.32)	0.39 (0.3)	0.53 (0.43)	0.04 (0.04)	0.4 (0.3)
HPCs (CFU/100 mL)	5.00	10.5	96.0	1.60 x10^4	4.50 x10^6	9.03 x10^5
Coliforms (MPN/ 100mL)	<1	<1	<1	<1	<1	<1
***E. coli* (MPN/100ml)**	<1	<1	<1	<1	<1	<1
1st Floor composite samples[b]						
Temperature (°C)	33.4	29.2	34.2	31.9	25.8	30.9
Conductivity (µS)	814	857	807	1256	845	915.8
Turbidity (NTU)	0.55	0.98	6.36	3.44	4.43	3.2
pH	7.3	7.4	7.5	7.2	7.6	7.4
Total (Free) Chlorine Residual (Mg/L)[b]	0.16 (0.13)	0.16 (0.02)	0.12 (0.03)	0.24 (0.17)	0.17 (0.1)	0.17 (0.09)
HPCs (CFU/100 mL)	5.00 x10^4	4.20 x10^4	1.45 x10^4	1.27 x10^4	3.20 x10^5	8.78 x10^4
Coliforms (MPN/100 mL)	<1	<1	<1	<1	<1	<1
***E. coli* (MPN/100 mL)**	<1	<1	<1	<1	<1	<1

Table 2.5.2. (cont'd)

Top Floor [b]						
Temperature (°C)[b]	32	27.1	36.7	35	N/A	32.7
Conductivity (µS)	794	904	794	819	N/A	827.8
Turbidity (NTU)	2.06	5.07	13.1	3.46	N/A	5.9
pH	7.3	7.5	7.5	7.4	N/A	7.4
Total (Free) Chlorine Residual (mg/L)[b]	0.29 (0.22)	0.33 (0.21)	0.1 (0.05)	0.1 (0.04)	N/A	0.2 (0.21)
HPCs (CFU/100 mL)	6.58 x10_4	2.75 x10_4	1.07 x10_4	3.29 x10_4	N/A	3.42 x10_4
Coliforms (MPN/100 mL)	<1	<1	<1	<1	N/A	<1
***E. coli* (MPN/100 mL)**	<1	<1	<1	<1	N/A	<1

[a] ERC, has one floor. [b] Composite cold and hot taps.

For the winter event, the cold- and hot-water taps were collected as separate samples; thus, the chemical-physical parameters are reflective of this as the interest was determining the difference of water quality by taps. In the winter, building influent water temperature ranged from 11.2 to 26.9°C, with an average of 17.9 $_o$C (Table 2.5.3). The average (21.4 ◦C for the first floor and 22.4 ◦C on the top floor) cold-water temperature for the buildings did not differ between floors; however, the buildings' hot-water was slightly warmer, on average, on the top floor (36.1◦C) compared to the first floor (31.6 ◦C). Free chlorine ranged from 0.17 to 1.46 mg/L (influent of FH Hall to influent of BPS) with an average of 0.6 mg/L. The buildings' mean for free chlorine (first and top floors) differed between the cold- (0.07 mg/L) and hot-water taps (0.04 mg/L) (Table 2.5.3). The buildings' average conductivity in winter ranged from 794 to 931 μS/cm in the influent with an average of 847 μS/cm. The conductivity of the cold- (947 μS/cm) and hot-water taps (931 μS/cm) on the first floor of the buildings were only slightly different. The conductivity on the top floors varied more between the cold- (890 μS/cm) and hot-water taps

(918 μS/cm). Turbidity ranged from 4.6 to 155 NTU (influent of FH Hall to influent of BPS) with an average of 58.3 NTU. The mean turbidity for the cold-water taps was 7.6, and 2.4 for the hot-water taps on the buildings' first floors. However, the mean turbidity slightly increased on the top floor for both taps, 20.6 NTU (cold-water tap), and 2.81 NTU (hot-water tap). The pH was approximately the same as the summer sampling, ranging from 7.3 (influent) to 7.6 (first-floor hot-water tap) and 7.5 (top-floor hot-water taps).

Table 2.5.3. Chemical-Physical and Microbial Data for Influents and Composite Tap Water Samples January 7th, 8th, 9th 14th and 15th, 2019.

Composite Tap Table						
	F	**BPS**	**M**	**FH**	**ERC[a]**	**Building Average**
Influent						
Temperature °C	14.5	23.6	13.3	11.2	26.9	17.9
Conductivity µS	914	794	931	799	797	847
Turbidity NTU	18.6	155	6.33	4.6	106.9	58.3
pH	7.3	7.5	7.3	7.3	7.3	7.3
Total (Free) Chlorine Residual Mg/L	0.24(0.23)	1.16(1.46)	0.09(0.18)	0.05(0.17)	1.19(1.33)	0.5(0.6)
Coliforms MPN/100ml	<1	<1	<1	<1	<1	<1
***E. coli* MPN/100ml**	<1	<1	<1	<1	<1	<1
1st Floor from composite samples						
Temperature °C (Cold and Hot taps)	17.8 37.6	22.8 31.3	23.3 36.3	21.8 28.2	21.1 24.7	21.4 31.6
Conductivity µS (Cold and Hot taps)	793 801	1161 1220	913 904	865 895	924 914	931 947
Turbidity NTU (Cold and Hot taps)	2.4 0.1	6.44 1.44	16.4 2.39	4.22 4.36	8.55 3.78	7.6 2.4
pH (Cold and Hot taps)	7.4 7.7	7.3 7.4	7.6 7.7	7.6 7.8	7.7 7.6	7.5 7.6
Total (Free) Chlorine Residual Mg/L (Cold and Hot taps)	0.04(0.03) 0.03(0)	0.14(0.1) 0.03(0.05)	0.14(0.13) 0.03(0.02)	0.05(0.16) 0.08(0.12)	0.07(0.09) 0.09(0.11)	0.09(0.1) 0.05(0.06)
Coliforms MPN/100ml	<1	<1	<1	<1	<1	<1
***E. coli* MPN/100ml**	<1	<1	<1	<1	<1	<1
Top Floor						
Temperature (°C) (Cold and Hot taps)	19.9 38.9	22 31.1	24.2 44.9	23.3 29.6	N/A	22.4 36.1
Conductivity µS (Cold and Hot taps)	786 802	977 1062	882 895	916 914	N/A	890 918
Turbidity NTU (Cold and Hot taps)	4.04 0.32	3.73 1.62	71 7.14	3.64 2.16	N/A	20.6 2.81
pH (Cold and Hot taps)	7.5 7.6	7.3 7.2	7.4 7.8	7.5 7.6	N/A	7.4 7.5

Table 2.5.3. (cont'd)

Total (Free) Chlorine Residual Mg/L (Cold and Hot taps)	0.05(0.07) 0.07(0.03)	0.05(0.05) 0.02(0.01)	0.4(0.4) 0.09(0.09)	0.03(0.05) 0.02(0.04)	N/A	0.13(0.14) 0.05(0.04)
Coliforms MPN/100ml	<1	<1	<1	<1	N/A	<1
***E. coli* MPN/100ml**	<1	<1	<1	<1	N/A	<1

[a]ERC, only has one floor.

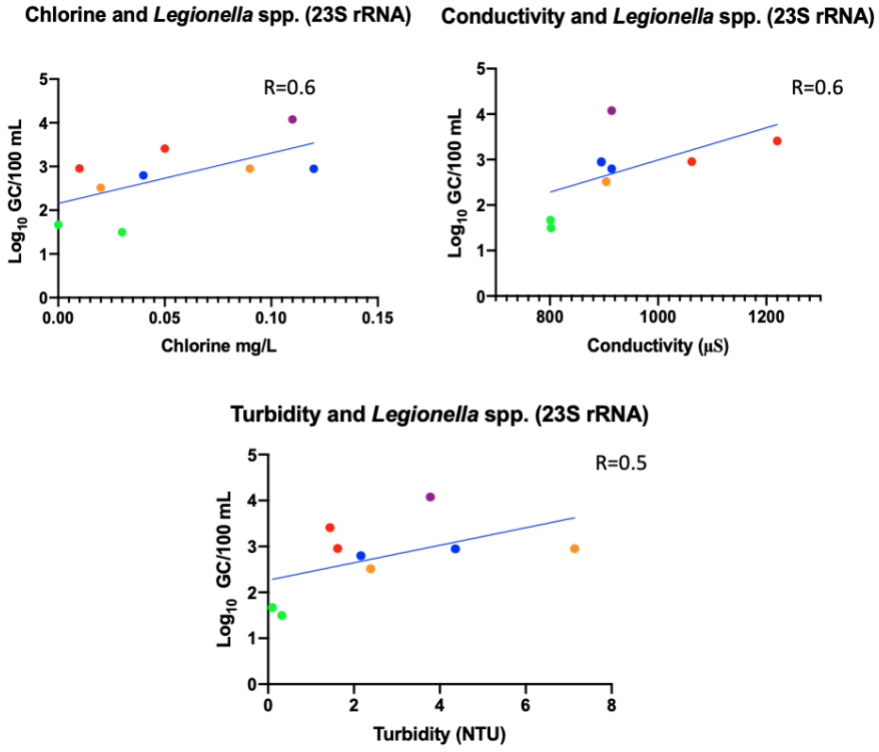
2.5.6 Relationship Between the Presence of *Legionella* and Water Quality Parameters

To determine which water quality parameters were associated with the detection and concentrations of general *Legionella* spp., *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae* in the different locations (influent, cold- and hot-water taps), a correlation analysis was performed. During the summer, there was not a relationship (positive or negative) between temperature, HPCs, chlorine, turbidity, pH, or conductivity with respect to general *Legionella* spp. (23S rRNA). Figure 2.5.5 shows the correlation between *Legionella* spp. 23S rRNA and three water quality parameters (free residual chlorine concentration, conductivity, and turbidity) during the winter, which were not significant. The positive trend between *L. anisa, L. bozemanii, L. micdadei, L. pneumophila, and L. longbeachae* (combined) positive samples (occurred in F, BPS and ERC influent, and FH and ERC first floor) and turbidity, pH, and HPCs, conductivity, and turbidity, during the winter, were not significant (p=0.6).

There were insufficient data to develop statistically significant relationships between the five targeted *Legionella* species and the water quality parameters. Observations found that *L. pneumophila* and *L. anisa* were both detected in the hot-water taps on the first floor of building F, and within this positive sample, no residual chlorine was detected, the conductivity was 801 μS/cm, and the turbidity was 0.1 NTU. In the *L. anisa* positive sample (in the hot-water tap on the second floor on building FH), the residual chlorine was 0.04 mg/L, the conductivity was 914

μS/cm, and the turbidity was 0.1 NTU. *Legionella bozemanii*, and *L. longbeachae* occurred in hot-water taps on both floors (except ERC) of all five buildings; in the positive samples, the conductivity, turbidity, and free residual chlorine ranged from 786 to 1220 μS/cm, 0.1 to 7.1 NTU, and 0 to 0.4 mg/L, respectively. It is interesting to note that free residual chlorine was below the minimal 0.2mg/L threshold (CDC, 2014) in all *Legionella*-positive samples, with the exception of the BPS influent sample.

Figure 2.5.5. Correlation Between Three Water Quality Parameters (Chlorine, Conductivity, and Turbidity) and *Legionella* spp. 23S rRNA During January 7th, 8th ,9th ,14th and 15th, 2019 Sampling Event. The Color Coding for Each Building is as Follows: Green: F; Red: BPS; Orange: M; Blue: FH; Purple: ERC



2.6 Discussion

This study reveals information about the distribution of general *Legionella* and five pathogenic species in buildings on a community drinking water system. This study has systematically evaluated the quantitative occurrence of *Legionella* spp. (23S rRNA), *L.*

pneumophila, L. anisa, L. micdadei, L. bozemanii, and *L. longbeachae* in the influent water pipe that enters into buildings and at distal points of use. The water quality in the influents of the buildings and at the points of use differ between buildings. The assays for specific species highlighted differences between cold- and hot-water taps as well as between summer and winter samplings likely due to water residence time (water age). But it is interesting to note that in general, the inconsistency detection of *Legionella* species is driven by water quality in the building such as, the differences in water temperature, the concentration of disinfectant residual, variable water usage patterns that lead to stagnation and the water age (as observed in all five buildings).

Increased water age in the distribution system has adverse downstream effects within the building water system (Masters et al., 2015). For example, the impact of increased water age increases water temperature and a loss of chemical residual (Ambrose et al., 2020). These changes combined (described previously) influences the occurrence and diversity of *Legionella* species (Wang et al., 2012; Rhoads et al., 2016; Lu et al., 2016). In this study, the influent of the ERC building had the greatest variety of *Legionella* species (*L. pneumophila*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae*). This may be due to the ERC building having increased water age as its influent water pipe is the furthest away from the water source (reservoir) at 19.4 km. This suggests that water age plays a role in the occurrence of *Legionella* species and warrants further exploration.

Legionella species can survive in cold-water taps (Donohue et al., 2014), but hot-water taps are known to be a source for their amplification (Bollin et al., 1985). This study detected the presence of specific *Legionella* species in both cold-water and hot-water taps with slightly higher concentrations seen in hot-water; this is in agreement with previous studies (Donohue et al.,

2014; Peter and Routledge et al., 2018; Totaro et al., 2017; Rhoads et al., 2016; Toyosada et al., 2017; Proctor et al., 2017). Recently, Donohue et al. (2019) found a difference in concentrations for *L. pneumophila* in hot-water taps compared to cold-water taps. The concentrations were higher (median concentrations: 4,201 CE/L) for *L. pneumophila* in the hot-water taps than in the cold-water taps (median concentrations: 341 CE/L). In this study, *L. pneumophila* was only detected in the hot-water tap at a concentration of 2.0 Log_{10} GC/100 mL.

When specific species were examined, the hot-water systems were more often found to be positive for both *L. bozemanii* and *L. longbeacha*e, which may be due to the premise plumbing recirculation loops —creating a conducive environment for *Legionella* growth (Rhoads et al., 2016). *Legionella pneumophila* was detected in 82% of samples from a hot-water system at a university hospital located in Sherbrooke, Canada, by culture and qPCR (Bédard et al., 2016). In 2019, Bédard et al. found *L. pneumophila* SG1 positive in 41%, and *L. pneumophila* serogroups 4 and 10 in 91% of the water samples in hot-water taps and connecting pipes in an undisclosed Canadian hospital by culture and sequence-based typing.

Several studies have shown that the concentrations of general *Legionella* spp. (23S rRNA) and *L. pneumophila* increases during the summer season (Whiley et al., 2015; Kao et al., 2015; Liu et al., 2019). While our study observed the opposite, it was not due to environmental conditions. The building water use data show that water use was higher in BPS followed by ERC, FH, M and, F; and that water use was higher in the summer than in winter for BPS and ERC. This seasonal variation in water use is likely due to increased use of the building cooling towers in the summer and possibly to seasonal variations in occupancy. Because water temperatures were not different between the two seasons, these data suggest that the water quality was affected more by water stagnation (low water use) than by other water quality

parameters. It is thought that water stagnation affects *Legionella* colonization in buildings' plumbing as the water sits in the pipes longer during times of low water use. The water temperature averages on the first and top floors for all the buildings sampled were close to the *Legionella*'s optimal growth temperatures (25 to 45°C, [Katz and Hammel, 1987]).

The potential amplification of *Legionella* (23S and target species) at the taps was seen during the winter sampling event and is further discussed. Higher *Legionella* (23S rRNA) concentrations seen in the exposure sites (taps) compared to influent samples of buildings FH and ERC suggest that *Legionella* spp. were potentially amplifying within the premise plumbing systems (Figure 2.6). When the five specific *Legionella* species were examined, three buildings (BPS, M and ERC) showed suspected amplification for two specific species, *L. bozemanii,* and *L. longbeachae.* Building F (20% of the buildings) showed potential amplification of *L. pneumophila, L. anisa, L. bozemanii,* and *L. longbeachae,* and FH showed suspected amplification for *L. anisa, L. bozemanii* and *L. longbeachae* (Table 2.6).

The points of use serve as an ideal environment for amplification and aerosolization of *Legionella*. Amplification of *Legionella* in premise plumbing occurs due to a variety of factors, such as a change in water temperatures (cold and hot) (Ohno et al., 2003; García Montero et al., 2019), reduced residual chlorine (Wang et al., 2012), increased number of free-living amoebae (Wang et al., 2015), and altered microbial community composition (Dai et al., 2018, Lautenschlager et al., 2010). Once amplified, *Legionella* can transmit from water to air from aerosol-generating features, such as the hot-water faucets, showers, decorative fountains, and building cooling towers. Since federal authority is restricted to the public water system in the US, there are no EPA requirements directly regulating *Legionella* in premise plumbing systems (EPA, 2020). However, *Legionella* water-related outbreaks and sporadic cases occur at the

premise plumbing level (office buildings or hospitals and cooling towers) even when residual chlorine concentrations are well maintained (Demirjian et al., 2015).

Health departments should consider the role of other *Legionella* species (*L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae*) in the presentation of pneumonia as they may pose a greater risk than *L. pneumophila* as they are widely distributed in the environment (Vaccaro et al., 2016; Mee-Marquet et al., 2006; Fleres et al., 2018; Dilger et al., 2017; Wullings et al., 2011; Wullings and van der Kooij, 2006; Fiume et al., 2005; Lesnik et al., 2016; Muder and Victor, 2002). The aging population and those with underlying conditions such as cancer, diabetes, or cardiac disease also increase the risk of LD (Boe et al., 2017; del Castillo et al., 2016; Viasus et al., 2013). Thus, individuals who are at greater risk (>55 or older, or immunocompromised) are driving the need for a rapid, quantitative approach to monitor and manage the risk from other *Legionella* species to better protect public health. Digital droplet PCR can be used to target low-copy DNA from highly contaminated environmental samples whereas the sample dilution requirements (standard curve) for qPCR may not detect the low target DNA in environmental samples (Rački et al., 2014; Verhaegen et al., 2016). Digital droplet PCR also overcomes the time delay from sampling to quantitative results (Baume et al., 2019), as seen with the "gold standard" culture method for *Legionella* detection and LD diagnosis (as reviewed by Mercante and Winchell, 2015). In this study, digital droplet PCR enumerated *L. pneumophila*, *L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae* in water samples duplex assays. Thus, it is crucial to utilize a method for the detection of *Legionella* in the environment and perform different assays to determine which species are causing LD.

Figure 2.6. *Legionella* spp. (23S rRNA) Concentrations at the Influent and the Tap During Both Seasons. A) Summer, Influent N=1 (error bars are indicative of technical replicate), Taps N=2. B) Winter, Influent N= 1 (error bars are indicative of technical replicate), Taps N=2. The Asterisks (***) Below Represent the Significance for F Tap vs FH Tap; P=0.0001

A B

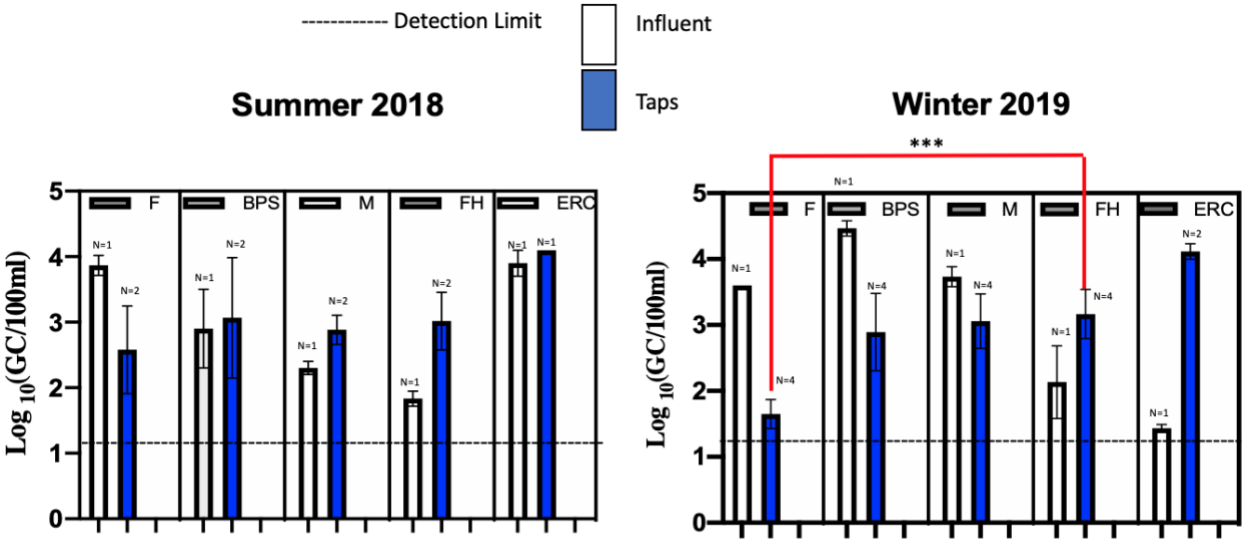


Table 2.6. Presence of Pathogenic *Legionella* Species at the Taps.

Building	August	January
	Amplification at Tap	Amplification at Tap
F	*L. anisa* (1st Floor)	*L. pneumophila*, *L. anisa*, *L. bozemanii* & *L. longbeachae* (1st Floor Hot Tap, 2nd Floor Cold & Hot Taps)
BPS	NA[a]	*L. bozemanii* & *L. longbeachae* (1st Floor Cold and Hot Taps, 6th Floor Cold and Hot Taps
M	NA	*L. bozemanii* & *L. longbeachae* (1st Floor Hot Tap, 2nd Floor Cold & Hot Taps)
FH	*L. anisa* (1st Floor)	*L. anisa*, *L. bozemanii* & *L. longbeachae* (1st Floor Cold & Hot Taps, 2nd Floor Cold & Hot Taps)
ERC	NA[a]	*L. bozemanii* & *L. longbeachae* (1st Floor Hot Tap)

[a] NA: Not applicable

2.7 Conclusions

This study focused on the occurrence and quantification of *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae* in a community drinking water system in the US. Our results from the winter sampling event provide evidence that four out of five targeted *Legionella* species showed signs of amplification between the influent and the points of use in various large educational buildings. The amplification of *Legionella* species was observed in conditions with water stagnation (low water use) and potentially increased water age. This suggests that the amplification of these species in a drinking water system is multifactorial. For example, *L. pneumophila, L. anisa, L. bozemanii,* and *L. longbeachae* potentially amplified in buildings with varying water usage (dependent on occupancy), and water age. Because each building has different risks at different times of the year, there needs to be a water management plan for various building types to reach optimization.

The examination of large volume (10-L) water samples using ultrafiltration within five large building water systems allowed for the detection of five individual *Legionella* species. The increased presence of specific *Legionella* species present downstream from the influent pipe, suggests that the bacteria are amplifying within the building water system. A monitoring scheme that includes composite, large-volume sampling, and rapid assessment by ddPCR could lead to better control of *Legionella* in building drinking water systems. Decreases in water flows that could lead to increased colonization and amplification within plumbing should be monitored beyond building influents. *Legionella* is part of the water microbiome and was found 100% of the time when sampling cold-and hot-water taps; thus, the monitoring of specific *Legionella* species using a large volume sample may be more appropriate when examining risk.

CHAPTER THREE

THE OCCURRENCE OF 5 PATHOGENIC *LEGIONELLA* SPECIES FROM SOURCE (GROUNDWATER) TO EXPOSURE (TAPS AND COOLING TOWERS) IN A COMPLEX WATER SYSTEM

3.1 Abstract

In this study, droplet digital PCR™ (ddPCR™) was used to characterize *Legionella* species from source (groundwater) to exposure sites (taps and cooling towers). A total of 42 samples were analyzed during this study: 12 from the reservoir, 24 from two buildings, and six from the cooling towers. Results demonstrated that gene copies of *Legionella* spp. (23S rRNA) was significantly higher in the cooling towers, relative to the reservoir and building Fa (closest to reservoir). *Legionella* spp. were found in 100% (42/42) of water samples at concentrations from 2.2 to 4.5 Log_{10} GC/100 mL. More specifically, *Legionella pneumophila* was found in 57% (24/42) of the water samples, followed by *L. bozemanii* 52% (22/42), *L. longbeachae* 36% (15/42), *L. micdadei* 23% (10/42), *L. anisa* 21% (9/42) at geomean concentrations of 1.4, 1.5, 1.3, 1.5 and 1.5 Log_{10} GC/100 mL respectively. Average gene copy numbers of *Legionella* spp. in the influent and the taps of the building furthest away from the reservoir (ERC) were higher than in the influent and taps of the building closest to the reservoir (Fa), and the difference was significant ($p < 0.05$). Positive Pearson correlations between pH, and HPCs and the gene copy number of *Legionella* spp. (23S rRNA) were observed (pH, R= 0.67, p= 0.0482; HPCs, R=0.9, p=0.0005). Based on this study, this data shows that water age in the distribution system and the premise-plumbing plays a major role in the increase of *Legionella* spp., as seen in the influent, and at the taps in the ERC building. Buildings furthest away from water utilities may face challenges in water quality, such as a loss of disinfectant residual, lower (<55) hot water temperatures, and higher (>20) cold-water temperatures.

3.2 Introduction

Legionella was first described and classified over 40 years ago (Fraser et al., 1977 and Brenner et al., 1979). Since its discovery, there have been 61 identified *Legionella* species (Zeng

et al., 2019), of which 28 have been isolated from human specimens (as reviewed by Zeng et al., 2019). *Legionella pneumophila* serogroup 1 is the most well-known and studied *Legionella* species, as it is most often identified as the etiologic agent of Legionnaires' Disease (LD). *Legionella pneumophila* accounts for more than 90% of pneumonia cases (Brady and Sundareshan, 2018; Waldron et al., 2015), followed by *L. micdadei, L. bozemanii, L. longbeachae* and other species such as *L. anisa* are rare (Sanchez et al., 2013, Lachant and Prasad, 2015, Miller et al., 2007).

Currently, in the United States (US), the incidence rate of LD is rapidly increasing with a rate of 300%, corresponding to a range of 0.4 to 1.6 reported cases per 100,000 population (Hicks et al., 2012; Adams et al., 2016). *Legionella pneumophila* is primarily responsible for drinking water disease outbreaks in the US (Brunkard et al., 2011).

A large percentage of the US population gets it's drinking water from groundwater (USGS, 2015). In the US, groundwater withdrawal for public supply accounts for approximately 39% of the total (USGS, 2015). Even in Michigan surrounded by the Great Lakes, total groundwater usage is about 700 million gallons per day (DEQ, 2018). Moreover, there is an estimation of 1.7 million people in Michigan that rely on municipal water supplies utilizing groundwater as their primary drinking water source (DEQ, 2018).

In groundwater, *Legionella* has been shown to range in concentrations from 10^2 to 10^5 CFU/L (Brooks et al., 2010). Groundwater sources are notorious for having iron concentrations, and this may be considered problematic (Johnson et al., 2018) as iron is a micronutrient for the growth of *Legionella* (Cianciotto et al., 2015). Because iron, however, is a secondary, EPA standard there is no maximum contaminant level (EPA, 2018).

Legionella bacteria are known to colonize engineered water systems such as premise plumbing, and cooling towers (Logan-Jackson et al., 2020, Donohue et al., 2014, Hamilton et al., 2018, Llewellyn et al., 2017). In these environments, *Legionella* can be aerosolized and potentially inhaled from showers, faucets, hot tubs/swimming pools, and cooling towers (as reviewed by Prussin et al., 2017).

There are a few limited studies on specific pathogenic *Legionella* species other than *L. pneumophila* in drinking water supply systems and cooling towers. For example, there has been a characterization study of pathogenic *Legionella* species in hot water systems by MALDI-TOF (Dilger et al., 2017), in tap water at hospitals by nested PCR assays (Fiume et al., 2005), and from kitchen sinks in private residences and restroom sinks in public buildings by PCR amplification, and sequencing (Richards et al., 2015). Lesnik et al., 2016 evaluated pathogenic *Legionella* species in a drinking water supply system by single-stranded conformation polymorphism. Pereira et al., 2017 evaluated *Legionella* species in cooling towers using universal primers 16S rRNA (PCR) and genus-specific deep sequencing (next-generation sequencing). Recently, Tsao et al., 2019 also evaluated *Legionella* species in cooling towers; using 16 and 18S rRNA gene amplicon sequencing. Although there has been an environmental surveillance of pathogenic *Legionella* species in a building water system and cooling towers, none of these six studies (mentioned above) have collectively investigated pathogenic *Legionella* spp. from source (groundwater) to exposure sites (taps and cooling towers).

The goal of this study was to detect and quantify five pathogenic *Legionella* species from groundwater to exposure sites (taps and cooling towers). Thus, this study examined the ecology of disease-relevant strains of *Legionella* in a whole water supply system. The following objectives was pursued: (i) characterization of total *Legionella* spp. (23S rRNA), *L.pneumophila*,

L.anisa, L. longbeachae, L. micdadei, and *L. bozemanii* in groundwater coming into the reservoir (untreated water), the reservoir (treated chlorinated water), the influent pipe within two buildings, the hot and cold-water taps and cooling towers and (ii) exploration of the associations of *Legionella* species with respect to, temperature, chlorine, conductivity, pH, HPCs, and water age.

3.3 Materials and Methods

3.3.1 Site Location and Sampling

Water samples were collected during the summer of 2019 from the reservoir (influent and effluent pipes), two research buildings (Fa, and ERC), and ten cooling towers on Michigan State University campus in East Lansing, Michigan. Sample collection was conducted in July, August, and September. The description of building age, water use, and distance from the reservoir are detailed previously (Logan-Jackson et al., 2020).

A large-volume composite sample was collected from each location to obtain water that was coming directly from the groundwater, storage tank, and the buildings. Ten liters were collected from the influent and effluent of the reservoir, both building's influent, cold- and hot-water taps, and cooling towers. Each carboy contained 10% sodium thiosulfate.

The first flush with equal total volumes from each tap was collected and composited into 10L for the first floor (cold and hot water taps, separately) and top floor (cold and hot water taps, separately). A total of 42 large-volume samples were collected: 12 from the reservoir (six influent and six effluent), 15 from building Fa, nine from building ERC, and 6 from the cooling towers. Three samples were collected from the influent, three hot and three cold from the first floor and top floor for building Fa. Building ERC only has one floor; thus, three samples were

collected from the influent, three hot and three cold from the first floor. Details on the number of taps on each floor are described in an earlier publication (Logan-Jackson et al., 2020).

3.3.2 Chemical-Physical and Microbiological Analysis

A 300ml sample was collected for physiochemical parameters. During sampling, the temperature and chlorine residuals (total and free) were measured using calibrated thermometers and the Test Kit Pocket Colorimeter II (HACH®, CO, USA) according to the manufacturer's instructions. After sampling, conductivity, pH, and turbidity were measured at the laboratory according to the manufacturers' instructions using a Russell RL060C Portable Conductivity Meter (Thermo Scientific, MA, USA), UltraBasic pH meter (Denver Instrument, NY, USA), and a Turbidity Meter code 1970-EPA (LaMottee Company, MD, USA).

After collecting the water samples, all samples were placed on ice and transported to the laboratory and immediately processed for heterotrophic plate count.

3.3.3 Water Sample Processing, DNA Extraction, and Molecular Analysis

The details for water processing, DNA extraction, and molecular analysis are detailed in a preceding article (Logan-Jackson et al., 2020). In brief, a high-pressure single-use elution fluid canister (INNOVAPREP LLC, MO, USA) was used to concentrate the 10L to ~50ml, and each ultrafiltration concentrate was split into several 10ml subsamples.

3.3.4 DNA Extraction and Quantitative Detection of *Legionella* Droplet Digital PCR

This study followed the procedure described by Logan-Jackson et al., 2020. In brief, a 10ml subsample was filtered on to a polycarbonate filter inside of a sterilized 0.47mm magnetic filter funnel (PALL Corporation, MI, USA), and one aliquot per water sample was later used for ddPCR analysis.

Droplet digital PCR technology was performed according to the manufacturer's instructions to analyze each sample for total *Legionella* spp. (23S rRNA), and five pathogenic species (*L.pneumophila, L. anisa, L. micdadei, L. bozemanii, and L. longbeachae*). The primers and probes used in this study are described in detail in a previous publication (Logan-Jackson et al., 2020).

Each amplification ddPCR reaction mixture consisted of 2X supermix (no dUTP) (Bio-Rad Laboratories CA, USA), mixed with a final concentration of 900nM forward and reverse primers and 250nM probes (Eurofins Genomics Co., AL, USA), and up to 330 ng of DNA template in a final volume of 20 μL. Droplets were generated by a QX200 Droplet Generator, and endpoint PCR was performed in a T100 Thermal Cycler (Bio-Rad Laboratories). Thermal cycle conditions are detailed previously (Logan-Jackson et al., 2020). The plate was cooled for at least 30 minutes, and droplets were then read using a QX200 droplet reader (Bio-Rad QX200TM Droplet Digital PCR System, CA, USA). For each assay: water without template served as a no-template control to detect environmental contamination; phosphate-buffer water served as a filtration blank; and there were five positive controls: *L. pneumophila, L. micdadei, L. anisa, L. bozemanii,* and *L. longbeachae,* were used to verify the assay performance.

Sample results were only considered for analysis when the reader accepted 10,000 or more droplets as part of the quality control, and unknown samples with three or more positive droplets per well were considered a true positive. All samples were performed in triplicate.

3.3.5 Statistical Analysis

Descriptive statistics were conducted in GraphPad Prism 8 software (GraphPad Software, CA, USA). Statistical analysis, including One-way ANOVA, Pearson Correlation, and simple linear regression, were used to determine the significance of the findings. Sample concentrations

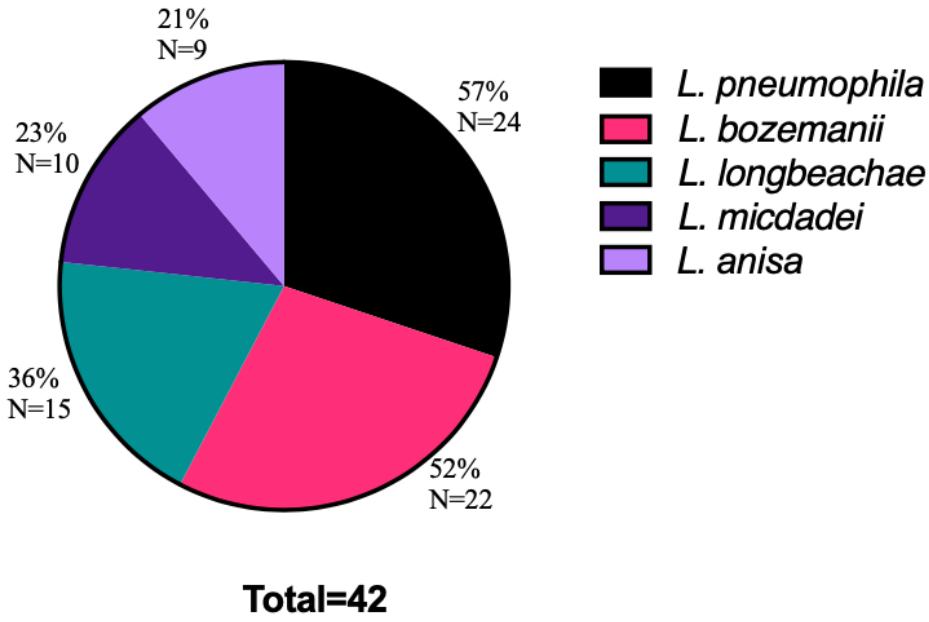
were transformed from gene copies (GC)/100 mL into $\log_{10}$ GC/100 mL for statistical analysis. The biological data were expressed as the geometric mean, and chemical data were shown as arithmetic means with standard deviation. A geometric mean for each sample was calculated using all values from technical and biological replicates. Statistical results were interpreted at the level of significance $p<0.05$.

3.4 Results

3.4.1 Characterization and Concentrations of *Legionella* 23S rRNA and Five Pathogenic *Legionella* Species

A total of 42 samples were analyzed during this study: 12 from the reservoir, 24 from the buildings, and six from the cooling towers (Table 3.4.2). *Legionella* spp. (23S rRNA) was found in 100% (42/42) of water samples at concentrations from 2.2 to 4.5 Log_{10} GC/100ml. *Legionella pneumophila* was found in 57% (24/42) of the water samples, followed by *L. bozemanii* 52% (22/42), *L. longbeachae* 36% (15/42), *L. micdadei* 23% (10/42), *L. anisa* 21% (9/42) at geomean concentrations of 1.6, 1.8, 1.4, 1.7 and 1.6 Log_{10} GC/100 mL respectively (Figure 3.4.1 and Table 3.4.2).

Figure 3.4.1 *Legionella* in All Water Samples Collected in Summer (August and September) of 2019



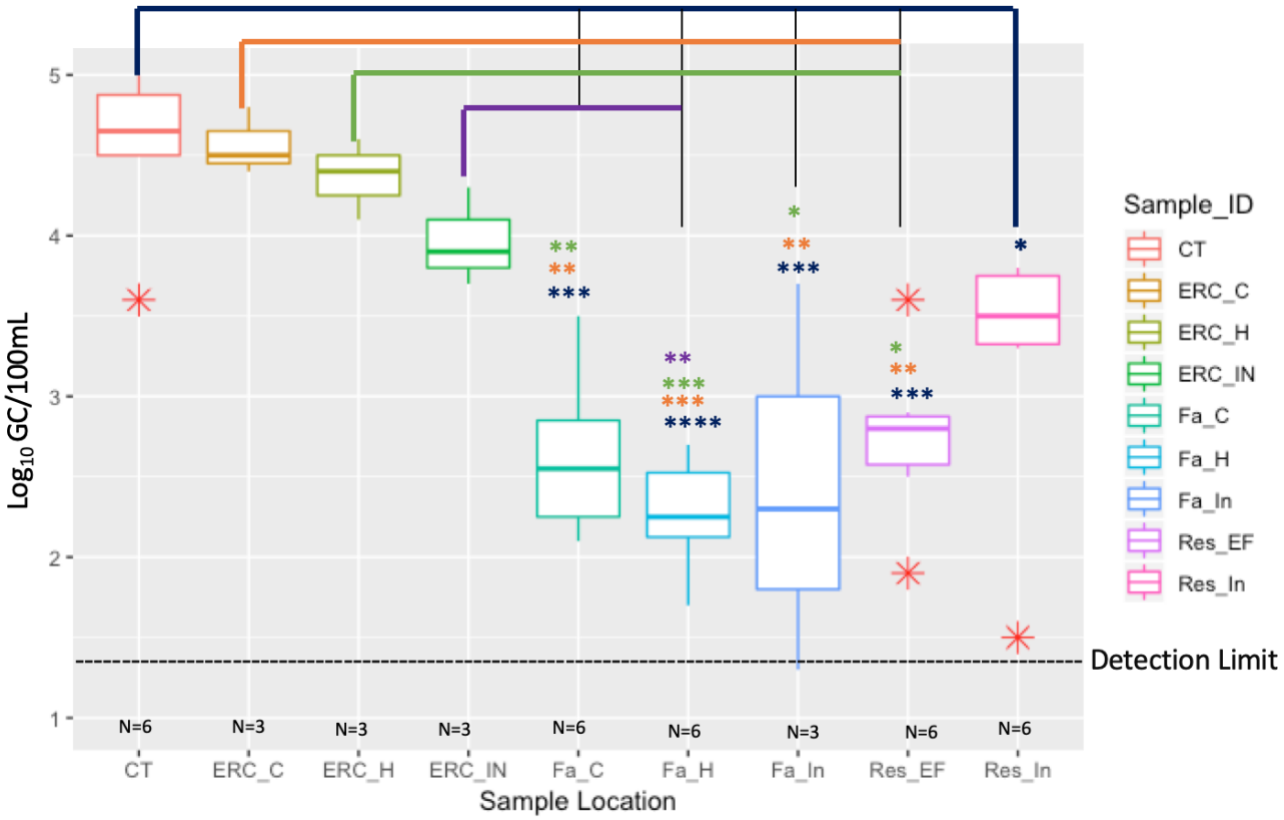
3.4.2. Detection of 23S rRNA and Five *Legionella* Species from Groundwater Source to the taps in the Buildings, to the Cooling Towers

Total *Legionella* spp. were found in all sampling sites at concentrations that ranged from 2.2.6 to 4.5 Log_{10} GC/100 mL. The concentration of total *Legionella* spp. in the influent of the reservoir was 3.2 Log_{10} GC/100 mL but when the water was treated with chlorine the concentration of *Legionella* in the effluent of the reservoir decreased to 2.7 Log_{10} GC/100 mL. The Log_{10} GC/100 mL of total *Legionella* spp. in the influent water pipe (2.4), the cold- (2.6), and hot-water taps (2.2) of building Fa (closest to the reservoir) was about the same as effluent of the reservoir (2.7). The ERC building had a water age of 20.8 hours, and the concentration of total *Legionella* spp. significantly increased. Building ERC is furthest away from the reservoir, and the concentrations in the influent water pipe, cold- and hot water taps were 4.0, 4.5, and 4.3 Log_{10} GC/100 mL, respectively. And as the water travel from the reservoir effluent to the

cooling towers, the concentrations of total *Legionella* spp. significantly (0.0003,) increased to 4.5 Log_{10} GC/100 mL.

Overall, the average concentrations of total *Legionella* spp. (23S rRNA) in the cooling towers and in the ERC building (both influent, hot and cold water taps) were statistically and significantly higher than what was found in the influent and effluent of the reservoir, influent, cold- and hot-water taps of building Fa. (Figure 3.4.2). The *p* values were as follows: CT vs Res_In, (0.0156); CT vs Res_EF (0.0003); CT vs Fa_In (0.0006); CT Vs Fa_H (<0.0001); CT vs Fa_C (0.0001); ERC_C vs Res_EF (0.0043); ERC_C vs Fa_In (0.0036); ERC_C vs Fa_H (0.0002); ERC_C vs Fa_C (0.0020); ERC_H vs Res_EF (0.0152); ERC_H vs Fa_In (0.0107); ERC_H vs Fa_H (0.0007); ERC_H vs Fa_C (0.0074); ERC_In vs Fa_H (0.0091) (Figure 3.4.2).

Figure 3.4.2. Comparison of *Legionella* spp. (23S rRNA) in the Reservoir (Influent and Effluent), Buildings: F and ERC, and the Cooling Towers. The Water Age (hrs) in Res_In (4.5), Res_EF (3.4), Fa (9.2), ERC (20.8), and CT (?)



The geometric means presented in the paragraphs below are for each species. Pathogenic *Legionella* species were detected in the reservoir (influent and effluent), the buildings, and the cooling towers. *Legionella bozemanii, L. micdadei,* and *L. pneumophila* 1.3 were detected in the influent of the reservoir (Res_IN) at concentrations of 1.7, 1.5, 1.6 Log_{10} GC/100mL, respectively. *Legionella pneumophila* and *L. bozemanii* were detected in the effluent of the reservoir at concentrations of 1.6 and 1.5 Log_{10} GC/100 mL. *Legionella longbeachae, L. pneumophila,* and *L. micdadei* were detected in the influent water pipes of buildings Fa at a concentration of 1.7, 1.6, and 1.6 Log_{10} GC/100 mL, respectively. *Legionella micdadei, L. bozemanii, L. pneumophila,* and *L. longbeachae,* and were detected in the influent water pipes of buildings ERC at a concentration

of 1.6, 1.4, 1.4, and 1.2 Log_{10} GC/100 mL. *Legionella anisa* was not detected in the influents of either building (Table 3.4.2).

In building Fa, *L. pneumophila, L. bozemanii, L. longbeachae, L. micdadei,* and *L. anisa* were detected in the cold-water taps at a concentration of 1.4, 1.4, 1.2, 1.1, and 1.1 Log_{10} GC/100 mL, respectively. *Legionella bozemanii, L. pneumophila, L. longbeachae,* and *L. anisa* were detected in the hot-water taps in building Fa at concentrations of 1.8, 1.6, 1.6, and 1.6 Log_{10} GC/100 mL, respectively. In building ERC, *L. pneumophila,* was only detected in the cold-water taps at a concentration of 1.4 Log_{10} GC/100 mL. *Legionella micdadei, L. bozemanii,* and *L. longbeachae* were detected in the hot-water taps in building ERC at a concentration of 2.2, 1.8, 1.7 Log_{10} GC/100 mL (Table 3.4.2).

Pathogenic *Legionella* species were detected in the cooling towers and the water concentrations were higher than the detectable *Legionella* species in the building water system. In the cooling towers, *L. bozemanii* had the highest concentration at 3.0 followed by *L. pneumophila* (2.8), *L. micdadei* (2.4), *L. anisa* (2.1), and *L. longbeachae* at 1.5 Log_{10} GC/100 mL (Table 3.4.2).

Depending on the location, pathogenic *Legionella* species were 10 to 1000-fold lower in concentration than total *Legionella* spp. (23S rRNA) in all water samples. For example, in the influent of the reservoir, *L. bozemanii, L. micdadei,* and *L. pneumophila* were 100-fold lower than total *Legionella* (23S rRNA). In the effluent of the reservoir, *L. pneumophila* and *L. bozemanii* were 10-fold lower than total *Legionella* (23S rRNA). The species were 10-fold lower in building Fa at any sampling site. In the ERC building, *L. pneumophila*, *L. bozemanii and L. longbeachae* were 1000-fold lower and *L. micdadei* was 100-fold lower than total *Legionella* (23S rRNA) spp. However, in the cooling towers, the five pathogenic species concentrations varied. For example, *L. longbeachae* were 1000-fold lower, *L. pneumophila, L. anisa,* and *L. micdadei,* were 100-fold

lower, and *L. bozemanii* 10-fold lower than total *Legionella* (23S rRNA) spp. Overall, five specific *Legionella* species does not account for the total *Legionella* spp. (23S rRNA) in either of the water samples (Table 3.4.2).

Table 3.4.2 *Legionella* Species in the Reservoir (Influent and Effluent), Influent, Cold- and Hot-water Taps of Buildings Fa, ERC, and the Cooling Towers (CT)

***Legionella* Species**	**CT (N=6)**	**ERC Hot Taps (N=3)**	**ERC Cold Taps (N=3)**	**ERC_IN (N=3)**	**Fa_Hot Taps (N=6)**	**Fa_Cold Taps (N=6)**	**Fa_IN (N=3)**	**Res_EF (N=6)**	**Res_In (N=6)**
***Legionella* spp. (23S rRNA) (%+)**	100% (6/6)	100% (3/3)	100% (3/3)	100% (3/3)	100% (6/6)	100% (6/6)	100% (3/3)	100% (6/6)	100% (6/6)
***Legionella* spp. (23S rRNA) Geomean (Log $_{10}$GC/100ml)**	4.5	4.3	4.5	4.0	2.2	2.6	2.4	2.7	3.2
***L. pneumophila* (%+)**	83% (5/6)	0% (0/6)	66% (2/3)	33% (1/3)	33% (2/6)	33% (2/6)	66% (2/3)	83% (5/6)	83% (5/6)
***L. pneumophila* Geomean (Log $_{10}$GC/100ml)**	2.8	ND	1.4	1.4	1.680oj9	1.4	1.6	1.6	1.3
***L. micdadei* (%+)**	33% (2/6)	66% (2/3)	0% (0/3)	66% (2/3)	0% (0/6)	16% (1/6)	66% (2/3)	0% (0/6)	16% (1/6)
***L. micdadei* Geomean (Log $_{10}$GC/100ml)**	2.4	2.2	ND	1.6	ND	1.1	1.6	ND	1.5
***L. bozemanii* (%+)**	100% (6/6)	33% (1/3)	0% (0/3)	100% (3/3)	16% (1/6)	16% (1/6)	0% (0/3)	100% (6/6)	66% (4/6)
***L. bozemanii* Geomean (Log $_{10}$GC/100ml)**	3.0	1.8	ND	1.4	1.8	1.4	ND	1.5	1.7

Table 3.4.2 (cont'd)

***L. longbeachae* (%+)**	50% (3/6)	33% (1/3)	0% (0/3)	100% (3/3)	50% (3/6)	66% (4/6)	33% (1/3)	0% (0/6)	0% (0/6)
***L. longbeachae* Geomean (Log $_{10}$GC/100ml)**	1.5	1.7	ND	1.2	1.6	1.2	1.7	ND	ND
***L. anisa* (%+)**	66% (4/6)	0% (0/3)	0% (0/3)	0% (0/3)	66% (4/6)	16% (1/6)	0% (0/3)	0% (0/6)	0% (0/6)
***L. anisa* Geomean (Log $_{10}$GC/100ml)**	2.1	ND	ND	ND	1.6	1.1	ND	ND	ND

3.4.3 Water Quality Parameters

The water quality characteristics of the reservoir, the buildings, and the cooling towers are presented in Table 3.4.3. Water temperature in the reservoir (influent and effluent) ranged from 11.6 to 12.3°C; the free chlorine residual in the reservoir influent was 0, and in the reservoir effluent, it ranged from 0.04 to 0.64. The conductivity ranged from 620 to 1032 μS/cm and the turbidity ranged from 1.08 to 9.55; the pH ranged from 7.1 to 7.4. The HPCs in the reservoir influent ranged from 1.50×10^1 to 7.80×10^1 CFU/100ml, and decreased in the reservoir effluent, and ranged from 1.0×10^0 to 6.0×10^0 CFU/100ml. The water quality parameters between the building influents of F and ERC were statistically different from each other. The water temperatures, turbidity, pH, and HPC (cold and hot) on both floors in building ERC were statistically different from building Fa (Table 3.6.3). A higher variance of the all water quality parameters was noted in the cooling towers. Interestingly, total coliforms and *E. coli* were seen in the cooling towers at 17.3 and 666.6 MPN/100 mL, respectively (Table 3.4.3).

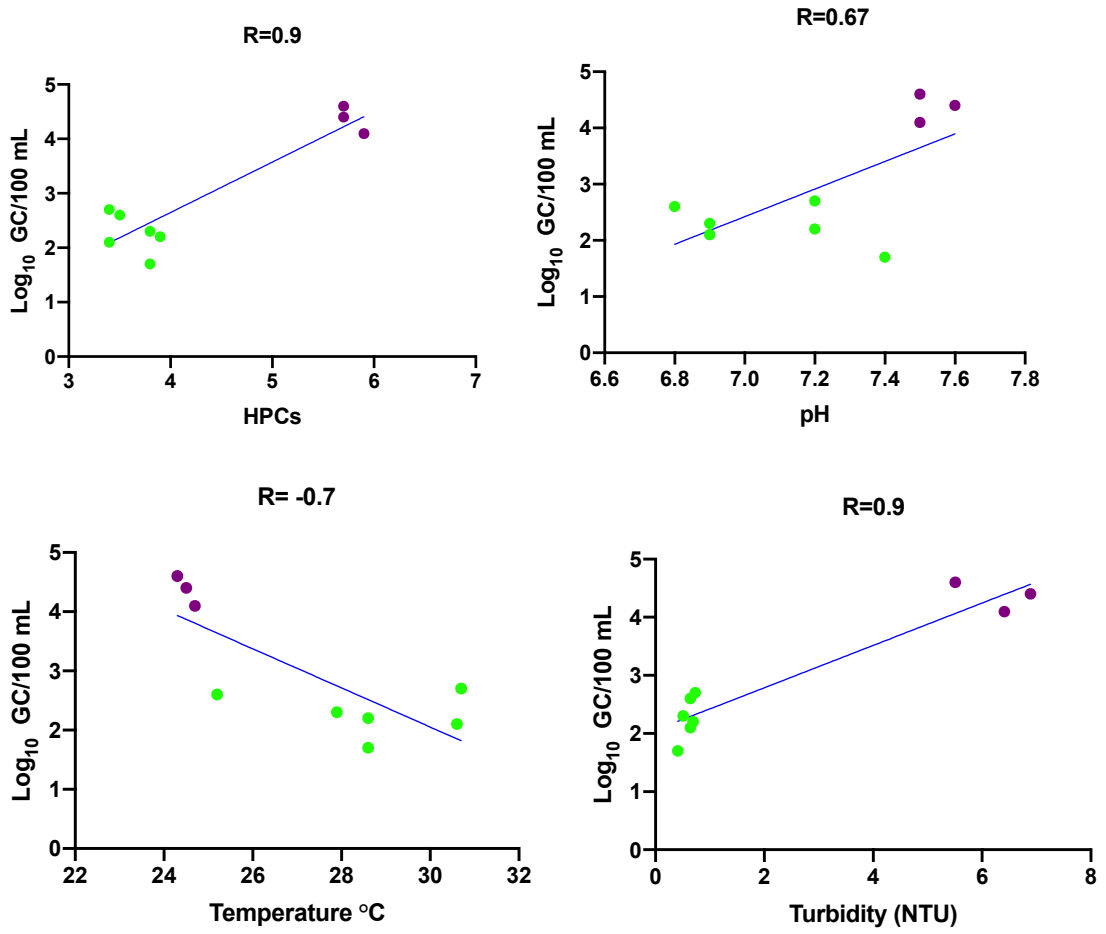
Table 3.4.3. Water Quality Parameters of the Reservoir (Influent and Effluent), the Buildings (Fa and ERC), and the Cooling Towers

Temperature (°C)	Total Chlorine (mg/L)	Free Chlorine (mg/L)	Turbidity NTU	pH	Conductivity (mS)	Composite CT1-2 HPC (CFU/100 mL)	Composite CT1-2 Total Coliforms (MPN/100 mL)	Composite CT1-2 *E. coli* (MPN/100 mL)
Reservoir Influent (N=6)								
12.1	0	0	4.1	7.2	851	3.52×10^{1}	<1	<1
Reservoir Effluent (N=6)								
11.9	0.64	0.33	3.85	7.2	855	2.10×10^{0}	<1	<1
Building F Influent (N=3)								
26.8	0.41	0.35	8.4	7.3	897	8.57×10^{4}	<1	<1
Building Fa 1st Floor Cold and (Hot Taps) (N=6)								
26.7 (28.6)	0.16 (0.04)	0.14 (0.02)	3.06 (0.53)	7.2 (7.1)	867 (815)	1.02×10^{4} (7.3×10^{3})	<1 (<1)	<1 (<1)
Building Fa 2ND Floor Cold and (Hot Taps) (N=6)								
26.8 (28.8)	0.05 (0.02)	0.03 (0)	3.37 (0.67)	7.0 (6.9)	856 (822)	2.00×10^{4} (3.15×10^{3})	<1 (<1)	<1 (<1)
Building ERC Influent (N=3)								
31.5	0.31	0.20	12.5	7.4	883	4.32×10^{5}	<1	<1
Building ERC 1St Floor Cold and (Hot Taps) (N=6)								
23.5 (24.5)	0.09 (0.04)	0.03 (0)	5.97 (6.27)	7.6 (7.5)	866 (847)	4.38×10^{5} (6.80×10^{5})	<1 (<1)	<1 (<1)
Cooling Towers								
25.3	0.49	0.08	1.94	8.2	2564	2.35×10^{7}	666.6	17.3

3.4.4 Relationship of *Legionella* 23S to Water Quality Parameters

Figure 3.4.4 shows the Pearson correlation between *Legionella* 23S rRNA and four water quality parameters (HPCs, pH, water temperature, and turbidity). In the hot-water taps in the premises of Fa and ERC, there was a strong positive correlation between *Legionella* 23S rRNA and HPCs (R= 0.9), and turbidity (R= 0.9). There was a moderate positive and a strong negative correlation between *Legionella* 23S rRNA and pH (R= 0.67), and water temperature (R= -0.7), respectively. The relationship between *Legionella* 23S rRNA and four water quality parameters (HPCs, pH, water temperature, and turbidity) was statistically significant (Figure 3.4.4).

Figure 3.4.4. Correlation Between 4 Water Quality Parameters (HPCs, pH, temperature and turbidity) and *Legionella spp.* 23S rRNA. The Color Coding for Each Building is as Follows: Green: Fa; Purple: ERC



3.5 Discussion

3.5.1 Identification and Quantification of Total *Legionella* (23S rRNA) and Five Pathogenic Species from Source to Exposure Sites

Legionella spp. are a consistent part of the water microbiome and have been previously described in groundwater (Brooks et al., 2004; Costa et al., 2005; Wullings and van der Kooij, 2006), influent water pipes (Peter and Routledge, 2018,;Wang et al., 2015; Pierre et al., 2019; Buse et al., 2017), cold- (Donuhue et al., 2014; Lu et al., 2017; Lesnik et al., 2016), and hot-water taps (Totaro et al., 2017; Lu et al., 2017; Lesnik et al., 2016), and cooling towers (Li et al.,

2015; Zhang et al., 2017; Hamiliton et al., 2018). The concentrations of *Legionella* spp. delivered to the taps from the water source may be due in part to the difference in disinfection used by the water utility (Hull et al., 2017), after delivery, there are key factors that have been suggested to influence the bacteria occurrence and concentrations in building water systems, such as water temperatures (Rhoads et al., 2015; Dilger et al., 2017), lower water usage (Rhoads et al., 2015) and water stagnation (Ji et al., 2015). *Legionella* spp. concentrations in groundwater (Costa et al., 2005; Riffard et al., 2001), and in building water systems (Pierre et al., 2019, Rodríguez-Martínez et al., 2015) have been described previously as high as 10^3 CFU/L. In contrast, *Legionella* concentration in cooling towers identified by the colony-forming unit has been shown to be 8.8 X 10^4 CFU/L (Li et al., 2015), but when identified by molecular analysis, *Legionella* densities reached up to 10^6 GU/L (Farhat et al., 2018). In this study, the concentrations (10^2 – 10^4 Log_{10} GC/100 mL) of total *Legionella* spp. (23S rRNA) in groundwater, building water systems, and cooling towers by molecular analysis were similar to Lagana et al., 2019; Valcina et al., 2019; Farhat et al., 2018; Wullings et al., 2011 and Wullings and van der Kooij, 2006). Rivera et al., 2007 found higher occurrence and concentrations of *Legionella* spp. in cooling towers 23.8% (112/373) relative to tap water 2.2% (2/373) but with little difference in concentrations of 3.60 X 10^3 and 3.17 X 10^3 Log_{10} CFU/mL. However, this study found a higher occurrence and concentration of *Legionella* species in the cooling towers compared to the building water system (reservoir and the taps).

While there have been some studies to reveal the differences of total *Legionella* spp. from finished water in a drinking water treatment plant (Ma et al., 2020), source water to taps (Hull et al., 2017; Lesnik et al., 2016), there are only a couple of studies to enumerate pathogenic *Legionella* spp. from groundwater (Wullings et al., 2011; Wullings and van der Kooij, 2006),

tap water (Lagana et al., 2019; Dilger et al., 2017; Leoni et al., 2005), cooling towers (Fragou et al., 2012), or a complete water supply chain (Lesnik et al., 2016) (Table 3.5.1).

The studies described below detected a variety of specific *Legionella* spp.; however, the present focus is on the species that are mostly associated with human disease, *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae*. *Legionella anisa, L. micdadei,* and *L. bozemanii,* were detected in water utilities that were supplied by surface water and groundwater; these species were identified by cloning and sequencing of *Legionella* specific 16S rRNA gene; however, the concentrations were not given (Wullings et al., 2011; Wullings and van der Kooij, 2006). In disagreement with this study, *L. pneumophila* was not detected in the water utility that was served by groundwater (Wullings et al., 2011; Wullings and van der Kooij, 2006), but its concentration in another utility supplied by surface water ranged from 1.0×10^3 to 2.0×10^4 (Wullings and van der Kooij, 2006). *Legionella pneumophila. L. bozemanii,* and *L. micdadei* were detected in tap water and identified by serology; the species consolidated concentrations ranged from 10^2 - 10^5 CFU/L^{-1} (Lagana et al., 2019). The Bruker MALDI Biotyper System was used to identify *L. pneumophila, L. anisa,* and *L. bozemanii* in tap water, but the individual concentrations were not given (Dilger et al., 2017). *Legionella pneumophila, L. anisa, L. bozemanii,* and *L. micdadei* were detected in tap water in apartments, hotels, and hospitals, by a serological test, and the concentrations were grouped into one category (Leoni et al., 2005). In apartments with a centralized water system, hotels, and hospitals, the concentration of non-*pneumophila* spp. was 400 – 3.3×10^3, 10^3 – 4.7×10^4, and 25 – 2.7×10^3 CFU/L^{-1}, respectively. Since non-*Legionella pneumophila* spp. account for a low percentage (~10%) of community-acquired LD, the majority of studies only focus on *L. pneumophila*, the etiological agent responsible for LD. Even when studies detect non-*Legionella pneumophila* spp. (as

described above) there isn't specific information on individual species percent positive or its concentrations, and the identified non-*Legionella pneumophila* spp. are consolidated into a single category (Table 3.5.1).

Similar to this study, Fragou et al., 2012 examined the occurrence and concentration of pathogenic *Legionella* spp. in taps of large buildings (hospitals and hotels) and cooling towers. *Legionella pneumophila, L. anisa, L. bozemanii,* and *L. longbeachae,* were detected in tap water, and cooling towers by an agglutination test and DNA sequencing (Fragou et al., 2012). *L. pneumophila* concentration ranged from $10^2 – 10^3$ CFU/ L^{-1} in hot and cold water, and interestingly the concentration was 10^2 CFU/ L- in the cooling towers. The data on the concentration of *L. anisa, L. bozemanii,* and *L. longbeachae* was not shown; these species were analyzed for phylogenetic purposes to determine the relationship between environmental species, thus there was less focus on specific species concentration (Fragou et al., 2012).

Lesnik et al., 2016 examined pathogenic *Legionella* species from source to taps in the premise. *Legionella longbeachae, L. pneumophila,* and *L. anisa* were detected in the raw water (untreated), treated water, and tap water (Lesnik et al., 2016). However, the average concentration of *L. longbeachae* (cold: 3.44 X 10^5; hot: 1.36 X 10^6), *L. pneumophila* (hot: 8.87 X 10^5), and *L. anisa* (cold: 2.11 X 10^5) were only given for the tap water (cold and/or hot). The concentrations of *L. longbeachae, L. pneumophila* and *L. anisa* are inconsistent with the present study; *L. pneumophila, L. anisa, L. micdadei, L. bozemanii* and *L. longbeachae* were near the detection limit (1.3 Log_{10} GC/100 ml) in the reservoir, and within the buildings influent and tap samples, but the concentrations were higher in the cooling towers, ranging from 1.5 to 3.0 Log_{10} GC/100 mL (Table 3.5.1).

Table 3.5.1. *Legionella* spp. in Raw Water, Treated Water, Tap Water, and Cooling Towers. Labeled Source Acronyms (*) Indicate Concentration Data Not Shown

Source	Water type	Concentrations	Reference:
Surface water	Finished water from four drinking water treatment plant	2.85, 4.35, 2.39, 3.01 Log GC/ mL	Ma et al., 2020
Surface water	Raw water*, finished water*, tap water*	Data not shown	Hull et al., 2017
Surface water	Raw water*, treated water*, cold and hot drinking water	3.44×10^5 1.36×10^6 cells liter^{-1}	Lesnik et al., 2016
Groundwater	Raw water Treated water	2.9×10^2 2.5×10^3 cells liter^{-1}	Wullings et al., 2011
Surface and Groundwater	Raw water (surface and ground water), Treated water (surface and groundwater)	2.5×10^6, 2.5×10^4 cells liter^{-1} 7.8×10^5, 9.8×10^4 cells liter^{-1}	Wullings and van der Kooij, 2006
Groundwater	Tap water	Range: 10^2 - 10^5 CFU/ L^{-1}	Lagana et al., 2019
Information not given	Tap water	Range: 10^2-10^4 CFU/100 mL	Dilger et al., 2017
Information not given	Tap water Cooling towers	Range: 10^2 - 10^4 CFU/ L^{-1}	Fragou et al., 2012
Surface water	Tap water	Range: 25–97,500 CFU/ L^{-1}	Leoni et al., 2005

3.5.2 *Legionella* spp. (23S rRNA) and water age

Previous studies (Hull et al., 2017; Wang et al., 2014; Wang et al., 2015; Rhoads et al., 2020) have shown how water age influences *Legionella* spp. on an experimental laboratory scale; however, there are only a couple of studies that demonstrated this observation in a natural system (Nguyen et al., 2012 and Rhoads et al., 2016). In contrast to this study, Nguyen et al., 2012 and Rhoads et al., 2014 determined water age by water usage patterns within premise plumbing systems. Nguyen et al., 2012 compared four sites and three out of four sites had toilets. Nguyen et al., 2012 did a flushing test (flushing the taps). Since one site lacked a toilet (demand of heavy water use from toilets is decreased), it was suggested lower water usage pattern (from the lack of toilet flushing) was directly related to higher water age. *Legionella* species were not detected in any water samples despite the water usage patterns (Nguyen et al., 2012). Rhoads et al., 2016 surveyed four buildings that were different in terms of water age. Each building had different estimates on water age; the hydraulic retention time for each house is as follows: one conventional house with no conservation features (~ one day), a healthcare facility (~8 days), a net-zero energy house (~2.7 days), and a net-zero water office building (30 to 180 days). Three buildings are green except for the conventional house. Quantitative PCR (qPCR) data showed that *Legionella* spp. were detected in all three green buildings, whereas it was not in the conventional building (Rhoads et al., 2016). Having a lower water age is consistent with no detection or a lower concentration of *Legionella* spp. in plumbing systems. The study described herein evaluated the water age as it relates to pipe mileage from the reservoir to the premise for buildings Fa and ERC, which are closer and furthest from the reservoir, respectively. Building ERC had an increase in *Legionella* 23S rRNA from the closest building (Fa). Interestingly, building ERC has a cooling tower (heavy water demand), and building Fa lacks a cooling tower,

(lack of heavy water demand), thus the increase in *Legionella* spp. observed in building ERC is not directly related to water usage, it related to the water age in the distribution and the premise plumbing system.

3.5.3 Correlation Between *Legionella* spp. and Water Quality Parameters

The results of the relationships between the bacteria and other water quality variables are mixed. Some reports also showed a statistically significant correlation between HPCs (De Filippis et al., 2018), turbidity (Valster et al., 2011), pH (Walczak et al., 2016), and water temperature (De Giglio et al., 2019) with *Legionella* spp. However, others found no correlations between *Legionella* spp. and HPCs, pH, and water temperature (Pierre et al., 2017), and turbidity (Liu et al., 2019). Like this study, Lesnik et al., 2016 did not observe a correlation between specific *Legionella* species (*L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae*) and water temperature; this null finding suggests the need for further exploration with larger data sets. There is not a correlation within buildings, but between buildings; thus, it is recommended that one examine the buildings' water quality with large volume composite sampling to begin to define further what characteristics are important to risk. Although there was not a correlation between *Legionella* spp. and physiochemical parameters in the cooling towers, this water type is a significant reservoir for the growth of *Legionella* due to the warm water temperatures. The concentrations of *Legionella* spp. (23S rRNA) were relatively abundant in the cooling towers (4.5 Log_{10} GC/ 100 mL), which is higher than the safety levels established in ASHRAE 2018-188. *Legionella* species associated with human disease have been detected in cooling towers (Bacigalupe et al., 2017; Thornley et al., 2017). *Legionella* spp. aerosolizing from cooling towers may be more likely to reach deep into the lungs; thus, a monitoring scheme is vital to assess whether a system is over the recommended threshold. Moreover, a monitoring

scheme could result in improved control measures for species (*L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae*) that are associated with human disease.

3.6 Conclusions

Overall, these data demonstrate that water age likely plays the most major role in the increase of *Legionella* spp., as seen in the ERC building. Buildings furthest away from water utilities may face challenges in water quality, such as a loss of disinfectant residual, lower (<55) hot water temperatures, and higher (>20) cold-water temperatures. The reduce chlorine and changes in water temperature over time consequently creates an optimal environment for the growth of *Legionella* spp. in a drinking water system. Thus, there needs to be strategies for monitoring drinking water systems to evaluate whether the distribution and premise plumbing systems are at risk for an increase of *Legionella*. Additionally, a routine monitoring scheme is critical to assess and verify the safety in drinking water as it relates to water-related pathogens, such as *Legionella*.

Since 2009, *L. pneumophila* has been on the United States Environmental Protection Agency (USEPA) Candidate Contaminant List (CCL); thus, there are reasons to believe that there should be federal regulations for monitoring and controlling this primary water-related bacterium. Ultimately, a routine monitoring scheme would decrease the morbidity and mortality rate of LD—caused by *L. pneumophila, L. anisa, L. bozemanii, L. micdadei, and L. longbeachae* in common exposure sites (taps and cooling towers) where conditions are favorable for their proliferation, and where *Legionella*-containing aerosols are generated.

CHAPTER FOUR

CO-OCCURRENCE OF FIVE PATHOGENIC *LEGIONELLA* SPP. AND TWO AMOEBAE SPP. IN A COMPLETE DRINKING WATER SYSTEM AND COOLING TOWERS

4.1 Abstract

Pathogenic *Legionella* species grow optimally inside free-living amoebae to concentrations that increase risks to those who are exposed. The aim of this study was to screen a complete drinking water system and cooling towers for the occurrence of *Acanthamoeba* spp., and *Naegleria fowleri* and their cooccurrence with general *Legionella* spp. (23S rRNA), *L. pneumophila, L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae.* A total of 42 large-volume water samples, including 12 from the reservoir (water source), 24 from two buildings (influents to the buildings and exposure sites (taps)), and six cooling towers were collected and analyzed using droplet digital™ PCR (ddPCR™). Altogether, 47% (20/42) of the water samples were positive for free-living amoebae, and Naegleria *fowleri* was the most detected species 40% (17/42) followed by *Acanthamoeba* spp. 19% (8/42). In 69% (29/42) of the samples (both positive and negative), *L. micdadei* and *L. bozemanii* cooccurred with *Naegleria fowleri.* In the building water system, the concentrations of *L. micdadei, L. bozemanii* and *Naegleria fowleri* ranged from 1.5 to 1.6 Log_{10} Gene Copies (GC)/100 mL, but and the concentrations of species in the cooling towers were higher. For instance, *L. bozemanii* and *Naegleria fowleri* was found at the highest concentrations, which was the same concentration for both species, 3.0 Log_{10} GC/100 mL. The data obtained in this study illustrates the ecology of pathogenic *Legionella* species in two exposure sites. Investigating *Legionella*'s ecology within exposure sites (taps and cooling towers) will hopefully lead to better control of these pathogenic species in the drinking water supply system and cooling towers.

4.2 Introduction

Free-living amoebae species are found in various natural and engineered water systems, such as surface water, groundwater, drinking water supply systems, hot springs, and cooling

towers (Armand et al., 2016; Lares-García et al., 2018; Baquero et al., 2014; Delafont et al., 2013; Retana-Moreira et al., 2014; Canals et al., 2015; Ren et al., 2018). *Acanthamoeba* and *Naegleria* are the two most common genera that are frequently isolated from aquatic environments (Liu et al., 2006; Thomas et al., 2006; Thomas et al., 2008; Rohr et al., 1998; Buse et al., 2013). Similarly, to free-living amoebae, *Legionella* spp. are found in natural water bodies (surface water, groundwater, and hot springs) (De Giglio et al., 2019; Shen et al., 2015; Ishizaki et al., 2016) and human-made systems (swimming pools, drinking water supply systems, and cooling towers) (as reviewed by Leoni et al., 2018).

Free-living amoebae play an important role in harboring *Legionella* in aquatic systems (Shaheen et al., 2019; Gomes et al., 2020). For example, the growth and survival habitat of *Legionella* in the environment is within free-living amoebae (Hsu et al., 2015; Abu Kwaik et al. 1998; Marciano-Cabral 2004). The symbiotic relationship confers protection from disinfectants at concentrations that kill *Legionella* spp. but not amoebae spp. in treated water supply systems (Kilvington and Price 1990; Thomas et al. 2004; Molmeret et al. 2005; Marciano-Cabral et al., 2010). Amoebae cysts are resistant to harsh environments due to the process of encystment; this process makes amoebae able to survive and proliferate in treated water supplies (Neff et al., 1964; De Jonckheere and van de Voorde 1976; Sarkar and Gerba, 2012; Coulon et al., 2010; Cursons et al., 1980; Greub et al., 2003; Marciano-Cabral et al., 2003; Majid et al., 2017). Particularly, *Acanthamoeba* spp. and *Naegleria fowleri* are suitable hosts for *Legionella* spp. because their cysts are resistant against disinfectants, changes in pH, temperature, osmolarity, and UV (Kilvington and Price 1990; Cervero-Aragó et al., 2014; Shaheen et al., 2019). For instance, the effect of UV irradiation on free-living *Legionella* and *Legionella* associated *Acanthamoeba* were examined to determine its effectiveness in controlling both pathogens

(Cervero-Aragó et al., 2014). UV treatment was effective against free-living *Legionella* and the trophozoite form of amoebae. Still, the results showed that the association of *L. pneumophila* with cyst-formed-*Acanthamoeba* decreases the effectiveness of UV irradiation (Cervero-Aragó et al., 2014). Another study shows a similar trend but with a different environmental condition. *Legionella* and *A. polyphaga* were inoculated together to determine the effect of temperature on both species (Shaheen et al., 2019). High temperatures of 40 °C induced the cyst form of *Legionella* associated *A. polyphaga* (Shaheen et al., 2019). Indeed, the cyst form serves as a protection for *Legionella.*

Several lines of evidence suggest that *Acanthamoeba* spp. are low-temperature amoebae while *Naegleria fowleri* is a thermotolerant amoeba (Kang et al., 2020; Lam et al., 2019; Xue et al., 2018; Nielsen et al., 2014; Marciano-Cabral et al., 2010). Temperature tolerance of the genera *Acanthamoeba* and *Naegleria* species was conducted using a temperature gradient block, which ranged from 29 to 48°C (Griffin et al., 1972). Two species of *Naegleria* (*N. fowleri* and *N. gruberi*), and six species of *Acanthamoeba* (*A. culbertsoni, A. rhysodes, A. polyphaga, A.castellanii, A.astronyxis,* and *A.palestinensis*) were tested (Griffin et al., 1972). *Acanthamoeba* species, except *A. culbertsoni,* did not grow pass 37°C and *Naegleria fowleri* was able to grow at 45°C (Griffin et al., 1972). Another study showed a similar trend by comparing clinical and environmental strains of free-living amoebae. Four clinical and two environmental strains of *A. castellanii* were examined for its growth rate and temperature tolerance by applying a temperature gradient that ranged between 22 to 36 (Nielsen et al., 2014). The optimal growth temperature for all six strains of *Acanthamoeba* species ranged between 30 to 33°C, and the generation time was ranged from 7-12 hours (Nielsen et al., 2014). Because *N. fowleri* actively grows at 42°C with a generation time of 3hr, there is

evidence to believe that this organism has a high optimal temperature for its growth (Goudout et al., 2012). Thus, temperature influences growth of *Acanthamoeba* and *Naegleria* (Nielsen et al., 2014; Goudout et al., 2012).

Water temperatures in man-made environments may also affect the detection of free-living amoebae. One study suggested that water temperatures via environmentally (seasonal influence) affected the dynamics of free-living amoebae species within a distribution system (Marciano-Cabral et al., 2010). Marciano-Cabral et al., 2010 surveyed tap water from a community drinking water system in March and September from two different geographical areas in the United States. Surface water supplied both water utilities, and the temperature in the distribution system ranged from 4 to 28°C (Marciano-Cabral et al., 2010). But the temperature of the tap water was undisclosed (Marciano-Cabral et al., 2010). Nevertheless, in both sampling events (March and September), *Legionella,* and free-living amoebae were detected. Interestingly, *Acanthamoeba* spp. was detected in the spring water samples, whereas *Naegleria* was detected in the autumn samples by PCR. Another study surveyed two chlormianted drinking water distribution systems in Virginia and Florida during the warmer months. The Virginia water utility supplying the water in the city uses surface water, and the Florida water utility uses a blend of surface, ground, and desalinated water. Water samples were collected from residential houses with various water ages (Wang et al., 2012). Wang et al., 2012 showed that *Acanthamoeba* spp. and *Legionella pneumophila* were less abundant from both sampling locations (Virginia and Florida), indicating that its low abundance may be relative to higher ambient temperature, humidity, and water chemistry (Wang et al., 2012). While both of these studies demonstrate that the difference in detection of free-living amoebae and *Legionella* may be seasonally influenced via water temperature, there was not any

comparison on their detection in a groundwater source (which would not be affected by ambient temperatures). Interestingly, either study did not determine whether or not the detection of free-living amoebae and *Legionella* cooccurred in the same water sample.

There are limited studies examining their cooccurrence in a complete drinking water system and cooling towers, collectively. The cooccurrence of *Legionella* spp. and free-living amoebae have been examined solely in a drinking water supply system (Valcina et al., 2019), a hospital water network (Muchesa et al., 2018), and cooling towers (Scheikl et al., 2014). Each study described below collected a small volume of the water sample, which ranged from 100 mL to 1000 mL (Valcina et al., 2019; Muchesa et al., 2018; Scheikl et al., 2014). In one study, a total of 268 water samples were collected, and *Legionella* and free-living amoebae were detected by culture and PCR methods (Valcina et al., 2019). Out of 268 drinking water supply samples, *Legionella* and amoebae spp. (*Acanthamoeba, Vermaoeba,* and *Naegleria*) cooccurred in 114, and both species were negative in 61 samples (Valcina et al., 2019). Valcina et al., 2019 also detected other *Legionella* species, such as *Legionella rubrilucens* (observed in seven samples, 6.1%), and *Legionella anisa* (found in two samples 1.8%). However, this study grouped all the *Legionella* species together and did not show the specific cooccurrence between *Legionella* and free-living amoebae. Nevertheless, there were no *Legionella* spp. positive samples in the absence of free-living amoebae (Valcina et al., 2019). A total of 98 water and biofilm samples were collected from the sterilization unit, theatres, neonatal ward, and intensive care units and analyzed by culture and PCR methods (Muchesa et al., 2018). Amoebae species were isolated from 71 of the 98 samples. More specifically, *Vermamoeba vermiformis* (n=68) and *Acanthamoeba* (n=30) were isolated from 69.4, and 30.6% of the bulk water and biofilm samples, respectively (Muchesa et al., 2018). Interestingly, *Legionella pneumophila* cooccurred

in 9.9% (7/71) of the amoebae positive samples by qPCR (Muchesa et al., 2018). Another study collected a total of 201 water samples from cooling towers, paper machines, and sewage plants (Scheikl et al., 2014). By cultivation, serology, and PCR methods, only 57 samples were positive for both *Legionella* and free-living amoebae (*Acanthamoeba, Vermaoeba*, and *Naegleria*). *Legionella* spp. cooccurred with *Acanthamoeba* (n= 19), *Vermaoeba* (n=16), and *Naegleria* (n=1) in 33.3, 28.1, and 1.75%, respectively (Scheikl et al., 2014). Among 57 cooccurrence samples, 70.1% (n=40) were specifically positive for *L. pneumophila.* Among the 40 samples, *L. pneumophila* cooccurred with *V. vermiformis* in 12.5% (n=5), and *Acanthamoeba* cooccurred with *L. pneumophila*, in 27.5% (n=11). Each study above focused mainly on the cooccurrence of *Legionella pneumophila* and various amoebae species by collecting a small volume (1L or less) of the sample. The differences in the cooccurrence of amoebae and *Legionella* species in the studies described above could potentially be due to a sampling bias. Thus, sampling a small volume of water may not represent the true distribution of pathogenic amoebae and coexisting *Legionella* spp. in the environment.

The present paper is aimed at filling in the knowledge gaps in the distribution of pathogenic amoebae spp. and *Legionella* spp. by collecting a large volume of bulk water from a drinking water source, building water system, and cooling towers, all served by a groundwater source. Utilizing ddPCR, this study addressed the following objectives (i) identify the cooccurrence of pathogenic *Legionella* spp. (*L. pneumophila, L. anisa, L. longbeachae, L. bozemanii,* and *L. micdadei*) and virulent amoebae spp. (*Naegleria fowleri* and *Acanthamoeba* pathogenic spp.) in a complete water supply chain and cooling towers, (ii) examine the difference in occurrence and concentration of *Naegleria fowleri* and *Acanthamoeba* pathogenic spp. in a groundwater source, building water system, and cooling towers.

4.3 Materials and Methods

4.3.1 Sampling

Details on the sample collection are described in an earlier publication (Logan-Jackson et al., 2020). In brief, a total of 42 large-volume samples were collected from the reservoir (influent and effluent pipes), two research buildings (F, and ERC), and ten cooling towers on Michigan State University campus in East Lansing, Michigan from July to August 2019. Ten liters were collected from each site location (reservoir, building, and cooling towers) in a carboy containing 10% sodium thiosulfate. There were six samples collected from the influent and effluent of the reservoir, 24 from the buildings (15 from building F, nine from building ERC), and six from the cooling towers. Each water sample was processed immediately after collection.

4.3.2 Chemical and Physical Analyses

A 300ml sample was collected separately from the large volume (mentioned above) for chemical and physical parameters. The water samples were analyzed for temperature and chlorine residuals (total and free) onsite using calibrated thermometers and the Test Kit Pocket Colorimeter II (HACH, CO, USA) according to the manufacturer's instructions. After sampling, conductivity, pH, and turbidity were measured offsite according to the manufacturers' instructions using a Russell RL060C Portable Conductivity Meter (Thermo Scientific, MA, USA), UltraBasic pH meter (Denver Instrument, NY, USA), and a Turbidity Meter code 1970-EPA (LaMotte Company, MD, USA).

4.3.3 Molecular Analysis of *Acanthamoeba* spp., *Naegleria fowleri*, and *Legionella* spp.

Water sample processing and DNA extraction were done using a protocol previously described (Logan-Jackson et al., 2020). Genomic DNA from *Acanthamoeba* castellani strain

Neff (ATCC® 30010™) and *Naegleria fowleri* (ATCC® 30174™) was obtained from American Type Culture Collection (ATCC, Va, USA).

Droplet digital PCR technology was performed according to the manufacturer's instructions to analyze each water sample for five *Legionella* pathogenic species (*L. pneumophila, L. anisa, L. micdadei, L. bozemanii, and L. longbeachae*), and two free-living amoebae species (*Acanthamoeba* spp. and *Naegleria fowleri*). The primers and probes used in this study are listed in Table 4.3.3. Duplex reactions for *Legionella* are detailed in a preceding article (Logan-Jackson et al., 2020). The description of the duplex reactions for free-living amoebae are listed in Table 4.3.3.

In brief, the reaction mixture consisted of 2X supermix (no dUTP), (Bio-Rad Laboratories CA, USA), 900nM forward and reverse primers and 250nM for each probe ((Eurofins Genomics Co., AL, USA), and up to 330 ng of DNA template, to a final volume of 20 ul. Droplets were generated using the QX200™ Droplet Generator Bio-Rad) and transfer of emulsified samples to a ddPCR 96-well plate (semi-skirted) was performed according to manufacturer's instructions (Instruction Manual, QX200™ Droplet Generator – Bio-Rad). The ddPCR™ plate was sealed with a pierceable foil heat seals using a PX1™ PCR Plate Sealer (Bio-Rad, Laboratories, CA USA). The plate was amplified using a Benchmark TC9639 thermal cycler (Benchmark Scientific Inc, NJ, USA). The cycling protocol was as follows: 95°C for 10 min, followed by 40 cycles of 94°C for 30 sec and 57°C for 1 min with a final 10 min cycle at 98°C for 10 min. After endpoint amplification, droplets were read using a QX200 droplet reader (Bio-Rad QX200™ Droplet Digital™ PCR System, CA, USA). Negative and positive controls were used to determine contamination (if any) and the efficiency of the assay.

Table 4.3.3. Free-living Amoebae Primers and Probes.

Target Species	Primer/Probe name	Primer/Probe Sequence	Reference
Acanthamoeba spp.	18S rRNAF 18S rRNAR 18S rRNAP	5'-CGACCAGCGATTAGGAGACG-3' 5'-CCGACGCCAAGGACGAC-3' 5'-FAM-TGAATACAAAACACCACCATCGGCGC-BHQ1-3'	Riviere et al., 2006;
Naegleri fowleri	ITSF ITSR ITSP	5'-GTGAAAACCTTTTTTCCATTTACA-3' 5'-AAATAAAAGATTGACCATTTGAAA-3' 5′-HEX-GTGGCCCACGACAGCTTT-BHQ1-3′	Pélandakis et al., 2000

4.3.4 Statistical Analysis

Statistical analysis was performed in GraphPad Prism 8 software (GraphPad Software, CA, USA). Principal component analyses, contingency tables, chi-squared tests, Pearson correlation, and regression analysis were used to evaluate the associations between the occurrence of *Legionella* spp. and free-living amoebae. Additionally, the principal component analysis was also conducted to assess the associations between the cooccurrence and water quality parameters.

Sample concentrations were transformed from gene copies (GC)/100 mL into Log_{10} GC/100mL. Biological data were expressed as geometric means, and chemical data were shown as arithmetic means with standard deviation. Statistical results were interpreted at the level of significance $p<0.05$.

4.4 Results

Free-living amoebae were detected in 47% (20/42) of the water samples. *Naegleria fowleri* occurrence rate was higher 40% (17/42) than detectable *Acanthamoeba* spp. 19% (8/42).

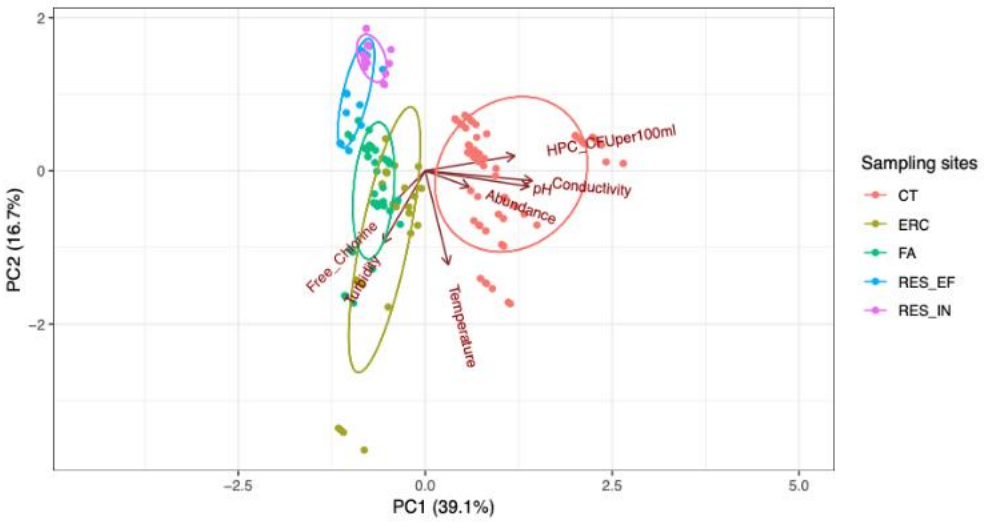
In the drinking water system (Fa, ERC, and RES_IN, and RES_EF), *Acanthamoeba* spp. only occurred in 8% (3/36), while *Naegleria fowleri* occurred in 33% (12/36). The concentrations of *Acanthamoeba* spp. in building F ranged from 1.1 to 1.4 Log_{10} GC/100 mL. The concentrations of *Naegleria fowleri* in the reservoir, and building ERC ranged from 1.3 to 1.7 and 1.1 to 1.8 Log_{10} GC/100 mL, respectively (Table 4.4.1). Water samples collected from the cooling towers were higher in percentage positives for *Acanthamoeba* spp. 83% (5/6) and *Naegleria fowleri* 83% (5/6) than the drinking water system (Fa, ERC, and RES_IN, and RES_EF). The concentrations of *Acanthamoeba* spp. and *Naegleria fowleri* in the cooling towers, ranged from 2.0 to 2.5 and 1.3 to 2.4 Log_{10} GC/100 mL, respectively (Table 4.4.1).

Table 4.4.1. The Occurrence of Two Amoebae spp. in 42 Water Samples Collected from the Reservoir, Buildings, and Cooling Towers. The Water Age in Res_In (4.5), Res_EF (3.4), Fa (9.2), ERC (20.8), and CT (?)

Free-living amoebae	**Site Location**				
	Res_In	**Res_EF**	**Fa**	**CT**	**ERC**
Acanthamoeba spp. (%+)	0/6 (0%)	0/6 (0%)	3/15 (20%)	0/9 (0%)	5/6 (83%)
Acanthamoeba spp. Min, and Max Geomean (Log_{10} GC/100ml)	ND	ND	1.1, 1.4	ND	2.0, 2.5
Naegleria fowleri (%+)	5/6 (83%)	1/6 (16%)	0/15 (0%)	6/9 (66%)	5/6 (83%)
Naegleria fowleri Min, and Max Geomean (Log_{10} GC/100ml)	1.3, 1.7	1.5[a]	ND	1.1, 1.8	2.3, 3.4

[a]Only value detected.

Figure 4.4.1. Principal Component Analysis (PCA) Biplot Showing the Clustering of the Data According to the Water Sampling Sites. Each Data Point Represents Each Species from a Particular Sampling Site (the observation). Samples from Five Sampling Locations, Color-coded Based on Sampling Location



The abundance represents the presence of *Legionella* and amoebae species in each sampling site. According to Figure 4.4.1, a clear separation was observed between water samples collected from the cooling towers (CT) and those collected from the drinking water system (Fa, ERC, and RES_IN, and RES_EF). The cooling towers are different from Fa, ERC, and the reservoir (RES_IN, and RES_EF). For instance, the cooling towers are associated with higher abundance, pH, conductivity, HPC, and lower turbidity, and free chlorine. Building Fa and ERC are characterized by free chlorine and turbidity, while the reservoir clustering (RES_IN and RES_EF) are associated with lower temperature, abundance, pH, HPC, and conductivity.

The Chi-squared test of independence showed a weak association between two amoebae spp. and four pathogenic *Legionella* spp. in all 42 water samples; *Acanthamoeba* spp. and *L. anisa* (χ^2 = 4.791, p=0.0286), *Naegleri fowleri* and *L. micdadei* (χ^2 = 4.748, p=0.0293), *Naegleri fowleri* and *L. pneumophila* (χ^2 = 4.356, p= 0.0369), *Naegleri fowleri* and *L. bozemanii*

(χ^2 = 6.645, p=0.0099). *Legionella anisa* positive samples were observed in the presence or absence of *Acanthamoeba* spp. *Legionella micdadei, Legionella pneumophila, and Legionella bozemanii* positive samples were also observed in the presence or absence of *Naegleria fowleri* (Table 4.4.2 to 4.4.5).

The concordance percentage between the presence of *Acanthamoeba* spp. and *L. anisa* was 9.52%. The absence percentage of *Acanthamoeba* spp. and *L. anisa* was higher (69.05%) than the presence percentage (9.52%) (Table 4.4.2). *Naegleria fowleri* and three *Legionella* spp. (*L. micdadei, L. pneumophila,* and *L. bozemanii*) concordance positive percentage was 16.67, 30.95, and 30.95%, respectively. The absence percentage of *Naegleria fowleri* and *L. micdadei, L. pneumophila,* and *L. bozemanii* were higher (52.38, 38.10, 33.33%, respectively) than the positive percentage (Table 4.4.3 to 4.4.5). Thus, the relationship that is observed between these two amoebae and *Legionella* species are driven by the absence percentage of both species (Table 4.4.2 to 4.4.5).

Table 4.4.2. Chi-squared Test Between *Acanthamoeba* spp. and *Legionella anisa* in 42 Water Samples

p value= 0.0286*	*L. anisa* Present	*L. anisa* Absent	Total
Acanthamoeba spp. Present	4 (9.52%)	4 (9.52%)	8
Acanthamoeba spp. Absent	5 (11.90%)	29 (69.05%)	34
Total	9 (21.42%)	33 (78.57%)	42

Table 4.4.3. Chi-squared Test Between *Naegleria fowleri* and *Legionella micdadei* in 42 Water Samples

[a]p value= 0.0293*	*L. micdadei* Present	*L. micdadei* Absent	Total
Naegleria fowleri Present	7 (16.67%)	10 (23.81%)	17
Naegleria fowleri Absent	3 (7.14%)	22 (52.38%)	25
Total	10 (23.8%)	32 (76.19%)	42

Table 4.4.4. Chi-squared Test Between *Naegleria fowleri* and *Legionella bozemanii* in 42 Water Samples

p value= 0.0099**	*L. bozemanii* Present	*L. bozemanii* Absent	Total
Naegleria fowleri Present	13 (30.95%)	4 (9.52%)	17
Naegleria fowleri Absent	9 (21.43%)	16 (38.10%)	25
Total	22 (52.38%)	20 (47.61%)	42

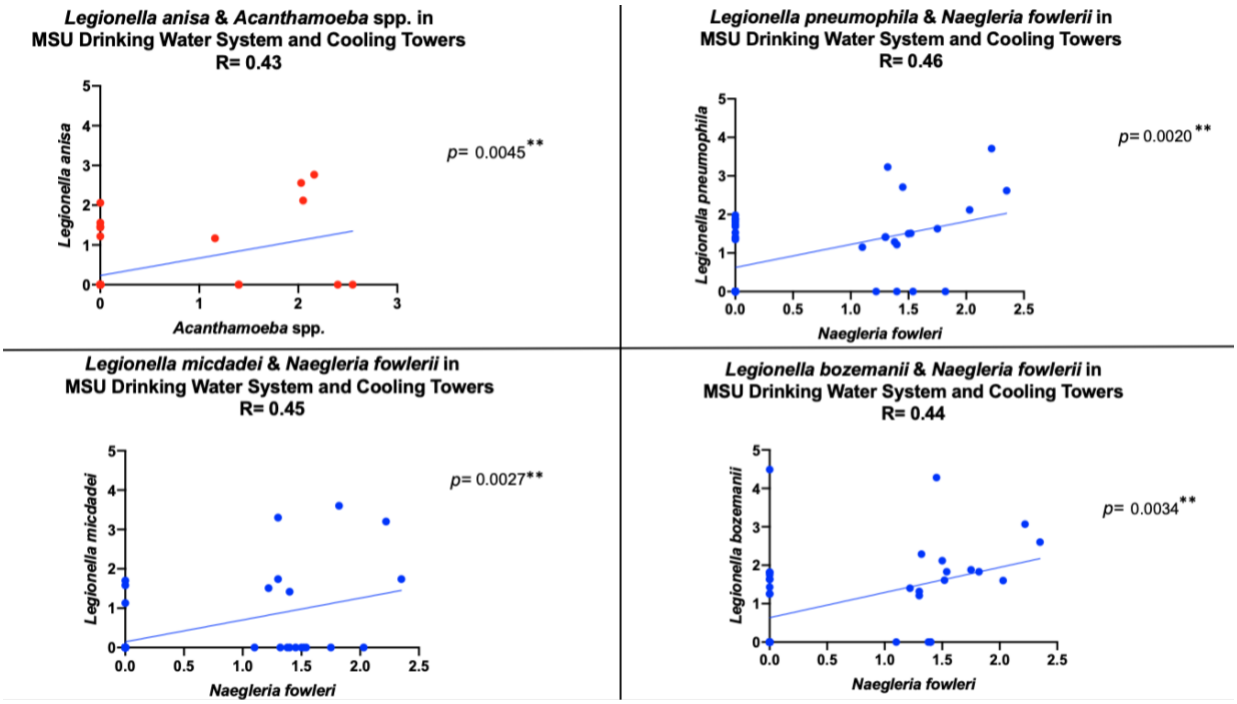
Table 4.4.5. Chi-squared Test Between *Naegleria fowleri* and *Legionella pneumophila* in 42 Water Samples

[a]p value= 0.0369*	*L. pneumophila* Present	*L. pneumophila* Absent	Total
Naegleria fowleri Present	13 (30.95%)	4 (9.52%)	17
Naegleria fowleri Absent	11 (26.19%)	14 (33.33%)	25
Total	24 (57.14%)	18 (42.85%)	42

[a]**Weak p value is <0.05 but >0.01**

Correlation analysis revealed a weak positive correlation (r = 0.43, p = 0.0045) between *Acanthamoeba* spp. and *L. anisa* in all water samples (Figure 4.4.2). There was also a weak positive correlation observed between *Naegleria fowleri* and *L. micdadei* (r= 0.45 p= 0.0027) *L. pneumophila* (r= 0.46, p= 0.0020), and *L. bozemanii* (r= 0.44, p=0.0034) in all 42 water samples (Figure 4.4.2).

Figure 4.4.2. Pearson Correlation Analyses Between Two Amoeba spp. and Four Pathogenic *Legionella* spp. in 42 Water Samples

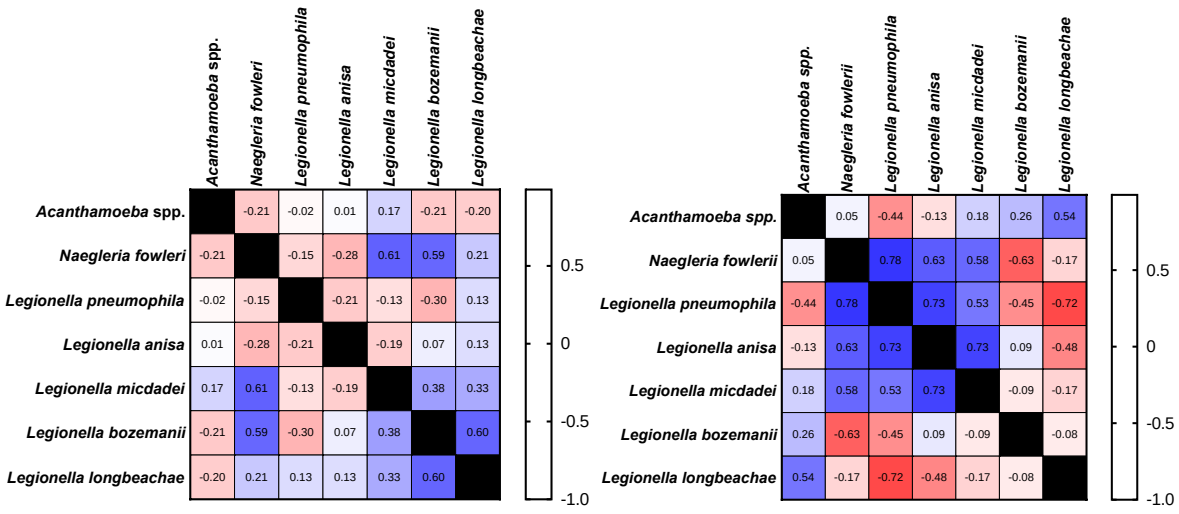


A Pearson correlation matrix was only observed for the building water system (Fa, ERC) and cooling tower samples, separately. Figure 4.4.3 shows a significant correlation between *Naegleria fowleri* and two *Legionella* spp. (*Legionella micdadei and Legionella bozemanii*) in buildings Fa and ERC. Correlation analysis revealed weak positive correlation in the building water system between *Naegleria fowleri and Legionella micdadei* (r = 0.61, p = 0.002) and *Naegleria fowleri and Legionella bozemanii* (r = 0.59, p = 0.003) (Figure 4.4.3a).In addition, correlation analysis revealed weak positive correlation between *Legionella bozemanii* and *Legionella longbeachae* (r = 0.60, p = 0.002) (Figure 4.4.3a). Correlation analysis revealed a positive correlation in the cooling towers between *Naegleria fowleri* and three *Legionella* spp. (*L. pneumophila, Legionella anisa, and L. micdadei*), which were not significant (Figure 4.4.3b).

In the building water system and cooling towers, *Acanthamoeba* spp. was observed in three and five water samples, respectively (Table 4.4.1), and this genus did not show a

statistically significant impact on the occurrence of *Legionella* spp. in either system (Figure 4.4.3). Out of three water samples in the building Fa, *Acanthamoeba* spp. and *Legionella* anisa only cooccurred in one sample (data not shown). And out of five cooling tower samples, *Acanthamoeba* spp. and *L.* anisa only cooccurred in three water samples. Thus, the relationship between *Acanthamoeba* spp. and *L.* anisa that was seen in Table 4.4.2 was driven by the absence of both species in the reservoir and building water samples, and the presence of both species in the cooling towers.

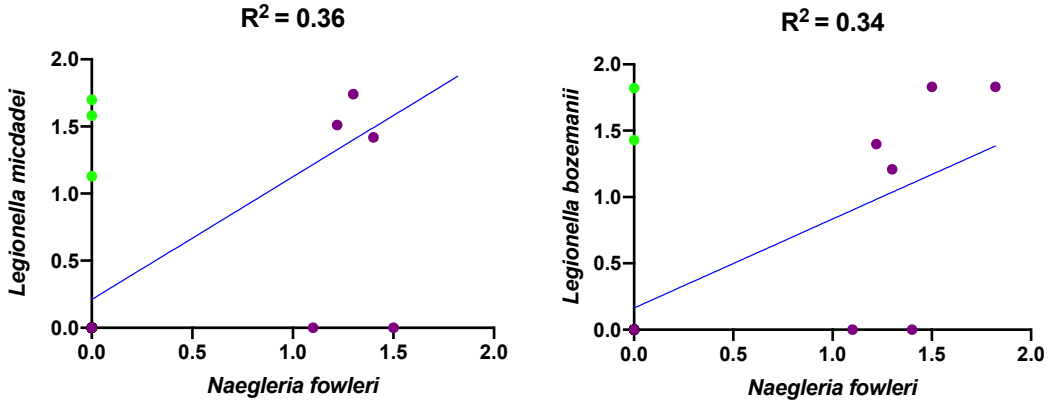
Figure 4.4.3. Pearson Correlation Matrix of Amoebae and *Legionella* spp. in Buildings Fa and ERC and Cooling Towers, Separately



There was a small dataset to develop statistically significant relationships between any of the five targeted *Legionella* species and amoebae species in the cooling towers. Thus, the rest of the paragraph will focus on *Legionella* species and amoebae species in the building water system (Fa and ERC). Simple linear regression analysis revealed a weak significant positive relationship (R_2 = 0.37, *p*= 0.0016) between *Naegleria fowleri* and *Legionella micdadei* and in the building water system (F and ERC) (Figure 4.4.4). Simple linear regression analysis also revealed a weak significant positive relationship (R_2 = 0.34, *p*= 0.0025) between *Naegleria fowleri* and

L.bozemanii. As *Naegleria fowleri* was undetected in building F, these two relationships (listed above) were driven by the ERC building (Table 4.4.1 and Figure 4.4.4). *Naegleria fowleri* affected *L. micdadei* in the influent pipe and hot-water taps of building ERC (Data not shown). There was not an inverse relationship observed between *Naegleria fowleri* and two *Legionella* spp. (*L. micdadei* and *L. bozemanii*) in the building water system (Figure 4.4.4).

Figure 4.4.4. Two *Legionella* spp. (*L. micdadei* and *L. bozemanii*) May Be Dependent on *Naegleria fowleri* in Buildings Fa and ERC



4.5 Discussion

Acanthamoeba spp. and *Naegleria* spp. are common in drinking water (Lu et al., 2017, Muchesa et al., 2017; Valcina et al., 2019; Liu et al., 2019; Üstüntürk-Onan et al., 2018) and cooling towers (Scheikl et al., 2014; Scheikl et al., 2016). Previous reports have shown that *Acanthamoeba* spp. were more frequently detected in drinking water supplies relative to *Naegleria fowleri* by molecular methods (Kim et al., 2018; Yousuf et al., 2013; Mahmoudi et al., 2012; Mahmoudi et al., 2015; Valcina et al., 2019; Scheikl et al., 2014; Üstüntürk-Onan et al., 2018; Liu et al., 2019). For example, *Acanthamoeba* spp. and *N. fowleri* were detected in 75% (39/52), 5.8%, respectively (3/52) of tap water samples by qPCR. Interestingly, *Acanthamoeba* spp. was detected in 88.5% (23/26) of water samples from cooling towers, while *N. fowleri* was detected in 3.8% (1/26) by qPCR (Scheikl et al., 2014). Consistent with this study, *Naegleria*

fowleri was frequently detected relative to *Acanthamoeba* spp. by culture and PCR molecular technique in untreated and treated water (Abdul Majid et al., 2017), tap water (Gabriel et al., 2019), and from municipal drinking waters and recreational water sources (Javanmard et al., 2017).

Previous studies have examined the cooccurrence of *Legionella* spp. with free-living amoebae in a drinking water supply system (Valcina et al., 2019) and cooling towers (Scheikl et al., 2014). A total of 268 water samples (1L each) were collected from different water sources (surface or ground) and cold and hot water taps located in apartments, hotels, and public buildings (Valcina et al., 2019). Culture and qPCR methods were used to quantify *Legionella, Acanthamoebae* spp. and *Naegleria fowleri* and different primer sets were used relative to this study (Valcina et al., 2019; Scheikl et al., 2014). The concordance rate between the presence of FLA and *Legionella* spp. were 55.1% (n=114), and correlation analysis revealed a weak positive correlation ($r = 0.467$, $p<0.01$) (Valcina et al., 2019). *Legionella* and free-living amoebae negative samples reached a concordance rate of 100% (n=61) (Valcina et al., 2019). Valcina et al., 2019, examined the cooccurrence of several *Legionella* spp. such as *Legionella pneumophila, Legionella anisa,* and *Legionella rubrilucens,* and several free-living amoebae (*Acanthamoeba, Vermaoeba, and Naegleria*); however, there was no mention of what *Legionella* spp. mostly cooccurred with specific amoeba. A total of 201 water samples (129 cooling waters and 72 process waters, and 30 cooling lubricants) were collected. Thirty-four percent (70/201) of the water samples were positive for *Legionella* spp. And by culture method, *Legionella* cooccurred with *Acanthamoeba* spp. in 27.1% (19/70) of water samples collected, while *Naegleria fowleri* concordance rate was 0.38% (1/70) (Scheikl et al., 2014). Unfortunately, this study did not mention the concordance rate of amoebae and *Legionella* species (Scheikl et al., 2014).

Several studies have shown that the concentrations of *Naegleria fowleri*, general *Legionella* spp. (23S rRNA) and *L. pneumophila* amplification in a distribution system may be seasonally influence via water temperature (Marciano-Cabral et al., 2010; Wang et al., 2012). This study observed the opposite, it was not due to environmental conditions. For example, pathogenic *L. micdadei, L. bozemanii* and *Naegleria fowleri* were detected more in a drinking water supply system that was supplied by a groundwater source, which is not affected by ambient temperatures like surface waters. However, the occurrence of free-living amoeba and *Legionella* spp. may be affected by the built environment. For example, water age has been observed to affect the detection of *Legionella* and amoebae species in a distribution system (Ambrose et al., 2020; Wang et al., 2012). Wang et al., 2012 evaluated two simulated water distribution systems with different water ages ranging from three to 12 days. Water age affected *Legionella* and *Acanthamoeba* in a simulated water distribution system with a water age greater than three days (Wang et al., 2012). However, the water age in each water distribution system and within systems is different (EPA, 2002). As observed in this study, the increase in water age in the distribution system, correlates with an increase in *Legionella* and amoebae species. Thus, water age may play a role in the occurrence of *Legionella* and amoebae species in a building with an increased water age, as seen in the ERC building (water age of 20.8 hours). Also, the regression analysis revealed that *Naegleria fowleri* affects the occurrence of *L. micdadei* and *L. bozemanii* in the ERC building, which is furthest away from the reservoir. However, the weak regression suggests that this observation will need to be further elucidated with a more extensive data set.

Since amoebae promote the growth of *Legionella* post-treatment in water systems, it is critical to gain an understanding of microbial-amoebae ecological relationships in large, complicated plumbing. Overall, this study found a greater association between *Naegleria fowleri*

and two *Legionella* species. Two lines of evidence revealed that *Naegleria fowleri* strongly associated with *L. bozemanii,* and *L. micdadei* in the building water system (Fa and ERC).

4.6 Conclusion

Most of the public health agencies and guidance documents have water management programs that are focused on the risk of *Legionella,* without considering their particular host, *Naegleria,* and *Acanthamoeba* spp. Utilizing ddPCR, *Naegleria fowleri* were detected more often in a drinking water supply system and cooling towers. The Chi-squared test showed that *Naegleria fowleri* significantly cooccurred with two pathogenic *Legionella* spp. (*L. micdadei,* and *L. bozemanii*) in a drinking water supply system and cooling towers. Moreover, regression analysis revealed that *Naegleria fowleri* may affect *Legionella micdadei* and *L. bozemanii* in a building water system. In this study, *Naegleria fowleri, L. micdadei,* and *L. bozemanii* are the most important species. Thus, by examining large volume (10-L) water ultrafiltrate concentrates from the groundwater source to exposure sites (taps and cooling towers) utilizing ddPCR, allowed for the detection of individual-specific *Legionella*, and *Naegleria fowleri*. Most importantly, the widespread of *Naegleria fowleri,* and *Legionella* species in the taps and cooling towers indicates an important health concern.

CHAPTER FIVE

CONCLUSION

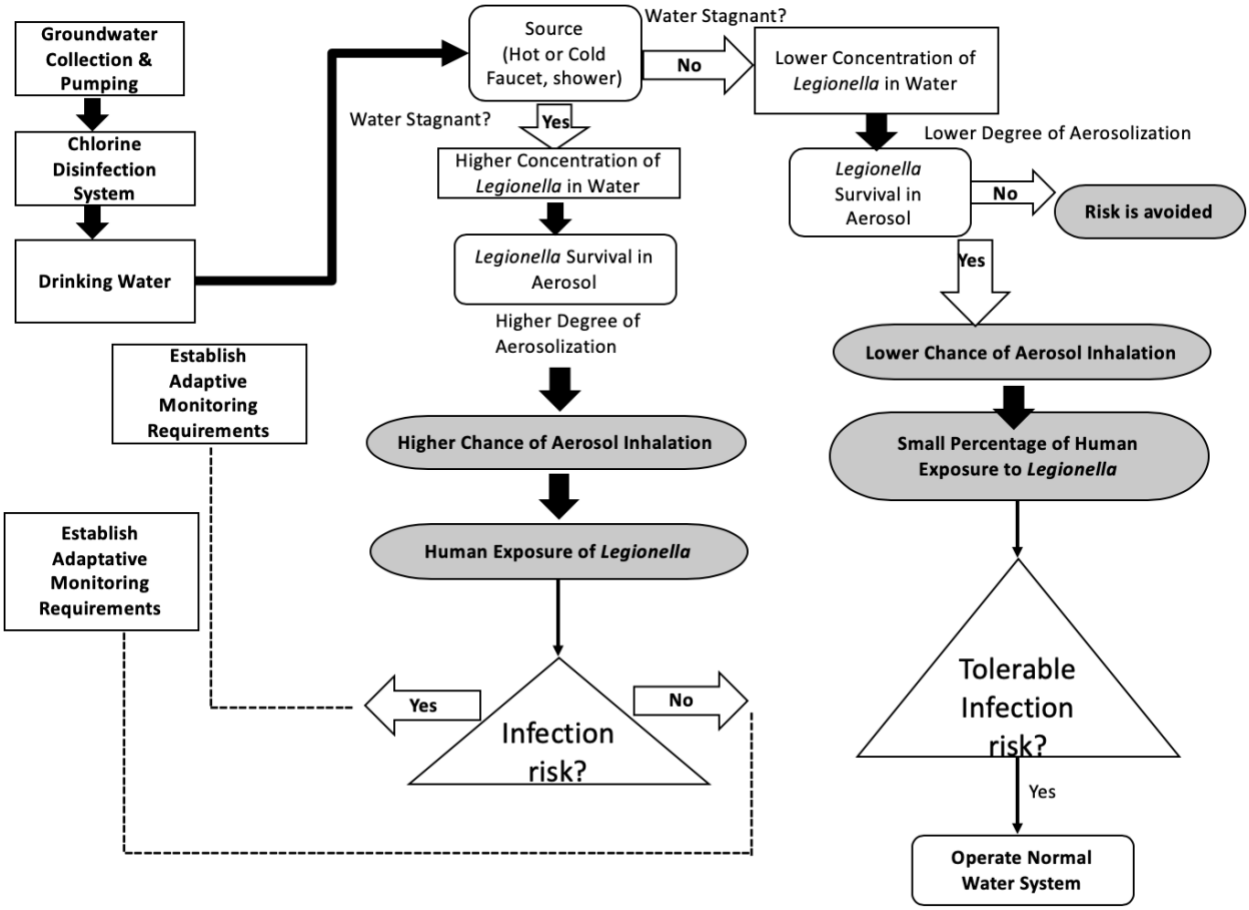
5.1 Limitations and Future Directions

Federal, state and local regulatory approaches to manage the risk of *Legionella* rely on maintaining the water supply system including a disinfection residual throughout the distributed water and minimizing exposure routes where possible (eg., fountains in hospitals). The literature and papers described here assessed the risk by examining the exposure at the point of use in the water supply system. The occurrence of *Legionella* bacteria and the probability of infection cannot be directly equated from one location to another within a water supply system or building. The literature lacks a detailed exposure assessment in the complete water supply chain from the source through the distribution system to the building taps or cooling towers. Understanding the wholistic view of the water supply system (reservoir tank, the distribution system, and the premise plumbing) may add insight in how to mitigate some of the uncertainties that go into the risk management plans (Figure 5.1).

There is also a lack of knowledge about how to manage the risk from the various other pathogenic *Legionella* species besides *L. pneumophila.* There has been a suggestion that *L. anisa* have a unique relationship with *L. pneumophila* such that both spp. co-occurred together in the environment (Mee-Marquet et al., 2006). *Legionella anisa* has caused LD (Vaccaro et al., 2016), but there is a detection limitation on other non-*pneumophila* spp., the diagnosis has been on *L. pneumophila* (the urinary antigen test is specific for *L. pneumophila* sg 1, as described above). Thus, there is a risk of LD caused by other *Legionella* spp. However, little is known about the concentrations in the complete drinking water supply system for *L. anisa, L. micdadei, L. bozemanii,* and *L. longbeachae*. Moreover, there should be a routine (monthly) sampling to highlight the occurrence and concentrations of other pathogenic *Legionella* species; this will give a building owner the ability to prevent *Legionella* growth in a drinking water supply system.

Primary prevention (proactive instead of reactive) starts with understanding the factors that encourages the growth of *Legionella* in a drinking water system. Factors that are associated with *Legionella* spp. in large, complex buildings are increased water age in the distribution system, water stagnation in the premise plumbing system, and the ability to live inside free-living amoebae throughout the whole water network. In such environments (listed above), water temperatures are warm and there's little to no disinfectant residuals available, these conditions create a conducive niche for the proliferation of *Legionella* intracellularly within free-living amoebae throughout the water network. The risks associated with an increase water age, and water stagnation (low water usage) could be reduced by implementing, for example, manual flushes. Thus, there is a critical need for a water management plan to implement strategies to better assess and manage water quality parameters (for ex: temperature, disinfection residuals, pH) and microbiological parameters (different *Legionella* spp., listed above).

Figure 5.1. A Schematic Linking Point of Use to Human Exposure



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